Effective Anti-Neu–Initiated Antitumor Responses Require the Complex Role of CD4⁺ T Cells

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Abstract

Purpose: Targeting oncogenic receptors with antibodies has been thought to suppress tumor growth mainly by interrupting oncogenic signals. Recently, the essential role for adaptive immunity, and CD8⁺ T cells in particular, has been established as a major factor for anti-HER2/neu–mediated tumor regression. However, the role of CD4⁺ T cells is still being defined. The purpose of this study was to explore whether and to what extent CD4⁺ T cells are involved in mediating the effects of anti-HER2/neu therapy.

Experimental Design: The role of CD4⁺ T cells was examined using a transplant model of the rat HER2/neu–overexpressing cell line TUBO. Tumor-bearing mice were treated with anti-neu therapy in conjunction with CD4 depletion or CD40L blockade. The effects of CD4 depletion on the antitumor response were examined by tumor growth analysis and enzyme-linked immunospot (ELISPOT).

Results: In addition to CD8⁺ T cells, CD4⁺ T cells are also essential for anti-neu antibody-mediated tumor regression, but B cells are not required. The role for CD4⁺ cells is necessary throughout anti-neu therapy and not limited to helping CD8⁺ T cells. Expression of IFN-γ is necessary for anti-neu therapy and IFN-γ induces MHC-II expression in TUBO cells promoting direct recognition by CD4⁺ T cells. Furthermore, intratumoral depletion of CD4⁺ T cells or blockade of the activating cell-surface protein CD40L inhibits the antitumor response.

Conclusions: This study reveals the essential role of CD4⁺ T cells for anti-neu–mediated tumor regression.

Cancer Therapy: Preclinical

Introduction

The use of antibody therapies against oncogenic receptors has changed the landscape for treating multiple cancer types. In fact, antibody-based therapeutics are now essential components of many cancer treatment regimens (1). Among the oncoproteins to which antibodies have been generated, the HER2/ErbB2 is among the most targeted with 2 molecular targeting agents approved by the U.S. Food and Drug Administration (FDA; ref. 2). HER2 is amplified or overexpressed in 25% to 30% of patients with breast cancer and is associated with aggressive disease, a high recurrence rate, and reduced patient survival (3, 4). Trastuzumab (Herceptin), a humanized monoclonal antibody targeting HER2, has been used in multiple clinical trials with various inclusion criteria but consistently increased patient survival (5). Thus, trastuzumab is established as an effective therapeutics for HER2-positive breast cancer, although its mechanism of action is still being defined.

Trastuzumab binds the extracellular region of HER2 (6), and multiple studies have shown different mechanisms by which this therapy inhibits tumor growth. First, HER2/neu activation initiates multiple signaling pathways, including the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades (7), and trastuzumab treatment reduces the activity of these cascades, leading to cell-cycle arrest and apoptosis (2, 8). Second, trastuzumab binding to HER2 prevents protease cleavage of the HER2/neu extracellular domain that usually results in a constitutively active receptor (9–11). Third, the ability of trastuzumab to engage Fc receptors and initiate antibody-dependent cell-mediated cytotoxicity (ADCC) has been shown to be essential for its antitumor activity in both in vitro and in vivo studies (2, 7, 12–14). Finally, the role of the adaptive immune system in anti-HER2/neu therapy has recently begun to be appreciated as an additional mechanism of action (15–18). However, the cellular and molecular components involved in this process are still being defined. Previous data from our laboratory established a role for the adaptive immune system in anti-neu therapy and defined an essential role for CD8⁺ T cells and the presence of neuspecific memory (15). In a separate study, anti-neu therapy...
When examining how CD4\(^+\) have been shown to play a major role in suppressing CTL (20). In established tumor models, however, regulatory T cells have been shown to play a major role in suppressing CTL (19). In established tumor models, however, regulatory T cells have been shown to play a major role in suppressing CTL (20). When examining how CD4\(^+\) T cells contribute to anti-neu vaccines, multiple studies focused on the role of CD4\(^+\) regulatory T cells in neu-positive tumor progression and show that CD4\(^+\) regulatory T cells mask effector CD8\(^+\) T-cell responses and promote metastasis (23) of neu-positive tumors. Here, using a CD4-depleting antibody during anti-neu therapy, we establish an unprecedented role for CD4\(^+\) T cells in supporting the antitumor function of anti-neu antibody therapy.

**Materials and Methods**

**Mice**

BALB/c, BALB/c Rag2-KO, and IFN-\(\gamma\)-KO mice were purchased from Jackson Laboratory. Tolerized F1 neu transgenic (Tg) mice (FVB/N MMTV-neu \(\times\) BALB/c) were bred and housed at the University of Chicago (Chicago, IL). All mice were maintained under specific pathogen-free conditions and used between 6 and 16 weeks of age in accordance to the animal experimental guidelines set by the Institutional Animal Care and Use Committee. All experiments have been approved by the Institutional Animal Care and Use Committee and conform to the relevant regulatory standards.

**Cell lines and reagents**

TUBO-cloned cell line derived from a spontaneous mammary tumor in a BALB-neuT transgenic mouse expressing transforming rat neu (24) was a gift from Joseph Lustgarten (Mayo Clinic, Scottsdale, AZ). TUBO was cultured in 5% CO\(_2\) and maintained in vitro in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FBS (Sigma), 10% NCTC 109 medium, 2 mmol/L L-glutamine, 0.1 mmol/L minimum essential medium nonessential amino acids, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin. The anti-neu monoclonal antibody (mAb) 7.16.4, anti-CD4-depleting antibody GK1.5, and CD40 agonist antibody FGK-45 were produced in house. The CD20-depleting antibody 18B12 and CD40L-blocking antibody MR1 were kindly provided by Biogen. The anti-neu antibody (7.16.4) recognizes the juxtamembrane region of rat neu and competes with 4D5, the precursor of trastuzumab, for binding and inhibition of tumor growth (25). All antibodies for analysis by flow cytometry were purchased from BD Biosciences or Biolegend.

**Tumor inoculation**

Adherent TUBO cells were removed from culture flasks by incubating for 3 to 5 minutes in 1 \(\times\) Trypsin EDTA (Mediatech, Inc.). Cells were washed 2 to 3 times in 1 \(\times\) PBS and counted by Trypan exclusion. TUBO cells (3\(\times\)10\(^5\)) were injected subcutaneously in the back of 6- to 8-week-old anesthetized mice. Tumor volumes were measured along 3 orthogonal axes \((x, y, z)\) and calculated as tumor volume = \((xyz)/2\).

**Antibody treatments**

Mice were treated with 2 or 3 intraperitoneal injections of 100 to 200 \(\mu\)g of anti-neu antibody (clone 7.16.4) diluted in 100 to 200 \(\mu\)L of 1 \(\times\) PBS. For CD4\(^+\), CD8\(^+\), and CD20 depletion experiments, 200 \(\mu\)g of anti-CD4 antibody (clone GK1.5), anti-CD8 antibody (clone YTS 169.4.2 or 53.6.4), or anti-CD20 antibody (clone 18B12) diluted in 100 to 200 \(\mu\)L of 1 \(\times\) PBS was administered intraperitoneally at the indicated time points. Blocking CD40L was achieved by intratumoral administration of 50 \(\mu\)g anti-CD40L (clone MR1) diluted in 30 to 50 \(\mu\)L of 1 \(\times\) PBS on the indicated days. Injection time points are indicated by different triangles along the x-axis of tumor growth curves graphs.

**Flow cytometry analysis**

A total of 1 to 2 \(\times\)10\(^6\) cells were added to flow cytometry tubes and washed in PBS with 0.5% bovine serum albumin (BSA). FcR were blocked with 20 to 50 \(\mu\)L of 2.4G2 and incubated for 15 minutes at room temperature. Surface proteins were labeled by adding 10 \(\mu\)L of an antibody cocktail in PBS with 0.5% BSA (i.e., \(\alpha\)CD3-PE, \(\alpha\)CD4-PercP-Cy5.5, \(\alpha\)CD8-PE-Cy7, and \(\alpha\)CD45-APC-Cy7). Cells were incubated for 30 minutes at 4 \(^\circ\)C in the dark. Following incubation, cells were washed and resuspended in 200 to 300 \(\mu\)L of PBS with 0.5% BSA and analyzed on a BD FACS Canto cytometer (BD Cytometry Systems).

**Measurement of IFN-\(\gamma\)-secreting cells by ELISPOT assay**

Tumor-reactive T cells were measured by enzyme-linked immunospot (ELISPOT) assay. Spleen or tumor cells (stimulatory cells) were suspended in RPMI-1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin. A total of 4 \(\times\)10\(^3\) spleen cells...
or purified CD8⁺ cells (CD8α Positive Selection Kit; Stem Cell Technologies) were added to each well of a 96-well HTS IP plate (Millipore) that was precoated with 2.5 μg/mL rat anti-mouse IFN-γ (clone R4-6A2; BD-PharMingen). CD8α purity following positive selection was 90.6% (±5.2%). TUBO cells were used as stimulatory cells. The ratio of responder to stimulatory cells was 10:1. After a 40- to 46-hour incubation at 37°C, the cells were removed, the wells were washed, and 2 μg/mL biotinylated rat anti-mouse IFN-γ (clone XMG 1.2; BD-PharMingen) was added. Plates were then either incubated for another 12 hours at 4°C or for 2 hours at 37°C, and then washed to remove unbound antibody. Bound antibody was detected by incubating the plates with the 0.9 μg/mL avidin-horseradish peroxidase (BD-PharMingen) for 2 hours at room temperature. The substrate 3-amino-9-ethylcarbazole (AEC; Pharmingen) diluted in 0.1 mol/L in acetic acid and 0.003% hydrogen peroxide was added, and the plate was incubated for 3 to 5 minutes. The AEC solution was discarded, and the plates were washed 6 × with water. The visualized cytokine spots were enumerated with the ImmunoSpot analyzer (CTL), and results were expressed as the number of cytokine-producing cells per the number of cells plated.

Analysis of tumor-infiltrating lymphocytes

Established TUBO tumors were resected on the days indicated. Single-cell suspensions were obtained by chopping tumors into small pieces and then incubating the pieces in 3 to 5 mL of digestion media (RPMI with 1.5 mg/mL collagenase and 0.4 mg/mL DNase) for 30 to 45 minutes on a rotator. Cells were then collected and washed 1 × PBS and counted by Trypan blue exclusion. For flow cytometry analysis, 1 to 2 × 10⁶ cells were incubated with fluorochrome-conjugated mAbs against surface markers. Cells were acquired on a FACSCanto flow cytometer.

Statistical analysis

Differences between groups were analyzed using an unpaired t test or repeated measurement two-way ANOVA. Repeated measurement two-way ANOVA analysis of tumor growth curves included data points after the administration of anti-neu therapy until where noted. Error bars represent ±SD. All statistical analyses were conducted using GraphPad Prism Version 4.0 for Macintosh (GraphPad Software). Unless specified, statistically significant differences of P < 0.05, 0.01, and 0.001 are noted with *, **, and ***, respectively. Differences that are not statistically significant are left unnoted.

Results

CD4⁺ T cells are necessary for anti-neu antibody therapy

The TUBO cell line is a well-characterized model of neu-positive breast cancer derived from a spontaneous mammary tumor in BALB-neuT mice expressing transforming rat neu (24). When transplanted into wild-type (WT) BALB/c mice, these cells establish tumors histologically similar to autochthonous tumors in BALB-neuT females (26). While the essential role for CD8⁺ T cells in anti-neu–mediated tumor regression has been clearly shown using this and similar transplant models, the role of CD4⁺ T cells remains controversial (16, 27). We observed that administering anti-neu therapy to TUBO tumor-bearing BALB/c mice resulted in increased CD4⁺ tumor-infiltrating lymphocytes (TIL) within the tumor microenvironment (Fig. 1A); moreover, CD4⁺ T cells outnumbered CD8⁺ T cells, raising the possibility that CD4⁺ T cells may play a major role in this model (Supplementary Fig. S1). To determine if CD4⁺ T cells were required for the tumor-suppressing function of anti-neu therapy, a CD4⁺-depleting antibody was coadministered with anti-neu systemically by intraperitoneal injection to tumor-bearing mice. This treatment resulted in greater than a 90% reduction in CD4⁺ T cells as assessed by the reduction of CD3⁺CD8⁻ cells in the peripheral blood (Supplementary Fig. S2). Loss of CD4⁺ T cells significantly reduced the antitumor effects of anti-neu antibody therapy (Fig. 1B). Notably, the reduced efficacy was not due to Fc receptor saturation, because mice receiving anti-neu antibody plus a control antibody responded in a similar manner to mice receiving anti-neu antibody alone (Fig. 1B).

We recently published that CD8⁺ T cells were necessary for anti-neu therapy (15). To directly compare the requirement of CD4⁺ and CD8⁺ T cells, tumor-bearing mice were depleted of either CD4⁺ or CD8⁺ T cells during anti-neu therapy. The loss of CD4⁺ T cells had a more dramatic effect on the efficacy of anti-neu therapy than the loss of CD8⁺ T cells (Fig. 1C). Thus, CD4⁺ T cells may either be involved earlier or have a more significant role than CD8⁺ T cells. In an effort to further define the requirement of CD4⁺ T cells in anti-neu–mediated tumor regression, we sought to address when these cells were necessary. To this end, a CD4⁺-depleting antibody was administered to tumor-bearing mice at the following time points during anti-neu therapy: at the time of inoculation (pre) that continued throughout anti-neu therapy; in conjunction with anti-neu therapy (during); and 3 to 5 days after the final anti-neu treatment (late). As previously observed, CD4⁺ T-cell depletion in conjunction with anti-neu therapy significantly inhibited the efficacy of anti-neu therapy (Fig. 1C, during). To a lesser extent, a decrease in effective anti-neu therapy was also observed when CD4⁺ T cells were depleted after the final anti-neu treatment (Fig. 1C, late). Similarly, CD4⁺ T-cell depletion at the time of tumor inoculation significantly inhibited anti-neu therapy effects; however, this effect was only observed after anti-neu therapy was initiated (Fig. 1C, pre). Collectively, these data suggest that CD4⁺ T cells are not only required after the initiation of but also contribute throughout anti-neu therapy.

IFN-γ is necessary for anti-neu therapy

Because cells from both the innate and adaptive immune systems produce IFN-γ and are involved in mediating the anti-neu therapy effects, we conducted ELISPOT analysis on cells harvested from Rag2-KO mice to examine whether, and to what extent, IFN-γ produced by innate cells was required. As expected, anti-neu therapy induced a
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Figure 1. Effective anti-neu therapy requires CD4+ T cells. A, tumor-bearing mice were treated with a single dose of mlg or anti-neu (7.16.4, 200 μg); 3 to 5 days later, tumors were resected and analyzed for the presence of CD4+ T cells via flow cytometry. To normalize for differences in tumor size between groups, data were represented as either the percentage of CD4+ T cells within the CD45+ population, or the ratio of CD4+ T cells to CD45+ cells. Statistical analysis was conducted using an unpaired t test. B, WT BALB/c mice (n = 5/group) were inoculated subcutaneously with 4 × 10⁶ TUBO cells and treated intraperitoneally with 150 μg of anti-neu (clone 7.16.4) or control (mlgG) antibody on day 11 and 18 (black triangles). Two hundred micrograms of CD4-depleting antibody (clone GK1.5) or control antibody (rIgG) was administered intraperitoneally on day 10 and 17 (gray triangles). Statistical analysis was conducted using a repeated-measurement two-way ANOVA on time points after anti-neu therapy. Data are representative of 3 independent experiments. C, WT BALB/c were inoculated as in (B) and treated intraperitoneally with 150 μg of anti-neu (clone 7.16.4) or control (mlgG) antibody on day 10 and 17 (black triangles). Two hundred micrograms of CD4-depleting antibody (clone GK1.5) or control antibody (rIgG) was administered intraperitoneally on day 10 and 17 (gray triangles). Statistical analysis was conducted using a repeated-measurement two-way ANOVA on time points after anti-neu therapy. Data are representative of 3 independent experiments. D, WT BALB/c mice were inoculated as in (B) and treated with mlg or anti-neu on day 10 and 17 (gray triangles); or day 20 and 24 (late; white triangles). Two hundred micrograms of CD4-depleting (clone GK1.5), CD8-depleting (clone 53.6.7) or control antibody (rIgG) was administered intraperitoneally on day 10 and 17 (gray triangles). Statistical analysis was conducted using a repeated-measurement two-way ANOVA on time points after anti-neu therapy, and compared anti-neu therapy to anti-neu therapy plus CD4 depletion early (•) or late (••). Data are representative of 3 independent experiments.

significant increase in the antitumor IFN-γ response in WT mice (Fig. 2A). However, anti-neu–treated tumor-bearing Rag2-KO mice did not produce a measurable amount of IFN-γ. These data suggest that anti-neu therapy promotes IFN-γ production, and that innate cells alone are not producing this cytokine. To test the necessity of IFN-γ in our model, we compared the effects of anti-neu therapy in WT and IFN-γ-KO BALB/c mice. The absence of IFN-γ significantly reduced the efficacy of anti-neu therapy, suggesting a necessary role for this cytokine (Fig. 2B). Although anti-neu therapy did delay tumor progression in IFN-γ-KO mice as compared with mouse IgG (mlgG)–treated controls, no tumor regression was observed and is most likely due to the direct antitumor effect of anti-neu therapy and other cytokines. Collectively, these data suggest that IFN-γ is crucial for effective anti-neu therapy.

B cells are not required for anti-neu therapy

As CD4+ T cells are known to provide B-cell help, we addressed whether CD4+ T cells promote endogenous anti-
After CD4 depletion, IFN-γ production by the TIL was also significantly reduced (Fig. 3B). These data suggest that the antitumor response initiated by anti-neu therapy is dependent on CD4+ T cells.

It has been previously reported that the TUBO cells express MHC-I but not MHC-II in vitro (24). Thus, the loss of IFN-γ production was assumed to be from a reduced CD8+ T-cell antitumor response after CD4+ T-cell depletion. However, because ELISPOT analysis of total splenocytes could not identify if the loss of IFN-γ production was from CD4+ or CD8+ T cells, this assay was repeated using purified CD8+ T cells. Surprisingly, CD4+ T-cell depletion did not alter the antitumor response of CD8+ T cells (Fig. 3C). Collectively, these data indicate that anti-neu therapy induces a tumor-specific response that is dependent on the presence of CD8+ T cells.

IFN-γ induces MHC-II expression for CD4+ T-cell recognition of TUBO cells

Given the necessity of CD4 cells in anti-neu therapy, we next wanted to examine the possibility of CD4+ T cells directly recognizing TUBO cells by evaluating the ability of IFN-γ to induce expression of MHC-II. As previously published, TUBO cells in culture did not express MHC-II in vitro (24). Thus, when treated with anti-neu antibody; however, addition of exogenous IFN-γ induced the expression of MHC-II in TUBO cells within 24 hours (Fig. 4A). Although, anti-neu alone did not induce MHC-II expression in vitro, the expression of MHC-II was induced in tumor cells in vivo after anti-neu therapy (Fig. 4B). The mean fluorescence intensity of MHC-II–expressing tumor cells was 140.3 ± 33.4 after mlgG treatment but increased to 594.3 ± 183.5 after anti-neu therapy. The expression of MHC-II in TUBO cells raised the possibility that CD4+ T cells may be able to identify the tumor directly. To test this, CD8+ T cells were depleted during anti-neu therapy and the antitumor response of CD4+ T cells was evaluated by ELISPOT. In the absence of CD8+ T cells, CD4+ T cells were still able to...
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induce an antitumor response (Fig. 4C). Moreover, this response was repeated using neu-Tg recipient that are tolerant to the neu antigen. T cells from the spleens of neu-Tg mice displayed a stronger antitumor response to TUBO after anti-neu treatment as determined by IFN-γ ELISPOT (IFN-γ+ cells/4 x 10^5: 97 ± 23 in control Ig-treated group compared with 590 ± 48 in the anti-neu–treated group). Collectively, these data suggest that IFN-γ within the tumor microenvironment induced by anti-neu therapy upregulates the expression of MHC-II on tumor cells allowing CD4+ T cells to directly engage the tumor in both WT and neu-Tg mice.

**CD40L expression within the tumor microenvironment is necessary for effective anti-neu–mediated tumor regression**

The requirement for CD4+ T cells in the antitumor response initiated by anti-neu therapy suggested a role for CD4+ T cells within the tumor microenvironment. Therefore, we next wanted to determine a mechanism, in addition to cytokine production, by which CD4+ T cells initiate an antitumor response. We first determined the requirement of CD4+ TIL and found that a reduced dose of a CD4-depleting antibody significantly reduced tumor growth when administered intratumorally to tumor-bearing mice in conjunction with anti-neu therapy (Fig. 5A); moreover, this suppression was more pronounced than intraperitoneal administration using a much higher dose of antibody. These data suggest that CD4+ T cells within the tumor microenvironment are important for mediating the antitumor effect initiated by anti-neu therapy.

The necessity of tumor-infiltrating CD4+ T cells suggested that close cell–cell interactions might be required for anti-neu–mediated tumor regression. A surface molecule important for CD4+ T-cell activation and effector function is CD40L (29–31). Its binding partner, CD40, is expressed in many cell types, including B cells, dendritic cells, macrophages, and epithelial cells (31). In the context of adaptive immune responses, binding of CD4+ T-cell–expressed CD40L with CD40 expressed in antigen-presenting cells is vital to provide help for cytotoxic CD8+ T-cell priming and memory function (31–34). To determine whether CD40L played a role in anti-neu antibody therapy, TUBO-bearing mice were treated intratumorally with a CD40L-blocking antibody. Intratumoral blockade of CD40L significantly inhibited tumor regression mediated by the anti-neu antibody (Fig. 5B). Because CD40L blockade has previously been reported to inhibit T-cell extravasation (35), we tested whether CD40L blockade prevented CD8+ T-cell infiltration by using flow cytometry to quantify the number of CD8+ TIL. We found that intratumoral blockade of CD40L did not prevent CD8+ T cells from entering the tumor microenvironment (Fig. 5C), which excluded the possibility that inhibition of tumor regression by CD40L blockade was due to reduced T-cell infiltration. Moreover, anti-CD40L blockade presumably does not directly affect tumor cells, as TUBO cells did not express CD40L in vitro, even in the presence of exogenous IFN-γ or anti-neu antibody (Fig. 5D). These data suggest that expression of CD40L and interaction with CD40 on nontumor cells within the tumor microenvironment is essential for anti-neu therapy.

We next wanted to examine whether and how CD40L blockade affected the antitumor response. To this end, tumor-bearing mice were treated with anti-neu antibody with or without intratumoral injection of a CD40L-blocking antibody; spleenocytes and TIL were then harvested and analyzed by IFN-γ ELISPOT as described earlier. While CD40L blockade did not significantly reduce the antitumor response within the spleen (Fig. 6A), it significantly reduced...
the anti-neu–induced IFN-γ response by the TIL (Fig. 6B). These data suggest that CD40L does not alter T-cell priming within the tumor-draining lymph node but that it is required for antitumor responses within the tumor microenvironment. Collectively, these data suggest that effective anti-neu antibody-mediated tumor regression requires CD40L expression within the tumor microenvironment.

Discussion

CD4+ T cells play a crucial role in antitumor immunity. Most studies examining the role of CD4+ T cell in immunity to neu-positive tumors have focused on regulatory CD4+ T-cell suppression of CD8+ effector T cells. Using similar tumor transplant models, multiple studies have focused on the role of CD4+CD25+ regulatory T cells in neu-positive tumor progression, where these T cells have been shown to mask effector CD8+ T-cell responses (21, 22) and promote neu-positive tumor metastasis (23). However, these previous studies examined the role of vaccine efficacy and did not evaluate their role in anti-neu antibody-mediated tumor regression. In our system, depleting total CD4+ T cells (which includes CD4+CD25+ regulatory T cells) increased resistance to anti-neu antibody-mediated tumor regression, suggesting that the overall antitumor CD4+ T-cell response initiated by this therapy outweighs suppression by regulatory T cells.

In terms of anti-neu therapy, 3 mechanisms may explain the role of CD4+ T cells in tumor regression: (i) after anti-neu therapy, these cells may provide help for endogenous antibody production against neu-positive tumors or

Figure 4. IFN-γ induces MHC-II expression for CD4+ T-cell recognition of TUBO cells. A, 4 x 10^5 TUBO cells were cultured in 12-well plates alone or in the presence of IFN-γ at the specified concentrations with or without anti-neu. After a 24-hour incubation, cells were collected and labeled with a phycoerythrin (PE)-conjugated anti-I-A/E antibody (clone M5/114.15.2), and then analyzed by flow cytometry. B, WT BALB/c mice (n = 3/group) were inoculated subcutaneously with 4 x 10^5 TUBO cells and treated intraperitoneally with 200 μg anti-neu (clone 7.16.4; open histograms) or control (mIgG; filled histograms) antibody on day 13. On day 18, tumors were resected and tumor cells were analyzed using flow cytometry by gating on live (7AAD−), large (FSC+), CD45+ cells. Data are representative of 2 independent experiments. C, WT BALB/c mice (n = 3/group) were inoculated subcutaneously with 4 x 10^5 TUBO cells and treated intraperitoneally with 200 μg anti-neu (clone 7.16.4) on day 14 with or without prior intraperitoneal administration of 200 μg of a CD8-depleting antibody (clone YTS 169.4.2) on day 13. On day 19, cells were isolated from the spleen for each mouse and used in an ELISPOT assay. Statistical analysis was conducted using an unpaired t test. Data are representative of 3 independent experiments.
Mechanistic studies of anti-neu therapy have been conducted in both transplant and autochthonous tumor models, and each has provided complementary data. Transgenic mice expressing mouse human neu fail to develop mammary tumors; but transgenic mice expressing rat neu under the control of the mouse mammary tumor virus (MMTV) promoter grow mammary carcinomas by 10 to 15 months of age. Although use of this promoter effectively promotes the induction of autochthonous tumor formation, the neu antigen is expressed in all mammary cells at higher levels and earlier stages of development than in humans or WT mice. This altered expression can potentially induce stronger tolerance to rat neu (36). Along these lines, T cells from WT mice and neu-Tg mice both displayed a stronger antitumor response to TUBO after anti-neu treatment. Thus, it is likely that human CD4+ T cells also respond to HER2/neu and other antigens in the patients. For example, endogenous anti-HER2/neu antibodies have been detected in humans and shown to suppress HER kinase activity and inhibit the transformed phenotype of HER2-expressing tumor cells (37).

Figure 5. CD40L expression within the tumor microenvironment is necessary for effective anti-neu therapy. A, WT BALB/c mice (n = 5/group) were inoculated subcutaneously with 4 × 10^6 TUBO cells and treated intraperitoneally with 150 μg anti-neu (clone 7.16.4) or control (mIg) antibody on day 10 and 17 (black triangles) with or without administration of a CD4-depleting antibody (clone GK1.5) intraperitoneally (200 μg) or intratumorally (50 μg) on day 10 and 15 (gray triangles). Statistical analysis was conducted using a repeated-measurement two-way ANOVA on time points after anti-neu therapy, and anti-neu therapy to anti-neu therapy plus CD4 depletion intratumorally (**) or intraperitoneally (*) were compared. Data are representative of 3 independent experiments. B, WT BALB/c mice were inoculated subcutaneously with 4 × 10^6 TUBO cells and treated intraperitoneally with 150 μg anti-neu (clone 7.16.4) or control (mIg) antibody on day 10 and 17 (black triangles) with or without intratumoral injection of 50 μg of a CD40L-blocking antibody (clone MR1) on days 10, 12, 14, and 17 (gray triangles). Statistical analysis was conducted using a repeated-measurement two-way ANOVA on time points after anti-neu therapy. Data are combined from 3 independent experiments. C, WT BALB/c mice (n = 3–4/group) were inoculated subcutaneously with 4 × 10^6 TUBO cells and treated intraperitoneally with 150 μg anti-neu (clone 7.16.4) antibody on day 10 and 15 with or without intratumoral injection of 50 μg of a CD40L-blocking antibody (clone MR1) on day 10, 12, 14, and 17. On day 19, tumors were resected and isolated TIL were analyzed for the presence of CD8+ and CD4+ T cells via flow cytometry. Statistical analysis was conducted using an unpaired t test. Data are combined from 3 independent experiments. D, 4 × 10^6 TUBO cells were cultured in 12-well plates alone or in the presence of IFN-γ at the specified concentrations with or without anti-neu antibody. After a 24-hour incubation, cells were collected and labeled with an anti-CD40L antibody (clone MR1) or isotype control (hamster Ig) then analyzed by flow cytometry. Splenocytes stimulated overnight with phorbol 12-myristate 13-acetate (PMA; 20 ng/mL) and ionomycin (500 ng/mL) were used as a positive control for CD40L expression. Data are representative of 2 independent experiments.
Murine and rat neu display a 95% homology at the amino acid levels, respectively (26, 38). The homologs share peptide motifs that can be presented on mouse H-2d to elicit CTL responses in mice (26, 27, 38). Moreover, when these tumors are transplanted into WT BALB/c mice, these cells establish tumors histologically similar to autochthonous tumors in BALB-neuT females (26). Thus, the antigenic differences between rat and murine neu may be considered more like mutated antigens. Furthermore, multiple mutated antigens have been identified in human breast cancer, which might present better to T cells after treatment. Our study suggests that CD4+ T cells contribute to anti-neu-mediated tumor control using both models.

As mentioned earlier, the presence of endogenous anti-HER2/neu antibodies has been documented in patients (37, 39). However, the actual contribution of endogenously generated antibodies to the efficacy of anti-neu therapy is unclear. One complication in answering this question through mouse models is separating endogenous antibody production from the exogenous antibody provided for therapy. This is particularly important, given that endogenous antibodies have been shown to enhance cross-presentation and bolster CD8+ T-cell activation (40). In the present study, total CD20+ cells were removed during anti-neu therapy at the time of inoculation to circumvent this issue, and we found that CD20+ B-cell depletion did not inhibit anti-neu-mediated tumor regression. Although endogenous antibodies are presumably generated after exogenous anti-neu treatment and may have antiproliferative impacts on tumor growth, these data suggest that endogenous antibodies are not the primary antitumor mechanism generated by anti-neu therapy. In addition, our finding that B cells are not necessary suggests that these cells may not be the major antigen-presenting cells in this system.

CD4+ T cells can exert direct effector functions on tumors. In some models, CD4+ T cells induce cellular senescence (41). Although this may be occurring in our model, markers of cellular senescence within the tumor microenvironment were not significantly increased after anti-neu therapy (data not shown). Alternatively, much focus has been directed toward the role of IFN-γ production by CD4+ T cells. In antitumor immunity, IFN-γ may act directly on tumor cells to induce MHC-II expression (42) or indirectly to inhibit angiogenesis (43–45). In this study, we showed, that CD4+ T-cell depletion significantly inhibited the effects of anti-neu therapy. Moreover, CD4+ T-cell depletion during anti-neu therapy reduced the antitumor response, and intratumoral CD40L blockade reduced the efficacy of anti-neu therapy. In addition, anti-neu therapy induced the production of IFN-γ, which upregulated the expression of MHC-II in tumor cells.

Although there is an increasing amount of literature supporting an independent role for CD4 cells in tumor clearance (42, 46), the role that these cells play in helping CD8+ T cells is often emphasized. Along these lines, the depletion of CD4+ T cells during anti-neu therapy significantly reduced the antitumor response. However, our data suggest that the role of CD4+ T cells in anti-neu therapy extends beyond helping the CD8+ T cells antitumor response. First, depletion of CD4+ T cells after cessation of anti-neu therapy resulted in tumor relapse. Given our previous study that CD8+ T-cell responses are initiated during anti-neu therapy (15), these data suggest that CD4+ T cells are necessary even after CD8+ T-cell responses are generated. Second, the loss of CD4+ T cells did not significantly alter IFN-γ production by CD8+ T cells. This observation suggested that CD4+ T cells might have a more direct role in anti-neu therapy. Indeed, IFN-γ was necessary for anti-neu therapy and induced the expression of MHC-II in TUBO cells both in vitro. Moreover, anti-neu therapy induced the expression of MHC-II in TUBO cells in vivo. Finally, CD4+ T cells were able to mount an antitumor...
response even in the absence of CD8+ T cells. Although these data do not exclude the possibility that CD4+ T cells are also helping CD8+ T cells in our tumor model, they do suggest that CD4+ T cells are necessary and have a role independent of CD8+ T cells for the antitumor effect of anti-neu therapy.

Inhibiting CD40/CD40L interactions also significantly reduced the efficacy of anti-neu therapy. CD40L blockade could function in 3 possible ways to repress tumor growth: (i) preventing the infiltration of CD8+ T cells into the tumor microenvironment; (ii) inhibiting CD40/CD40L interactions between CD4+ T cells and either dendritic cells or CD8+ T cells within the tumor; or (iii) inhibiting CD40 activation of macrophages. Because CD40L blockade has been reported to inhibit T-cell extravasation (35), reduced T-cell infiltration could account for the reduced efficacy of anti-neu therapy when CD40L is administered intratumorally. In our model, however, CD8+ and CD4+ T-cell infiltration was not inhibited by CD40L blockade, which suggests that CD40L interaction is working by one of the other possible mechanisms.

CD8+ T-cell activation via CD40/CD40L interactions is commonly indirect and involves both dendritic cells and CD4+ T cells, respectively, within the draining lymph node (33, 47, 48). In addition, CD40 expression directly by CD8+ cells has been reported to be important in memory formation (49). Although we found that intratumoral CD40L blockade did not decrease antitumor responses in the spleen, it did significantly reduce the antitumor response in the TIL. Thus, these data argue that (i) intratumoral anti-neu antibody administration does not have significant systemic effects, and (ii) CD40/CD40L interactions within the tumor microenvironment are essential for effective anti-neu therapy. It remains to be determined whether CD40L engagement is acting to enhance antigen presentation or activating macrophages.

A recent study confirmed our observation that CD8+ T cells are necessary for anti-neu therapy, but they also reported that CD4+ T cells are not required (16). This seemingly differing requirement for CD4+ T cells may be due to differences in experimental models. First, our anti-neu treatment regimen uses less total antibody and is seemingly differing requirement for CD4+ T cells and CD40L in enhancing antitumor response within the tumor microenvironment. Moreover, our findings support on-going preclinical and clinical efforts aimed at enhancing the adaptive immune response to neu-positive tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.D. Mortenson, S. Wang, Y.-X. Fu
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