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Effective anti-neu initiated anti-tumor 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 anti-tumor response were examined by tumor growth analysis and 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 on 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 anti-tumor response.

Conclusions—This study reveals essential role of CD4⁺ T cell for anti-neu mediated tumor regression.

Keywords

CD4; HER2; neu; antibody; Herceptin

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 human epidermal growth receptor-2 (HER2/*ErbB2*) is among the most targeted with two molecular targeting agents approved by the FDA (2). HER2 is amplified or overexpressed in 25–30% of breast cancer patients 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 therapeutic 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 demonstrated different mechanisms by which this therapy inhibits tumor growth. First, HER2/neu activation initiates multiple signaling pathways, including the PI3 kinase and MAP kinase 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 cellular cytotoxicity (ADCC) has been demonstrated to be essential for its anti-tumor 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 lab 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 neu-specific memory (15). In a separate study, anti-neu therapy was shown to require CD8⁺ T cells and interferons, but not CD4⁺ T cells, perforin, or FasL (16). Taken together, these results challenged the current notion that antibody-dependent cell-mediated cytotoxicity (ADCC) is the main Fc-mediated mechanism for anti-neu therapy.

CD4⁺ T cells play a major role in orchestrating the adaptive immune response to infection by aiding antibody production by B cells, enhancing and maintaining CD8⁺ T cells responses, and regulating macrophage function (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⁺CD25⁺ regulatory T cells in neu-positive tumor progression, and show that CD4⁺CD25⁺ regulatory T cells mask effector CD8⁺ T cell responses (21,22) and promote metastasis (23) of neu-positive tumors. Here, using a CD4-depleting antibody during anti-neu therapy, we establish an unexpected but necessary role for CD4⁺ T cells in supporting the anti-tumor function of anti-neu antibody therapy.

Materials and Methods

Mice

BALB/c, BALB/c *Rag2*-KO, and *IFN* γ -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. 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. 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 Tg mouse expressing transforming rat *neu* (24), and were a gift from Joseph Lustgarten, Mayo Clinic, Arizona. TUBO was cultured in 5% CO₂, and maintained *in vitro* in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (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. The anti-neu mAb 7.16.4, anti-CD4 depleting antibody GK1.5, and CD40 agonist 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 3–5 minutes in 1× Trypsin EDTA (Mediatech, Inc., Manassas, VA). Cells were washed 2–3 times in 1× PBS and counted by trypan exclusion. TUBO cells ($3\text{--}5 \times 10^5$) were injected s.c. in the back of 6 to 8-week-old anesthetized mice. Tumor volumes were measured along three orthogonal axes (x, y, and z) and calculated as tumor volume = (xyz)/2.

Antibody Treatments

Mice were treated with two or three i.p. injections of 100–200 µg of anti-neu antibody (clone 7.16.4) diluted in 100–200 µL of 1× PBS. For CD4, CD8 and CD20 depletion experiments, 200 µg 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 100–200 µL of 1× PBS was administered i.p. at the indicated time points. Blocking CD40L was achieved by intratumoral administration of 50 µg anti-CD40L (clone MR1) diluted in 30–50 µL of 1× 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

$1\text{--}2 \times 10^6$ cells were added to flow cytometry tubes and washed in PBS with 0.5% BSA. FcR were blocked with 20–50 µL of 2.4G2 and incubated for 15 min at room temperature. Surface proteins were labeled by adding 10 µL of an antibody cocktail in PBS with 0.5% BSA (i.e. αCD3-PE, αCD4-PercP-Cy5.5, αCD8-PE-Cy7, and αCD45-APC-Cy7). Cells were incubated for 30 min at 4°C in the dark. Following incubation, cells were washed and re-suspended in 200–300 µL of PBS with 0.5% BSA and analyzed on a BD FACSCanto cytometer (BD Cytometry Systems).

Measurement of IFNγ secreting cells by ELISPOT assay

Tumor reactive T cells were measured by enzyme-linked immunospot (ELISPOT) assay. Spleen or tumor cells (responder cells) were suspended in RPMI 1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. A total of 4×10^5 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 pre-coated with 2.5 µg/mL rat anti-mouse IFNγ (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 cells to stimulatory cells was 10:1. After a 40–46 h incubation at 37°C, the cells were removed, the wells 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 h at 4°C or for 2 h 37°C, and 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 h 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 3–5 minutes. The AEC solution was discarded, and the plates were washed 6 $\times$ 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 (TIL)

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 3–5 mL of digestion media (RPMI with 1.5 mg/mL collagenase and 0.4 mg/mL DNase) for 30–45 min on a rotator. Cells were then collected and washed in 1 $\times$ PBS and counted by trypan blue exclusion. For flow cytometry analysis, 1–2 $\times 10^6$ 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 standard deviations ($\pm$ SD). All statistical analyses were performed using GraphPad Prism Version 4.0 for Macintosh (GraphPad software, San Diego, CA). Unless specified, statistically significant differences of $P < 0.05$, $P < 0.01$, and $P < 0.001$ are noted with *, **, ***, 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 BALB/c mice, these cells establish tumors histologically similar to autochthonous tumors in BALB-neuT females (26). Whereas 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 (Supplemental Fig 1). To determine if CD4 $^{+}$ T cells were required for the tumor-suppressing function of anti-neu therapy, a CD4-depleting antibody was co-administered with anti-neu systemically by intraperitoneal (i.p.) 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 (Supplemental Fig 2). Loss of CD4 $^{+}$ T cells significantly reduced the anti-tumor 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, this data suggests 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 performed 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 significant increase in the anti-tumor IFN γ response in wild-type 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 wild-type 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 compared to mIgG treated controls, no tumor regression was observed, and is most likely due to the direct anti-tumor 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-neu antibody production after anti-neu therapy by examining whether B cells were necessary for the anti-tumor effect. Because plasma cells down-regulate CD20 from the plasma membrane (28), a CD20-depleting antibody regimen was initiated at the time of tumor inoculation to prevent the formation of antibody-producing cells primed during tumor inoculation. While CD19⁺ cells were absent from circulating blood after administration of the CD20 antibody (data not shown), loss of CD20⁺ cells during anti-neu therapy did not affect the ability of anti-neu therapy to suppress tumor growth (Supplemental Fig 3), indicating that B cells are not necessary for anti-neu therapy. Furthermore, these data suggest that CD4⁺ T cells are necessary for the anti-tumor effect of anti-neu therapy in a way that is independent of providing B cell help for endogenous anti-neu antibody production.

The anti-neu induced IFN γ production is CD4 $^{+}$ T cell dependent

Given that our data thus far indicated that effective anti-neu therapy requires CD4 $^{+}$ T cells and IFN γ , but not B cells, and previously published data demonstrated the necessity of CD8 $^{+}$ T cells (15,16), we hypothesized that CD4 $^{+}$ T cells were required to help CD8 $^{+}$ T cell responses. Using an ELISPOT assay, we first examined the role CD4 $^{+}$ T cells play in the anti-tumor CD8 $^{+}$ T cell response. CD4 $^{+}$ T cell depletion significantly reduced IFN γ production by splenocytes (Fig 3a). To examine if CD4 depletion also reduced the IFN γ response within the tumor microenvironment, TIL were isolated and analyzed by an IFN γ ELISPOT assay. After CD4 depletion, IFN γ production by the TIL was also significantly reduced (Fig 3b). These data suggest that the anti-tumor response initiated by anti-neu therapy is dependent on CD4 $^{+}$ T cells.

It has been previously reported 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 anti-tumor 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 anti-tumor 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 CD4 $^{+}$ 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*, even when treated with anti-neu antibody; however, addition of exogenous IFN γ induced the expression of MHC-II on 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 on 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 mIgG treatment, but increased to 594.3 ± 183.5 after anti-neu therapy. The expression of MHC-II on 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 anti-tumor response of CD4 $^{+}$ T cells was evaluated by ELISPOT. In the absence of CD8 $^{+}$ T cells, CD4 $^{+}$ T cells were still able to induce an anti-tumor response (Fig 4c). Moreover, this response was repeated using neu-Tg recipient that are tolerized to the neu antigen. T cells from the spleens of neu-Tg mice displayed a stronger anti-tumor response to TUBO after anti-neu treatment as determined by IFN γ ELISPOT (IFN γ^{+} cells/ 4×10^5 : 97 ± 23 in control Ig treated group compared to 590 ± 48 in the anti-neu treated group). Collectively these data suggest that IFN γ within the tumor microenvironment induced by anti-neu therapy up-regulates 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 anti-tumor 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 anti-tumor 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 anti-tumor 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 on 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 on 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). Since 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⁺ or CD4⁺ 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 non-tumor cells within the tumor microenvironment is essential for anti-neu therapy.

We next wanted to examine whether and how CD40L blockade affected the anti-tumor response. To this end, tumor-bearing mice were treated with anti-neu antibody with or without intratumoral injection of a CD40L blocking antibody; splenocytes and TIL were then harvested and analyzed by IFN γ ELISPOT as described above. While CD40L blockade did not significantly reduce the anti-tumor 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 anti-tumor 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 anti-tumor 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 anti-tumor CD4⁺ T cell response initiated by this therapy outweighs suppression by regulatory T cells.

In terms of anti-neu therapy, three mechanisms may explain the role of CD4⁺ T cells in tumor regression: (1) after anti-neu therapy, these cells may provide help for endogenous antibody production against neu-positive tumors or enhance B cell mediated tumor control;

(2) they could exhibit a direct effector function against the tumor; (3) they could be required to prime and/or maintain effector CD8⁺ T cells. In this study, we define an essential role for CD4⁺ T cells whereby the production of IFN γ allows these cells directly engage the tumor via MHC-II during anti-neu mediated tumor regression. In addition, we propose that expression of CD40L is essential within the tumor microenvironment for effective anti-neu therapy, and that blockade of this surface protein significantly reduces the anti-tumor response.

Mechanistic studies of anti-neu therapy have been performed in both transplant and autochthonous tumor models, and each has provided complementary data. Transgenic mice expressing mouse and 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 10–15 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 wild-type 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 anti-tumor 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).

Murine and rat neu display a 95% homology at the amino acid levels respectively (26,38). The homologues share peptide motifs that can be presented on mouse H-2d to elicit CTL responses in humans (26,27,38). Moreover, when these tumors are transplanted into wild-type 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 above, 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 anti-proliferative impacts on tumor growth, these data suggest that endogenous antibodies are not the primary anti-tumor 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). Though 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 anti-tumor 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 demonstrated, that CD4⁺ T cell depletion significantly inhibited the effects of anti-neu therapy. Moreover, CD4⁺ T cell depletion during anti-neu therapy reduced the anti-tumor 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 on tumor cells.

Although there is an increasing amount of literature supporting an independent role for CD4 cells in tumor clearance (42,46), the role 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 anti-tumor response. However, our data suggests that the role of CD4⁺ T cells in anti-neu therapy extends beyond helping the CD8⁺ T cells anti-tumor 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 on TUBO cells both *in vitro*. Moreover, anti-neu therapy induced the expression of MHC-II on TUBO cells *in vivo*. Finally, CD4⁺ T cells were able mount an anti-tumor 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 from CD8⁺ T cells, for the anti-tumor effect of anti-neu therapy.

Inhibiting CD40/CD40L interactions also significantly reduced the efficacy of anti-neu therapy. CD40L blockade could function in three possible ways to repress tumor growth: (1) preventing the infiltration of CD8⁺ T cells into the tumor microenvironment, (2) inhibiting CD40/CD40L interactions between CD4⁺ T cells and either dendritic cells or CD8⁺ T cells within the tumor, or (3) inhibiting CD40 activation of macrophages. Since 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). Additionally, 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 anti-tumor responses in the spleen, it did significantly reduce the anti-tumor response in the TIL. Thus, these data argue that (1) intratumoral anti-neu antibody administration does not have significant systemic effects, and (2) 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 utilizes less total antibody and is given over a shorter time period. Because anti-neu therapy does display limited tumor growth inhibition in *Rag*-KO mice (15), as well as in xenograph models in nude mice (12), the lower antibody dose

and shorter treatment duration in our study may limit the direct effect of anti-neu therapy on tumor cells, and instead allow the effects of anti-neu therapy on the adaptive immune system to be more pronounced. Second, our tumor model demonstrates a significant increase in the number of CD4⁺ TILs after anti-neu therapy. Using adoptive transfer experiments, Stagg *et al.* demonstrated that transfer of both CD4⁺ and CD8⁺ T cells was more effective than CD8⁺ cells alone. This result suggests that CD4⁺ T cells are involved, and may have a bystander effect on CD8⁺ mediated anti-tumor responses. This is consistent with data presented here as well as elsewhere in the literature, including a study showing that the absence of CD4⁺ T cells diminishes the efficacy of anti-human CD20 antibody therapy targeting hCD20-transfected cells (50).

In conclusion, this study extends the understanding of the cellular mechanisms involved in adaptive immune response initiated after anti-neu therapy. We define a contributing role for CD4⁺ T cells and CD40L in enhancing anti-tumor 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

Inhibition of tumor growth after FDA approved anti-HER2/neu therapy has been thought to occur 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. This study describes the essential role of CD4⁺ T cells for antibody-mediated tumor regression and mechanisms within the tumor microenvironment, and supports on-going preclinical and clinical efforts aimed at enhancing the adaptive immune response to fight neu-positive tumors and reduce metastatic disease.

Figure 1. Effective anti-neu therapy requires CD4⁺ T cells

A) Tumor bearing mice were treated with a single dose of mIg or anti-neu (7.16.4, 200 μ g); 3–5 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 percent of CD4⁺ T cells within the CD45⁺ population, or the ratio of CD4⁺ T cells to CD45⁺ cells. Statistical analysis was performed using an unpaired t-test.

B) WT BALB/c mice (n=5/group) were inoculated s.c. with 4×10^5 TUBO cells and treated i.p. with 150 μ g of anti-neu (clone 7.16.4) or control (mIgG) antibody on day 11 and 18 (black triangles). 200 μ g of a CD4-depleting antibody (clone GK1.5) or control antibody (rIgG) was administered i.p. on day 10 and 15 (grey triangles). Statistical analysis was performed using a repeated-measurement Two-Way ANOVA on time points after anti-neu therapy. Data are representative of three independent experiments.

C) WT BALB/c were inoculated as in B, and treated i.p. with 150 μ g of anti-neu (clone 7.16.4) or control (mIgG) antibody on day 10 and 17 (black triangles). 200 μ g of a CD4-depleting antibody (clone GK1.5), CD8-depleting (clone 53.6.7) or control antibody (rIgG) was administered i.p. on day 10 and 17 (grey triangles). Statistical analysis was performed using a repeated-measurement Two-Way ANOVA on time points after anti-neu therapy. Data are representative of three independent experiments.

D) WT BALB/c mice were inoculated as in B, and treated with mIg or anti-neu on day 10 and 17 (black triangles). The CD4-depleting antibody (GK1.5, 200 μ g) was administered on days 0, 5, 10, and 15 (pre; hashed triangles); 10 and 15 (during; grey triangles); or day 20 and 24 (late; white triangles). Statistical analysis was performed 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 three independent experiments.

Figure 2. IFN γ is necessary for anti-neu therapy

A) WT and *Rag2*-KO BALB/c mice (n=3/group) were inoculated s.c. with 4×10^5 TUBO cells and treated i.p. with 150 μ g anti-neu (clone 7.16.4) or control (mIgG) antibody on day 15 and 20. Ten days later, spleen cells were isolated and used in an ELISPOT assay. Statistical analysis was performed using an unpaired t-test. B) WT and *IFN γ* -KO BALB/c (n=5/group) mice were inoculated s.c. with 4×10^5 TUBO cells and treated i.p. with 150 μ g of anti-neu (clone 7.16.4) or control (mIgG) antibody on day 10 and 17 (black triangles). Tumor growth was then monitored. Statistical analysis was performed using a repeated-measurement Two-Way ANOVA on time points after anti-neu therapy. Data are combined from two of three independent experiments.

Figure 3. The anti-neu induced IFN γ production is CD4⁺ T cell dependent

WT BALB/c mice (n=3–4/group) were inoculated s.c with 4×10^5 TUBO cells and treated i.p. with 200 μ g anti-neu (clone 7.16.4) on day 14 with or without prior i.p. administration of 200 μ g of a CD4-depleting antibody (clone GK1.5) on day 13. On day 19, cells were isolated from the spleen (A) for each mouse and tumors from each treatment group combined (B), and used in an ELISPOT assay. Statistical analysis was performed using an unpaired t-test. Data are representative of three independent experiments. C) WT BALB/c mice (n=3/group) were inoculated and treated as in A. 5 days after anti-neu therapy, CD8⁺ cells were isolated from the spleen for each mouse and used in an ELISPOT assay. Statistical analysis was performed using an unpaired t-test. Data are representative of three independent experiments.

Figure 4. IFN γ induces MHC-II expression for CD4⁺ T cell recognition of TUBO cells

A) 4×10^5 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-h incubation, cells were collected and labeled with a PE-conjugated anti-IA/IE antibody (clone M5/114.15.2) then analyzed by flow cytometry. B) WT BALB/c mice (n=3/group) were inoculated s.c with 4×10^5 TUBO cells and treated i.p. with 200 μ g 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^{hi}), CD45⁻ cells. Data are representative of two independent experiments. C) WT BALB/c mice (n=3/group) were inoculated s.c with 4×10^5 TUBO cells and treated i.p. with 200 μ g anti-neu (clone 7.16.4) on day 14 with or without prior i.p. administration of 200 μ g 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 performed using an unpaired t-test. Data are representative of three independent experiments.

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 s.c. with 4×10^5 TUBO cells and treated i.p. 150 μ g anti-neu (clone 7.16.4) or control (mlgG) antibody on day 10 and 17 (black triangles) with or without i.p. administration of a CD4-depleting antibody (clone GK1.5) i.p. (200 μ g) or i.t. (50 μ g) on day 10 and 15 (grey triangles). Statistical analysis was performed 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 i.t. (**) or i.p. (*) were compared. Data are representative of three independent experiments. B) WT BALB/c mice were inoculated s.c. with 4×10^5 TUBO cells and treated i.p. with 150 μ g anti-neu (clone 7.16.4) or control (mlgG) antibody on day 10 and 17 (black triangles) with or without intratumoral injection of 50 μ g of a CD40L blocking antibody (clone MR1) on days 10, 12, 14, and 17 (grey triangles). Statistical analysis was performed using a repeated-measurement Two-Way ANOVA on time points after anti-neu therapy. Data are combined from three independent experiments. C) WT BALB/c mice (n=3–4/group) were inoculated s.c. with 4×10^5 TUBO cells and treated i.p. with 150 μ g anti-neu (clone 7.16.4) antibody on day 10 and 15 with or without intratumoral injection of 50 μ g of a CD40L blocking antibody (clone MR1) on day 10, 12, and 14. 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 performed using an unpaired t-test. Data are combined from three independent experiments. D) 4×10^5 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 antibody. After a 24-h 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 PMA (20 ng/mL) and ionomycin (500ng/mL) were used as a positive control for CD40L expression. Data are representative of two independent experiments.

Figure 6. CD40L expression within the tumor microenvironment is necessary for effective CD8⁺ T cell anti-tumor responses

A,B) WT BALB/c (n=3–4/group) mice were inoculated s.c. with 4×10^5 TUBO cells and treated i.p. with 250 μ g anti-neu (clone 7.16.4) on day 14 with or without intratumoral injection of 50 μ g of a CD40L blocking antibody (clone MR1) on day 13 and 15. On day 20, cells were isolated from the spleen (A) for each mouse and tumors from each treatment group combined (B), and used in an ELISPOT assay. Statistical analysis was performed for using an unpaired t-test. Data are representative of three independent experiments.