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Clinical Cancer Research

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Cancer Therapy: Preclinical

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

Eric D. Mortenson[1,2], SaeGwang Park[3], Zhujun Jiang[1], Shengdian Wang[1], and Yang-Xin Fu[1,2]

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 cell for anti-neu–mediated tumor regression. *Clin Cancer Res; 19(6); 1476–86. ©2013 AACR.*

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/E*rbB2* 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 neu-specific memory (15). In a separate study, anti-neu therapy

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Translational Relevance

Inhibition of tumor growth after U.S. Food and Drug Administration (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.

was shown to require CD8$^+$ T cells and IFNs, but not CD4$^+$ T cells, perforin, or FasL (16). Taken together, these results challenged the current notion that 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-cell 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 antitumor 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 F_1 neu transgenic (Tg) mice (FVB/N MMTV-neu × 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 μg/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× Trypsin EDTA (Mediatech, Inc.). Cells were washed 2 to 3 times in 1× PBS and counted by Trypan exclusion. TUBO cells ($3–5 \times 10^5$) were injected subcutaneously in the back of 6- to 8-week-old anesthetized mice. Tumor volumes were measured along 3 orthogonal axes (x, y, and z) and calculated as tumor volume $= (xyz)/2$.

Antibody treatments

Mice were treated with 2 or 3 intraperitoneal injections of 100 to 200 μg of anti-neu antibody (clone 7.16.4) diluted in 100 to 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 to 200 μL of 1× PBS was administered intraperitoneally at the indicated time points. Blocking CD40L was achieved by intratumoral administration of 50 μg anti-CD40L (clone MR1) diluted in 30 to 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

A total of 1 to 2×10^6 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 μL of 2.4G2 and incubated for 15 minutes 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 minutes at 4°C in the dark. Following incubation, cells were washed and resuspended in 200 to 300 μL of PBS with 0.5% BSA and analyzed on a BD FACSCanto cytometer (BD Cytometry Systems).

Measurement of IFN-γ–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 μg/mL streptomycin. A total of 4×10^5 spleen cells

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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 cells 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 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 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 in 1× PBS and counted by Trypan blue exclusion. For flow cytometry analysis, 1 to 2 × 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 ±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 *, **, ***, 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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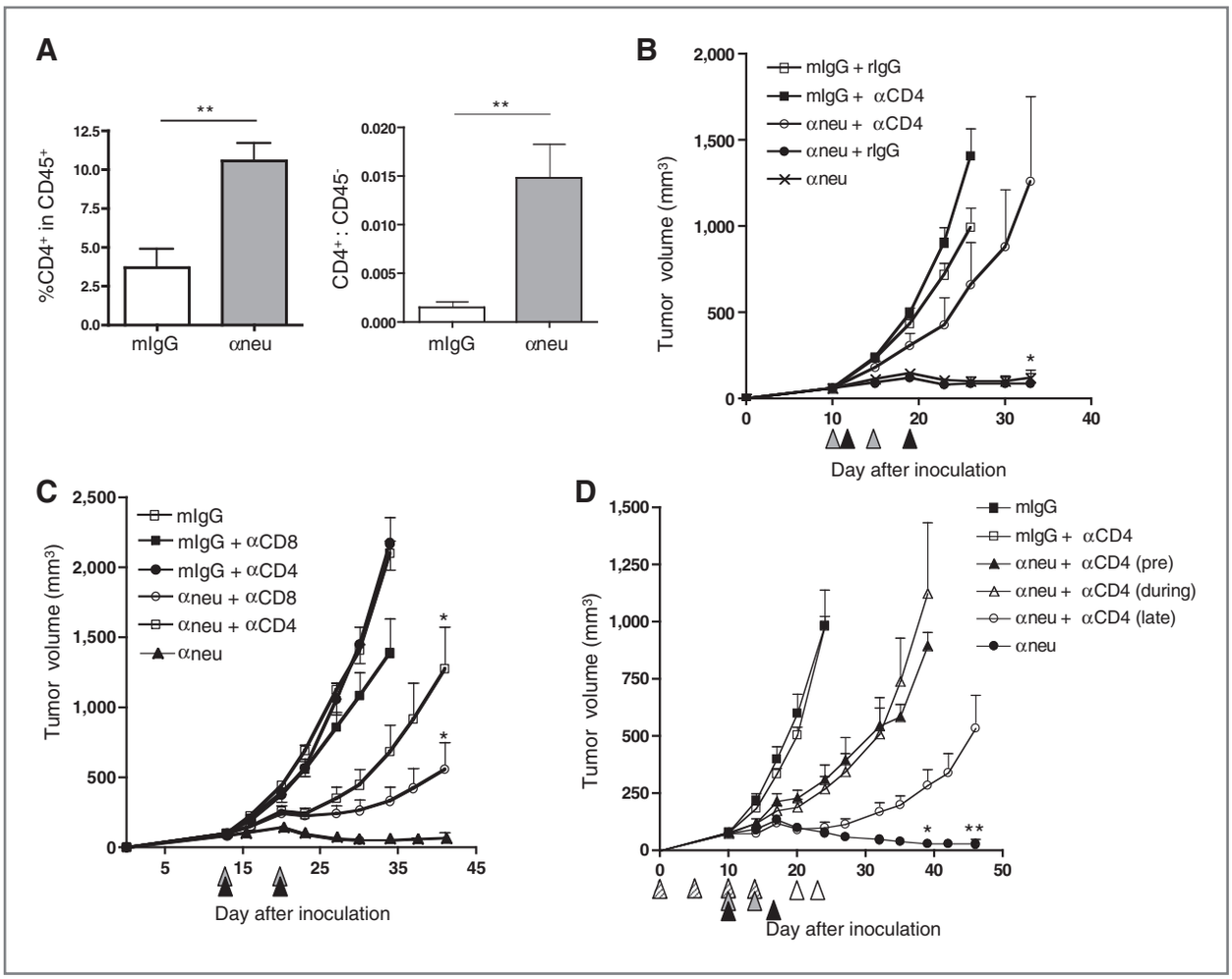

Figure 1. Effective anti-neu therapy requires CD4$^+$ T cells. A, tumor-bearing mice were treated with a single dose of mIg or anti-neu (7.16.4, 200 μg); 3 to 5 days later, tumors were resected and analyzed for the presence of CD4$^+$ T cells via flow cytometry. To normalize for differences in tumor 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 4×10^5 TUBO cells and treated intraperitoneally with 150 μg of anti-neu (clone 7.16.4) or control (mIgG) 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 15 (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 (mIgG) antibody on day 10 and 17 (black 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. Data are representative of 3 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 μg) was administered on days 0, 5, 10, and 15 (pre; hashed triangles); days 10 and 15 (during; gray triangles); or day 20 and 24 (late; white 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 (mIgG)–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-

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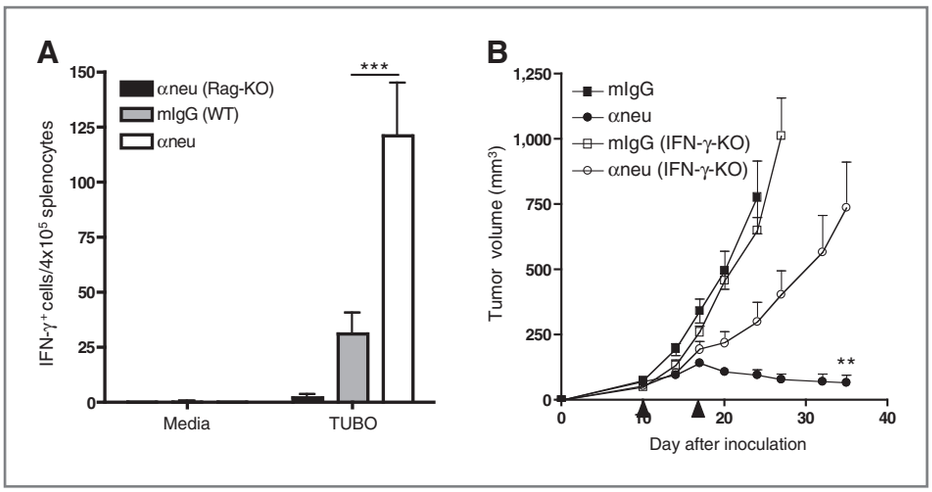

Figure 2. IFN-γ is necessary for anti-neu therapy. A, WT and *Rag2*-KO BALB/c mice ($n = 3$/group) were inoculated subcutaneously with 4×10^5 TUBO cells and treated intraperitoneally with 150 μg anti-neu (clone 7.16.4) or control (mIgG) antibody on day 15 and 20. Ten days later, spleen cells were isolated and used in an ELISPOT assay. Statistical analysis was conducted using an unpaired *t* test. B, WT and IFN-γ-KO BALB/c ($n = 5$/group) mice were inoculated subcutaneously with 4×10^5 TUBO cells and treated intraperitoneally with 150 μg 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 conducted using a repeated-measurement two-way ANOVA on time points after anti-neu therapy. Data are combined from 2 of 3 independent experiments.

neu antibody production after anti-neu therapy by examining whether B cells were necessary for the antitumor effect. Because plasma cells downregulate 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 (Supplementary Fig. S3), indicating that B cells are not necessary for anti-neu therapy. Furthermore, these data suggest that $CD4^+$ T cells are necessary for the antitumor 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 showed 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 antitumor $CD8^+$ 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 antitumor 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 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 $CD4^+$ 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 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 mIgG 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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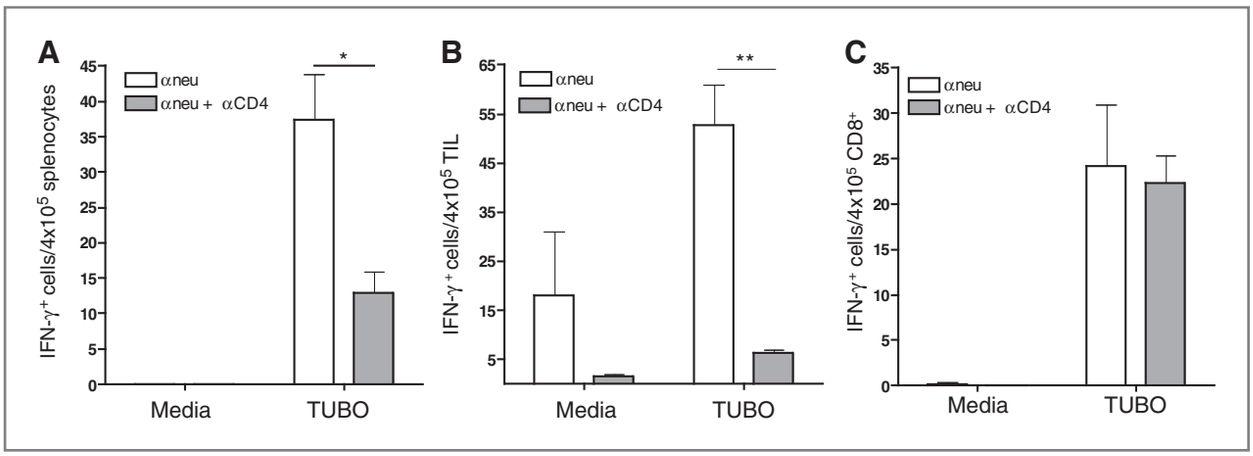

Figure 3. The anti-neu–induced IFN-γ production is CD4+ T-cell dependent. WT BALB/c mice ($n = 3$–4/group) were inoculated subcutaneously with 4×10^5 TUBO cells and treated intraperitoneally with 200 μg anti-neu (clone 7.16.4) on day 14 with or without prior intraperitoneal administration of 200 μg of a 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 conducted using an unpaired *t* test. Data are representative of 3 independent experiments. C, WT BALB/c mice ($n = 3$/group) were inoculated and treated as in (A). Five days after anti-neu therapy, CD8+ 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.

induce an antitumor 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 antitumor 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 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+ 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 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; splenocytes 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

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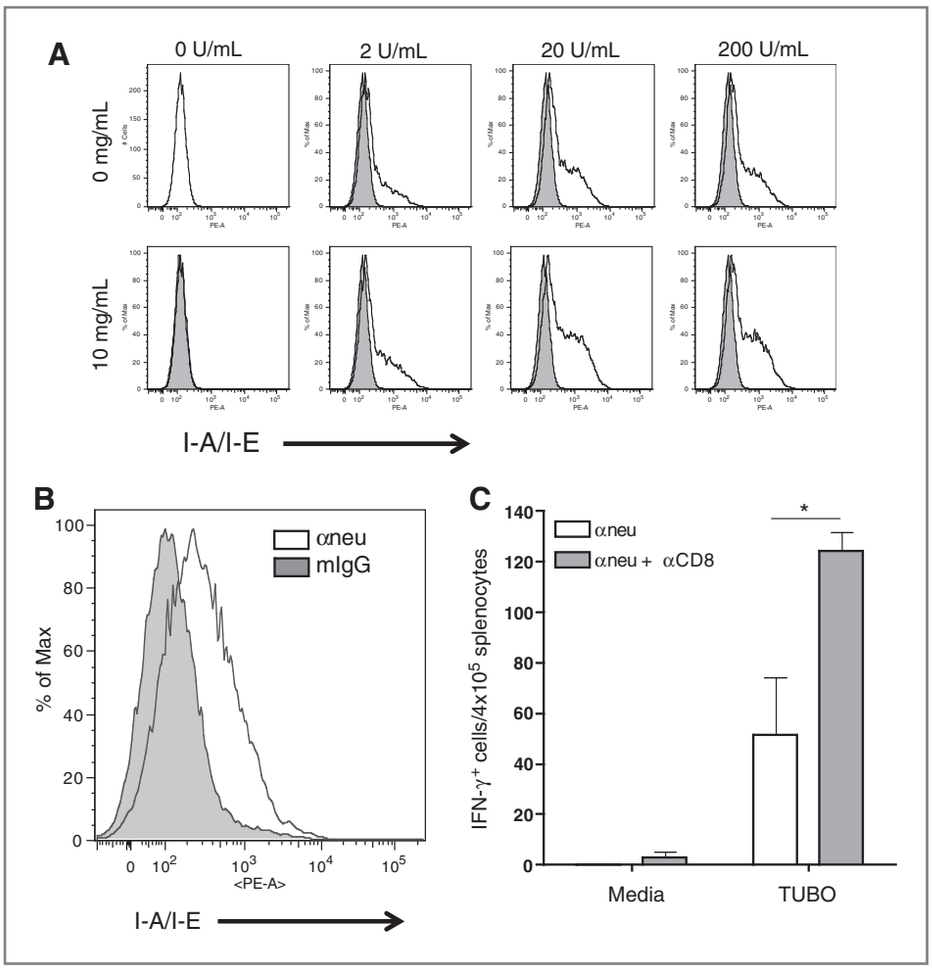

Figure 4. IFN-γ induces MHC-II expression for $CD4^+$ T-cell recognition of TUBO cells. A, 4×10^5 TUBO cells were cultured in 12-well plates alone or in the presence of IFN-γ at the specified concentrations with or without anti-neu. After a 24-hour incubation, cells were collected and labeled with a phycoerythrin (PE)-conjugated anti-IA/IE antibody (clone M5/114.15.2), and then analyzed by flow cytometry. B, WT BALB/c mice ($n = 3$/group) were inoculated subcutaneously with 4×10^5 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^{hi}), $CD45^-$ cells. Data are representative of 2 independent experiments. C, WT BALB/c mice ($n = 3$/group) were inoculated subcutaneously with 4×10^5 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.

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

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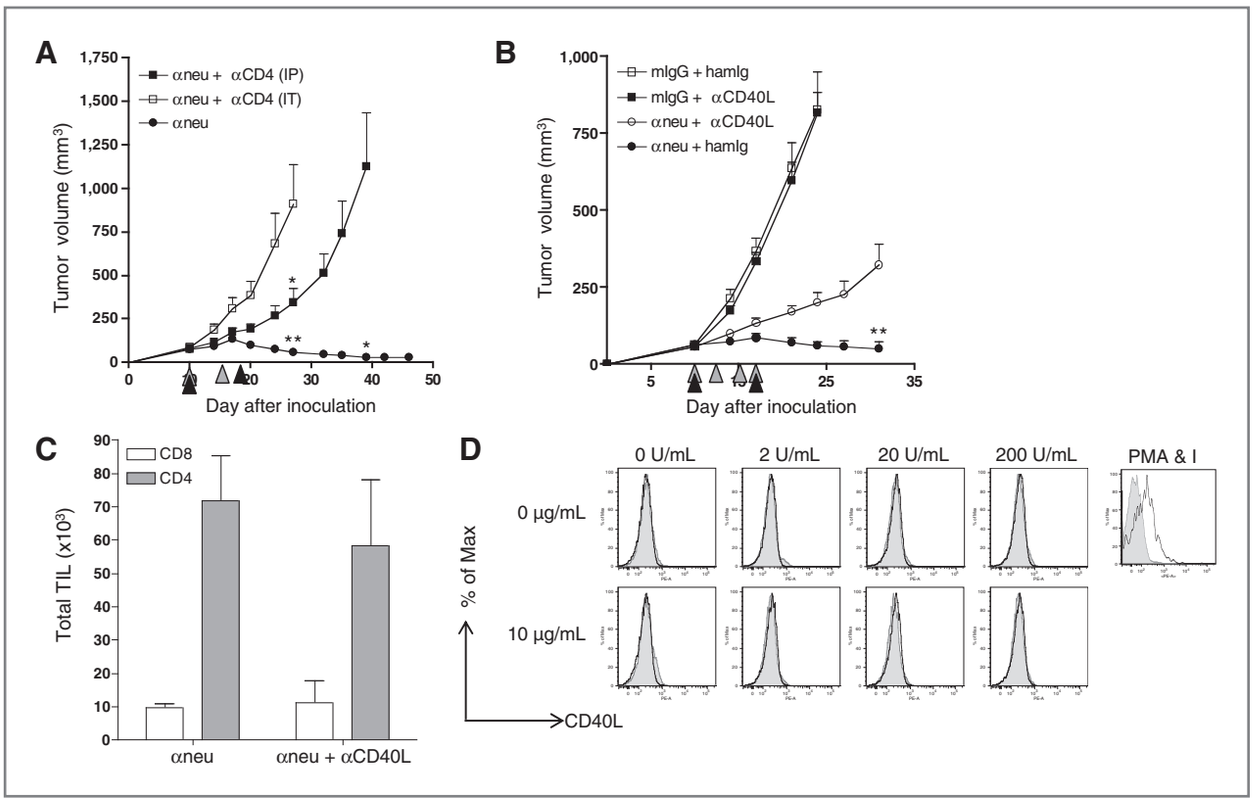

Figure 5. CD40L expression within the tumor microenvironment is necessary for effective anti-neu therapy. A, WT BALB/c mice ($n = 5$/group) were inoculated subcutaneously with 4×10^5 TUBO cells and treated intraperitoneally with 150 μg anti-neu (clone 7.16.4) or control (mIgG) antibody on day 10 and 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 4×10^5 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 4×10^5 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, 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 conducted using an unpaired t test. Data are combined from 3 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-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.

enhance B-cell–mediated tumor control; (ii) they could exhibit a direct effector function against the tumor; (iii) 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 antitumor response.

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 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 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).

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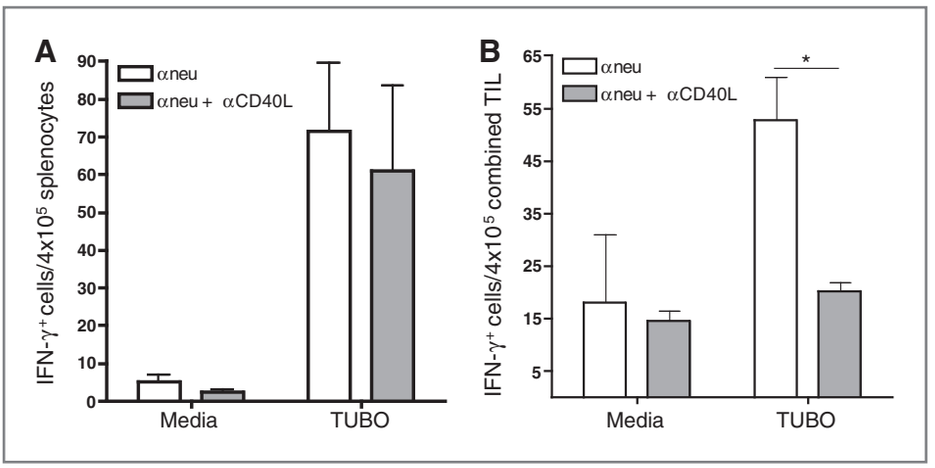

Figure 6. CD40L expression within the tumor microenvironment is necessary for effective $CD8^+$ T-cell antitumor responses. A and B, WT BALB/c ($n = 3$–4/group) mice were inoculated subcutaneously with 4×10^5 TUBO cells and treated intraperitoneally with 250 μg anti-neu (clone 7.16.4) on day 14 with or without intratumoral injection of 50 μg 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 conducted for using an unpaired *t* test. Data are representative of 3 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

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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 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 shows a significant increase in the number of CD4$^+$ TILs after anti-neu therapy. Using adoptive transfer experiments, Stagg and colleagues showed 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 antitumor 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 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.

Authors' Contributions

Conception and design: E.D. Mortenson, Y.-X. Fu
Development of methodology: E.D. Mortenson, S. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.D. Mortenson, S.G. Park, Z. Jiang, S. Wang, Y.-X. Fu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.D. Mortenson, S. Wang, Y.-X. Fu
Writing, review, and/or revision of the manuscript: E.D. Mortenson, S.G. Park, S. Wang, Y.-X. Fu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.D. Mortenson, S. Wang, Y.-X. Fu
Study supervision: S. Wang, Y.-X. Fu

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References

1. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. Nat Biotechnol 2005;23:1147–57.
2. Kruser TJ, Wheeler DL. Mechanisms of resistance to HER family targeting antibodies. Exp Cell Res 2010;316:1083–100.
3. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.
4. Hudis CA. Trastuzumab—mechanism of action and use in clinical practice. N Engl J Med 2007;357:39–51.
5. Gasparini G, Gion M, Mariani L, Papaldo P, Crivellari D, Filippelli G, et al. Randomized phase II trial of weekly paclitaxel alone versus trastuzumab plus weekly paclitaxel as first-line therapy of patients with Her-2 positive advanced breast cancer. Breast Cancer Res Treat 2007;101:355–65.
6. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci U S A 1992;89:4285–9.
7. Nahta R, Esteva FJ. Herceptin: mechanisms of action and resistance. Cancer Lett 2006;232:123–38.

Published OnlineFirst January 30, 2013; DOI: 10.1158/1078-0432.CCR-12-2522

8. Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL. Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res 2002;62:4132–41.
9. Christianson TA, Doherty JK, Lin YJ, Ramsey EE, Holmes R, Keenan EJ, et al. NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer. Cancer Res 1998;58:5123–9.
10. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. Cancer Res 2001;61:4744–9.
11. Liu X, Fridman JS, Wang Q, Caulder E, Yang G, Covington M, et al. Selective inhibition of ADAM metalloproteases blocks HER-2 extracellular domain (ECD) cleavage and potentiates the anti-tumor effects of trastuzumab. Cancer Biol Ther 2006;5:648–56.
12. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumor targets. Nat Med 2000;6:443–6.
13. Varchetta S, Gibelli N, Oliviero B, Nardini E, Gennari R, Gatti G, et al. Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2. Cancer Res 2007;67:11991–9.
14. Carson WE, Parihar R, Lindemann MJ, Personeni N, Dierksheide J, Meropol NJ, et al. Interleukin-2 enhances the natural killer cell response to Herceptin-coated Her2/neu-positive breast cancer cells. Eur J Immunol 2001;31:3016–25.
15. Park S, Jiang Z, Mortenson ED, Deng L, Radkevich-Brown O, Yang X, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. Cancer Cell 2010;18:160–70.
16. Stagg J, Loi S, Divisekera U, Ngiow SF, Duret H, Yagita H, et al. Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. Proc Natl Acad Sci U S A 2011;108:7142–7.
17. Loi S, de Azambuja E, Pugliano L, Sotiriou C, Piccart MJ. HER2-overexpressing breast cancer: time for the cure with less chemotherapy? Curr Opin Oncol 2011;23:547–58.
18. Yang X, Mortenson ED, Fu Y-X. Targeting and utilizing primary tumors as live vaccines: changing strategies. Cell Mol Immunol 2012;9:20–6.
19. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Ann Rev Immunol 2010;28:445–89.
20. Sakaguchi S, Powrie F. Emerging challenges in regulatory T cell function and biology. Science 2007;317:627–9.
21. Ercolini AM, Ladle BH, Manning EA, Pfannenstiel LW, Armstrong TD, Machiels J-PH, et al. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. J Exp Med 2005;201:1591–602.
22. Ambrosino E, Spadaro M, Iezzi M, Curcio C, Forni G, Musiani P, et al. Immunosurveillance of Erbb2 carcinogenesis in transgenic mice is concealed by a dominant regulatory T-cell self-tolerance. Cancer Res 2006;66:7734–40.
23. Tan W, Zhang W, Strasner A, Grivennikov S, Cheng JQ, Hoffman RM, et al. Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling. Nature 2011;470:548–53.
24. Rovero S, Amici A, Carlo ED, Bei R, Nanni P, Quaglino E, et al. DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. J Immunol 2000;165:5133–42.
25. Zhang H, Wang Q, Montone KT, Peavey JE, Drebin JA, Greene MI, et al. Shared antigenic epitopes and pathobiological functions of anti-p185 (her2/neu) monoclonal antibodies. Exp Mol Pathol 1999;67:15–25.
26. Whittington PJ, Piechocki MP, Heng HH, Jacob JB, Jones RF, Back JB, et al. DNA vaccination controls Her-2+ tumors that are refractory to targeted therapies. Cancer Res 2008;68:7502–11.
27. Wei W-Z, Jacob JB, Zielinski JF, Flynn JC, Shim KD, Alsharabi G, et al. Concurrent induction of antitumor immunity and autoimmune thyroiditis in $CD4^+CD25^+$ regulatory T cell-depleted mice. Cancer Res 2005;65:8471–8.
28. Uchida J, Lee Y, Hasegawa M, Liang Y, Bradney A, Oliver JA, et al. Mouse CD20 expression and function. Int Immunol 2004;16:119–29.
29. van Essen D, Kikutani H, Gray D. CD40 ligand-transduced co-stimulation of T cells in the development of helper function. Nature 1995;378:620–3.
30. Grewal IS, Xu J, Flavell RA. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. Nature 1995;378:617–20.
31. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol 1998;16:111–35.
32. Noelle RJ. CD40 and its ligand in host defense. Immunity 1996;4:415–9.
33. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. Nature 1998;393:480–3.
34. Borrow P, Tishon A, Lee S, Xu J, Grewal IS, Oldstone MB, et al. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory $CD8^+$ CTL response. J Exp Med 1996;183:2129–42.
35. Chiodoni C, Iezzi M, Guiducci C, Sangaletti S, Alessandrini I, Ratti C, et al. Triggering CD40 on endothelial cells contributes to tumor growth. J Exp Med 2006;203:2441–50.
36. Park JM, Terabe M, Steel JC, Forni G, Sakai Y, Morris JC, et al. Therapy of advanced established murine breast cancer with a recombinant adenoviral ErbB-2/neu vaccine. Cancer Res 2008;68:1979–87.
37. Montgomery RB, Makary E, Schiffman K, Goodell V, Disis ML. Endogenous anti-HER2 antibodies block HER2 phosphorylation and signaling through extracellular signal-regulated kinase. Cancer Res 2005;65:650–6.
38. Nagata Y, Furugen R, Hiasa A, Ikeda H, Ohta N, Furukawa K, et al. Peptides derived from a wild-type murine proto-oncogene c-erbB-2/HER2/neu can induce CTL and tumor suppression in syngeneic hosts. J Immunol 1997;159:1336–43.
39. Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA. High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. J Clin Oncol 1997;15:3363–7.
40. van Montfoort N, Mangsbo SM, Camps MGM, van Maren WWC, Verhaart IEC, Waisman A, et al. Circulating specific antibodies enhance systemic cross-priming by delivery of complexed antigen to dendritic cells *in vivo*. Eur J Immunol 2012;42:598–606.
41. Rakhra K, Bachireddy P, Zabuawala T, Zeiser R, Xu L, Kopelman A, et al. CD4(+) T cells contribute to the remodeling of the microenvironment required for sustained tumor regression upon oncogene inactivation. Cancer Cell 2010;18:485–98.
42. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive $CD4^+$ T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med 2010;207:637–50.
43. Müller-Hermelink N, Braumüller H, Pichler B, Wieder T, Mailhammer R, Schaak K, et al. TNFR1 signaling and IFN-gamma signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis. Cancer Cell 2008;13:507–18.
44. Qin Z, Blankenstein T. $CD4^+$ T cell–mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity 2000;12:677–86.
45. Mumberg D, Monach PA, Wanderling S, Philip M, Toledano AY, Schreiber RD, et al. CD4(+) T cells eliminate MHC class II-negative cancer cells *in vivo* by indirect effects of IFN-gamma. Proc Natl Acad Sci U S A 1999;96:8633–8.
46. Perez-Diez A, Joncker NT, Choi K, Chan WFN, Anderson CC, Lantz O, et al. CD4 cells can be more efficient at tumor rejection than CD8 cells. Blood 2007;109:5346–54.
47. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 1998;393:478–80.
48. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. Nature 1998;393:474–8.
49. Bourgeois C, Rocha B, Tanchot C. A role for CD40 expression on $CD8^+$ T cells in the generation of $CD8^+$ T cell memory. Science 2002;297:2060–3.
50. Abès R, Gélizé E, Fridman WH, Teillaud J-L. Long-lasting antitumor protection by anti-CD20 antibody through cellular immune response. Blood 2010;116:926–34.