Tolerance to an Endogenous Tumor Antigen


*J Immunol* 2006; 176:974-983; doi: 10.4049/jimmunol.176.2.974

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OX40 Costimulation Synergizes with GM-CSF Whole-Cell Vaccination to Overcome Established CD8+ T Cell Tolerance to an Endogenous Tumor Antigen*1

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T cell costimulation via OX40 is known to increase CD4+ T cell expansion and effector function and enhances the development of T cell memory. OX40 costimulation can also prevent, and even reverse, CD4+ T cell anergy. However, the role of OX40 in CD8+ T cell function is less well defined, particularly in the setting of immune tolerance. To determine the effects of OX40 costimulation on the induction of the host CD8+ T cell repertoire to an endogenous tumor Ag, we examined the fate of CD8+ T cells specific for the immunomodulating rat HER-2/neu epitope, RNEU420–429, in FVB MMTV-neu (neu-N) mice, which express rat HER-2/neu protein in a predominantly mammary-restricted fashion. We show that the RNEU420–429-specific T cell repertoire in neu-N mice expands transiently after vaccination with a neu-targeted GM-CSF-secreting whole-cell vaccine, but quickly declines to an undetectable level. However, OX40 costimulation, when combined with GM-CSF-secreting tumor-targeted vaccination, can break established CD8+ T cell tolerance in vivo by enhancing the expansion, and prolonging the survival and effector function of CD8+ T cells specific for RNEU420–429. Moreover, we demonstrate that OX40 expression is up-regulated on both CD4+ and CD8+ T cells shortly after administration of a GM-CSF expressing vaccine. These studies highlight the increased efficacy of OX40 costimulation when combined with a GM-CSF-secreting vaccine, and define a new role for OX40 costimulation of CD8+ T cells in overcoming tolerance and boosting antitumor immunity. The Journal of Immunology, 2006, 176: 974–983.

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Received for publication August 5, 2005. Accepted for publication November 1, 2005.

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1 This work was supported by Grant BCTR0201973 from the Susan G. Komen Breast Cancer Foundation and Grant RSG-01-080-01-LIB from the American Cancer Society (to R.T.R.). This work is also supported by the Department of Defense Grant W81XWH-04-1-0595 (to E.M.J. and L.A.E.), Grant U19CA72108 from the National Cooperative Drug Discovery Group, Grant P50CA88843 from the Breast Specialized Program of Research Excellence, Grant M030375 from the Avon Foundation (to R.T.R.), and by the National Institutes of Health Grant 1K23CA098409 (to L.A.E.).

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5 Abbreviations used in this paper: Treg, regulatory T cell; NP, nucleoprotein; ICS, intracellular cytokine staining; LN, lymph node.

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with other model Ags, is not subject to the same tolerance-imposed barriers to T cell activation that limit the induction of potent antitumor immunity. Thus, it is not clear at present whether OX40 costimulation can enhance the activation and persistence of a previously tolerized endogenous tumor-reactive CD8\(^+\) T cell repertoire.

In transgenic mice expressing the wild-type rat HER-2/neu (neu) protein (neu-N mice) there exists established immune tolerance to neu (1, 23). Previous reports in this model indicate that OX40 costimulation, when given in conjunction with peptide- or tumor lysate-pulsed dendritic cells, does not generate a sufficient immune response to eliminate tumor (24, 25). However, because data from other groups have suggested a synergistic relationship between systemic GM-CSF administration and OX40 costimulation in the generation of antitumor immunity (14, 16), we sought to explore the effect of combining OX40 costimulation with a neu-targeted GM-CSF-secreting whole cell vaccine in the generation of potent antitumor immunity against the immunodominant CD8\(^+\) T cell epitope associated with tumor rejection in neu-N mice (26).

We report that the combination of GM-CSF whole cell vaccine conjugation with agonist anti-OX40 mAb (anti-OX40) effectively induces a durable neu-specific CD8\(^+\) T cell response despite established immune tolerance to the target Ag. The activated tumor-specific CD8\(^+\) T cells demonstrate potent effector function in vitro and in vivo assays, and eliminate established tumors in neu-N mice. This effect occurs through the GM-CSF-dependent up-regulation of OX40 expression of bulk CD4\(^+\) and CD8\(^+\) T cells shortly after vaccination, and the anti-OX40-dependent persistence of neu-specific CD8\(^+\) T cells specific for the immunodominant RNEU420–429 epitope. These data support OX40 as an important costimulatory molecule for overcoming CD8\(^+\) T cell tolerance to a tumor-encoded immunodominant Ag.

**Materials and Methods**

**Mice**

FVB mice were purchased from Taconic Farms. Neu-N mice (27), generously provided by Dr. W. Muller (McMaster University, Hamilton, Ontario, Canada), were bred to homozygosity, and a breeding colony was maintained by Taconic Farms. All experimental animals were housed under pathogen-free condition at Johns Hopkins University (Baltimore, MD). All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

**Cell lines and media**

NIH-3T3 (American Type Culture Collection), 3T3/neu/GM, 3T3/neu, NT2, and T2D\(^1\) cells have been previously described (23, 26). The 3T3 nucleoprotein (NP)/granulocyte-macrophage (GM) cells were generated by transfection of 3T3/GM cells (23) with a plasmid encoding the lymphocytic choriomeningitis virus NP and were maintained under selection with 150 \(\mu\)g/ml hygromycin (Sigma–Aldrich). GM-CSF production by NIH-3T3 and 3T3/neu cell lines was undetectable, whereas the 3T3/GM and 3T3/neu/GM cell lines produced 180 and 110 ng/10\(^6\) cells/24 h, respectively. Purified GK1.5 (anti-CD4) or isotype control (PK136) Abs were generously provided by Dr. T. C. Wu (The Johns Hopkins University, Baltimore, MD).

**Peptides and Abs**

RNEU420–429 (PDSDRLDLSVF) and NP118–126 (RPQASGVYM) peptides were synthesized using either an Apex 396 or ACT 90 automated peptide synthesizer (AAPPTec) and purified by HPLC (model 1100; Agilent Technologies) to >95% purity by the Oncology Peptide Synthesis Facility (The Johns Hopkins University). The anti-OX40 mAb was purified from the supernatant of OX86 hybridoma (European Collection of Cell Cultures) grown in Protein Free Hybridoma Media II (Invitrogen Life Technologies) and purified over a protein G column (BD Pharmingen). Purified rat IgG reagent (Sigma–Aldrich) was used as an irrelevant IgG Ab. All Abs were dialyzed into PBS and sterile-filtered before administration.

**Flow cytometric (FACS) analysis**

Anti-CD4 CyChrome, anti-CD8 CyChrome, anti-IFN-\(\gamma\) PE, purified rat anti-mouse OX40, and PE-conjugated goat anti-rat IgG were purchased from BD Biosciences. Intracellular cytokine staining (ICS) was performed using the mouse ICS kit obtained from BD Biosciences for murine IFN-\(\gamma\) and was performed as previously described (1). Flow cytometric data were collected using BD FACSCalibur cytometer (BD Biosciences). Data were analyzed using CellQuest (BD Biosciences) and FlowJo software.

**Tumor treatment experiments**

Inoculation with the NT2 neu-expressing mammary tumor cell line, vaccination, and CD4\(^+\) T cell depletion protocols have been previously described (23). FVB mice received NT2 cells at a dose of 5 \(\times\) 10\(^5\) cells. neu-N mice were given NT2 cells at a dose of 2 \(\times\) 10\(^5\) cells. OX40, or control rat IgG, was administered i.p. on days 3 and 7 after vaccination (300 \(\mu\)g per injection).

**In vivo CTL assay**

In vivo cytolytic activity was determined using FVB splenocyte target cells differentially labeled with CFSE. RBC-depleted spleen cells were pulsed with either RNEU420–429 (2.5 \(\mu\)g/ml) or NP118–126 (2.5 \(\mu\)g/ml) for 90 min at 37°C, 5% CO\(_2\). Excess peptide was removed with five washes and cells were resuspended at 1 \(\times 10^7\) cells/ml in PBS/0.1% BSA. RNEU420–429 peptide-pulsed cells were then labeled with 3 \(\mu\)M CFSE (CFSE\(_{3\mu\text{M}}\) cells) in PBS/0.1% BSA. NP118–126 peptide-pulsed cells were labeled with 0.5 \(\mu\)M CFSE (CFSE\(_{0.5\mu\text{M}}\) cells) for 10 min at 37°C. Labeling reactions were terminated by the addition of ice-cold PBS followed by two washes in cold PBS. Recipient neu-N mice were given an i.v. injection containing a mixture of 2.5 \(\times\) 10\(^5\) CFSE\(_{3\mu\text{M}}\) cells and 2.5 \(\times\) 10\(^5\) CFSE\(_{0.5\mu\text{M}}\) cells in 200 \(\mu\)l of HBSS. After 18 h, spleens from recipient mice were harvested, and CFSE\(_{3\mu\text{M}}\) and CFSE\(_{0.5\mu\text{M}}\) cells were quantified by flow cytometry. Values for CFSE\(_{3\mu\text{M}}\) and CFSE\(_{0.5\mu\text{M}}\) cells were first normalized to those harvested from naive neu-N mice. The percentage of decrease in the CFSE\(_{3\mu\text{M}}\) peak was then determined for each animal relative to the mean CFSE\(_{3\mu\text{M}}\) value obtained in the five naive neu-N mice.

**Partial splenectomy procedure**

Mice were anesthetized with 2% isofluorane and an 8-mm incision made in the left flank. After cutting the peritoneum right above the spleen, the spleen was exposed. The spleen was clamped by a medium-sized titanium ligating clip (Weck Closure Systems) at the pancreas side of the spleen. Then, to seal the splenic blood flow, the splenic vessel was clamped at the pancreatic tail by two small titanium ligating clips. To remove 30–40% of the spleen, the splenic vessels and splenic parenchyma were cut down with a small margin from the surgical clips. The peritoneum was sutured with a 4–0 absorbable surgical suture, and skin was stapled with 2 skin clips. The partial spleen was then used for immune assays.

**Statistical analysis**

Unpaired Student’s \(t\) tests were performed using the Statview software program. Kaplan–Meier analyses were used to analyze tumor-free survival, and the log-rank test was used for comparisons. A value of \(p < 0.05\) was considered as statistically significant. Nonlinear regression analyses were performed on peptide titration data to yield the EC\(_{50}\) values.

**Results**

Anti-OX40 mAb induces tumor rejection in vaccinated neu-N mice

Several studies have shown that OX40 costimulation can augment both CD4\(^+\) and CD8\(^+\) T cell antitumor immunity in nontolerance models (14, 17, 19, 28, 29). However, it is not known whether T cell costimulation via OX40 is sufficient to elicit an effective antitumor immune response targeted to an endogenously expressed Ag. To address this issue, we determined the antitumor effect of neu-targeted vaccination given in conjunction with anti-OX40 mAb in nontolerized (FVB) and tolerant (neu-N) mice. The neu-expressing mammary tumors, established at a dose of 5 \(\times\) 10\(^5\) cells given 3 days before vaccination, grew progressively in FVB mice treated with a control vaccine (irradiated 3T3NP/GM cells) (Fig. 1A). However, tumor regression was seen in FVB mice given neutargeted vaccine (irradiated 3T3/neu/GM cells) plus either rat IgG
or anti-OX40. Although not statistically different, tumor regression was more rapid in vaccinated FVB given anti-OX40 relative to those that received rat IgG. In FVB mice, tumor regression was also seen in animals given a control, 3T3NP/GM (Fig. 1A) or 3T3/GM (data not shown) vaccine plus anti-OX40.

As in FVB mice, progressive tumor growth was seen in control vaccinated neu-N mice, even at a 25-fold lower inoculation dose of $2 \times 10^5$ tumor cells given 3 days before vaccine (Fig. 1B). However, the antitumor response to neu-targeted vaccination in neu-N mice was greatly diminished compared with that of FVB mice and mediated only a slight delay in tumor-free survival in neu-N mice given 3T3 neu-GM vaccine plus rat IgG ($p < 0.01$ vs 3T3NP/GM plus rat IgG). Neu-N mice given neu-targeted vaccine and anti-OX40 had a significantly improved tumor-free survival relative to animals given neu-targeted vaccine plus rat IgG or 3T3/GM vaccine plus rat IgG ($p < 0.001$), and 17% of these mice completely eradicated the established tumor burden. These animals remained tumor-free well beyond the 100-day endpoint of the experiment (data not shown). The neu-N mice given anti-OX40 in conjunction with 3T3NP/GM vaccination showed delayed tumor growth similar to that of mice treated with 3T3 neu-GM plus rat IgG ($p = 0.01$ for 3T3NP/GM plus anti-OX40 vs 3T3NP/GM plus rat IgG). However, unlike in FVB mice, the tumor-free survival of neu-N mice treated with 3T3NP/GM plus anti-OX40 did not approach that achieved with the combination of 3T3NP/GM plus anti-OX40 ($p < 0.01$). These data underscore the more stringent requirements necessary for the induction of potent antitumor immunity to an endogenous Ag and suggest that OX40 costimulation breaks tolerance to neu in a proportion of neu-N mice, resulting in the elimination of established neu-expressing tumors.

**GM-CSF production by vaccine cells induces OX40 expression on T cells**

To explore the potential interactions between our GM-CSF-secreting whole cell vaccine and OX40 costimulation, we characterized OX40 expression on splenic and lymph node (LN)-derived CD4$^+$ and CD8$^+$ T cells after vaccination alone. The neu-N mice were vaccinated with untransduced NIH-3T3 cells (unmanipulated NIH-3T3 cells), 3T3/GM cells (GM-CSF-transduced NIH-3T3 cells), 3T3 neu cells (neu-transfected NIH-3T3 cells), or 3T3 neu/GM cells (GM-CSF-transduced, neu-transfected NIH-3T3 cells). T cells were then isolated from the spleen and LN by nylon wool depletion on various days after vaccination. The expression of OX40 on CD4$^+$ and CD8$^+$ T cells after vaccination with CD4$^+$ and CD8$^+$ T cells was more rapid in vaccinated FVB given anti-OX40 relative to those that received rat IgG. In FVB mice, tumor regression was also seen in animals given a control, 3T3NP/GM (Fig. 1A) or 3T3/GM (data not shown) vaccine plus anti-OX40.

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**OX40 costimulation augments the CD8$^+$ T cell response to an endogenous Ag**

To determine the effects of OX40 costimulation on neu-specific CD8$^+$ cells, we first characterized the vaccine-induced activation of CD8$^+$ T cells in the absence of anti-OX40. Wild-type FVB and neu-N mice bearing a neu-expressing tumor burden were given a neu-targeted vaccine (irradiated 3T3NP/GM cells) or control vaccine (irradiated 3T3NP/GM cells). ICS was used to quantify CD8$^+$ T cells producing IFN-γ in response to the immunodominant neu-derived RNU420–429 peptide (26) at specific times after vaccination. The number of CD8$^+$ T cells producing IFN-γ in response to the immunodominant neu-derived RNU420–429 peptide increased slightly in neu-N mice vaccinated with 3T3 neu/GM, reaching peak levels between 5 and 9 days after vaccine (Fig. 3A). However, as evidenced by the large SD in about the day-7 percentage of RNU420–429-specific T cells in 3T3 neu/GM-vaccinated neu-N mice, RNU420–429-specific T cells were detected in only 2 of 10 neu-N mice analyzed. The percentage of RNU420–429-specific T cells then declined to below background levels by day 9 after neu-targeted vaccination. IFN-γ release in...
response to RNEU_{420-429} after day 7 was undetectable in vaccinated neu-N mice, confirming Ag-specific tolerance in this model. Similar results were obtained using non-tumor-bearing neu-N mice (data not shown). In contrast, NP_{118-126}-specific CD8^+ T cells (26) showed robust expansion in neu-N mice in response to NP-targeted vaccination, reached peak levels between days 7 and 9 after vaccine, and gradually contracted to a low but detectable level by day 21 (Fig. 3B). Similarly, in FVB mice, in which both rat neu protein and NP are exogenous immunogenic Ags, RNEU_{420-429}-specific T cells expanded in response to neu-targeted vaccination. RNEU_{420-429}-specific T cell numbers peaked between 7 and 9 days after neu-targeted vaccination, and then contracted to a low but stable population by 21 days after vaccine (Fig. 3C). In FVB mice, NP_{118-126}-specific T cells expanded in response to NP-targeted vaccination with the same dynamics that were observed in neu-N mice (Fig. 3D). The large and sustained T cell response to NP_{118-126} observed in neu-N mice is evidence that there is no global immunosuppression in tumor-bearing neu-N mice. Rather, neu-N mice show clear evidence of RNEU_{420-429}-specific CD8^+ T cell tolerance.

We next determined the effects of OX40 costimulation on the fate of host CD8^+ T cells in response to both exogenous (NP) and endogenous (neu) Ag. In a series of experiments, neu-N mice bearing neu-expressing tumors were given neu-targeted vaccine plus anti-OX40 or control rat IgG. At specific time points, animals were sacrificed and splenic CD8^+ T cells isolated for enumeration by ICS. In total, 4 of 33 neu-N mice (12.1%) treated with 3T3 neu/GM plus rat IgG and sacrificed at day 7 after vaccine had detectable RNEU_{420-429}-specific CD8^+ T cells, defined as a 2-fold or greater response to RNEU_{420-429} relative to the ICS response elicited using NP_{118-126} (irrelevant) peptide-pulsed targets (Fig. 4). The finding that not all neu-N mice mount a detectable RNEU_{420-429}-specific T cell response after neu-targeted vaccination is consistent with the large SD depicted in Fig. 3A for the RNEU_{420-429}-specific T cell response measured at day 7. Again, we were unable to detect RNEU_{420-429}-specific CD8^+ T cells in any neu-N mice given neu-targeted vaccine plus rat IgG at any time point after day 7. A similar frequency of RNEU_{420-429}-specific CD8^+ T cell induction was seen in neu-N mice given neu-targeted vaccine plus anti-OX40 mAb (6 of 38, 15.7%). In contrast to neu-N mice given neu-targeted vaccine plus control IgG, however, RNEU_{420-429}-specific T cells persisted in 15–20% of neu-N mice given neu-targeted vaccine plus anti-OX40. These T cells were detectable for as long as 83 days after treatment with neu-targeted vaccine and anti-OX40. The data indicate that the neu-targeted vaccination induces an RNEU_{420-429}-specific T cell response in ~15–20% of neu-N mice, irrespective of OX40 costimulation, by day 7 after vaccination. However, in neu-N mice in which neu-targeted vaccine elicits an RNEU_{420-429}-specific T cell response, OX40 costimulation prolongs the persistence of functional RNEU_{420-429}-specific CD8^+ T cells beyond day 7, despite the persistent endogenous expression of neu.

OX40 costimulation increases CD8^+ T cell expansion

Having demonstrated that OX40 costimulation enables the persistence of CD8^+ T cells to an endogenous Ag, we sought to determine whether the addition of OX40 costimulation to vaccine also increased CD8^+ T cell expansion. Using data from the animals depicted in Fig. 4, we quantified NP_{118-126} and RNEU_{420-429}-specific T cells by ICS. In neu-N mice treated with 3T3NP/GM plus anti-OX40 mAb, we measured a 2-fold increase in the number of NP_{118-126}-specific T cells between days 7 and 14 after vaccine when compared with neu-N mice treated with 3T3NP/GM vaccine plus rat IgG (p < 0.001 on days 7, 9, and 11, and p = 0.015 on day 14) (Fig. 5A). This difference was maintained throughout the contraction phase (days 12–22) of the immune response. These data demonstrate that OX40 costimulation increases CD8^+ T cell proliferation to an exogenous Ag.

We next characterized the effects of OX40 costimulation on the expansion of CD8^+ T cells specific for the endogenous RNEU_{420-429} Ag in tolerant neu-N mice. For these and subsequent characterizations of RNEU_{420-429}-specific T cell function we have restricted our analysis to only those neu-N mice in which neu-targeted vaccination induced a detectable RNEU_{420-429}-specific T cell response, termed responder mice. On day 7 following neu-targeted vaccine, the percentage of IFN-γ-producing CD8^+ T cells among responder mice was ~2-fold higher in mice treated with anti-OX40 (2.1%) compared with those given rat IgG (1.0%; p <
OX40 directly costimulates CD8+ T cells and abrogates the need for T cell help

We have demonstrated that OX40 costimulation can facilitate the expansion and persistence of RNEU420–429-specific T cells in neu-N mice. However, it is not clear whether this occurs by direct OX40 costimulation of CD8+ T cells, or indirectly through the enhanced Th function of OX40-costimulated CD4+ T cells in vivo. A role for direct OX40 costimulation of clonotypic CD8+ T cells specific for an exogenous Ag has been demonstrated (22). We sought to establish whether the direct costimulation of CD8+ T cells by OX40 similarly augments an endogenous host CD8+ T cell repertoire in the absence of CD4+ T cells. However, because of the low frequency of RNEU420–429-specific T cell activation in neu-N mice, we were unable to address this question in neu-N mice. Rather, we examined the effects of CD4+ T cell depletion on both the RNEU420–429-specific CD8+ T cell response and the overall antitumor effect of OX40 costimulation combined with neu-targeted vaccination in nontolerogenic FVB mice. Consistent with our previous data (23), CD4+ T cell depletion of FVB mice before neu-targeted vaccination plus rat IgG completely abrogated the protective effect of the vaccine; tumors grew progressively in all mice in this treatment group (Fig. 6A). In FVB mice that were mock-depleted, neu-targeted vaccination in conjunction with rat IgG induced complete regression of the 7-day established neu-expressing mammary tumors by day 60 after tumor inoculation. OX40 costimulation of undepleted FVB mice after neu-targeted vaccination significantly enhanced the rate of tumor rejection, resulting in complete tumor regression in all animals within 30 days of tumor inoculation. In FVB mice given neu-targeted vaccine and anti-OX40 in the context of CD4+ T cell depletion, tumors regressed in all animals within 60 days of inoculation. Notably, the rate of tumor regression seen in CD4-depleted mice given vaccine plus anti-OX40 was nearly indistinguishable from that of mock-depleted FVB mice given neu-targeted vaccination plus rat IgG (Fig. 6A), although not as rapid as that seen in mock-depleted mice given vaccine plus anti-OX40. This outcome is supported by intracellular cytokine staining detecting RNEU420–429-specific T cells performed at day 14 after vaccination (Fig. 6B). These data demonstrate that CD4+ T cell depletion inhibited the generation of RNEU420–429-specific T cells in response to neu-targeted vaccination, reducing the frequency of these T cells to near the level of background. Consistent with our earlier findings (Fig. 2), there was a 2-fold increase in the frequency of RNEU420–429-specific T cells in mock-depleted, vaccinated mice given anti-OX40 relative to those that received rat IgG. However, in CD4+ T cell depleted animals, the provision of OX40 costimulation resulted in a 2-fold increase in RNEU420–429-specific T cell frequency relative to vaccinated CD4-depleted mice. These data demonstrate that OX40
costimulation enhances the neu-specific CD8+ T cell response as well as the overall antitumor response in FVB mice even in the absence of CD4+ T cells.

**OX40-costimulated CD8+ T cells induced in neu-N mice are functional**

RNEU420-429-specific T cells expand after neu-targeted vaccination in fewer than 20% of neu-N mice. When provided with OX40 costimulation, RNEU420-429-specific T cells in these responder neu-N mice expand and persist at levels similar to what is observed in nontolerized FVB mice. We next sought to define the functional ability of the RNEU420-429-specific T cell population that persists in responder mice given anti-OX40. First, peptide titration ICS was performed on day 63 after vaccination. Splenic RNEU420-429-specific T cells from vaccinated FVB mice responded maximally to RNEU420-429 peptide concentrations above 1 µg/ml, but responses were measured even at peptide concentrations below 1 ng/ml (Fig. 7A). The peptide concentration that elicited the half-maximal T cell response (EC50) was determined to be 47 ± 12 ng/ml. Surprisingly, the endogenous RNEU420-429-specific T cell repertoire from responder neu-N mice given anti-OX40 showed a response profile that was indistinguishable from that of vaccinated FVB mice (EC50 = 31 ± 14 ng/ml). No detectable response was measured using splenocytes from vaccinated neu-N mice that received rat IgG. FVB mice are known to possess a high avidity and highly lytic RNEU420-429-specific T cell repertoire (1). These data would suggest that the RNEU420-429-specific T cells that persist in neu-N mice after neu-targeted vaccine plus OX40 costimulation exhibit similar function when compared with those obtained in FVB mice, which lack endogenous rat neu expression.

To determine whether the RNEU420-429-specific T cells that persist in responder neu-N mice given anti-OX40 are also lytically competent, we performed an in vivo cytotoxicity analysis in tumor-inoculated neu-N mice treated with neu-targeted vaccine plus anti-OX40 mAb. On day 20 after vaccination, FVB splenocytes were pulsed with either NP118-126 or RNEU420-429 differentially labeled with CFSE, and injected i.v. into recipient mice. After 18 h, RNEU420-429 peptide-specific in vivo cytotoxicity was assessed by flow cytometry. Only minimal lysis of RNEU420-429 peptide-pulsed targets was seen in neu-N mice given vaccine plus rat IgG (Fig. 7B). However, OX40 costimulation combined with neu-targeted vaccination lead to the development of in vivo lysis of RNEU420-429-pulsed targets (p < 0.01). Consistent with the results of the peptide titration studies, the degree of in vivo lysis of RNEU420-429-pulsed targets in OX40-costimulated responder neu-N mice was similar to that of nontolerized FVB mice.

The persistence of an RNEU420-429-specific CD8+ T cell repertoire predicts survival

The tumor-free survival rate seen in neu-N mice treated with neu-targeted vaccine plus anti-OX40 mAbs (16.9%) (Fig. 1) is very similar to the frequency of RNEU420-429-specific T cell persistence among these mice (Fig. 3). Because a similar association between tumor eradication and the persistence of high-avidity RNEU420-429-specific T cells has been reported by our group (1), we sought to determine whether there was a direct and predictive correlation between the activation and persistence of RNEU420-429-specific T cells and the elimination of neu-expressing tumors. This was accomplished using the partial splenectomy technique, which allowed us to measure RNEU420-429-specific T cell responses in individual animals early in the course of the in vivo tumor treatment experiment and to follow these animals for the duration of the experiment. Groups of 24 tumor-bearing, vaccinated neu-N mice were treated with anti-OX40. On day 14 after vaccine (17

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**FIGURE 4.** RNEU420-429-specific T cells persist in OX40-costimulated neu-N mice. Tumor-bearing neu-N mice were vaccinated with 3T3 neu/GM cells on day 0. A total of 300 µg of rat IgG (light gray) or anti-OX40 (dark gray) was administered on days 3 and 7 after vaccination. A minimum of 15 animals were sacrificed at each indicated time point, and IFN-γ production was determined in response to RNEU420-429. Data shown are the average (± SD) of at least three animals per time point and are an additional readout for the data presented in Fig. 4. **A**, neu-N mice received 3T3 NP/GM vaccine plus rat IgG (●) or anti-OX40 (○). IFN-γ production was determined in response to NP118-126. **B**, neu-N mice received 3T3 neu/GM vaccine plus rat IgG (●) or anti-OX40 (○). IFN-γ production was determined in response to RNEU420-429 peptide-pulsed T2Dq cells. Note the discontinuous scale on the x-axis.

**FIGURE 5.** CD8+ T cell expansion to exogenous and endogenous Ags is increased with OX40 costimulation. Tumor-bearing neu-N mice were vaccinated, and splenic CD8+ T cells isolated for ICS at the indicated time points. Data shown are the average (± SD) of at least three animals per time point and are an additional readout for the data presented in Fig. 4. **A**, neu-N mice received 3T3NP/GM vaccine plus rat IgG (●) or anti-OX40 (○). IFN-γ production was determined in response to NP118-126. **B**, neu-N mice received 3T3 neu/GM vaccine plus rat IgG (●) or anti-OX40 (○). IFN-γ production was determined in response to RNEU420-429 peptide-pulsed T2Dq cells. Note the discontinuous scale on the x-axis.
days after tumor inoculation), a partial splenectomy was performed on each animal and splenic CD8$^{+}$ T cells were evaluated for RNEU420–429-specific responses by ICS. After partial splenectomy, the animals recovered and were observed for the development of palpable tumors. In total, 7 of 24 mice had a measurable RNEU420–429-specific T cell response on day 14 after vaccination (Fig. 8A). Only those seven mice that developed a detectable RNEU420–429-specific T cell response, referred to as responder mice, completely rejected established HER-2/neu-expressing tumors (Fig. 8B). In contrast, vaccinated neu-N that received OX40 costimulation but failed to demonstrate persistence of RNEU420–429-specific T cells uniformly developed progressive tumors. At the conclusion of the experiment, ICS for IFN-γ was again performed to assess the ability of RNEU420–429-specific T cells to respond to peptide. Again, functional RNEU420–429-specific T cells were detected only in responder mice (data not shown). These data demonstrate that the OX40-mediated persistence of RNEU420–429-specific T cells is predictive of the rejection of neu-expressing tumors.

**Discussion**

The role of OX40 in the enhancement of CD4$^{+}$ T cell function is well characterized. OX40 costimulation is known to increase CD4$^{+}$ T cell proliferation and effector function (5, 6), enhance the development of the CD4$^{+}$ T cell memory pool (7, 10), and can prevent or reverse CD4$^{+}$ T cell tolerance (11). However, the effects of OX40 costimulation on the CD8$^{+}$ T cell response, particularly in the setting of established immune tolerance, have not been defined. Our results highlight two new findings regarding the role of OX40 costimulation in CD8$^{+}$ T cell antitumor immunity.
after tumor-targeted vaccination. First, we have shown that OX40 costimulation bypasses established tolerance to an endogenous Ag, leading to the activation and persistence of functional tumor-specific CD8+ T cells capable of causing regression of established mammary tumors. Second, we have demonstrated that the administration of GM-CSF-secreting vaccines results in the up-regulation of OX40 expression on host CD4+ and CD8+ T cells, suggesting a mechanism of interaction between GM-CSF and the OX40 T cell signaling pathway.

OX40 costimulation, provided by the systemic administration of anti-OX40 agonist mAb, increased the expansion of CD8+ T cells to exogenous Ags: RNEU420–429 in FVB mice and NP118–126 in neu-N mice. This outcome is consistent with data in other systems demonstrating the enhancement of the CD8+ T cell response to exogenous Ags by OX40 costimulation (20, 21). Similarly, studies have shown that OX40 costimulation, given in the absence of tumor-targeted vaccination, augments antitumor immunity to poorly immunogenic tumors or tumors expressing model Ags (13–16, 18). In tumor-bearing FVB mice, we also observed anti-OX40-mediated enhancement of the antitumor immune response in mice given a control vaccine. These findings suggest that in the absence of tolerance, Ags derived from the tumor itself initiate the antitumor T cell response without requiring Ag-targeted vaccines, and that this response is augmented by OX40 costimulation (17, 28, 29). However, other studies offer a contrasting view; indicating that OX40 costimulation does not play a role in CD8+ T cell expansion and antitumor immunity when an exogenous Ag is targeted (30, 31). It is likely that the discrepancy regarding a role for OX40 in augmenting CD8+ T cell responses to exogenous Ags derives from the strong antigenic response elicited by most exogenous Ags. In such a case, OX40 costimulation may be unnecessary or redundant for maximal T cell activation. The requirement for both neu-targeted vaccination and OX40 costimulation to mediate tumor regression in neu-N mice, relative to mice given control vaccine plus anti-OX40, underscores the more stringent requirements for the generation of effective antitumor immunity in a tolerant host.

Our findings suggest that the effects of OX40 costimulation are much more pronounced in the setting of immune tolerance. The provision of OX40 costimulation in vaccinated neu-N mice resulted in a 2-fold increase in the RNEU420–429-specific T cell population at its peak, relative to mice given only neu-targeted vaccine. This 2-fold increase in RNEU420–429-specific T cell expansion mirrored the anti-OX40-mediated increase in the expansion of T cells to the exogenous NP118–126 Ag. These data indicate that OX40 costimulation, directly or via CD4+ T cell help, provides an equivalent proliferative enhancement to self-reactive CD8+ T cells as well as T cells against an exogenous Ag. However, whereas the proportion of RNEU420–429-specific T cells making IFN-γ decreased to undetectable levels beyond day 7 after neu-targeted vaccine alone, RNEU420–429-specific T cells in neu-N mice given neu-targeted vaccine and anti-OX40 maintained effector function for >80 days after vaccination. Although OX40 costimulation has previously been shown to reverse CD4+ T cell tolerance (11, 12), this is the first report demonstrating that OX40 costimulation, when combined with a GM-CSF-secreting vaccine, can overcome CD8+ T cell tolerance to an endogenous Ag. Characterization of the vaccine-induced neu-specific CD8+ T cell response indicated an expansion of RNEU420–429-specific T cells in only ~15–20% of neu-N mice, irrespective of anti-OX40 administration. In mice given neu-targeted vaccine plus anti-OX40, RNEU420–429-specific T cells persisted in a similar 15–20% of neu-N mice. Thus, only in those mice in which the GM-CSF-secreting, neu-targeted vaccine successfully activates RNEU420–429-specific T cells, OX40 costimulation leads to the persistence of effector function within this T cell population. These data suggest that OX40 costimulation does not influence the outcome of the initial encounter between T cell and RNEU420–429-bearing APC, as RNEU420–429-specific T cell activation occurs with similar frequency after neu-targeted vaccination in both anti-OX40- and rat IgG-treated mice. Rather, OX40 costimulation may be exerting its effect during the early stages of T cell proliferation. It is not clear why all neu-N mice do not respond equivalently to neu-targeted vaccination. We have previously documented a similar effect in neu-N mice treated with immune-modulating doses of cyclophosphamide (1). This may reflect differences in the degree of tolerance to neu due to differential Ag expression levels, differences in the mechanism of peripheral immune tolerance that predominates in...
any given animal, or a low probability that recombination events during T cell development will give rise to an RNEU420-429-specific TCR that can escape central tolerance. We are currently investigating the source of this differential T cell response.

Others have demonstrated that OX40 costimulation of CD4+ T cells can contribute indirectly to enhanced CD8+ T cell function (20, 21, 25). More recently, the absence of OX40 specifically on OT-I CD8+ T cells resulted in decreased OT-I cell proliferation in response to OVA peptide in CFA (22), indicating that direct costimulation of Ag-specific CD8+ T cells by OX40 is important in the response to an exogenous Ag. Similarly, the in vitro expansion of splenic CD8+ T cells in response to anti-CD3 activation was augmented with the addition of anti-OX40 agonist Ab (32), again suggesting that OX40 costimulation can directly contribute to CD8+ T cell function. Using CD4+ T cell depletion we have shown that both the percentage of RNEU420-429-specific T cells and the tumor-free survival of tumor-bearing FVB mice in which CD4+ T cells were depleted before neu-targeted vaccination and OX40 costimulation were indistinguishable from those of vaccinated FVB mice. The fact that CD4+ T cell depletion reduced both the tumor-free survival and the generation of RNEU420-429-specific T cells indicates that CD4+ T cells play a role in the antitumor immune response in FVB mice. Nevertheless, these data demonstrate that anti-OX40 can directly costimulate the expansion and persistence of Ag-specific CD8+ T cells in vivo. Although T cell frequencies prevented a similar characterization of CD4-independent, OX40-mediated persistence of RNEU420-429-specific T cells in neu-N mice, these data support a mechanism of direct costimulation of neu-specific CD8+ T cells in the tolerance setting. It is important to note, however, that these data do not preclude a role for OX40 costimulation of CD4+ T cells in the antitumor immune response in neu-N mice.

OX40 costimulation has been shown to enhance CD4+ T cell expansion (10) and increase the expression of prosurvival factors in CD4+ T cells (8), thus facilitating the generation of a larger memory T cell pool. It is possible that the persistence of RNEU420-429-specific T cells in neu-N mice occurs through a similar up-regulation of bcl-2 and bcl-xL. However, alternative hypotheses implicate OX40 costimulation in the reversal on CD8+ T cell anergy or the prevention of CD8+ T cell suppression by CD4+CD25+ Treg cells. OX40 has been shown to prevent and even reverse CD4+ T cell anergy (11, 12), though the mechanism is unclear at present. Our data may reflect a reversal of established CD8+ T cell anergy similar to that reported for CD8+ T cell costimulation via 4-1BB (33). Alternatively, OX40 has been shown to prevent Treg-mediated suppression of CD4+ T cell proliferation either by directly altering Treg function or by rendering the CD4+CD25+ Treg cells to Treg suppression (34, 35). We recently demonstrated that the elimination of CD4+CD25+ Treg in neu-N mice through low-dose cyclophosphamide permits the activation of RNEU420-429-specific CD8+ T cells in ~20% of neu-N mice (1), suggesting that the OX40-mediated attenuation of CD8+ T cell suppression may play a key role in this case.

Although we cannot comment on the avidity of the RNEU420-429-specific T cell repertoire that persisted in anti-OX40-treated neu-N mice, peptide dilution ICS and in vivo cytotoxicity assays demonstrated that the RNEU420-429-reactive T cell repertoire that persisted in anti-OX40-treated neu-N mice was functional and capable of responding to peptide in a comparable dose range to that of nontolerized FVB T cells. RNEU420-429-specific T cells isolated from neu-N mice given neu-targeted vaccine plus anti-OX40 also demonstrated in vivo cytotoxicity that was equivalent to that achieved in nontolerized FVB mice. Previous studies by our group have linked tumor eradication in neu-N mice given immunomodu-

atory doses of chemotherapy before neu-targeted vaccination with the development of a high-avidity RNEU420-429-specific T cell repertoire. In the present study, the persistence of RNEU420-429-specific T cells in anti-OX40-treated neu-N mice was predictive of tumor rejection, suggesting that RNEU420-429-specific T cells may play a major role in tumor eradication. Notably, the persistence of RNEU420-429-specific T cells occurred despite the continued endogenous expression of neu on normal tissues in neu-N mice. Taken together, our data demonstrate that OX40 costimulation, when given in combination with a neu-targeted GM-CSF-secreting whole cell vaccine, can bypass mechanisms of neu-specific CD8+ T cell tolerance to mediate the elimination of established tumors in ~20% of neu-N mice. Although previous studies in neu-N mice have demonstrated an OX40-dependent delay in tumor growth (24, 25), this is the first report in which OX40 costimulation given in conjunction with tumor-targeted vaccination led to the elimination of an established tumor burden due to the expansion of CD8+ T cells directed at the immunodominant neu epitope.

The likely reason for the increased efficacy in our study relative to the previous work is the choice of a GM-CSF-secreting whole cell vaccine to activate neu-specific T cells. In this study, we show that the administration of GM-CSF-secreting vaccine cells resulted in the expression of OX40 on CD4+ and CD8+ T cells obtained from the spleen and LN. The proportion of CD8+ and CD4+ T cells expressing OX40 peaked between 2 and 5 days after GM-CSF vaccination. In addition to a GM-CSF-dependent increase in the OX40 expression among CD4+ and CD8+ T cell subsets, we also measured an increase in the proportion of OX40-positive T cells in mice given 3T3/neu/GM relative to mice vaccinated with 3T3/GM, suggesting that neu-specific T cells comprise a percentage of the OX40-positive T cells. The increased proportion of OX40-positive T cells likely underlies the synergy between GM-CSF administration and OX40 costimulation that has been reported previously (14, 16). It is not yet clear whether GM-CSF directly induces OX40 expression on T cells, or whether this is an indirect consequence. However, these data support the use of OX40 agonists in conjunction with GM-CSF-producing vaccines. Additional studies are underway to more clearly delineate the association between GM-CSF administration and the up-regulation of OX40 on peripheral T cells.

In conclusion, the data presented demonstrate that costimulation via OX40 leads to increased tumor-specific CD8+ T cell expansion, persistence, and effector function. Data obtained in FVB mice indicate that this can occur via the direct costimulation of CD8+ T cells by OX40 (22, 32), but we cannot rule out the possibility that the OX40-mediated enhancement of CD8+ T cell function also plays a role (20, 21, 31). As a whole, the data indicate that in the setting of established immune tolerance, OX40 costimulation provided in the context of an Ag-specific GM-CSF-secreting vaccine can bypass CD8+ T cell tolerance and mediate the persistence of tumor-reactive CD8+ T cells. These data define a role for OX40 in the costimulation of CD8+ T cells and implicate costimulation of CD8+ T cells via TNFR family members as a means to overcome tolerance and boost the response to antitumor vaccination.

Acknowledgments

We thank Dr. Andrew Weinberg for OX86 mAb used in pilot studies for these experiments, Dr. T. C. Wu for providing the depletion mAbs, Dr. Drew Pardoll and Dr. Jonathan Powell for valuable discussions and critical reading of this manuscript, and Dr. Kiyoshi Yoshimura for technical assistance with the partial splenectomy procedure.
Disclosures

The authors have no financial conflict of interest. This work describes the use of a GM-CSF-secreting tumor vaccine. Although none of the authors have financial interests in the work, the Johns Hopkins University receives milestone payments and has the potential to receive royalties in the future.

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