Nondominant CD8 T Cells Are Active Players in the Vaccine-Induced Antitumor Immune Response

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Abstract

We previously reported that $CD8^+$ T cells are directed predominantly toward the immunodominant Her-2/neu (neu) epitope $RNEU_{420-429}$ in nontolerized FVB/N but not tolerized HER-2/*neu* (*neu*-N) mice. In this study, we screened overlapping peptides of the entire neu protein and identified six new epitopes recognized by vaccine-induced *neu*-N–derived T cells. Evaluation of individual nondominant responses by tetramer staining and IFN-γ secretion demonstrate that this repertoire is peripherally tolerized. To address the role that the complete $CD8^+$ T cell repertoire plays in vaccine-induced antitumor immunity, we created a whole-cell vaccine-expressing *neu* cDNA that has been mutated at the $RNEU_{420-429}$ anchor residue, thereby abrogating activation of immunodominant epitope responses. Studies comparing the mutated and nonmutated vaccines indicate that nondominant $CD8^+$ T cells can induce antitumor immunity when combined with regulatory T cell-depleting agents in both *neu*-N and FVB/N mice. Collectively, these studies demonstrate that the neu-directed T cell repertoire is not intrinsically incapable of eradicating tumors. Rather, they are suppressed by mechanisms of peripheral tolerance. Thus, these studies provide new insights into the function of the complete T cell repertoire directed toward a clinically relevant tumor Ag in tumor-bearing hosts.

The success of cancer immunotherapy will depend on the development of vaccine approaches that specifically activate T cell responses against a panel of tumor-associated Ags (TAAs). Tumor-specific $CD8^+$ T cells, in particular, recognize epitopes that derive from

these Ags when bound to MHC class I molecules on the tumor cell surface. Early research in animal and human studies demonstrated the activation of CD8^{+} T cells against single epitopes contained within the amino acid sequence of one TAA (1–3). More recently, other nondominant CD8^{+} T cell responses have been identified as noted in *Cancer Immunity*'s peptide database. However, addressing the role that the complete CD8^{+} T cell repertoire plays in antitumor immunity is fundamental to the development of cellular-based vaccines and immunotherapy.

Immunodominance refers to the lymphocyte response that is predominantly directed toward one or a few epitopes in a protein rather than all of the potential determinants contained within that protein. Factors that lead to the development of immunodominance include Ag availability and processing, TAP specificity, peptide binding affinity to MHC class I, TCR affinity/avidity, the available precursor CD8^{+} T cell repertoire, and immunodomination (T cell–T cell competition) (4, 5). These factors ultimately generate CD8^{+} T cell responses that are larger and more robust than responses to other epitopes when evaluated by TCR avidity, effector function, and population size. The immunodominance of antigenic epitopes has been studied best in infectious disease models. Although immunodominant high-avidity CD8^{+} T cell responses have also been shown to be important in some cancer models (6–8), other models suggest that only low-avidity/nondominant TAA-specific T cells are available for activation in tolerized hosts. Often high-avidity immunodominant epitope-specific responses are tolerized due to expression of the Ag on nontumor tissues (9), whereas low-avidity/nondominant CD8^{+} T cell responses are thought to escape central and peripheral tolerance (10–12). Several reports have demonstrated that activation of low-avidity/nondominant CD8^{+} T cells are capable of expanding and lysing tumors (13–15). However, other studies suggest that there may be additional functional characteristics of the nondominant T cell repertoire that prevent effective T cell activation and function capable of causing tumor regression (6, 16, 17).

We have used the clinically relevant Her-2/*neu* transgenic (*neu*-N) mice to evaluate Her-2/neu (neu)–directed CD8^{+} T cell responses under tolerizing conditions. These mice overexpress the nontransforming rat *neu* cDNA under the mammary-specific mouse mammary tumor virus promoter and develop spontaneous neu-expressing mammary carcinomas (18). We previously reported that neu-targeted vaccination induces a predominantly high-avidity CD8^{+} T cell response to the immunodominant epitope RNEU$_{420–429}$, which is capable of clearing neu-expressing tumors in the parental FVB/N nontolerized mice (8, 19, 20). In contrast, the RNEU$_{420–429}$-specific CD8^{+} T cell response is tolerized in *neu*-N mice, which leads to the prevalence of a polyclonal but less functional T cell repertoire that is specific for other neu-derived epitopes (6, 19, 21). Interestingly, immune-modulating doses of cyclophosphamide (Cy) administered prior to vaccination to deplete T regulatory cells (Tregs) induces high-avidity RNEU$_{420–429}$-specific CD8^{+} T cells that cause tumor regression in ~20% of *neu*-N mice (6).

In this report, we screened an overlapping 15-aa peptide library spanning the complete neu protein and identified six new epitopes that are recognized by both the FVB/N and *neu*-N CD8^{+} T cell repertoires. We now establish that T cells responses against this expanded panel of neu epitopes are indeed subdominant to RNEU$_{420–429}$ in the nontolerized FVB/N mice. However, T cell responses against these epitopes in the tolerant *neu*-N mice form a new dominance hierarchy based on effector function. Additionally, we demonstrate that functional dominance does not always correspond with relative TCR avidity. Furthermore, we demonstrate that vaccination with a modified neu-targeted vaccine lacking expression of the RNEU$_{420–429}$ immunodominant epitope allows for enhanced function of the nondominant CD8^{+} T cell repertoire and tumor recognition.

Materials and Methods

Cell lines and medium

The NT2.5 tumor cell line was originally derived from naturally arising spontaneous tumor from *neu*-N mice. These cells were created and cultured as previously described (16). The 3T3neuGM vaccine and 3T3GM mock vaccine cells lines were generated and cultured as previously reported (16). The cell lines used as T cell targets, that is, T2-D^q, NIH-3T3, and NT2.5B7-1, were also produced and maintained as previously described (21, 22).

Mice, tumor, and immunization protocols

Mice 7–12 wk of age were used in all experiments in accordance with protocols approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine. FVB/N mice were purchased from Harlan Laboratories, The Jackson Laboratory, and Taconic. *Neu*-N mice (18) were originally provided by William Muller and The Jackson Laboratory and were bred and housed at The Johns Hopkins University. These mice were bred to homozygosity as verified by Southern blot analysis (16).

In all experiments, mice were treated with 100 mg/kg Cy (Bristol-Myers Squibb) and 50 μg PC61 (American Type Culture Collection) per mouse 1 d prior to vaccination. Cy and PC61 were diluted in PBS and individually administered i.p. in 500 μl doses. Mice were vaccinated with either a total of 3×10^6 irradiated (5000 rad from a [^{137}Cs] source) 3T3neuGM or 3T3GM cells or 6×10^6 3T3neumut vaccine cells with 3×10^6 3T3GM cells. All vaccine cells were distributed into three 100 μl s.c. injections and admininstered in both arms and one leg to include immunization of three different lymph node regions as previously described (23). Tumor treatment experiments were conducted by injecting *neu*-N mice with 5×10^4 and FVB/N mice with 2×10^6 NT2.5 cells in a 100 μl volume in the mammary fat pad 3 d prior to vaccination.

Peptides

All peptides were synthesized (>95% purity) by either the Peptide Synthesis Facility or the Biosynthesis and Sequencing Facility at The Johns Hopkins University. Peptides were designed as either 15-mer overlapping by nine amino acids or 10-mer overlapping by one amino acid. The nucleoprotein (NP)$_{118–126}$ (RPQASGVYM) peptide is from the lymphocytic choriomeningitis virus nucleoprotein (24) and was used as an irrelevant H2-D^q–binding peptide.

Ex vivo splenic stimulation and CD8$^+$ T cell isolation

Splenocytes and lymphocytes were isolated from either *neu*-N or FVB/N mice 1 wk postvaccination. Spleens and draining lymph nodes were mashed, strained using a 100-μm mesh (BD Falcon), and RBCs were removed using ACK lysis buffer (Quality Biological). Splenocytes were cultured for 1 wk at 37°C and 5% CO$_2$ with either 25 μg/ml peptide pool containing 10 overlapping peptides or 2.5 μg/ml individual peptides in CTL media consisting of RPMI 1640 (Invitrogen), 10% FBS (HyClone), 0.5% L-glutamine (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.1% 2-ME (Invitrogen). One week postpeptide stimulation, CD8$^+$ T cells were separated from dead cell debris using a Ficoll (GE Healthcare) density gradient and isolated using a Dynal mouse CD8 cell negative isolation kit (Invitrogen).

GM-CSF release ELISA

Neu-specific peptides, RNEU$_{420-429}$ and NP$_{118-126}$, were pulsed onto T2-D^q cells at 26°C for 2 h in CTL cell media at a final concentration of 2.5 μg/ml. Pulsed targets (3×10^4) were

then incubated with 3×10^5 isolated CD8$^+$ T cells in 96-well flat bottom plates with a final volume of 150 μl CTL cell media at 37°C and 5% CO$_2$. The supernatant was collected 24 h later and analyzed in a murine GM-CSF ELISA (R&D Systems). Graphed data depict peptide data minus background data (NP$_{118-126}$).

Abs, tetramer, and flow cytometric analysis

The mouse anti–IL-2 Rα-chain (CD25) mAb, PC61, is isolated from the PC61.5.3 hybridoma (American Type Culture Collection) grown in protein-free hybridoma medium II (Invitrogen) supplemented with 0.1% penicillin/streptomycin (Invitrogen) at 37°C and 10% CO$_2$. Expression of neu was analyzed using 7.16.4 (IgG2a), a neu mAb specific for the extracellular domain of neu. 7.16.4 was obtained from the M. Greene hybridoma (University of Pennsylvania, Philadelphia, PA) and purified from ascites prepared by Harlan Bioproducts for Science. The H-2D^q–specific Ab was isolated from the supernatant of the 30.5.7s hybridoma (American Type Culture Collection). Cells stained by either the neu or H-2D^q–specific Abs were labeled with a FITC-conjugated goat anti-mouse IgG (SouthernBiotech).

H-2D^q/neu–specific tetramer was assembled as previously described (25). PE-conjugated tetramer at varying dilutions was incubated with 5×10^5 isolated CD8$^+$ T cells for 15 min at 4°C. CD8$^+$ T cells were further incubated with anti–CD8-allophycocyanin and tetramer for an additional 15 min at 4°C. The percentage of cells that were CD8$^+$tetramer$^+$ was calculated from the total CD8$^+$ population, and the percentage of Ag-specific cells was calculated by subtracting the H-2D^q/NP$_{118-126}$ value from the neu-specific value.

IFN-γ cytokine staining was performed using the BD Biosciences mouse intracellular cytokine stain kit. Isolated T cells were incubated with either NT2.5B7-1 or T2-D^q cells that were pulsed with 2.5 μg/ml peptide at an equal ratio in the presence of GolgiStop. After a 5-h incubation, cells were stained with CD8-CyChrome, fixed and permeabilized, and stained with IFN-γ-PE and CD8-CyChrome or an isotype control. The percentage of CD8$^+$IFN-γ$^+$ was calculated from the total CD8$^+$ population, and the percentage of Ag-specific cells was calculated by subtracting the NP$_{118-126}$ background value from the neu-specific value.

All samples were read on the BD FACSCalibur cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Peptide binding studies

T2-D^q cells are washed twice in RPMI 1640 (Life Technologies) and resuspended at 2×10^5 cells in 250 μl RPMI 1640 supplemented with 3 μg/ml human β$_2$-microglobulin (Sigma-Aldrich) and varying concentrations of peptide (0, 0.1, 1.0, 10, 100 μg). Cells are incubated for 18 h, shaking, at 26°C. Cells are washed with 1× PBS (Life Technologies) and stained for the MHC molecule H-2D^q using the mAb 30.5.7S (American Type Culture Collection). Samples were analyzed based on their mean fluorescence intensity.

Rat Her-2/*neu* mutagenesis and cloning

Rat Her-2/*neu* was mutated using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The mutagenic primers used were designed to generate a leucine to alanine substitution at position four of RNEU$_{420-429}$: forward, 5′-GCCAGACAGTGCCCGTGACCTCAGTGTCTTCCAGAACCTTC-3′ and reverse, 5′-GAAGGTTCTGGAAGACACTGAGGTCACGGGCACTGTCTGGC-3′. These primers were used to amplify the mutated pSV2/*neu*-N plasmid using the following conditions: an initial step of 95°C for 1 min, 18 cycles of 95°C for 50 s, 60°C for 50 s, 68°C for 10 min, and a final step of 68°C for 7 min. Mutated rat *neu* was excised from pSV2 using HindIII,

EcroRI, and SalI (New England Biolabs) restriction enzymes and re-ligated into pcDNA3 expression vector (Invitrogen) using HindIII and XhoI (New England Biolabs).

Transfection and cloning of mutated Her-2/*neu* vaccine sorting

pcDNA3/*neu*mut was linearized by PvuI (New England Biolabs) digestion and purified by phenol-chloroform extraction and ethanol precipitation. NIH-3T3 cells were transfected with linearized pcDNA3/*neu*mut by electroporation ($30 \, \mu g/1 \times 10^7$ cells). 3T3neumut cells were selected and cloned using 700 μg neomycin or G418 (Invitrogen).

Statistics

A Student *t* test was applied to compare statistical significance between peptide-specific responses and treatment groups of tumor-challenged mice, with $p < 0.05$ being significant. All analysis was performed using Excel software (Microsoft Office).

Results

Screening of an overlapping 15-aa peptide library of the entire neu protein identifies six new CD8^{+} T cell epitopes

We previously reported that vaccine-induced neu-directed CD8^{+} T cells are predominantly directed at the immunodominant RNEU$_{420-429}$ epitope in FVB/N mice (18). However, follow-up studies suggested that the *neu*-N CD8^{+} T cell repertoire is more polyclonal, less functional, and mainly specific for other alternate epitopes. To elucidate the differences between the different neu-directed CD8^{+} T cell repertoires that are predominant in tolerant versus nontolerant environments, we first identified the additional epitopes recognized by each T cell population within the *neu*-N CD8^{+} T cell repertoire. To accomplish this, we used immunized lymphocytes from *neu*-N mice treated with a vaccine consisting of 3T3 cells genetically modified to express neu and secrete GM-CSF (3T3neuGM) and immune-modulating doses of Cy to deplete Tregs to screen the entire neu protein for additional CD8^{+} T cell epitopes. *Neu*-N mice treated with this vaccine approach can mount CD8^{+} T cell responses specific for multiple epitopes that are associated with tumor regression ~20% of the time (6). A library containing two hundred nine 15-aa peptides that overlapped by 9 aa was synthesized to cover the entire sequence of neu and divided into 21 pools containing ten 15-aa peptides each for initial screening. Splenocytes were isolated 1 wk following vaccination and stimulated in vitro for 7 d with each of the 21 peptide pools. CD8^{+} T cells stimulated with the individual pools of peptide were subsequently evaluated by ELISA for GM-CSF secretion. Initial studies demonstrated activation of the *neu*-N CD8^{+} T cell repertoire by many peptide pools (Fig. 1*A*). The corresponding 15-aa peptides in positive pools were then tested individually using the same procedure. With this approach, 21 individual 15-aa peptides tested positive in multiple ELISA assays (Fig. 1*B*). Next, 10-aa peptides overlapping by 9 aa corresponding to each positive 15-aa peptide were tested using either a GM-CSF ELISA assay or an IFN-γ intracellular cytokine stain assay. After extensive screening, six 10-aa peptides and one 9-aa peptide were confirmed to be recognized by the *neu*-N CD8^{+} T cell repertoire (Table I). Analysis of the primary structure of the neu protein revealed that five of the identified epitopes are located within the intracellular domain, and one 10-mer and the 9-mer are located within the extracellular domain (Table I). A comparison of the rat neu epitope sequence homology to mouse neu demonstrated that five of the identified epitopes are 100% homologous to mouse neu (Table I). Thus, a full TAA peptide screen is feasible and can identify an extended panel of epitopes recognized by a polyclonal T cell repertoire. These data further support our hypothesis that an Ag-specific T cell repertoire is primarily specific for nondominant CD8^{+} T cell epitopes in a tolerized host.

Nonimmunodominant CD8^{+} T cell epitopes bind H-2D^q

A series of additional analyses were performed on the six 10-mer and one 9-mer epitopes to confirm that these peptides induced neu-specific T cell responses rather than nonspecific activation of CD8^{+} T cells due to peptide cross-reactivity. Validation was carried out by first assessing the ability of each epitope to bind to H-2D^q. With these dilutional peptide binding assays, we observed that the epitopes 24F1 (9-aa), 131E, 134C, 158B, and 202F bind to H-2D^q with an affinity range similar to RNEU$_{420-429}$. In contrast, 24F (10-aa) and 184E epitopes have weaker binding affinities for H-2D^q in comparison with RNEU$_{420-429}$ (Fig. 2). Next, CD8^{+} T cells specific for each peptide were evaluated for IFN-γ production in a peptide titration assay. These functional T cell assays demonstrated that there are differences in the magnitude of CD8^{+} T cell responses to each epitope and identified a new hierarchy of functional dominance among the non–RNEU$_{420-429}$-specific CD8^{+} T cell repertoire. The CD8^{+} T cell responses to 24F1, 131E, and 134C were the most robust based on the percentage of CD8^{+}IFN-γ^{+} detected at nanomolar concentrations of peptide (Fig. 3*A*). These responses were followed by T cell responses specific for the 158B and 184E epitopes. Finally, the weakest CD8^{+} T cell responses were to the 24F and 202F epitopes (Fig. 3*A*). FVB/N CD8^{+} T cell responses specific for each epitope were also evaluated. Interestingly, CD8^{+} T cell responses directed at these non-dominant epitopes were observed, but the magnitude of these responses was 10-fold less than the immunodominant RNEU$_{420-429}$ response (Fig. 3*B*). Thus, these experiments demonstrate the relative binding affinities of the nondominant CD8^{+} T cell epitopes. Importantly, these studies identify a functional hierarchy among the nondominant CD8^{+} T cell repertoire.

The nondominant CD8^{+} T cell responses are less avid than RNEU$_{420-429}$ T cell responses in both FVB/N and neu-N mice

TCR avidity has been shown to be an important measure of the quality of a T cell response (26). Furthermore, we previously reported that Cy given as a Treg-depleting agent with the 3T3neuGM vaccine induces higher avidity T cell responses against the immunodominant RNEU$_{420-429}$ epitope that are associated with tumor regression in *neu*-N mice (6). We therefore used dilutional tetramer staining to compare RNEU$_{420-429}$-specific T cell avidity with the relative TCR avidities of the nondominant CD8^{+} T cell populations specific for the four epitopes against which the most robust responses are induced. We used our functional hierarchy data to limit our analysis to the 24F, 131E, 134C, and 158B 10-mer epitopes and the one 9-mer epitope because these four epitopes represent a range of functional avidity responses among the six 10-mer identified. We developed individual tetramers for each, and for the positive and negative control epitopes, RNEU$_{420-429}$ and NP$_{118-126}$, respectively, were used (for analysis, H-2D^q/NP$_{118-126}$ was subtracted from the percentage CD8^{+}tetramer^{+} as background). These tetramers were used to stain CD8^{+} T cell populations that were directly isolated from tumor-bearing FVB/N and *neu*-N mice treated with the 3T3neuGM vaccine (T cells populations were analyzed straight ex vivo without an in vitro stimulation as previously described by us; see Ref. 6). As expected, the RNEU$_{420-429}$ response was the most avid and prevalent, and it could be detected down to a dilution of 1:1000 (1 μg/ml tetramer protein). Among the nondominant repertoire, the response to the 9-aa and 10-aa peptide 24F1 and 24F were the most avid and prevalent, followed by the response to peptide 134C. CD8^{+} T cell responses to 131E and 158B were too weak to be detected via tetramer staining in FVB/N mice (Fig. 4*A*, 4*B*, Supplemental Fig. 1). The CD8^{+} T cell responses detected in the *neu*-N mice were similar to those observed in FVB/N mice in that the hierarchy of epitope-specific responses based on relative avidities remained the same. Responses directed against the 24F1, 24F, and RNEU$_{420-429}$ epitopes were the most avid, followed by the less avid responses toward the 134C, 158B, and 131E epitopes. However, the *neu*-N responses directed against 24F1, 24F, 134C, and RNEU$_{420-429}$ were not as prevalent or avid in comparison with their counterparts in FVB/N mice. The

exceptions to this observation were the responses to 131E and 158B. Unlike in FVB/N mice, these responses were detected in *neu*-N mice, but remained the least avid of the epitope-specific responses (Fig. 4*C*, 4*D*, Supplemental Fig. 1). Thus, the nondominant CD8^{+} T cell responses are functionally distinct and weaker than the immunodominant CD8^{+} T cell response against $RNEU_{420-429}$. Additionally, both immunodominant and nondominant relative TCR avidities and T cell population sizes are affected by the presence of peripheral tolerance.

Generation of a neu-directed vaccine that does not induce $RNEU_{420-429}$-specific responses allows further functional characterization of the nondominant neu-specific CD8^{+} T cell repertoire

Next, we wanted to address whether the nondominant CD8^{+} T cell repertoire is capable of generating antitumor immunity in the absence of the dominant $RNEU_{420-429}$ epitope in both FVB/N and *neu*-N mice. To accomplish this, we created a mutated vaccine, with an amino acid substitution at one of the anchor residues used to bind the immunodominant $RNEU_{420-429}$ epitope to $H-2D^q$. This substitution should prevent binding of the epitope to $H-2D^q$, and thus presentation to and activation of $RNEU_{420-429}$-specific T cells. We previously published that the substitution of an alanine residue into either position 4 or 10 of $RNEU_{420-429}$ abrogates the ability of the peptide to bind $H-2D^q$ (21). We therefore mutated the fourth anchor residue from leucine to alanine using PCR-based site-directed mutagenesis. The mutated rat *neu* cDNA was then electroporated into NIH-3T3 cells, and clones with the highest neu expression were sorted (Fig. 5*A*). To validate that the mutated vaccine (3T3neumut) was ineffective at inducing $RNEU_{420-429}$ T cell responses, we compared lymphocytes isolated from vaccinated FVB/N mice for recognition of both the $RNEU_{420-429}$ and the 134C epitopes, following a week of in vitro stimulation with the corresponding epitope. As expected, there was a significant decrease in detection of $RNEU_{420-429}$-specific T cell responses in mice receiving the mutated vaccine ($p = 0.004$), as great as 100-fold less when compared with the wild-type vaccine. In contrast, responses directed at the 134C epitope were not significantly altered ($p = 0.8$) with the mutated vaccine (Fig. 5*B*). Next, immunized lymphocytes from both FVB/N and *neu*-N mice treated with either the wild-type or mutated vaccine were stimulated for 1 wk in vitro with each of the nondominant epitopes and $RNEU_{420-429}$ and further assessed for their activation with and without the presence of $RNEU_{420-429}$-specific T cells. Unexpectedly, we found that the nondominant T cell repertoire is not significantly altered in either FVB/N or *neu*-N mice (Fig. 5*C*). This finding led us to address whether the nondominant repertoire was functional enough to compensate for the loss of $RNEU_{420-429}$-specific responses by eradicating neu-expressing tumors in tumor-bearing mice. In tumor treatment experiments, tumor-bearing FVB/N mice were treated with either the wild-type or mutated vaccine given alone or with Cy and PC61 (to more completely deplete Tregs) and assessed for tumor progression. Although all mice treated with the wild-type vaccine alone cleared tumor, mice treated with the mutated vaccine did not. However, mice treated with the mutated vaccine alone to induce nondominant CD8^{+} T cell responses demonstrated a stabilization of their tumor burden when compared with mice treated with mock vaccine that is statistically significant ($p < 0.003$). Interestingly, Cy administered with the mutated vaccine induced complete tumor clearance in 10–20% of FVB/N mice (Fig. 6*A*). Similar tumor treatment studies conducted in the *neu*-N mice demonstrated that the mutated vaccine given with Cy and PC61 can also induce significant tumor protection in tolerized mice when compared with mock vaccine (data not shown), neu vaccine, and mutated vaccine alone ($p < 0.002$) or mock vaccine given with Cy and PC61 ($p < 0.01$, data not shown) (Fig. 6*B*). Importantly, tumor outgrowth and survival kinetics remained similar in *neu*-N mice with or without $RNEU_{420-429}$-specific CD8^{+} T cell responses, thus demonstrating that the nondominant CD8^{+} T cells do play a role in antitumor immunity in tolerized hosts (Fig. 6*B*).

To better understand the function of the T cells directed at the nondominant epitopes, we analyzed the ability of this repertoire, in the absence of $RNEU_{420-429}$-specific $CD8^+$ T cell responses, to recognize neu-expressing mammary tumor cells straight ex vivo as well as after a 1-wk in vitro stimulation with each specific non-dominant epitope. Lymphocytes from tumor-bearing mice treated with Cy and PC61 and either mock or mutated vaccine were isolated and analyzed for IFN-γ secretion after incubation with NT2.5B7-1 tumor cells. The $CD8^+$ T cell repertoire from the mutated vaccine-treated mice demonstrated enhanced recognition of tumor cells in three of the five mice in comparison with mock-treated mice (range, 0.14–0.23%) (Fig. 7*A*). To demonstrate that the nondominant $CD8^+$ T cells we identified can recognize naturally processed neu epitopes on neu-expressing mammary tumors, we stimulated splenocytes from both the mock and mutated vaccine-treated mice in Fig. 7*A* with five of our identified epitopes. Results from these studies show that these nondominant $CD8^+$ T cell responses do indeed recognize naturally processed epitopes on the mammary tumor cells (Fig. 7*B*). Additionally, we compared tetramer staining of four individual T cell populations from tumor bearing hosts straight ex vivo. Lymphocytes from tumor-bearing mice treated with or without Cy and PC61 and either the wild-type or mutated vaccine were also analyzed to dissect how Treg-mediated immune tolerance affects the *neu*-N $CD8^+$ T cell repertoire. These studies revealed that 24F epitope-specific T cell responses are the only nondominant T cells enhanced after immune modulation with both the wild-type and mutated vaccines as measured by tetramer staining. All other nondominant responses tested were not significantly enhanced except for the $RNEU_{420-429}$-specific responses in wild-type vaccine-treated mice (Fig. 8). Interestingly, the response to the 9-aa peptide 24F1 was not enhanced in comparison with the responses specific for the 10-aa peptide 24F (data not shown). These data suggest that only some epitope-specific T cell responses (likely the higher avidity responses such as $RNEU_{420-429}$ and 24F targeted responses) are regulated by Tregs, whereas other immune regulatory mechanisms may alter the other epitope-specific T cell responses.

Discussion

The studies presented in this work report four new findings. First, we identified six novel 10-mer and one novel 9-mer neu-specific epitopes using vaccine-induced lymphocytes to screen an overlapping 15-aa peptide library of the entire rat neu protein. Second, this nondominant T cell repertoire is capable of recognizing tumors in vitro and in vivo following vaccination in sequence with immune-modulating agents that alleviate Treg suppression. Third, the higher avidity nondominant $CD8^+$ T cell responses are also subject to the Treg suppression that regulates the dominant $RNEU_{420-429}$ T cell response in *neu*-N mice. Fourth, analysis of this T cell repertoire revealed a hierarchy of dominance among the *neu*-N $CD8^+$ T cell repertoire based on both functional and relative TCR avidities.

The identification and validation of six 10-mer and one 9-mer neu-specific epitopes was the first step in understanding the role that the nondominant $CD8^+$ T cell repertoire plays in the neu-directed antitumor response. It was also important to screen the entire neu sequence to obtain a complete epitope analysis to better comprehend how different $CD8^+$ T cell populations respond to tumor-directed vaccination and therapy. Based on the size of neu (185 kDa) and the lack of previously identified $H-2D^q$ binding epitopes in databases, the most effective strategy was to use an overlapping 15-aa peptide library. This methodology has been extensively used in viral research to fully characterize HIV- (27, 28), hepatitis C virus- (29), and human papillomavirus-specific (30) epitopes. To our knowledge, this is the first example employing this approach to identify the epitopes contained within a cancer Ag that are recognized by the complete $CD8^+$ T cell repertoire. This screening process was effective in identifying epitopes located throughout all domains of the neu protein. Singh and Paterson (31, 32) previously reported the identification of eight new neu-specific $CD8^+$

T cell epitopes recognized by lymphocytes isolated from FVB/N mice following vaccination with a *Listeria monocytogenes* bacterial delivery system, with each vector expressing an overlapping *neu* gene fragment. Five overlapping fragments were screened, and additional studies demonstrated a hierarchy of T cell responses based on a dilutional in vitro lytic assay. Interestingly, the epitopes identified using the *L. monocytogenes* vaccine approach were distinct from those identified with our vaccine platform (31–33). Importantly, in contrast to our findings, T cell responses induced by the *L. mono-cytogenes* vaccines against these epitopes did not correlate with tumor regression in the *neu*-N mice (32).

Several factors may account for the difference in epitopes identified by the two different vaccine approaches. First, it is possible that the two different vaccine approaches provide different mechanisms of Ag access to dendritic cells (DCs), the most likely APC involved in processing and presenting epitopes for priming of CD8$^+$ T cells. In addition to the classical MHC class I presentation of Ag, at least two different intracellular pathways involved in cross-presentation of antigenic epitopes have already been described (34). There are several examples of differential epitope presentation based on which processing pathway the Ag enters (35–37). *L. monocytogenes* is known to initially gain entry through the lysosomal compartment and subsequently releases Ag directly into the cytosol via the enzyme listeriolysin O (38–40). Our group has shown that whole-cell GM-CSF–secreting vaccine cells are taken up exogenously and induce cross-priming by DCs in both animal (41) and human studies (42), although the intracellular mechanism by which Ags are processed for presentation to CD8$^+$ T cells is still not understood. A second potential explanation is that the two different vaccine approaches predominantly access different subpopulations of DCs or other APCs. *L. monocytogenes* vaccines are known to access macrophages as well as DCs, and there is the potential for differential Ag processing and presentation among these two cell populations (43, 44). Finally, the cytokine milieu induced by the two approaches may activate the APCs differently, resulting in different mechanisms of Ag processing and presentation. *L. monocytogenes* vaccines are known to engage the innate immune response quite effectively through signals such as TLRs, but they do not induce B cell responses. In contrast, the GM-CSF–secreting vaccine cells produce a predominant adaptive response including B cell responses that even facilitate cross-priming of CD8$^+$ Ag-specific immunity (45–47). Abs specific for neu have been shown to bind neu on the vaccinating cells, facilitating tumor cell uptake by CD8$^-$ DCs and resulting in enhanced in vivo tumor clearance (45). In fact, this mechanism might also explain why the GM-CSF–secreting vaccine cell approach induces more potent in vivo tumor regressions than observed with the *L. monocytogenes* approach (data not shown) (32). Additionally, our approach failed to induce T cell responses against the *L. monocytogenes* vaccine-derived epitopes (data not shown).

The use of an overlapping peptide library to screen an entire protein to identify a more complete panel of CD8$^+$ T cell epitopes proved both effective and timely, and it identified additional epitopes that play a role in the in vivo eradication of tumors. Interestingly, T cell responses against these epitopes are regulated by tolerance mechanisms, including Tregs, even in the FVB/N mice in which the neu protein should be viewed as foreign. Additionally, the T cell responses against the alternative epitopes are less avid than the T cell responses to the immunodominant epitope, RNEU$_{420–429}$, which are only subject to significant regulation by Tregs in the tolerized *neu*-N mice (6). These findings suggest that other T cell parameters, in addition to avidity, provide signals to induce mechanisms of T cell suppression in tumor-bearing hosts. Additional support for this concept comes from the fact that the T cell populations specific for the epitopes identified with the *L. monocytogenes* approach were also subject to Treg suppression in the nontolerant mice even though these populations were of higher avidity than the T cell populations identified by our approach. In contrast to our findings, the inhibition of Tregs did not result in enhanced in vivo tumor

eradication with the *L. monocytogenes* vaccine approach (32). Additional studies are underway to better understand the in vivo mechanisms regulating these alternative T cell populations.

One potential technical issue that occurred was the observation of nonspecific cross-reactivity between positively identified 15-aa peptides that bound to MHC class I and were recognized by neu-specific T cells. However, pulsing micromolar concentrations of peptide onto APCs bypasses the endogenous processing and presentation machinery of APCs, which has been shown to lead to false-positives (48). This problem is easily addressed by a peptide validation scheme that eliminates epitopes that are lost to detection in assays that aim to determine the limits of detection by measures of avidity and function. Thus, peptides identified through this screening process must be further validated functionally as previously reported (49).

Analyses of this more complete CD8$^+$ T cell repertoire also revealed a hierarchy of dominance among the *neu*-N CD8$^+$ T cell repertoire based on both functional and relative TCR avidity. Specifically, we demonstrated that three of the six 10-aa epitopes were more dominant based on their binding affinities to H-2D^q and activation by low concentrations of Ag. Interestingly, one of the 10-aa peptide responses identified (24F) has a corresponding 9-aa peptide that shows enhanced IFN-γ secretion and MHC class I binding. This finding was surprising to us since we have previously shown that the amino acids at positions 4 and 10 are the critical amino acids for peptide binding to H-2D^q using an alanine scan that replaced each amino acid within RNEU$_{420-429}$ (21). These data indicate that the T cell responses specific for the 9-aa peptide may have a proliferative advantage over the 10-aa–specific responses even though both responses have similar relative TCR avidities. A hierarchy of CD8$^+$ T cell populations based on functional avidity has been shown in several other models, including an influenza viral model and a hepatocellular carcinoma model (49, 50). Interestingly, although we originally hypothesized that the relative TCR avidities of the nondominant CD8$^+$ T cell responses would correlate with this functional avidity hierarchy (6, 51–53), tetramer staining analyses revealed that functional dominance may not always correlate with relative TCR avidities for all responses. These findings are supported by several other studies (54–58). In particular, Derby et al. (54) suggested that these discrepancies may be due to variable TCR expression and TCR signaling efficiency. However, this would not account for our findings because expression of CD8a and TCR remains similar among the nondominant neu epitope-specific T cell populations (data not shown). However, this does not rule out the possibility that TCR signaling efficiency may be diminished for each of these T cell responses based on changes in the localization of accessory molecules at the immunological synapse or other internal signaling molecules. Thus, the explanation for these differences remains unclear and warrants further investigation. Importantly, these data also highlight the need to better characterize more complete Ag-specific CD8$^+$ T cell populations based on multiple functional parameters. These studies also caution against vaccination with a single peptide based on our current understanding of the function of different epitope-specific CD8$^+$ T cell populations within the complete T cell repertoire.

Further evaluation of the nondominant CD8$^+$ T cell hierarchies in the absence of the immunodominant T cell response were carried out to uncover functionally important T cell populations that might be suppressed by the dominant response. We theorized that the elimination of the immunodominant CD8$^+$ T cell response would create a new hierarchy of CD8$^+$ T cell responses, where the nondominant T cell responses would not be outcompeted for either immunologic resources or space. Evaluation of CD8$^+$ T cell responses in both the *neu*-N and FVB/N mice demonstrate that regardless of whether the immunodominant response is present, the relative functional hierarchy and magnitude of T cell responses

directed against nondominant epitopes remain unchanged. Similar observations were made by both Andreansky et al. (59) and Kedzierska et al. (60) in influenza A viral infection models. These finding may be explained by the correlations between TCR avidity and the T cell population burst size and duration of the expansion period (61). However, this does not rule out the possibility that the mutated vaccine may uncover other, unidentified responses that may shape and alter the nondominant CD8$^+$ T cell repertoire. Additionally, it is possible that repeated boosting vaccinations might further alter the T cell repertoire toward a larger, more avid and functional overall response. The identification of these additional epitopes will allow further exploration of this question.

There is also evidence supporting the concept that TCR avidity directly affects the in vivo efficacy of CD8$^+$ T cell responses, where more avid responses correlate with enhanced antitumor activity (62, 63). However, many of the Ags that are expressed on tumors are also expressed at low levels on normal tissue, which results in either thymic depletion or peripheral suppression of the most avid T cell responses in cancer patients, leaving only a blunted T cell response for activation by immunotherapies (64). Along these lines, both our previously published and unpublished data suggest that the nondominant *neu*-N CD8$^+$ T cell repertoire is dysfunctional, and that only uncovering high-avidity RNEU$_{420-429}$-specific responses confers tumor protection (6). However, these new findings evaluating vaccination with a mutated vaccine lacking the dominant T cell epitope suggest that the nondominant CD8$^+$ T cell repertoire does indeed generate antitumor immune responses when Tregs are first inhibited. These data are further supported by the ability of the nondominant CD8$^+$ T cell repertoire to recognize tumor in our in vitro assays. These observations are in contrast to data from Dobaño et al. (65), who showed that subdominant CD8$^+$ and CD4$^+$ T cell responses cannot compensate for the loss of the immunodominant response after *Plasmodium yoelii* circum-sporozoite protein DNA vaccination against *P. yoelii*. In their studies, the subdominant repertoire could only partially compensate against parasitic challenge after vaccination. However, in the *neu*-N model, the kinetics of tumor outgrowth and tumor-free survival of mice treated with either the mutated or nonmutated vaccine overlap. These data are supported by other published studies demonstrating the efficacy of low-avidity T cell responses against developing tumors (13–15). It is still not clear why tumor-free survival is not enhanced either with or without the presence of the immunodominant T cell response. We suspect that this may be partially due to the available CD8$^+$ T cell precursor frequency. Another possibility may be the need to prolong the length of time that Tregs are suppressed, a question difficult to address without more specific Treg-depleting agents that will not also deplete activated T cells following vaccination. Treg populations recover 2 wk after administration of Cy (6) and the IL-2 receptor Ab PC61 (66).

It is also possible that additional peripheral tolerance mechanisms are providing inactivation signals to the nondominant T cell populations. Several published studies suggest that the non-dominant, or low-avidity, T cell responses escape the effects of central and peripheral tolerance (10–12). However, our tumor survival curves and tetramer data propose that the nondominant CD8$^+$ T cell repertoire is regulated by peripheral tolerization mechanisms even in the absence of RNEU$_{420-429}$-specific responses. Additional support for our findings come from Lyman et al. (17), where they demonstrated that low-avidity HA-specific CD8$^+$ T cells that are adoptively transferred into InsHA mice are also tolerized. These data further emphasize the importance of addressing and blocking immune regulatory pathways to generate more effective cancer vaccines. Studies are currently underway to better elucidate the differences in T cell function between *neu*-N mice that demonstrate tumor regression versus progression.

In summary, these data provide a detailed characterization of a CD8$^+$ T cell repertoire specific for a novel panel of neu-derived nondominant antigenic epitopes. The findings

presented identify a functional hierarchy of T cell subpopulations when defined by both functional capability and TCR avidity measures. These findings also demonstrate the complex interactions that exist within an expanded T cell repertoire specific for just one Ag. These data disprove the prior dogma that high-avidity T cell responses for dominant tumor Ags are irreversibly tolerized, leaving in place for vaccine induction only the nontolerized, low-avidity, and nondominant antigenic T cell responses. Finally, these data provide strong support for the requirement of immune-modulating agents to enhance vaccine induction of all components of the T cell repertoire. These findings also caution against the use of immunization approaches targeting only one antigenic epitope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used in this article

|	
Cy	cyclophosphamide
DC	dendritic cell
neu	Her-2/neu
neu-**N**	Her-2/*neu* transgenic
NP	nucleoprotein
TAA	tumor-associated Ag
Treg	T regulatory cell

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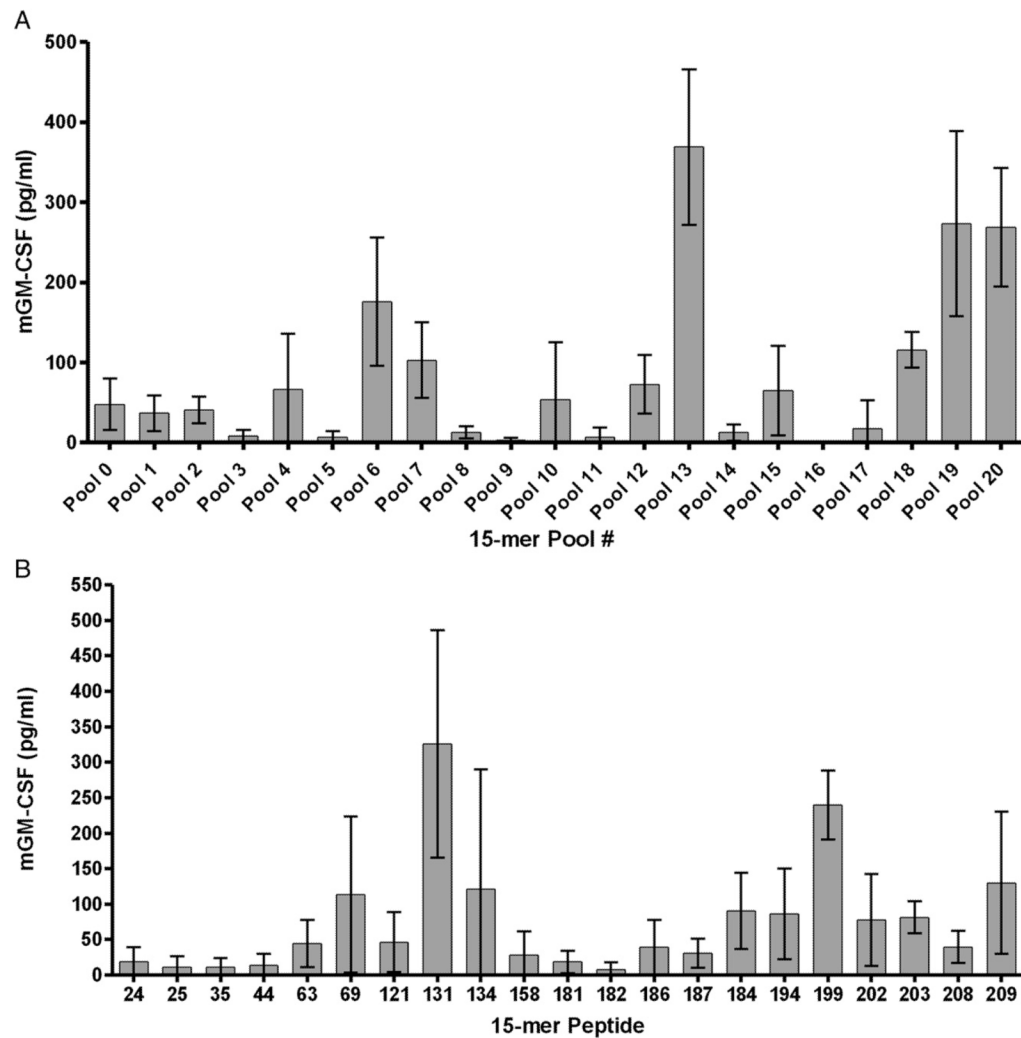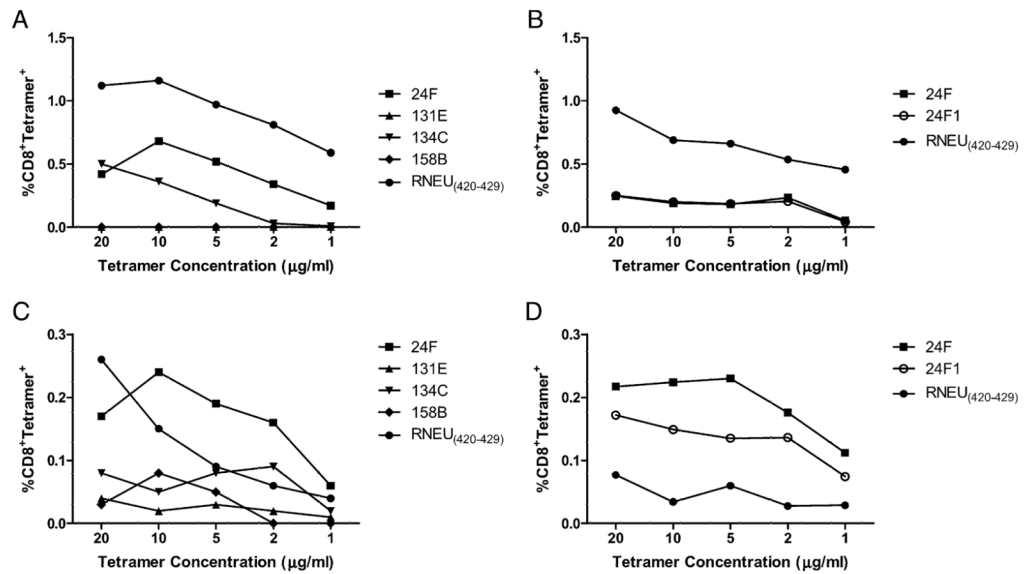
FIGURE 1.
Screening of overlapping 15-aa peptides spanning the entire length of the Her-2/neu protein with neu-specific CD8+ T cells from vaccinated *neu*-N mice identifies six new epitopes. Splenocytes from five vaccinated *neu*-N mice were pulsed with each peptide pool or a single peptide for 1 wk. CD8+ T cells were isolated and incubated with T2-D^q cells pulsed with the corresponding peptide(s) or the irrelevant LCMV nucleoprotein peptide NP$_{118–126}$. *A*, Detection of GM-CSF secretion by neu-specific CD8+ T cells after stimulation with peptide pools containing 10 overlapping 15-aa peptides. *B*, Individual 15-aa peptides elicit responses from neu-specific CD8+ T cell repertoire that correlate with the peptide pool data. Plotted are the mean GM-CSF ELISA values for five mice after background NP$_{118–126}$ levels were subtracted.
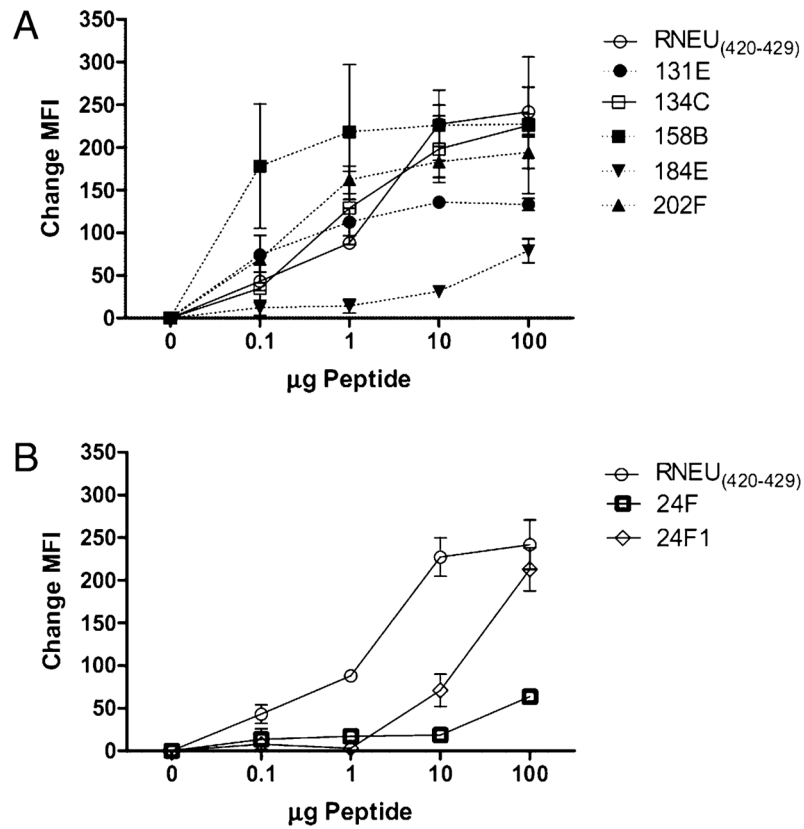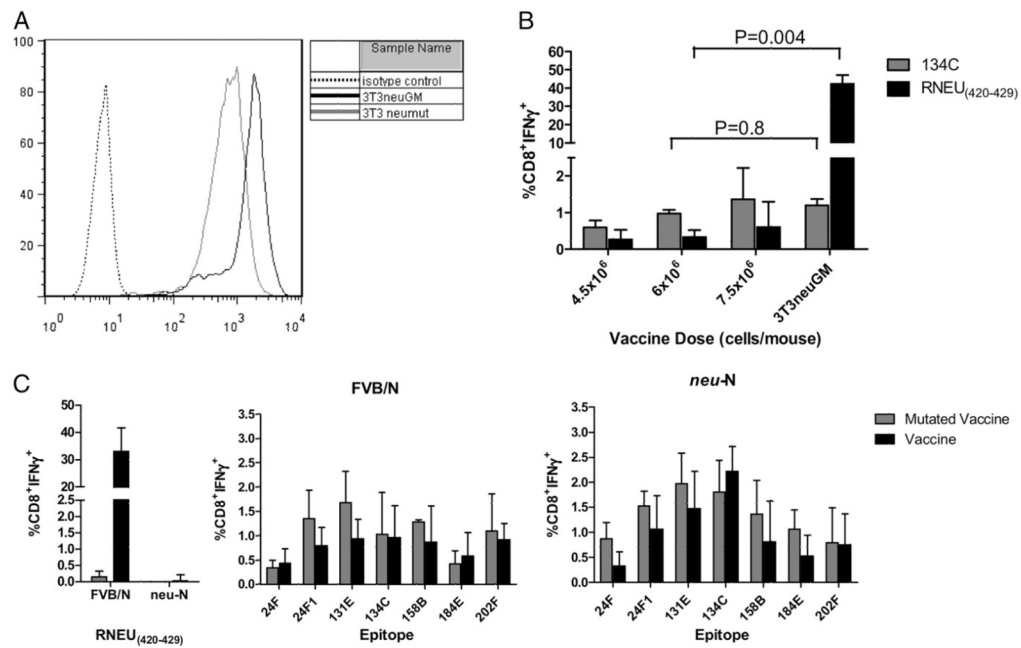
FIGURE 2.
Alternate epitopes bind to H-2D^q with varying affinities when compared with RNEU$_{420–429}$. T2-D^q cells were pulsed with each 10-aa epitope at varying concentrations of peptide and β$_2$-microglobulin for 18 h at room temperature. Samples were washed, stained with the H-2D^q mAb 30.5.7S, and analyzed based on the mean fluorescence intensity (MFI). The change in MFI from the no peptide control indicates stabilization of surface MHC class I by the peptide and relative strength of peptide binding to MHC class I. Shown is the average of triplicates after the no peptide control was subtracted. These experiments were repeated at least twice with similar results.
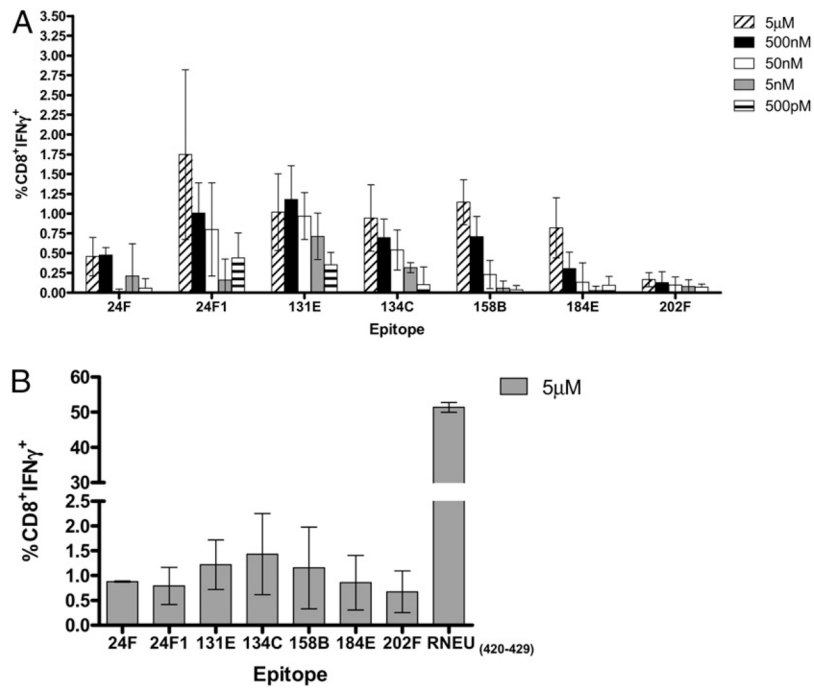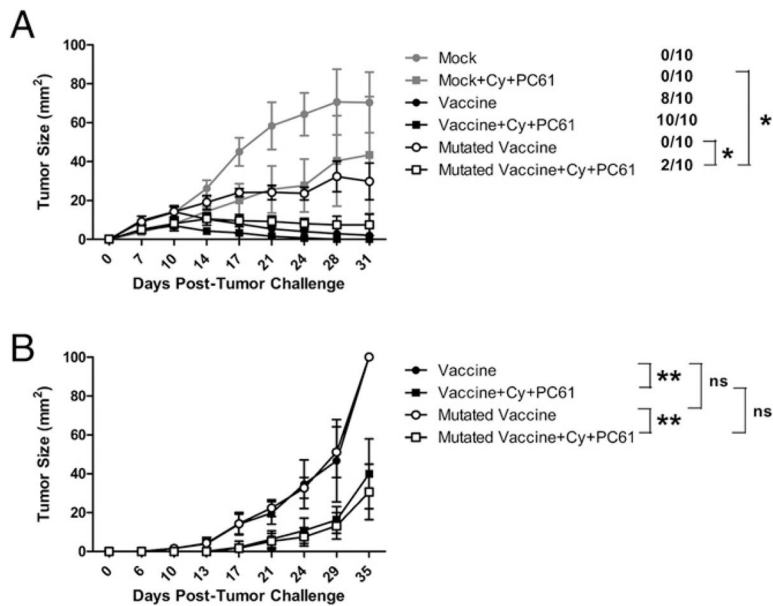
FIGURE 3.
Emergence of a functional hierarchy of neu-specific nondominant CD8$^+$ T cell responses isolated from vaccinated *neu*-N and FVB/N mice. One week following vaccination, splenocytes and vaccine draining lymph nodes were collected from five *neu*-N (*A*) or FVB/N (*B*) mice and incubated with 2.5 μg/ml peptide for 7 d. Stimulated lymphocytes were collected and incubated for T2-D^q cells pulsed with the corresponding peptide or NP$_{118-126}$ at varying concentrations. Cells were stained for CD8 and IFN-γ and analyzed by intracellular cytokine staining. *A*, CD8$^+$ T cell responses directed against 24F1, 131E and 134C are the most prevalent in vaccinated *neu*-N mice as determined by peptide titration studies. *B*, Vaccinated FVB/N mice mount CD8$^+$ T cell responses specific for nondominant epitopes that are significantly weaker than responses to RNEU$_{420-429}$. Shown for both are the mean percentage of CD8$^+$ T cells that are IFN-γ$^+$ after subtraction of background NP$_{118-126}$ responses. These experiments were repeated at least twice with similar results.
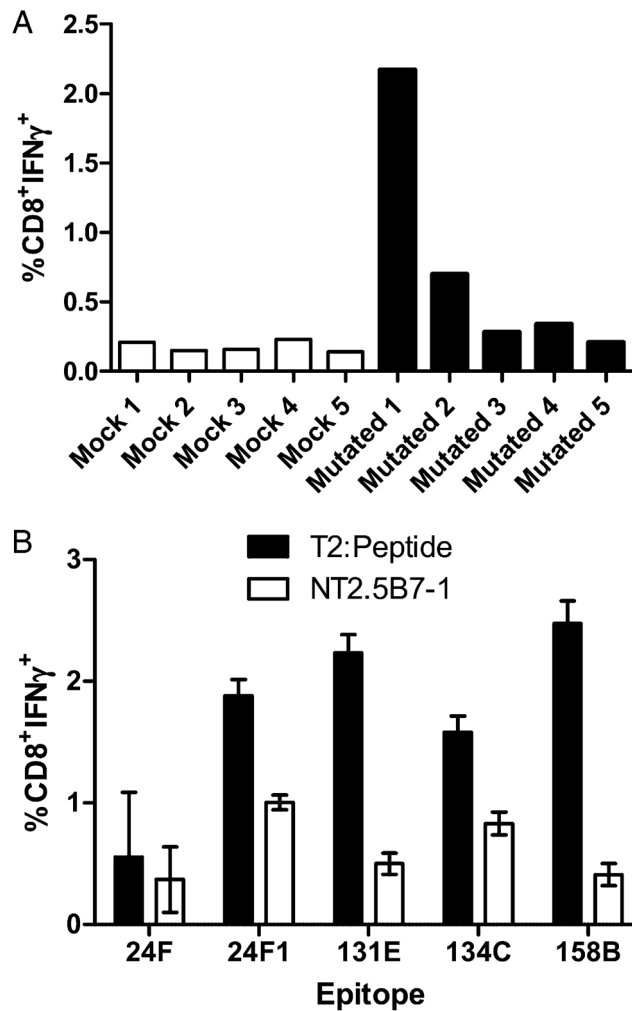
FIGURE 4.
Lower relative avidities are detected among nondominant CD8^{+} T cell responses when compared with RNEU$_{420-429}$ T cell responses in FVB/N and *neu*-N mice. *A* and *B*, RNEU$_{420-429}$-specific responses are the most abundant and avid in FVB/N mice. *C* and *D*, 24F- and 24F1-specific responses are the most avid in *neu*-N mice. Lymphocytes were isolated 14 d after tumor challenge from 10 tumor-bearing mice treated with the 3T3neuGM vaccine and stained with increasing dilutions of H-2D^q/peptide tetramer and anti-CD8 straight ex vivo. Tetramer dilutions were made from 1 mg/ml tetramer stock solutions. Graphed data depict the percentage of CD8^{+} T cells that are tetramer positive after subtraction of background tetramer staining with H-2D^q/NP$_{118-126}$. These experiments were repeated at least twice with similar results.
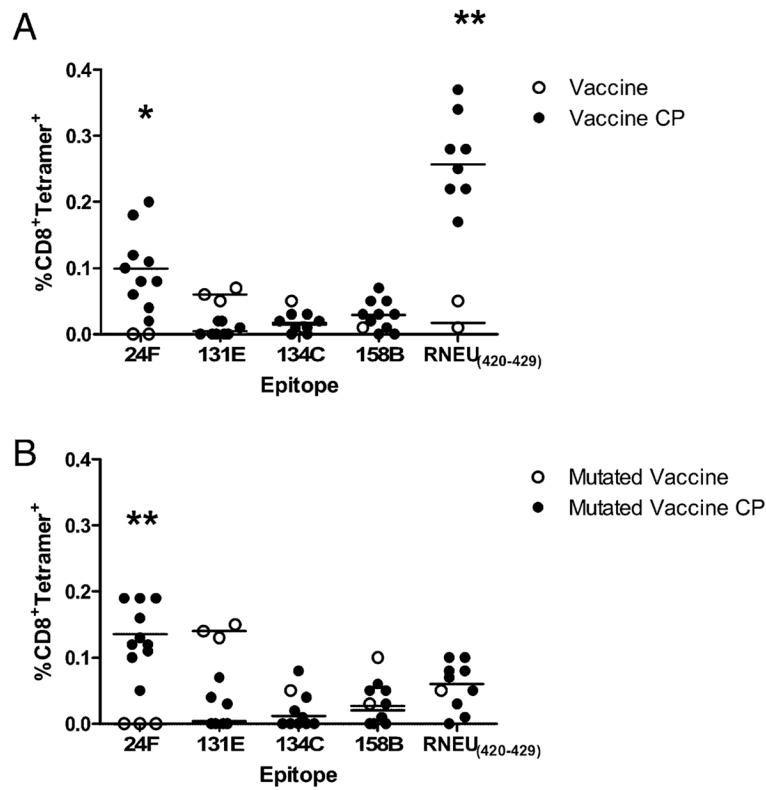
FIGURE 5.
Alanine substitution at anchor residue four within $RNEU_{420-429}$ eliminates $RNEU_{420-429}$-specific but maintains nondominant epitope-specific T cell responses following treatment of *neu*-N and FVB/N mice with the mutated vaccine. *A*, The 3T3neumut cell line expresses half the level of neu as 3T3neuGM vaccine cells. Full-length rat neu cDNA was mutated using PCR mutagenic primers and transfected into NIH-3T3 cells. Transfected cells were screened and sorted based on neu expression. *B*, Vaccination with 3T3neumut generates responses specific for 134C but not $RNEU_{420-429}$. Three FVB/N mice were vaccinated with varying doses of 3T3neumut along with 3×10^6 3T3GM cells or 3×10^6 3T3neuGM cells. One week later, splenocytes were pulsed with either $RNEU_{420-429}$ or the 134C epitope for 7 d. Lymphoctyes were isolated and incubated with T2-D^q cells pulsed with the corresponding peptide or $NP_{118-126}$. Plotted is the mean percentage of $CD8^+$ T cells that are IFN-γ^+ after subtraction of $NP_{118-126}$ values. *C*, Nondominant $CD8^+$ T cell responses are not significantly altered when comparing FVB/N and *neu*-N mice vaccinated with either the mutated or nonmutated vaccines. Experiments were carried out as described in *B* using 3×10^6 vaccine or 6×10^6 3T3neumut with 3×10^6 3T3GM cells. These experiments were repeated at least twice with similar results.

FIGURE 6.
Nondominant CD8^{+} T cell repertoire induces antitumor immunity. *A*, Combining immune-modulating agents (Cy and PC61) with vaccination leads to tumor clearance in FVB/N mice treated with either 3T3neuGM or 3T3neumut/3T3GM. Listed next to each curve is the proportion of mice that were tumor free at the end of the experiment (day 31). *$p < 0.002$ for selected groups 14 d posttumor challenge. *B*, Significant protection in tumor-challenged *neu*-N mice when treated with Cy and PC61 in combination with the mutated or nonmutated vaccines. Groups of 10 FVB/N (*A*) and *neu*-N (*B*) mice were treated with or without 100 mg/kg Cy and 50 μg PC61/mouse 2 d after tumor injection (2×10^6 and 5×10^4 NT2.5 cells, respectively). Mice were vaccinated 3 d posttumor injection with either 3T3neuGM or 3T3neumut/3T3GM cells. All experiments were repeated three times with similar results. **$p < 0.002$.

FIGURE 7.
Nondominant CD8^{+} T cell repertoire recognizes neu-expressing tumors. *A*, Enhanced tumor-specific CD8^{+} T cells responses are observed in tumor-challenged FVB/N mice that were depleted of Tregs and treated with the mutated vaccine. Tumor-bearing FVB/N mice were treated with 100 mg/kg Cy and 50 μg PC61/mouse 2 d after tumor injection. Mice were vaccinated 3 d posttumor injection with either 3T3GM or 3T3neu-mut/3T3GM cells. Fourteen days posttumor challenge, CD8^{+} T cells were isolated and stained with anti-CD8 and anti–IFN-γ after a 5-h incubation with NT2.5B7-1 tumor cells. *B*, Nondominant CD8^{+} T cell responses recognize neu-expressing tumors. Splenocytes isolated from mice in *A* were stimulated with peptide for 1 wk in vitro. Lymphoctyes were isolated and incubated with either T2-D^q cells pulsed with the corresponding peptide or NT2.5B7-1 tumor cells. Plotted is the mean percentage of CD8^{+} T cells that are IFN-γ [+] after subtraction of the mock values. These experiments were repeated at least twice with similar results.

FIGURE 8.
Nondominant CD8^{+} T cell repertoire is tolerized by Tregs in *neu*-N mice. *A*, Responses to 24F and RNEU$_{420-429}$ epitopes are enhanced in tumor-bearing *neu*-N mice treated with Cy and PC61 in combination with 3T3neuGM vaccine. *B*, Responses to the 24F epitope are enhanced in tumor-bearing *neu*-N mice treated with Cy and PC61 in combination with the mutated vaccine (3T3neumut/3T3GM). Mice were tumor challenged and treated as described in Fig. 6. Fourteen days posttumor challenge, CD8^{+} T cells were isolated and stained with anti-CD8 and a 1:200 dilution (5 μg/ml of tetramer protein) of H-2D^q/peptide tetramer straight ex vivo. These experiments were repeated at least twice with similar results. $*p = 0.014$, $**p < 0.0005$. CP, Cy plus PC61.

Table I

Nondominant epitopes recognized by the neu-specific CD8^{+} T cell repertoire of vaccinated *neu*-N mice

15-mer	Protein Location	9/10-mer	Sequence	Amino Acids	Homology to Mouse Her-2/neu (%)
24	ECD	24F	LQLRSLTEIL	144–153	10/10 (100)
24	ECD	24F1	LQLRSLTEI	144–152	10/10 (100)
RNEU	ECD	—	PDSLRDLSVF	420–429	7/10 (70)
131	ICD	131E	YVSRLLGICL	785–794	10/10 (100)
134	ICD	134C	VTQLMPYYGCL	797–806	10/10 (100)
158	ICD	158B	RLPQPPICTI	944–953	9/10 (90)
184	ICD	184E	QSLSPHDLSP	1103–1112	10/10 (100)
202	ICD	202F	HPSPAFSPAF	1212–1221	10/10 (100)