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13. ABSTRACT (Maximum 200 Words)

We sought to develop a transgenic mouse model for breast cancer that would allow the *in vivo* activities of tumor-specific T cell clones to be tracked through all stages of tumorigenesis and after various immunotherapies. We "tagged" the *neu* oncogene with two defined T cell epitopes, which conferred recognition by OT-I and OT-II T cell receptor (TCR) transgenic T cells. When expressed as a transgene in mammary epithelium, epitope-tagged *neu* (designated $neu^{OT1/OT2}$) was expected to induce mammary adenocarcinomas that express the epitope tags and hence are recognizable by OT-I and OT-II T cells. We generated three $neu^{OT1/OT2}$ transgene-positive founder lines, and expression of $neu^{OT1/OT2}$ in mammary epithelium was confirmed by Northern blot, western blot, and by immunological responses to the epitope tags. On its own, $neu^{OT1/OT2}$ induced tumors in a minority of mice and with a long latency (12-16 months). Fortunately, when $neu^{OT1/OT2}$ mice were crossed to mice expressing a mutant p53 transgene, tumors developed in the majority (7/8) of mice with a reasonable latency (7-9.5 months). We are currently using this model to analyze the T cell response to spontaneous mammary tumors and to develop novel immunotherapies for breast cancer. Thus, we fully accomplished the goals of this project.

14. SUBJECT TERMS
transgenic mouse, tumor immunology, immunotherapy, CD4+ and CD8+ T lymphocytes, adoptive transfer, HER2/neu

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BC990655 Final Report, June 2004

PI: Brad H. Nelson, Ph.D.

Title of Project: Development of a transgenic mouse model for breast cancer that is optimized for the study of T cell based therapeutic strategies

Introduction:

Currently, the development of immune-based therapies for breast cancer is impeded by the lack of an animal model that both mimics spontaneous human disease and is amenable to detailed monitoring of the activities of multiple, defined T cell clones that recognize tumor antigens. In this project, we created transgenic mice that are genetically programmed to develop spontaneous mammary tumors expressing defined T cell epitopes. Once such mice develop tumors, we will adoptively transfer CD4+ and CD8+ T cell clones with specificity for the engineered epitopes. These T cells will then be tracked in vivo and analyzed for functional responses to tumor cells. This system will be used to evaluate the mechanisms and efficacy of immune-based therapeutic and preventative strategies. The specific aims of this proposal were:

(1) To construct a dual epitope-tagged version of the oncogene *neu* that is recognized by two epitope-specific T cell clones while retaining transforming potential;

(2) To assess the ability of epitope-tagged *neu*, upon expression in transgenic mice, to induce the formation of mammary tumors that are recognized by epitope-specific CD4+ and CD8+ T cell clones.

Body:

Aim 1: To construct a dual epitope-tagged version of the oncogene neu *that is recognized by two epitope-specific T cell clones while retaining transforming potential.*

As originally proposed, the OT-1 epitope from chicken ovalbumin was placed at the C-terminus of the activated allele of *neu*. We originally proposed to place the IEα epitope from the MHC Class II gene at the N-terminus of *neu*, however this strategy had to be changed in two ways. First, we decided to use the OT-2 epitope from ovalbumin instead of the IEα epitope due to concerns that there might be some expression of the endogenous IE-α gene in B6 mice, which would complicate the interpretation of T cell responses. Like IE-α, the OT-2 epitope is presented to CD4+ T cells by MHC Class II molecules, is well characterized, and is recognized by a readily available TCR transgenic CD4+ T cell clone. However, the OT-2 epitope has the added advantage of being of non-murine origin and thus absolutely cannot be expressed by normal tissues in these mice. The second change we made was to place the OT-2 epitope at the C terminus of *neu* instead of the N terminus. This was necessitated by our unexpected finding that placement of epitopes near the N terminus of *neu* severely disrupted expression of the molecule. Thus, our final construct has both the OT-1 and OT-2 epitopes in tandem at the C terminus of neu, with the OT-2 epitope occupying the most C-terminal position (Fig. 1). This version of epitope-tagged *neu* was very well expressed in multiple cell lines (data not shown). In addition, we were able to demonstrate by in vitro proliferative assays (described in Aim 1 of the proposal) that both the OT1 and OT2 epitope tags were processed and presented to the appropriate CD8+ and CD4+ TCR transgenic T cells (Fig. 2). Finally, using an *in vitro* transformation assay with NR6 fibroblasts (described in Aim 1 of the proposal), we showed that the OT1 and OT2 epitopes do not interfere with the transforming properties of *neu* (data not shown). Thus, all aspects of Aim 1 were successfully completed on schedule, yielding a dual epitope-tagged version of *neu* that was suitable for generating transgenic mice. This corresponds to completion of all items (a-c) listed under Task 1 in the Statement of Work.

Aim 2: To assess the ability of epitope-tagged neu, upon expression in transgenic mice, to induce the formation of mammary tumors that are recognized by epitope-specific CD4+ and CD8+ T cell clones.

As described in previous annual reports, we have successfully generated three C57Bl/6 founder lines that stably transmit the $neu^{OT1/OT2}$ transgene. We demonstrated expression of the transgene by Northern blot.

For each transgenic line, mammary tissue was harvested from two lactating females and subjected to standard Northern blot analysis using a probe corresponding to the *neu*$^{OT1/OT2}$ cDNA. Tissue from a transgene-negative littermate served as a negative control. All three founder lines were found to express the *neu*$^{OT1/OT2}$ mRNA, whereas control tissue was negative for expression, as expected. All tissue samples were positive for expression of the housekeeping gene GAPDH.

For each of the three *neu*$^{OT1/OT2}$ founder lines, we monitored 5-12 females for the spontaneous development of mammary tumors. In line "A", 1/5 mice developed a mammary tumor, and with a latency of 11.3 months. In line "B", 0/6 mice developed tumors. In line "C", 4/12 mice developed tumors, with a latency of 12-16 months. While these results were encouraging, we felt that the latency and incidence of tumor development were far from ideal. Therefore, we undertook two alternative strategies to try to decrease the latency and increase the incidence of tumor formation:

(1) Some *neu*$^{OT1/OT2}$ mice were bred to mice that express a dominant-negative p53 transgene (DN-p53) in mammary epithelium (under the control of the whey acidic protein [WAP] promoter). This dominant-negative form of p53 has been shown by other groups to greatly accelerate *neu*-induced tumorigenesis. For each of the three *neu*$^{OT1/OT2}$ founder lines, we generated 5-8 females that were doubly transgenic for *neu*$^{OT1/OT2}$ and DN-p53 and monitored these mice for the spontaneous development of mammary tumors. This proved to be a successful strategy: for one of the *neu*$^{OT1/OT2}$ x DN-p53 lines (line "C"), 7/8 females developed mammary tumors with a latency of 7-9.5 months. This is a reasonable latency and incidence to satisfy the original goals of this project.

(2) Other *neu*$^{OT1/OT2}$ mice were bred to mice that express the SV40 middle T antigen (mTAG) under the control of the MMTV promoter. This transgene has been shown by other groups to strongly induce mammary tumors on the C57Bl/6 background. This strategy was also successful: we found that any female mice expressing mTAG developed multiple mammary tumors with a latency of about 4.5 months, irrespective of whether they expressed mTAG alone or in combination with *neu*$^{OT1/OT2}$. Thus, it seems that the mTAG oncogene is both necessary and sufficient for tumor formation.

For each mouse that developed tumors, tumor lysates were prepared and subjected to western blotting and flow cytometry to assess the expression of *neu*$^{OT1/OT2}$ at the protein level. As shown in Fig. 3, *neu*$^{OT1/OT2}$ was expressed at high levels in tumors derived from the "*neu*$^{OT1/OT2}$ line C alone" or " *neu*$^{OT1/OT2}$ line C x DN-p53" genotypes. By contrast, tumors derived using the mTAG oncogene in combination with *neu*$^{OT1/OT2}$ (lines A, B, or C) showed negligible expression of *neu*$^{OT1/OT2}$. Similar results were seen when expression of *neu*$^{OT1/OT2}$ was assessed by flow cytometry using a monoclonal antibody specific for the extracellular domain of *neu* (data not shown). Thus, the mTAG oncogene is useful for inducing mammary tumors with short latency and high incidence, but unfortunately those tumors fail to express the transgene of interest (*neu*$^{OT1/OT2}$), presumably because the mTAG oncogene is sufficient for tumorigenesis. By contrast, and most importantly, the combination of *neu*$^{OT1/OT2}$ (line C) and DN-p53 fully satisfies the objectives of this proposal: tumors arise with a reasonable latency (7-9.5 months) and incidence (7/8 females), and express *neu*$^{OT1/OT2}$ at high levels.

Expression of the *neu*$^{OT1/OT2}$ transgene at the protein level was also demonstrated by assessing the proliferative response of CD4+ OT-II T cells after adoptive transfer into a tumor-bearing, transgene-positive female. The premise underlying this experiment is that OT-II T cells should proliferate upon recognition of the OT-II epitope tag encoded within *neu*$^{OT1/OT2}$. T-cell proliferation was measured by pre-staining T cells with the flourescent dye CFSE and measuring incremental loss of the dye after cell division had occurred in vivo. Importantly, adoptively transferred OT-II T cells proliferated when transferred into mice expressing a tumor of the *neu*$^{OT1/OT2}$ x DN-p53 genotype, but not tumors of the *neu*$^{OT1/OT2}$ x mTAG or mTAG alone genotypes (data not shown).

Key Research Accomplishments:

The following items have been completed or are underway:

Task 1. To construct a dual epitope-tagged version of the oncogene *neu* that is recognized by two epitope-specific T cell clones while retaining transforming potential (Months 1-12). ***completed***

a. Construct plasmids encoding single (neu^{OT-I} and neu^{OT-II}) and dual ($neu^{OT-I/OT-II}$) epitope-tagged versions of *neu*; verify DNA sequence (Months 1-3). ***completed***
b. Evaluate signaling and transforming properties of epitope-tagged and untagged versions of *neu* in cell lines; if problems noted, modify epitopes as needed (Months 4-12). ***completed***
c. In vitro assays to evaluate recognition of OT-I and OT-II epitopes by CD4+ and CD8+ T cells from TCR-transgenic mice (Months 4-12). ***completed***

Task 2. To assess the ability of epitope-tagged *neu*, upon expression in transgenic mice, to induce the formation of mammary tumors that are recognized by epitope-specific CD4+ and CD8+ T cell clones (Months 13-36). ***underway***

a. Construct MMTV vector with human growth hormone gene at 3' end into which to introduce *neu* transgenes (Months 9-12). ***completed***
b. Insert dual tagged ($neu^{OT-I/OT-II}$) cDNAs into MMTV vector (Month 13). ***completed***
c. Provide transgenes to the Dept. of Immunology at the University of Washington and have C57Bl/6 transgenic founder mice generated (Months 14-17). ***completed***
d. Perform PCR on tail DNA of pups (approximately 60 animals); breed transgene-positive founders (10-12 animals) (Months 18-19). ***completed***
e. Expect birth of F2 generation (50-100 animals); perform PCR on tail DNA; cull males (Month 20). ***completed***
f. Monitor female F2 mice for tumor formation (25-50 animals); as tumors develop, perform autopsies and tumor histology with the Dept. of Comparative Medicine at the Univ. of Washington (Months 22-32). ***completed***
g. Perform preliminary adoptive T cell transfer experiments on animals that develop tumors (approximately 35 $neu^{OT-I/OT-II}$ mice, 20 OT-I-specific TCR transgenic mice, and 20 OT-II-specific TCR transgenic mice (Months 30-36). ***underway***

Reportable Outcomes:

New funding obtained with assistance from this grant:

U.S. Department of Defense
B.H. Nelson, P.I.

7/01/01-6/30/04
Direct total costs: $299,631
Direct annual costs: $99,877

Eliciting Autoimmunity to Ovarian Tumors in Mice by Genetic Disruption of T Cell Tolerance Mechanisms

The specific aims of this proposal are:
1. To generate an ovarian tumor cell line that is recognized by antigen-specific CD4+ and CD8+ T cell clones from TCR transgenic mice.
2. To define the mechanisms by which ID8 ovarian tumors evade rejection by tumor-specific CD4+ and CD8+ T cells.

3. To determine whether tumor-specific CD4+ and CD8+ T cells lacking the Cbl-b gene show enhanced functional responses to ovarian tumors.

Posters:

The elements that augment and limit tumor-specific CD8+ lymphocyte responses in vivo. Richard M. Tempero, Marc D. Coltrera, and Brad H. Nelson. Abstract #5497, 93rd Annual Meeting of the American Association for Cancer Research, San Francisco CA, April 6-10, 2002.

Identifying the signaling pathways that drive T-cell proliferation in response to tumors. Ryan M. Teague, Richard M. Tempero, and Brad H. Nelson. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orlando, FL, September 2002.

Primary in vivo expansion of naïve $CD8^+$ T cells in the absence of IL-2 receptor and STAT5 signaling Ryan M. Teague, Richard M. Tempero, and Brad H. Nelson. Annual Meeting of the American Association of Immunologists, Denver CO, 2003.

Proliferation and Differentiation of CD8+ T Cells in the Absence of IL-2/IL-15 Receptor beta Chain Expression or STAT5 Activation. Ryan M. Teague, Richard M. Tempero, Sunil Thomas, Murali-Krishna Kaja and Brad H. Nelson. Annual Meeting of the American Association for Cancer Research, Orlando, March 2004.

Proliferation and Differentiation of CD8+ T Cells in the Absence of IL-2/IL-15 Receptor beta Chain Expression or STAT5 Activation. Ryan M. Teague, Richard M. Tempero, Sunil Thomas, Murali-Krishna Kaja and Brad H. Nelson. 12th International Congress of Immunology and 4th Annual Conference of the Federation of Clinical Immunology Societies (FOCIS), Montreal, July 2004, Publication Number: (76PM) W8.29

Submitted papers:

Teague, R.M., Tempero, R.M., Thomas, S., Kaja, M.-K. and Nelson, B.H. 2004. Proliferation and Differentiation of $CD8^+$ T Cells in the Absence of IL-2/15 Receptor β Chain Expression or STAT5 Activation. *Currently being revised for Journal of Immunology.*

Invited presentations:

Molecular control of T cell proliferation in response to tumors. Brad H. Nelson. Annual Meeting of the British Columbia Cancer Agency, Vancouver, BC, Canada, November 23-24, 2001.

Identifying the signaling pathways that drive T-cell proliferation in response to tumors. Brad H. Nelson. 10th Annual SPORE Investigators Workshop, Chantilly VA, July 2002.

The immune response to cancer. Brad H. Nelson. Annual Meeting of the British Columbia Cancer Agency, Vancouver, BC, Canada, November 2002.

The immune response to cancer. Brad H. Nelson. Fox Chase Cancer Center, Philadelphia PA, November 2002.

Career advancement:

The PI, Brad Nelson, has been appointed Director of the Research Laboratories for the British Columbia Cancer Agency's Vancouver Island Centre (Victoria, BC). His work in this animal tumor model was integral to his success in this competition. He started his new position on July 1, 2003.

Personnel:

The following personnel received pay from this research effort:
Brad Nelson, Ph.D.
Patricia Theiss, Ph.D.
Ryan Teague, Ph.D.
Julie Stewart, B.Sc.
Meghan Crawford, B.Sc.
Timothy Martyak, B.Sc.
Karen Aiken, B.Sc.

Conclusions:

The mouse model we have developed should lead to an improved understanding of the immune response to breast cancer and may facilitate the development of novel immune-based therapies or immunopreventive strategies for this disease. Toward this goal, we have now created a dually epitope-tagged version of *neu* that is recognized by the appropriate CD4+ and CD8+ T cells while retaining transforming potential. Three transgenic founder lines have been established that express this version of *neu* in mammary epithelium. Expression of epitope-tagged *neu* has been demonstrated by Northern blot, by western blot, and by immunological responses of OT-I and OT-II T cells to epitope-tagged *neu* in vivo. Alternative strategies involving mutant p53 and SV40 middle T antigen transgenes were successfully applied, which lead to the development of spontaneous mammary tumors in mice with a variety of genotypes. Of the various genetic crosses tried, the combination of $neu^{OT1/OT2}$ (line C) and DN-p53 fully satisfies the objectives originally laid down in this proposal: tumors arise with a reasonable latency (7-9.5 months) and incidence (7/8 females), and express $neu^{OT1/OT2}$ at high levels. Thus, this will be the model with which we move forward in future. Currently, adoptive T cell studies are underway using such mice to address the immunological issues described in the original proposal. We expect to submit a publication describing the results of these studies within the next year. In summary, we are pleased to report that we completed this project in its entirety and now have on hand what we believe will become a major new animal model for developing novel immunotherapies for breast cancer.

References:

None.

Appendices:

See accompanying Figures 1-3.

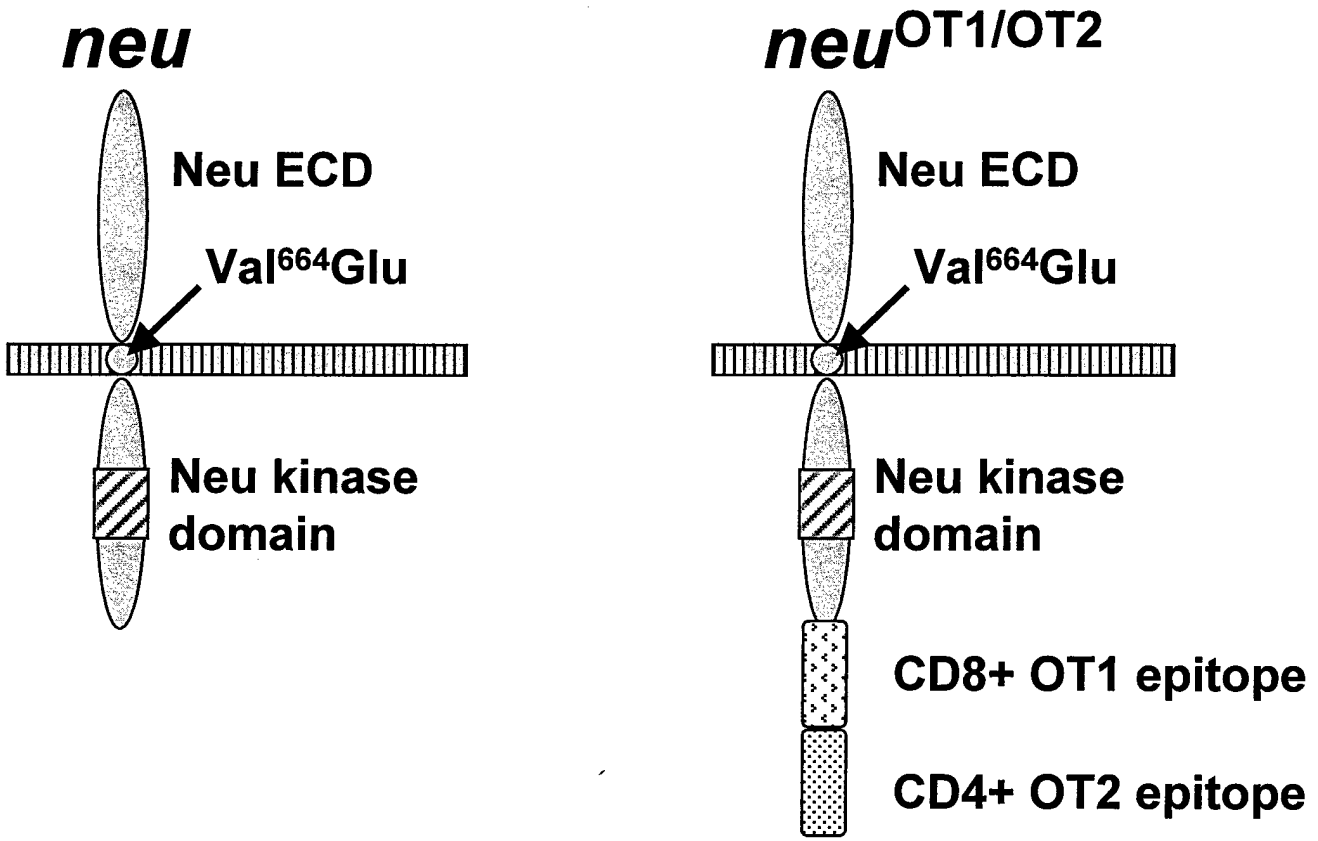

Figure 1. Schematic of the epitope-tagged *neu* oncogene. *Left*, unmodified *neu*. *Right*, epitope tagged *neu*. ECD = extracellular domain.

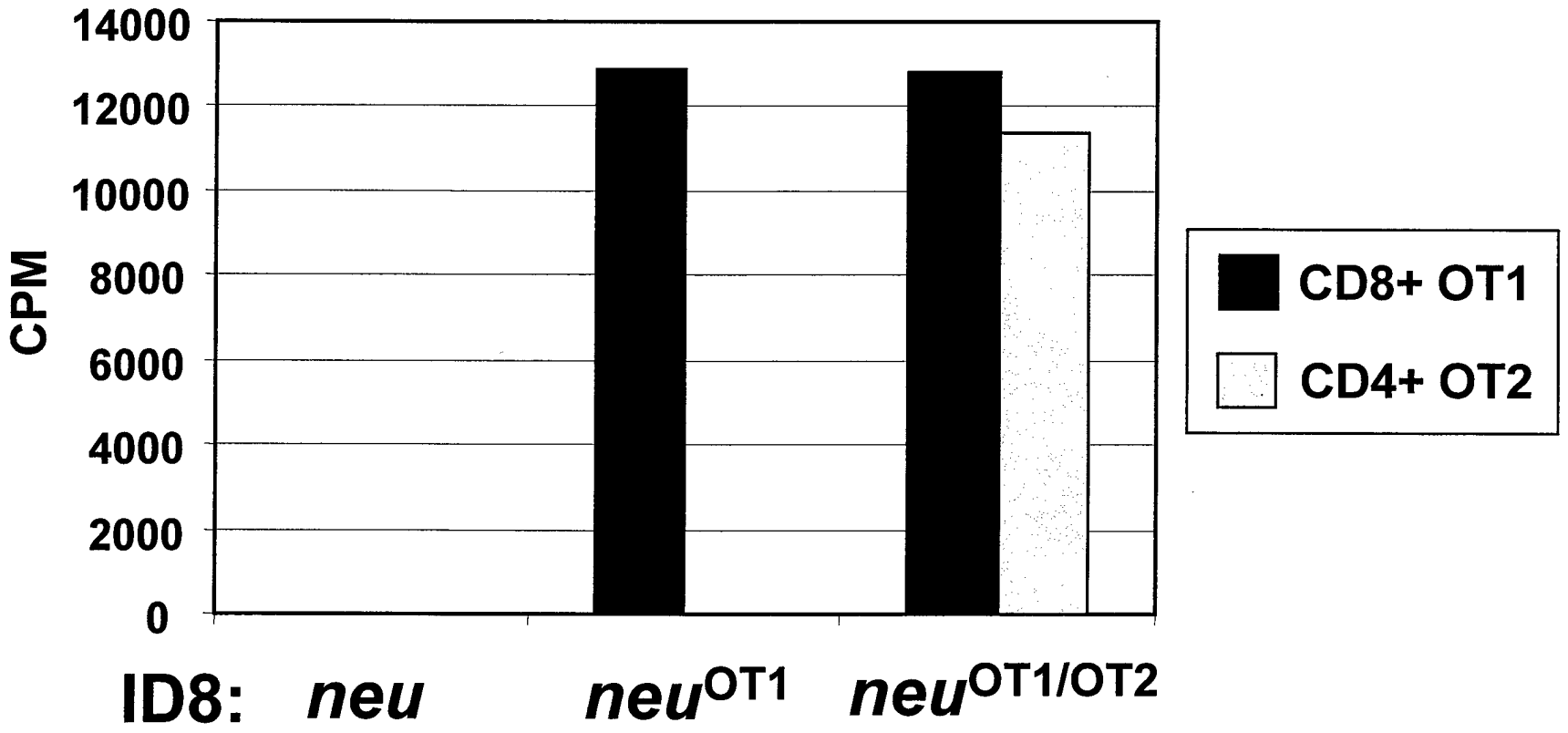

Figure 2. Recognition of epitope-tagged *neu* by CD8+ OT1 and CD4+ OT2 TCR transgenic T cells. Splenocytes from OT1 (black bars) or OT2 (gray bars) TCR transgenic mice were incubated with an epithelial tumor cell line (ID8) that had been transfected to express either untagged *neu*, *neu* bearing a single OT1 epitope tag (neu^{OT1}), or dually tagged *neu* ($neu^{OT1/OT2}$). Cultures were incubated for 40h, pulsed with tritiated thymidine for 8h, and subjected to liquid scintillation counting.

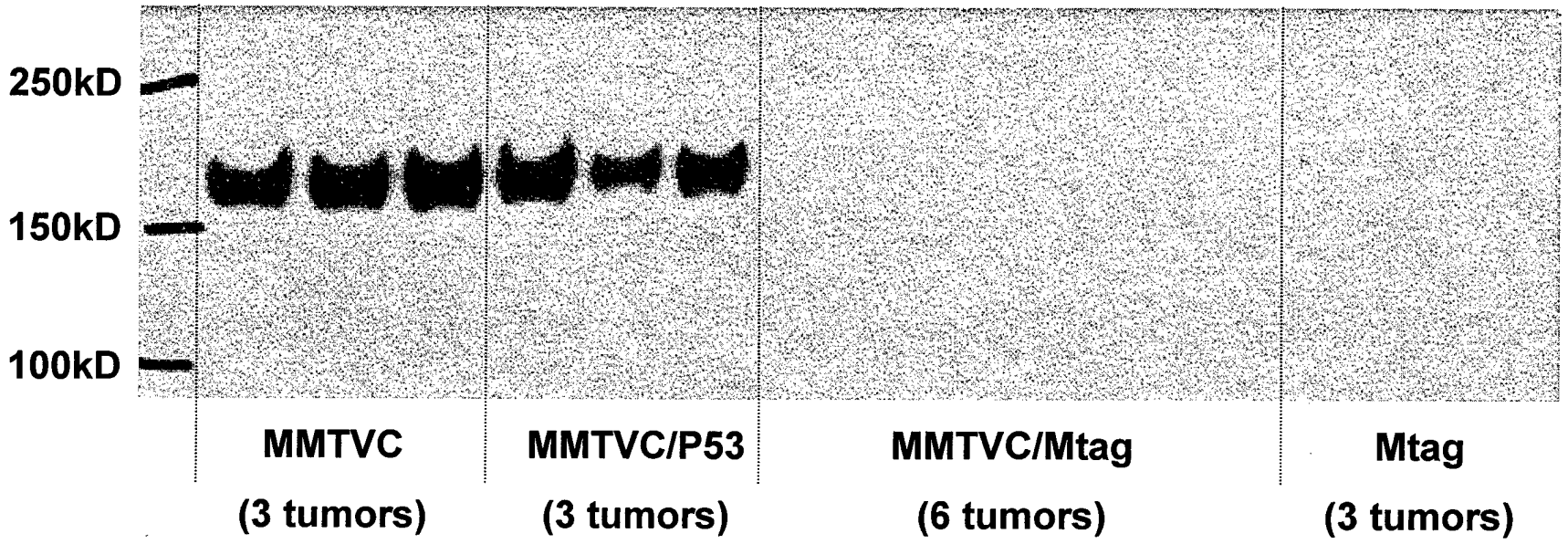

Figure 3. Expression of ***neu***$^{OT1/OT2}$ in mammary tumors from transgenic mice of different genotypes. Tumor cell lysates were prepared using a standard NP-40 lysis buffer, separated by SDS-PAGE, and subjected to western blotting with an antibody to rat *neu*. Strong expression of ***neu***$^{OT1/OT2}$ is seen in tumors from mice expressing ***neu***$^{OT1/OT2}$ alone or in combination with dominant-negative p53.