AD_____

Award Number: W81XWH-04-1-0546

TITLE: Amplification of Anti-Tumor Immunity Without Autoimmune Complications

PRINCIPAL INVESTIGATOR: Wei-Zen Wei, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
Detroit, MI 48202-3622

REPORT DATE: May 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2007	Final	15 Apr 2004 – 14 Apr 2007

4. TITLE AND SUBTITLE

Amplification of Anti-Tumor Immunity Without Autoimmune Complications

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0546

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Wei-Zen Wei, Ph.D.

E-Mail: weiw@karmanos.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wayne State University
Detroit, MI 48202-3622

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

See next page for abstract.

15. SUBJECT TERMS
No subject terms provided

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	36	**19b. TELEPHONE NUMBER** *(include area code)*

ABSTRACT

The hypothesis is that inactivation of Treg cells accompanied by Neu DNA vaccination will overcome tolerance in BALB NeuT mice and inhibit spontaneous mammary tumorigenesis or reject an established s.c. tumor. The anticipated tumor growth inhibition may be achieved at the risk of developing autoimmunity. Thyroiditis will be measured to indicate the level of risk. We further hypothesize that DNA vaccines encoding both Neu and GITR ligand will stimulate effector T cells via conventional TCR interaction and inhibit suppressor activity via GITR signaling, thus inducing anti-tumor immunity without systemic Treg cell inactivation and the inadvertent induction of autoimmune diseases.

To test the hypothesis that anti-tumor but not autoimmunity can be induced by DNA vaccine encoding Neu-TM and GITRL, we will (A) construct and test DNA plasmids encoding NeuTM and GITRL and (B) perform in vitro and in vivo testing of pVIVO-NeuTM/GITRL. Toward sub-task B, we will (1) establish the read-outs for NeuTM DNA vaccination, including humoral and cellular immunity, (2) establish the read-outs for autoimmune response by measuring immune reactivity to mTg and inflammatory infiltration in the thyroid and (3) measure anti-Neu and anti-mTg reactivity in mice immunized with DNA encoding NeuTM/GITRL.

Table of Contents

INTRODUCTION

The project was funded for two years. The tasks were modified to reflect new understanding in immune regulation and they are

Task 1 Test the hypothesis that anti-tumor but not autoimmunity will be induced by DNA vaccine encoding pNeuTM and GITRL

(A) Month 1-24 Construction and biochemical testing of DNA plasmids encoding GITRL or GITRL-ecd

(B) Month 1-24 In vitro and in vivo testing of pGITRL or pGITRL-ecd in association with pNeuTM vaccination.

I. Establish the read-outs for NeuTM DNA vaccination, including antibody production and T cell response measured by ELISPOT.

II. Establish the read-outs for autoimmune response, including antibody and T cell reactivity to mouse thyroglobulin and inflammatory infiltration in the thyroid.

III. Measure anti-neu and anti-mTg reactivity in mice immunized with DNA encoding NeuTM and GITRL.

BODY

Objective 1A Construction and biochemical testing of DNA plasmids encoding NeuTM/GITRL

Full length mouse GITRL in pMTF was provided by Dr. Herman Waldmann. The extracellular domain was amplified by PCR and cloned in pVIVO. Expression of recombinant mouse GITR and the ecd portion of GITR was verified by transient transfection of mouse mammary tumor cells D2F2, followed by staining with goat anti-GITRL serum with PE conjugated 2nd Ab.

Results from Objectives 1B-I and II have been reported and the manuscripts are attached in the appendices.

Objective 1B-I Establish the read-outs for neuTM DNA vaccination, including antibody production and T cell response measured by ELISPOT, and

Objective 1B-II Establish the read-outs for autoimmune response, including antibody and T cell reactivity to mouse thyroglobulin and inflammatory infiltration of the thyroid.

Wei,WZ., Jacob, J.B., Zielinski, J.F., Flynn, J.C., Shim, K.D., Alsharabi, G., Giraldo, A.A., Kong, Y.M. Concurrent induction of anti-tumor immunity and autoimmune thyroiditis in CD4+CD25+ regulatory T cell depleted mice, Cancer Research, 65:8471-8478, 2005.

Jacob, J.B., O. Radkevich, G. Forni, J. Zielinski, D. Shim, R.F. Jones, WZ. Wei. 2006. Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. Cellular Immunology 240:96-106.

J.B. Jacob, Y.M. Kong, C. Meroueh, D.P. Snower, C.S. David, YS. Ho, WZ. Wei. 2007. Control of Her-2 tumor immunity and thyroid autoimmunity by MHC and regulatory T cells. Cancer Research 67(14), In press.

Objective 1B-III Measure anti-neu and anti-mTg reactivity in mice immunized with DNA encoding neuTM/GITRL

To determine whether co-vaccination with pGITRL or pGITRL-ecd increases immune response to Her-2/neu, normal mice were electro-vaccinated twice, 2 wks apart, with pE2TM encoding the extracellular and transmembrane domains of human Her-2 and either pGITRL or pGITRL-ecd. Following the second immunization, antibody and T cell responses were analyzed. Mice vaccinated with pE2TM and either pGITRL or pGITRL-ecd produced high levels of anti-Her-2 antibodies at 174 $\pm$ 33 and 168 $\pm$ 65 μg/ml, respectively. In T cell response, mice vaccinated with pE2TM and pGITRL showed 89 $\pm$ 32 IFN-γ-producing T cells/10^6 spleen cells when stimulated in vitro with Her-2 expressing antigen presenting cells. When compared with mice vaccinated with pE2TM and pGM-CSF, the anti-Her-2 antibody and T cell responses were comparable whether mice received pGM-CSF or pGITRL. The effect on tumor growth was tested in human Her-2 transgenic mice produced in our lab. Tumor growth was comparable whether mice received pE2TM/pGM-CSF or pE2TM/pGITRL. Therefore, increase in anti-neu or anti-tumor immunity is comparable whether pGM-CSF or pGITRL was co-injected. These results are consistent with recent reports showing that stimulation of GITR triggers co-stimulatory signals in both effector and regulatory T cells, but the activity of Treg may not be reduced (1-3). Alternative measures may be required to amplify anti-tumor immunity without systemic Treg depletion which would trigger autoimmunity.

We started to test a new strategy to amplify anti-tumor immunity with minimal systemic immune modulation by expressing exogenous antigens or cytokines in the tumor itself. The results were reported in the 2007 Annual meeting of the American Association for Cancer Research.

"Expression of a foreign antigen by intratumoral DNA electroporation to enhance anti-tumor immune response, Olga Radkevich-Brown, Jayanth Panyam, Wei-Zen Wei"

We hypothesized that a strong immune reactivity to a foreign recall antigen expressed intratumorally will cause tumor cell destruction and lead to enhanced immune priming to TAA. To test the feasibility of expressing a foreign antigen in a solid tumor, we monitored the activity of firefly luciferase introduced into BALB/c mammary tumors D2F2 by DNA electroporation. Mice were inoculated subcutaneously with $1x10^5$ D2F2 cells. When tumors reached 3x3 mm in size, mice received 100-200 μg of luciferase DNA in 50-100 μl of PBS intratumorally (i.t.) twice, 3 days apart. Each DNA injection was followed immediately by electroporation at the injection site using a tweezer electrode to deliver 8 pulses, each at 100 V for 25 msec. Luciferase activity was measured twice a wk starting on d.7 after the 1st DNA electroporation. Mice received i.v. injection of 1.6 mg D-luciferin, the luciferase substrate, and were imaged with the Kodak IS4000MM small animal imager. The intratumoral luciferase activity was maximal between d.7 and d.10 after the 1st DNA electroporation and declined progressively until no activity was detected on d.21. Intratumoral expression of luciferase was verified by in

vitro luciferase assay of the tumor lysate. To test whether i.t. DNA electroporation induced immune reactivity to the expressed foreign protein, pE2TM DNA was electroporated into D2F2 tumor. pE2TM encodes the extracellular and transmembrane domains of human ErbB-2 (Her-2), a TAA overexpressed in several types of human cancers. Her-2 is the human homolog of mouse ErbB-2 and a foreign, heterologous Ag in mice. After two i.t. DNA electroporations, Her-2-specific antibodies and T cells were detected by flow cytometry and IFN-γ ELISPOT, respectively. These results demonstrate the feasibility of expressing a foreign antigen by i.t. DNA electroporation and its recognition by the immune system, suggesting the possibility of converting a solid tumor into a cancer vaccine reservoir.

KEY RESEARCH ACCOMPLISHMENTS

1. Subclone GITRLecd and verify the proper expression of recombinant GITRL and GITRL-ecd.
2. Establish the read-outs for accessing humoral and cellular immunity to neu.
3. Establish the read-outs for accessing humoral and cellular immunity to mTg.
4. Concurrent induction of tumor regression and autoimmune thyroiditis following depletion of regulatory T cells.
5. Establish intratumoral gene expression for modulation of the tumor microenvironment.

REPORTABLE OUTCOMES

WZ Wei, J.B. Jacob, J.F. Zielinski, J.C. Flynn, K.D. Shim, G. Alsharabi, A.A. Giraldo, Y.M. Kong. 2005. Concurrent induction of antitumor immunity and autoimmune thyroiditis in CD4+CD25+ regulatory T cell-depleted mice. Cancer Research 65:8471-8478.

Jacob, J.B., O. Radkevich, G. Forni, J. Zielinski, D. Shim, R.F. Jones, WZ. Wei. 2006. Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. Cellular Immunology 240:96-106.

J.B. Jacob, Y.M. Kong, C. Meroueh, D.P. Snower, C.S. David, YS. Ho, WZ. Wei. 2007. Control of Her-2 tumor immunity and thyroid autoimmunity by MHC and regulatory T cells. Cancer Research 67(14), In press.

J.B. Jacob, Y.M. Kong, J.C. Flynn, D. Shim, J. Zelinski, and W.-Z. Wei Concurrent induction of anti-tumor immunity and autoimmunity – A new model for testing tumor immunotherapy and assessing thyroiditis induction. Proc. AACR. 2004

Jennifer B. Jacob, Yi-chi M. Kong, Olga Radkevich, Chella S. David, Ibrahim Mansoor, Daniel Snower, Wei-Zen Wei. Regulatory T cell status and MHC II haplotypes determine

the balance between overcoming Her-2 tolerance by vaccination and inducing autoimmune thyroiditis. Proc. AACR, 2005.

Jennifer B. Jacob, Yi-chi M. Kong, Daniel Snower, and Wei-Zen Wei. Tumor regression and reactivity to thyroglobulin in BALB NeuT mice following $CD25^{+}$ regulatory T cell depletion and immunization. AAI, 2006.

J.B. Jacob, Y.M. Kong, I. Nalbantoglu, D.P. Snower, WZ. Wei. Th1 and Th17 in tumor immunity versus autoimmunity. International Conference of the American Association of Immunologists, Miami Beach, FL. May 18-22, 2007.

CONCLUSIONS

We have established a test system to induce, concurrently, tumor regression and EAT after Treg depletion. A synergy between anti-neu and anti-mTg immunity was observed in BALB/c mice experiencing tumor regression and mouse thyroglobulin immunization at the same time (5).

Co-vaccination with pE2TM and pGITRL induced comparable anti-neu immunity as did pneuTM and pGM-CSF. These results are consistent with the co-stimulatory activity of GITRL, but there may not be a direct effect on Treg activity as described in several recent reports. To enhance anti-tumor immunity without excessive systemic modulation of Treg, we are testing the expression of foreign antigens in the tumor by intratumoral DNA electroporation.

REFERENCES

(1) Esparza EM, Arch RH. Signaling triggered by glucocorticoid-induced tumor necrosis factor receptor family-related gene: regulation at the interface between regulatory T cells and immune effector cells. Front Biosci. 2006, 11:1448-65. Review.

(2) Kanamaru F, Youngnak P, Hashiguchi M, Nishioka T, Takahashi T, Sakaguchi S, Ishikawa I, Azuma M. Costimulation via glucocorticoid-induced TNF receptor in both conventional and CD25+ regulatory CD4+ T cells. J Immunol. 2004, 172(12):7306-14.

(3) Ronchetti S, Zollo O, Bruscoli S, Agostini M, Bianchini R, Nocentini G, Ayroldi E, Riccardi C. GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. Eur J Immunol. 2004, 34(3):613-22.

(4) Stephens GL, McHugh RS, Whitters MJ, Young DA, Luxenberg D, Carreno BM, Collins M, Shevach EM. Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells. J Immunol. 2004,173(8):5008-20.

(5) Wei,WZ., Jacob, J.B., Zielinski, J.F., Flynn, J.C., Shim, K.D., Alsharabi, G., Giraldo, A.A., Kong, Y.M. Concurrent induction of anti-tumor immunity and autoimmune

thyroiditis in CD4+CD25+ regulatory T cell depleted mice, Cancer Research, 65:8471-8478, 2005.

(6) Jacob, J.B., O. Radkevich, G. Forni, J. Zielinski, D. Shim, R.F. Jones, WZ. Wei. 2006. Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. Cellular Immunology 240:96-106.

(7) J.B. Jacob, Y.M. Kong, C. Meroueh, D.P. Snower, C.S. David, YS. Ho, WZ. Wei. 2007. Control of Her-2 tumor immunity and thyroid autoimmunity by MHC and regulatory T cells. Cancer Research 67(14).

APPENDICES

WZ Wei, J.B. Jacob, J.F. Zielinski, J.C. Flynn, K.D. Shim, G. Alsharabi, A.A. Giraldo, Y.M. Kong. 2005. Concurrent induction of antitumor immunity and autoimmune thyroiditis in CD4+CD25+ regulatory T cell-depleted mice. Cancer Research 65:8471-8478.

Jacob, J.B., O. Radkevich, G. Forni, J. Zielinski, D. Shim, R.F. Jones, WZ. Wei. 2006. Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. Cellular Immunology 240:96-106.

J.B. Jacob, Y.M. Kong, C. Meroueh, D.P. Snower, C.S. David, YS. Ho, WZ. Wei. 2007. Control of Her-2 tumor immunity and thyroid autoimmunity by MHC and regulatory T cells. Cancer Research 67(14), In press.

Concurrent Induction of Antitumor Immunity and Autoimmune Thyroiditis in CD4$^+$CD25$^+$ Regulatory T Cell–Depleted Mice

Wei-Zen Wei,[1] Jennifer B. Jacob,[1,2] John F. Zielinski,[1] Jeffrey C. Flynn,[2] K. David Shim,[1] Ghazwan Alsharabi,[3] Alvaro A. Giraldo,[3] and Yi-chi M. Kong[2]

[1]Karmanos Cancer Institute and [2]Department of Immunology and Microbiology, School of Medicine, Wayne State University, and [3]Division of Immunopathology, St. John Hospital and Medical Center, Detroit, Michigan

Abstract

When CD4$^+$CD25$^+$ regulatory T cells are depleted or inactivated for the purpose of enhancing antitumor immunity, the risk of autoimmune disease may be significantly elevated because these regulatory T cells control both antitumor immunity and autoimmunity. To evaluate the relative benefit and risk of modulating CD4$^+$CD25$^+$ regulatory T cells, we established a new test system to measure simultaneously the immune reactivity to a tumor-associated antigen, neu, and an unrelated self-antigen, thyroglobulin. BALB/c mice were inoculated with TUBO cells expressing an activated rat neu and treated with anti-CD25 monoclonal antibody to deplete CD25$^+$ cells. The tumors grew, then regressed, and neu-specific antibodies and IFN-γ–secreting T cells were induced. The same mice were also exposed to mouse thyroglobulin by chronic i.v. injections. These mice produced thyroglobulin-specific antibody and IFN-γ–secreting T cells with inflammatory infiltration in the thyroids of some mice. The immune responses to neu or thyroglobulin were greater in mice undergoing TUBO tumor rejection and thyroglobulin injection than in those experiencing either alone. To the best of our knowledge, this is the first experimental system to assess the concurrent induction and possible synergy of immune reactivity to defined tumor and self-antigens following reduction of regulatory T cells. These results illustrate the importance of monitoring immune reactivity to self-antigens during cancer immunotherapy that involves immunomodulating agents, and the pressing need for novel strategies to induce antitumor immunity while minimizing autoimmunity. (Cancer Res 2005; 65(18): 8471-8)

Introduction

CD4$^+$CD25$^+$ regulatory T (Treg)–like cells have been described in patients with different types of cancers (1–3). We and others have shown that removal of CD4$^+$CD25$^+$ cells from tumor-bearing mice resulted in the regression of certain mouse tumors (4, 5), suggesting that Treg may negatively regulate antitumor immunity and depletion of Treg may be a powerful way to control tumor growth. In addition to CD4 and CD25, Treg express CTLA-4 (6), a glucocorticoid-induced tumor necrosis factor receptor family member (TNFRSF18; ref. 7), CD80 (8), CD62L, membrane-bound transforming growth factor β (9), as well as the transcription factor scurfin, encoded by *foxp3* (10). They do not proliferate when stimulated *in vitro* via CD3. Treg suppressive activity is triggered through the T-cell receptor by specific antigen and can inhibit T-cell activation in an antigen-specific (11) or nonspecific (12, 13) manner through a contact-dependent mechanism.

In this study, rat neu is used as the model tumor-associated antigen. Overexpression of erbB-2 or Her-2/neu in a number of common cancers, such as breast, ovarian, colorectal, prostate, and pancreatic adenocarcinoma (14–17), is correlated with a more aggressive course of disease (18, 19), rendering Her-2 an important target of cancer therapy. The therapeutic effect of anti–Her-2 monoclonal antibody (mAb), Herceptin, in stage IV breast cancer patients further distinguishes this molecule as an exceptional target of immunotherapy and vaccination. Because of self-tolerance, it is difficult to elicit strong immune responses to Her-2, as we showed in Her-2 transgenic mice (20), and Treg depletion may be a plausible strategy to amplify anti–Her-2/neu immunity.

Depletion of CD4$^+$CD25$^+$ cells combined with CTLA-4 blockade has been shown to enhance the efficacy of B16 melanoma cell vaccine with an increase in autoimmune skin depigmentation, demonstrating the concurrent induction of antitumor immunity and autoimmunity directed at common antigens (21). Autoimmunity induced through modulation of regulatory T cells is, however, not restricted to such common antigens. Autoimmune thyroiditis and a spectrum of other autoimmune diseases have been observed in cancer patients receiving melanoma gp100 or Her-2 peptide vaccines with immunomodulating agents (22, 23). In this study, we examined the induction of autoimmunity in the thyroid which does not share common antigens with Her-2.

We have shown that depletion of Treg in CBA/J mice increased their susceptibility to experimental autoimmune thyroiditis (24), the murine model of Hashimoto's thyroiditis. Hashimoto's thyroiditis, the leading cause of hypothyroidism, is characterized by mononuclear cell infiltration and destruction of the thyroid, elevation of thyroid-stimulating hormone, and decrease of thyroid hormones (T3 and T4). The production of autoantibodies (25) and T-cell proliferation to thyroid antigens (26) are indicators of autoreactivity. Susceptibility to thyroiditis is strongly influenced by the haplotype of class II MHC. For example, human *HLA-DRB1*0301* (DR3) transgene (27) and murine $H2^k$ (CBA/J) confer susceptibility to autoimmune thyroiditis, whereas murine $H2^d$ (BALB/c) is associated with resistance (28).

In genetically susceptible mice, experimental autoimmune thyroiditis is induced by injection of mouse thyroglobulin (mTg), usually in the presence of a strong adjuvant (e.g., complete Freund's Adjuvant or lipopolysaccharide; ref. 29), or by repeated injections of mTg for 4 weeks (30). Like Hashimoto's thyroiditis, experimental

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Wei-Zen Wei, Karmanos Cancer Institute, Wayne State University, 110 East Warren Avenue, Detroit, MI 48201. Phone: 313-833-0715 Ext 2360; Fax: 313-831-7518; E-mail: weiw@karmanos.org.

doi:10.1158/0008-5472.CAN-05-0934

autoimmune thyroiditis is also characterized by mononuclear cell infiltration, autoantibody production, and T-cell proliferation. Susceptible mice can be tolerized to mTg by short-term elevation of circulating mTg (31). This induced tolerance is abrogated by depletion of $CD25^+$ cells and mice again become susceptible to experimental autoimmune thyroiditis (24).

In this study, we assessed the risk of experimental autoimmune thyroiditis in genetically resistant BALB/c mice undergoing Treg depletion to induce tumor regression. Our results show that in a Treg-deprived environment, tumor cells effectively prime the immune system, resulting in tumor regression and persistent immunologic memory. The same depletion enhanced autoimmunity to mTg in resistant BALB/c mice. Concurrent tumor regression and mTg immunization resulted in further elevation of both antitumor and anti-mTg immunity. This report describes the first test system to analyze simultaneous antitumor and anti-self immunity while reducing immune regulatory mechanisms as a form of cancer therapy.

Materials and Methods

Mice and cell lines. Six- to eight-week-old female BALB/c mice ($H2^d$) were obtained from Charles River Laboratory (Frederick, MD). All animal procedures were conducted in accordance with accredited institution guidelines and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/olaw.htm#pol). D2F2 is a mouse mammary tumor line derived from a spontaneous mammary tumor that arose in the BALB/c hyperplastic alveolar nodule line D2 (32). The TUBO cell line, kindly provided by Dr. Guido Forni (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy), was derived from a spontaneous mammary tumor which arose in a BALB NeuT transgenic mouse expressing a transforming rat neu (33, 34). TUBO cells grow progressively in normal BALB/c mice and give rise to tumors which are histologically similar to those in BALB NeuT mice. All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise specified. Cell lines were maintained *in vitro* in DMEM supplemented with 5% heat-inactivated cosmic calf serum (Hyclone, Logan, UT), 5% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 10% NCTC 109 medium, 2 mmol/L L-glutamine, 0.1 mmol/L MEM nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin. D2F2 cells were cotransfected with pRSV2/neo and pCMV/neu, which encodes wild-type rat *neu*. Stable clones of D2F2/neu were selected and the expression of neu protein on the cell surface was verified by flow cytometry. Transfected cell lines were maintained in medium containing 0.8 mg/mL G418 (Geneticin, Sigma).

Depletion of $CD25^+$ T cells with anti-CD25 monoclonal antibody, PC61. The hybridoma line PC61 which produces rat anti-mouse CD25 immunoglobulin G1 [IgG1; American Type Culture Collection (ATCC), Manassas, VA] was propagated in severe combined immunodeficient mice. BALB/c mice were injected i.p. with ~0.5 mg of PC61 or normal rat immunoglobulin (or PBS). Depletion of $CD25^+$ cells was verified by flow cytometry. Lymph node cells were prepared and washed with PBS containing 0.1% sodium azide and 2% cosmic calf serum. All samples were treated with Fc receptor blocker (rat antibody to CD16/CD32; PharMingen, San Diego, CA) for 15 minutes on ice, then washed once. Cells were incubated with goat anti-GITR IgG (R&D Systems, Minneapolis, MN) for 20 minutes on ice followed by allophycocyanin-rat anti-CD4 (RM4-5), FITC-rat anti-CD25 (7D4), and phycoerythrin-donkey anti-goat IgG. Controls were stained with FITC-rat immunoglobulin M, allophycocyanin-rat IgG2b, or normal goat IgG with phycoerythrin-donkey anti-goat IgG. mAb RM4-5, 7D4, and their isotype controls were from PharMingen. Normal goat IgG and phycoerythrin-donkey anti-goat IgG were from Jackson ImmunoResearch Lab (West Grove, PA). Single- and double-stained samples were used for instrument setup. Lymph node cells from naïve animals were measured in parallel. Flow cytometric analysis was done with a FACSCalibur (Becton Dickinson, Mountain View, CA).

Tumor growth and measurement. To measure tumor growth, mice were challenged s.c. with 2×10^5 cells in the flank. Tumor growth was monitored by weekly palpation. Tumor diameters were measured in two dimensions and mice were sacrificed when any one dimension reached 15 mm. Tumor volume was calculated as $X^2Y / 2$, where X and Y represent the short and long dimension, respectively, of the tumor. Comparison of tumor-free mice was analyzed with the log-rank test.

Measurement of anti-neu antibodies. Antibody response to rat neu was determined by flow cytometry as we previously described (35). BALB/c 3T3 cells (ATCC) were stably transfected with rat neu to establish 3T3/N cells. The mouse anti-rat neu mAb (IgG2a, clone 7.16.4), which recognizes an extracellular domain of rat neu protein (Oncogene Research Products, Cambridge, MA), was serially diluted and used to stain 3T3/N cells to establish a standard binding curve. FITC-goat anti-mouse IgG was the secondary antibody (Jackson ImmunoResearch). To determine antibody concentration in test sera, serially diluted test sera were used as primary antibody. Antibody concentration was calculated by regression analysis as we previously reported (35). Normal mouse immunoglobulin was used as negative control. The isotype of bound antibody was measured with FITC-goat anti-mouse IgG1 or IgG2a (Caltag, Burlingame, CA) and the results were expressed as mean channel fluorescence. Flow cytometric analysis was done with a FACSCalibur (Becton Dickinson). The results were analyzed by two-tailed Student's t test and are presented as mean ± SD.

Enumeration of cytokine-producing cells by ELISPOT assay. Spleen cells were suspended in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, and 100 units/mL penicillin and 100 μg/mL streptomycin. Three to four hundred thousand cells were added to each well of the 96-well high-throughput screening immunoprecipitation plates (Millipore, Bedford, MA), which were precoated with rat anti-mouse IFN-γ (IgG1, clone R4-6A2) or rat anti-mouse interleukin 4 (IL-4; IgG1, clone 11B11), and incubated for 24 to 48 hours at 37°C in 5% CO_2. Test wells also contained the engineered antigen-presenting cells 3T3/NKB, which were 3T3 cells expressing rat neu, H2-K^d and CD80. The control 3T3/K cells were 3T3 cells expressing H2-K^d. The ratio of spleen cells to 3T3/NKB was 10:1 and 3T3/NKB were added after spleen cells had been plated. After incubation, cells were discarded and biotinylated rat anti–IFN-γ (IgG1, clone XMG 1.2) or rat anti–IL-4 (IgG1, clone BVD6-24G2) was added. All antibodies were purchased from BD PharMingen. Plates were incubated for 12 hours at 4°C, then washed to remove unbound antibody. Bound antibody was detected by incubating the plates with 0.9 μg/mL avidin-horseradish peroxidase (Sigma) for 2 hours at room temperature. Following washing, the substrate 3-amino-9-ethylcarbazole in 0.1 mol/L acetic acid and 0.003% hydrogen peroxide was added and the plates were incubated for 5 minutes. The substrate was discarded and the plates were washed six times with water. The visualized cytokine spots were enumerated with the ImmunoSpot analyzer (CTL, Cleveland, OH) and the results expressed as the number of cytokine-producing cells per 10^6 spleen cells. Data were analyzed by Student's t test.

Immunization with mouse thyroglobulin. mTg was prepared from frozen thyroids by fractionation on a Sephadex G-200 column as previously described (36, 37) and diluted in nonpyrogenic saline before use. The presence of lipopolysaccharide was measured by *Limulus* amoebocyte assay (Associates of Cape Cod, Woods Hole, MA; ref. 30). A 40 μg dose of mTg contained <0.5 ng of lipopolysaccharide.

Mice were injected i.v. with 40 μg mTg, followed in 3 hours with 20 μg *Salmonella enteritidis* lipopolysaccharide. The injections were repeated in 7 days. Alternatively, 40 μg mTg was injected i.v. on 4 successive days with 3 days of rest (30). This treatment was repeated for 3 more weeks.

Measurement of anti–mouse thyroglobulin antibody. Anti-mTg antibody titers were determined by ELISA as previously described (38). Briefly, Immulon I microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with mTg at 1 μg/well and serially diluted test sera were added. After washing, bound antibody was detected with alkaline phosphatase–labeled goat anti-mouse IgG and enzyme substrate.

Measurement of anti–mouse thyroglobulin T-cell response. T-cell proliferation was measured by [^{3}H]thymidine incorporation. Splenocytes were cultured in triplicate in RPMI + 1% normal mouse serum, 2 mmol/L L-glutamine, 5×10^{-5} mol/L β-mercaptoethanol, 100 units/mL penicillin,

and 100 μg/mL streptomycin, in 96-well plates at 6×10^5 cells/well, either with or without 40 μg/mL mTg for 4 days at 37°C, 6% CO_2. The cells were pulsed with 1.2 μCi/well of [^{3}H]thymidine and incubated for 18 hours before harvest onto glass fiber filter paper (Tomtec Mach3Man Cell Harvester, LKB Wallac, Gaithersburg, MD). [^{3}H]Thymidine was measured with a Microbeta Plus 1450 liquid scintillation counter (LKB Wallac). Data were analyzed by two-tailed Student's *t* test.

IFN-γ– and IL-4–producing cells were enumerated by ELISPOT assay using 40 μg/mL mTg as the antigen. In some experiments, a two-step ELISPOT assay was done to amplify mTg-specific T-cell response. Spleen cells and mTg were incubated in 96-well tissue culture plates for 3 days before the content of the wells was transferred to high-throughput screening immunoprecipitation plates. The high-throughput screening immunoprecipitation plates were incubated for 24 hours. The detection and enumeration of cytokine spots was done as described earlier.

Histologic evaluation of experimental autoimmune thyroiditis. Thyroid specimens were sectioned vertically through both lobes and 50 to 60 histologic sections were prepared from 10 to 15 step levels. The extent of mononuclear cell infiltration was scored based on the pathology index scale of 0 to 4 and presented as percent thyroid infiltration: 0, no infiltration; 0.5, >0% to 10% thyroid infiltration consisting of perivascular foci without follicular destruction; 1.0, >10% to 20% thyroid infiltration with follicular destruction; 2.0, >20% to 40% diffuse thyroid infiltration; 3.0, >40% to 80% thyroid destruction; and 4.0, >80% to 100% thyroid destruction (36). The sections were scored without knowledge of the groups. Statistical differences were analyzed by the nonparametric Mann-Whitney *U* test.

Statistical analyses. Tumor growth expressed as percent tumor-free mice was analyzed with the log-rank test. Thyroiditis expressed as percent infiltration of thyroid was analyzed using the nonparametric Mann-Whitney *U* test. The number of IFN-γ–producing cells and the antibody levels were analyzed by Student's *t* test.

Results

Duration of CD4$^+$CD25$^+$GITR$^+$ T-cell depletion by anti-CD25 monoclonal antibody treatment. To establish the time window of Treg depletion by anti-CD25 mAb, BALB/c mice were injected i.p. on 2 consecutive days with 0.5 mg of anti-CD25 mAb, PC61. CD4$^+$CD25$^+$GITR$^+$ T cells in the lymph nodes were enumerated on days 0 to 12 after the second injection. On each test day, three test mice were sacrificed and their lymph node cells analyzed individually. Control cells were prepared by pooling lymph node cells from three mice which received normal rat immunoglobulin or PBS. Cells were stained with allophycocyanin-anti-CD4, FITC-anti-CD25, and goat anti-GITR with phycoerythrin-conjugated secondary antibodies and analyzed by flow cytometry. Figure 1 shows representative T-cell profiles on days 0, 3, 5, 9, and 12. CD4$^+$ T cells were gated (Fig. 1*A*) and percentages of CD25$^+$GITR$^+$ cells in gated CD4$^+$ cells are shown in density plots (Fig. 1*B*). The results from all test and control mice are summarized in a table and provided in the Supplementary data. In CD4$^+$ T cells, the majority of CD25$^+$ cells were also GITR^{hi}. These triple-positive cells were reduced from 11 ± 0.1% to 6.2% ± 0.5% on day 1, reached their nadir of 3.0 ± 0.7% by day 5, and began to reappear after day 9. Therefore, treatment with anti-CD25 mAb maximally reduced CD4$^+$CD25$^+$GITR$^+$ cells between days 5 and 7.

TUBO tumor regression in Treg-depleted mice. *In vivo* priming to tumor-associated antigens after Treg depletion was tested with TUBO tumor, which was established from a spontaneous mammary tumor in BALB NeuT mice expressing a transforming rat neu (33, 34). Consequently, TUBO cells express neu on their cell surface (Fig. 2*A, inset*). Anti-CD25 mAb was administered twice, either 5 and 6 days before or 1 and 3 days after tumor cell inoculation. In naïve mice, TUBO tumors grew progressively to reach >500 mm^3 (not shown) when mice were sacrificed. In anti-CD25 mAb–treated mice, all mice developed tumors which started to regress when they were 15 to 180 mm^3 in size, and regressed completely by week 11 (Fig. 2*A*), suggesting *in vivo* priming by a growing TUBO tumor. The course of tumor growth and regression was nearly identical whether anti-CD25 mAb was administered before or after tumor cell inoculation. This experiment was repeated at least twice with similar results.

Anti-neu immunity in mice undergoing TUBO tumor regression. To characterize the immune responses induced during TUBO tumor regression, sera were collected at 5 and 9 weeks following tumor cell inoculation and anti-neu antibody was measured by flow cytometry as we previously reported (35). As shown in Fig. 2*B*, in naïve mice, tumor growth did not induce detectable anti-neu antibody at week 5. The tumor volume exceeded 500 mm^3 at week 9 (not shown), at which time 11.4 ± 3.5 μg/mL of anti-neu IgG was detected. Mice treated with anti-CD25 mAb before and after tumor cell inoculation displayed 11.7 ± 8.6 and 8.9 ± 6.4 μg/mL, respectively, of anti-neu IgG at week 5, when the tumors were ~35 mm^3. At week 9, when the tumors were almost completely eliminated, these same mice had 16.7 ± 9 and 34 ± 9 μg/mL of anti-neu IgG, respectively (P = 0.01). Thus, depletion of CD25$^+$ T cells either before or after TUBO cell inoculation resulted in a more prompt and elevated antibody response, with the highest level of anti-neu IgG observed at week 9 in mice receiving anti-CD25 after TUBO cell inoculation (Fig. 2*B*). The isotypes of antibodies were further analyzed. Figure 2*C* shows the representative week 5 results from mice treated with α-CD25 before tumor inoculation. Both IgG1 and IgG2a were elevated, suggesting activation of both T-helper (Th) 1 and Th2 cells.

To measure neu-specific T-cell response, four tumor-free mice which received anti-CD25 after TUBO cell inoculation were sacrificed at week 9. Their spleen cells were isolated and incubated individually with the engineered 3T3/NKB cells. IFN-γ– and IL-4–producing cells were enumerated by ELISPOT assay after a 2-day culture and similar results were obtained from the four mice. Figure 2*D* shows the result from a representative animal. There were 36 ± 2

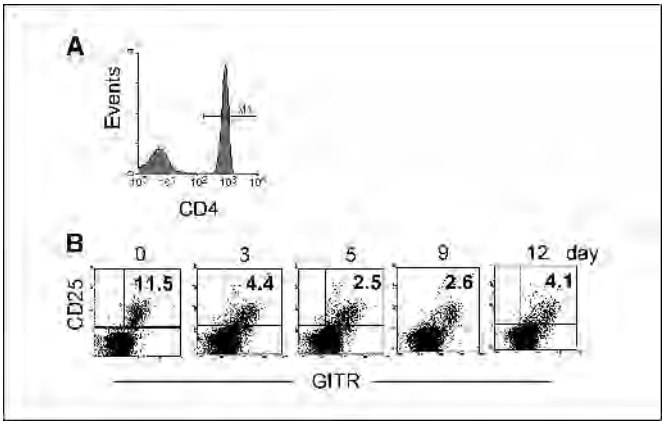

Figure 1. Depletion of Treg with anti-CD25 mAb PC61. BALB/c mice were injected i.p. with 0.5 mg of anti-CD25 mAb on 2 consecutive days. Lymph node cells were collected from treated and control mice on days 0, 1, 3, 5, 9, and 12 after antibody treatment. The cells were stained with allophycocyanin-anti-CD4, FITC-anti-CD25, and goat anti-GITR with phycoerythrin-anti-goat IgG. Positively stained CD4$^+$ T cells were gated (*A*) and CD25 and GITR profiles of gated CD4$^+$ cells harvested on days 0, 3, 5, 9, and 12 were depicted as dot plots (*B*). The number in the top right quadrant represents the percentage of CD25$^+$GITR$^+$ cells within the CD4$^+$ T-cell population.

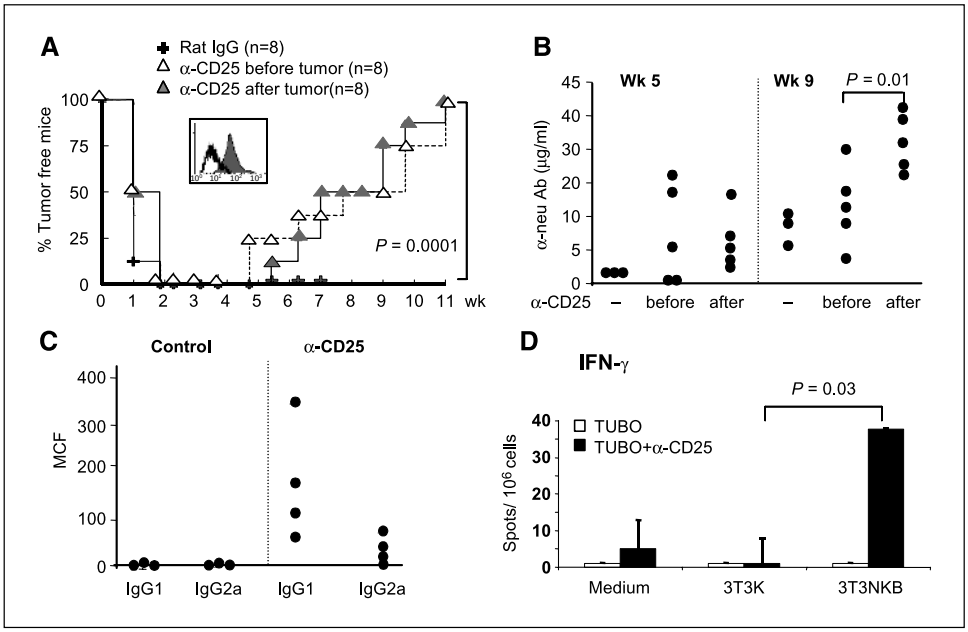

Figure 2. Tumor regression following Treg depletion. BALB/c mice were injected i.p with 0.5 mg anti-CD25 mAb either 5 and 6 days before or 1 and 3 days after they received 2×10^5 TUBO cells. Control mice were treated with normal rat immunoglobulin or PBS. There were eight mice in each group. Tumor growth was monitored for 11 weeks. *A,* TUBO tumor growth in mice treated with anti-CD25 before (△) or after (▲) tumor cell inoculation or in control mice (+). The results are expressed as percentage of tumor-free mice. *Inset,* expression of neu on TUBO cells was verified by flow cytometry using mouse anti-rat neu mAb (7.16.4, *filled histogram*) followed by phycoerythrin-anti-mouse immunoglobulin, and compared with isotype controls (*open histogram*). *B,* anti-neu IgG levels in mice treated with anti-CD25 before or after TUBO cell inoculation were measured by flow cytometry and calculated by regression analysis. *C,* IgG1 and IgG2a levels in mice treated with anti-CD25 mAb after TUBO cell inoculation are reported as mean channel fluorescence (*MCF*). *D,* T-cell response to neu antigen. Following anti-CD25 mAb treatment and tumor regression, tumor-free mice were sacrificed at week 9 and their spleen cells isolated and cultured with 3T3/K or 3T3/NKB cells. IFN-γ–producing cells were enumerated by ELISPOT assay, and normalized to express the number of spots per 10^6 spleen cells.

neu-specific IFN-γ–producing cells per 10^6 spleen cells. Neu-specific IL-4–producing cells were not detected (not shown), suggesting a more prominent Th1 response. Stimulation with concanavalin A typically resulted in over 1,000 spots per 10^6 spleen cells (not shown). Incubation with 3T3/K cells did not induce IFN-γ or IL-4 production.

Immunologic memory to tumor-associated antigen. To assess the strength of immunologic memory to tumor-associated antigens, the group of eight mice which rejected TUBO tumors by anti-CD25 mAb treatment postinoculation were rechallenged with TUBO or D2F2/neu cells at week 14 (week 0 in Fig. 3*A*). TUBO tumors were rejected by all three mice receiving this cell line as the second challenge (Fig. 3*A*). The other five mice received D2F2/neu cells, which were generated by transfecting a prolactin-induced BALB/c mammary tumor D2F2 with the wild-type rat neu (Fig. 3*B*; ref. 39). D2F2/neu cells were rejected by 4 of 5 (80%) mice. Mice which rejected TUBO tumors had 34 ± 9 μg/mL anti-neu antibody just before they received the second tumor challenge. The level of antibody cross-reactive with D2F2 was below 1 μg/mL and remained low after these mice rejected D2F2/neu, suggesting that the second challenge of D2F2/neu cells was rejected primarily through anti-neu immunity.

In summary, depletion of Treg in mice carrying TUBO tumors resulted in anti-neu antibodies, neu-specific IFN-γ–producing T cells, eradication of the growing tumor, as well as immunologic memory to neu and possibly other tumor-associated antigens.

Experimental autoimmune thyroiditis induced in resistant BALB/c mice. We have shown that elimination of Treg in mTg-tolerized CBA/J mice resulted in their reversion to the susceptible phenotype (4, 24). To test whether depletion of Treg in BALB/c mice, an experimental autoimmune thyroiditis–resistant mouse strain, would render them susceptible, mice were treated with anti-CD25 mAb twice, 4 days apart. On days 5 and 12 after the second mAb injection, mice received 40 μg of mTg and 20 μg of lipopolysaccharide (29), and they were sacrificed 4 weeks after the second immunization. Anti-mTg antibody was measured by ELISA. Depletion of Treg before mTg and lipopolysaccharide treatment resulted in a significant increase in anti-mTg antibodies ($P = 0.003$; Fig. 4*A*), including both IgG1 and IgG2a. To measure T-cell response, splenocytes were cultured with mTg for 4 days and cell proliferation was measured by [^{3}H]thymidine incorporation. A significant increase ($P = 0.002$) in T-cell response was detected in mice depleted of Treg (Fig. 4*B*). The incidence and severity of thyroiditis was significantly increased ($P = 0.01$) with Treg depletion (Fig. 4*C*). Compared with control mice without anti-CD25 mAb treatment,

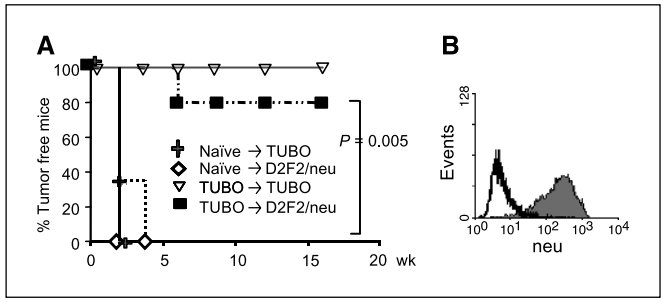

Figure 3. Induction of memory T cells after Treg depletion and tumor regression. *A,* BALB/c mice which had been treated with anti-CD25 at 1 and 3 days following TUBO cell inoculation, and of which tumors had regressed, were rechallenged with 2×10^5 TUBO or D2F2/neu cells. Three mice were given a second challenge with TUBO (▽) and five mice with D2F2/neu tumor cells (■). Control groups were naïve mice inoculated with D2F2/neu (◇) or TUBO (+) cells. *B,* flow cytometric analysis of D2F2/neu cells (*filled histogram*). Normal mouse immunoglobulin was used as control (*open histogram*).

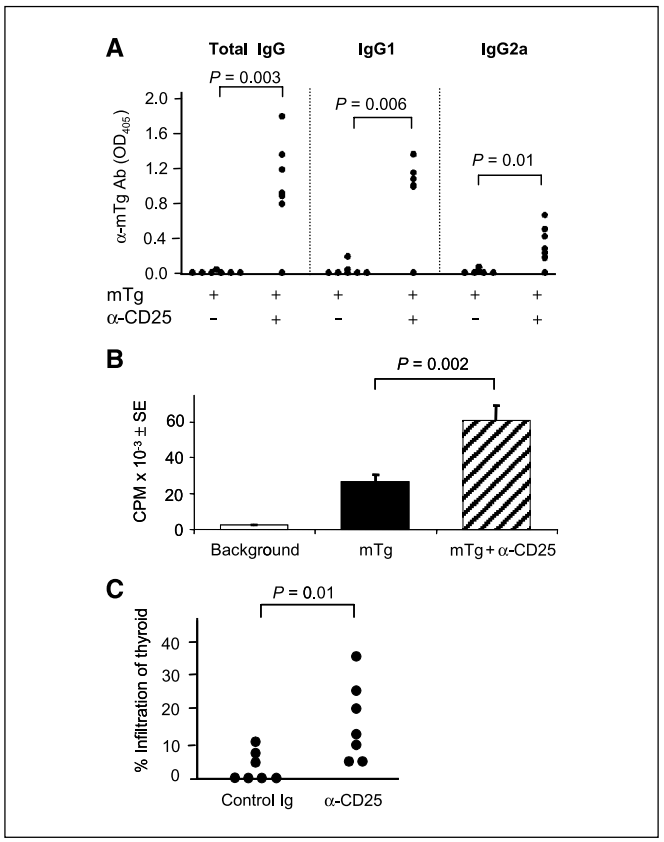

Figure 4. Enhanced anti-mTg immunity following Treg depletion. BALB/c mice were injected with 0.5 mg anti-CD25 mAb 4 days apart. On days 5 and 12 after the second injection, each mouse received i.v. 40 μg mTg and 20 μg lipopolysaccharide. On day 40, thyroids, sera, and spleen cells were collected for evaluation. *A,* mTg antibody measured by ELISA. Serum samples were tested at 1:2,500 dilution. *B, in vitro* proliferative response to mTg. A total of 6×10^5 spleen cells were incubated in the presence of 40 μg/mL mTg for 4 days and [^{3}H]thymidine uptake was measured. *C,* infiltration of individual thyroids by mononuclear cells in histologic sections was recorded. There were seven mice in each group.

the incidence of thyroid infiltration was 100% (7 of 7) versus 43% (3 of 7). Follicular destruction which is associated with >10% mononuclear infiltration of the thyroid was detected only in Treg-depleted mice. Therefore, removal of Treg enabled "resistant" BALB/c mice to develop moderate experimental autoimmune thyroiditis.

Concurrent induction of anti-neu and anti–mouse thyroglobulin response in Treg-depleted mice. To evaluate the risk of autoimmunity in mice undergoing Treg depletion to enhance antitumor immunity, we inoculated BALB/c mice with TUBO cells, followed on days 1 and 3 with anti-CD25 mAb (Fig. 5*A*). The same mice also received mTg to assess the concurrent induction of anti-mTg immunity. Rather than immunizing with mTg and lipopolysaccharide, because the latter may complicate antitumor immunity, mice received 40 μg of mTg i.v. on 4 consecutive days each week, for 4 weeks. In our previous report, this regimen induced experimental autoimmune thyroiditis in about 50% of susceptible CBA/J mice (30). All mice inoculated with tumor cells developed solid tumors in 2 weeks (Fig. 5*A* and *B*). In naïve mice, the tumors grew progressively and mice were sacrificed by week 7. All mice receiving TUBO cells and mTg without Treg depletion developed tumors by week 3. None of the tumors regressed (Fig. 5*A* and *B*). In mice depleted of Treg, tumors started to regress when they reached ~100 mm^3 and three of four tumors regressed completely. In all seven mice which received anti-CD25 mAb and mTg, TUBO tumors completely regressed. A repeated experiment showed similar results. Therefore, TUBO tumors were rejected following Treg depletion regardless of the presence of mTg.

To characterize the immune response, anti-neu antibodies were measured at week 10 or at time of sacrifice. Anti-neu IgG in untreated, tumor-bearing mice averaged 6 ± 2.4 μg/mL (Fig. 5*C*). With Treg depletion and tumor regression, average antibody level increased to 28.7 ± 26 μg/mL. Although the difference did not reach statistical significance, an increase in anti-neu antibody was observed consistently after Treg depletion. Serum samples from these two groups did not react with mTg when tested by ELISA (not shown), showing the absence of cross-reactivity between neu and mTg. Interestingly, exposure to mTg in Treg-depleted mice which undergo TUBO tumor regression resulted in an average of 78.6 ± 56 μg/mL of anti-neu IgG, demonstrating an up-regulation of anti-neu response in about 50% of the mice responding to both neu and mTg ($P = 0.05$). Neu-specific T-cell response was measured by ELISPOT following *in vitro* stimulation with the engineered antigen-presenting cells 3T3/NKB. Low, but detectable, IFN-γ–producing cells were detected following tumor regression. Figure 5*D* shows the result of a representative experiment from four independent analyses. Reactive T cells increased to ~45 per 10^6 spleen cells after the mice were also immunized with mTg. Neu-specific IL-4–producing cells were not detected (not shown). Therefore, injection of mTg in TUBO cell–bearing mice without anti-CD25 mAb treatment did not result in enhanced anti-neu immunity (Fig. 5*C* and *D*), demonstrating that exposure to mTg, a self-antigen, per se did not enhance antitumor immunity. Rather, depletion of regulatory T cells resulted in tumor regression with anti-neu immunity, which is further enhanced when anti-mTg reactivity is also triggered with this regimen.

To assess anti-mTg response in Treg-depleted mice which received mTg immunization after TUBO cell inoculation, serum antibody to mTg was measured. In three of seven mice, the antibody level was higher than that in mice which were immunized with mTg after Treg depletion, but were not inoculated with tumor cells, although the difference between these two groups was not statistically significant with this sample size (Fig. 6*A*). Mice inoculated with TUBO cells and treated with anti-CD25 mAb did not produce anti-mTg antibody. Anti-mTg antibody in Treg-depleted, mTg-immunized mice did not interact with neu when measured by flow cytometry (not shown), showing the absence of cross-reactivity between mTg and neu. mTg-specific T-cell proliferation or cytokine production was not detected at week 9 (not shown). To amplify possible T-cell responses, spleen cells were cultured with mTg for 3 days before they were transferred to ELISPOT plates precoated with anti–IFN-γ (Fig. 6*B*) or anti–IL-4 (Fig. 6*C*). Using this two-step ELISPOT assay, 612 ± 31 IFN-γ–producing and 79 ± 10 IL-4–producing cells per 10^6 spleen cells were detected in Treg-depleted mice which received TUBO and mTg. Mice inoculated with TUBO cells and injected with mTg did not develop T-cell response to mTg (not shown). Histologic analysis of the thyroid glands revealed low level of mononuclear cell infiltration, consisting mostly of T lymphocytes and macrophages as we previously reported (36, 40), in 3 of 7 mice (43%; Fig. 6*D*). Photomicrograph showing an area of thyroid with mononuclear cell infiltration is shown in Fig. 6*E*. There were no pathologic changes in thyroids of mice which received mTg without experiencing tumor. Therefore, depletion of Treg enhanced autoreactivity to mTg with further elevation after tumor regression, leading to thyroid infiltration.

Discussion

The observed tumor regression in Treg-depleted mice was paralleled by the induction of anti-neu immunity. The accelerated and heightened anti-neu antibody response may contribute significantly to TUBO tumor regression because BALB NeuT tumors, like TUBO, are effectively controlled by anti-neu antibody (41). This may be due to antibody-mediated down-regulation of neu protein and neu-mediated signaling, which is critical to their survival. Antibody-dependent, cell-mediated cytotoxicity may also contribute to tumor destruction. Significant anti-neu T-cell response was shown. Unlike TUBO cells, the rejection of D2F2/neu cells is controlled primarily by T cells as we previously described (42, 43). Rejection of D2F2/neu supports the presence of neu-specific memory T cells after TUBO tumor regression. Therefore, both humoral and cellular immune responses to tumor-associated antigen were significantly elevated following Treg depletion.

Anti-neu and anti-mTg IgG1 and IgG2a were detected in Treg-depleted mice, suggesting the activation of both Th1 and Th2 cells. When T-cell response was tested, neu-specific IFN-γ–producing cells, but not IL-4–producing cells, were detected. mTg-specific IFN-γ– and IL-4–producing T cells were detected in the amplified two-step ELISPOT assay with greater number in IFN-γ–producing cells. These results show that depletion of Treg enhanced both Th1 and Th2 immunity.

T-cell response to mTg is usually evaluated by [^{3}H]thymidine incorporation because, unlike anti-mTg antibody response, *in vitro* proliferation generally correlates with thyroid infiltration (36). Compared with T-cell proliferation, ELISPOT assay for IFN-γ–producing cells is more sensitive in detecting T-cell response. It is possible to further amplify the sensitivity of ELISPOT assay by the two-step incubation. Immune cells were expanded by preculturing with the antigen for 3 days before they were subjected to ELISPOT assay. If we estimate that effector cells divide every 12 to 16 hours on antigen stimulation, the cell number would increase between 16- and 64-fold in 3 days. Every 16 to 64 spots detected in the amplified ELISPOT assay would represent one responding cell in the starting population. The sensitivity of ELISPOT is increased significantly by preincubation, yet the difference between the control and test groups is unequivocal.

Induction of experimental autoimmune thyroiditis in Treg-depleted BALB/c mice indicated that resistance to experimental autoimmune thyroiditis determined by MHC class II was influenced in part by Treg because mononuclear cell infiltration was much less pronounced in resistant BALB/c mice carrying intact Treg (Fig. 4*C*). After depletion, greater humoral and cellular immunity to mTg was induced by the conventional immunization with mTg and lipopolysaccharide (Fig. 4*A* and *B*). When immunized by repeated mTg injection without lipopolysaccharide as adjuvant, a mild response to mTg was induced, but the response was significantly elevated in mice undergoing tumor regression such that more mice developed inflammatory infiltration in their thyroids (Fig. 6*D*). The mononuclear cell infiltration of ~10% in this experiment was insufficient to cause thyroid hormonal changes. Nevertheless, this synergy between anti-neu and anti-mTg responses was both intriguing and alarming. Although the specific mechanisms remain to be delineated, it is possible that repeated injections of the self-antigen mTg in Treg-depleted mice not only stimulated mTg-specific T cells, but that the attendant inflammatory cytokines released systemically may also enhance antitumor response. From the tumor standpoint, in Treg-depleted mice, TUBO tumors grew to palpable size before sufficient immune effectors were generated to commence tumor regression. At the tumor site, tumor cell destruction can be envisioned following immunologic attack.

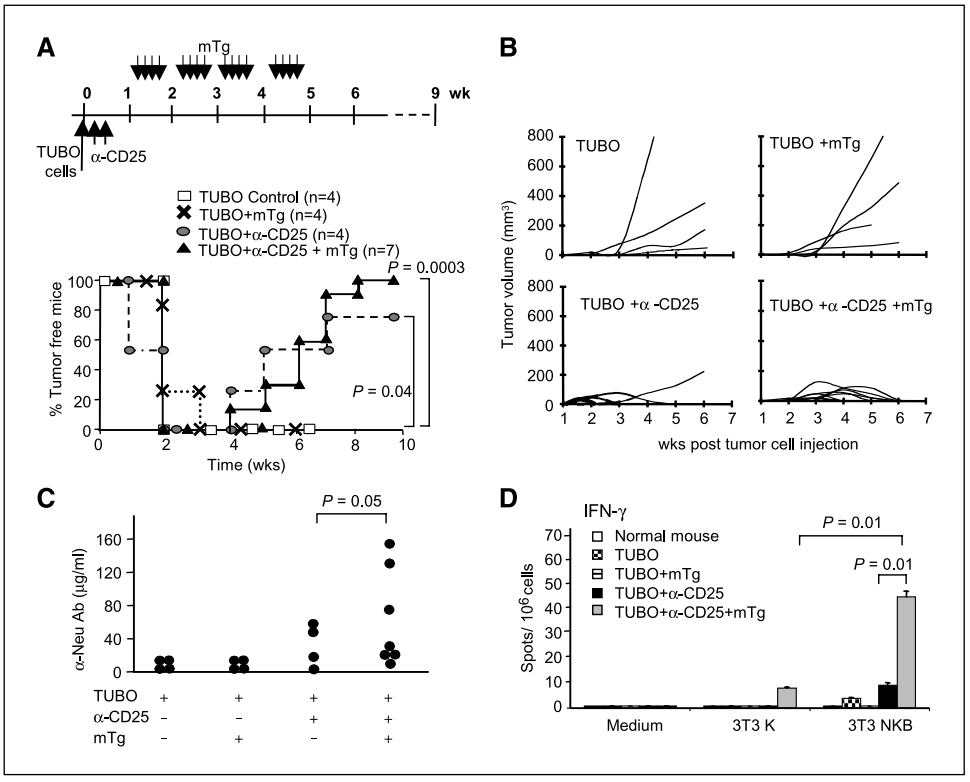

Figure 5. Effect of Treg depletion and mTg immunization on anti-neu immunity in TUBO cell–inoculated mice. BALB/c mice were inoculated with TUBO cells on day 0 and treated with anti-CD25 mAb on days 1 and 3. Starting on day 10 and continuing for 4 weeks, mice were injected with 40 μg of mTg daily for 4 days followed by a 3-day rest before the next cycle of injections. There were three other groups of mice which received TUBO cells and anti-CD25 mAb or mTg immunization or untreated. *A,* tumor growth in Treg-depleted mice with (▲) or without (●) mTg treatment. Mice in the control group received PBS (□) or mTg (×). *B,* tumor volume of individual mice in *A*. *C,* at week 10, anti-neu antibody was measured by flow cytometry; *D,* IFN-γ–producing cells were analyzed by ELISPOT assay. There were four mice in each group except seven mice in the group receiving TUBO cells, anti-CD25, and mTg.

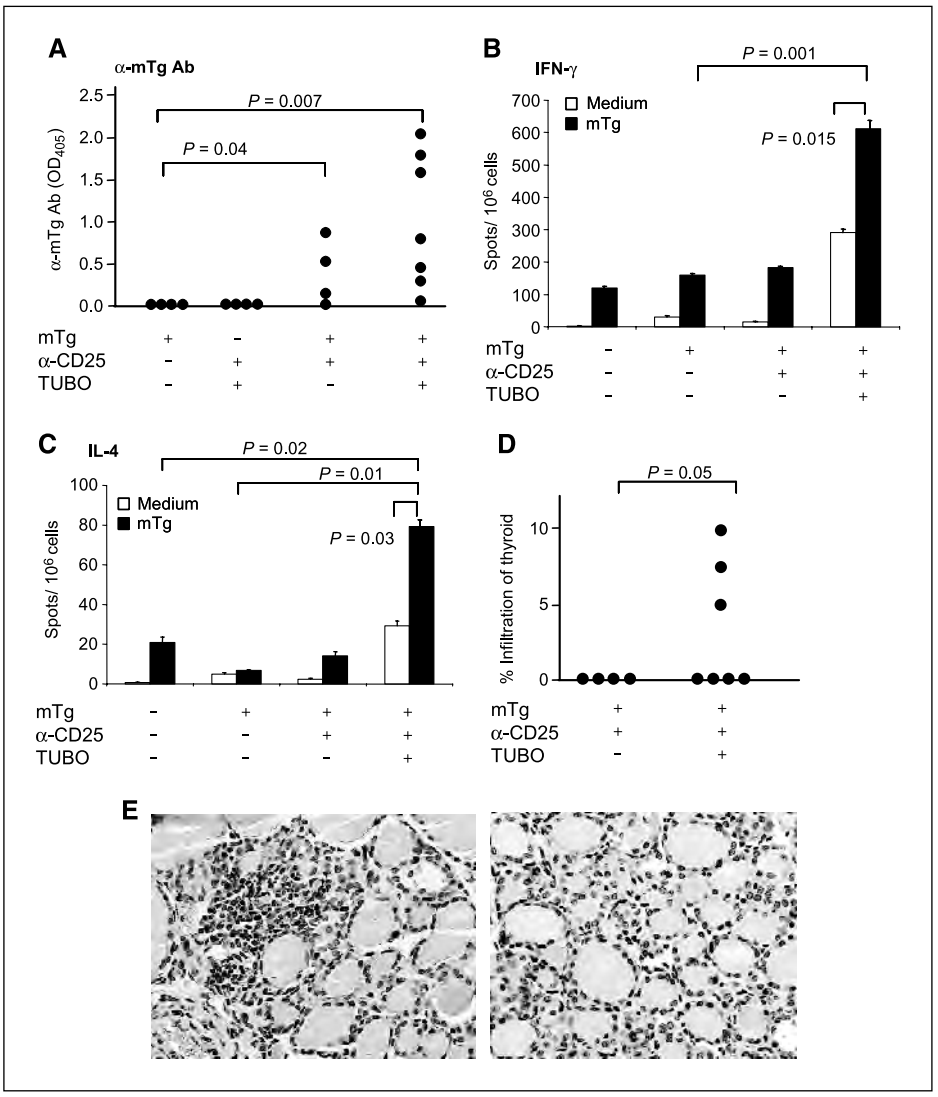

Figure 6. Effect of Treg depletion and TUBO tumor regression on anti-mTg immunity. There were four groups of mice; two groups were the same mice as described in Fig. 5 (i.e., mice injected with TUBO cells, treated with anti-CD25, and immunized with mTg, and the control mice). There were two additional groups of mice receiving mTg with or without anti-CD25, but not inoculated with TUBO cells. *A,* serum samples were diluted 1:2,500 and anti-mTg IgG measured by ELISA. IFN-γ–producing (*B*) and IL-4–producing (*C*) T cells were determined by the two-step ELISPOT assay. *D,* infiltration of individual thyroids by mononuclear cells was recorded. *E,* photomicrographs showing a normal thyroid section from an untreated mouse (*right*) and mononuclear cell infiltration with thyroid follicular destruction involving >10% of the thyroid from a Treg-depleted mouse which experienced TUBO tumor regression and mTg immunization (*left*); original magnification, ×200.

A cascade of immunologic events may lead to elevated reactivity to both mTg and tumor-associated antigens.

In cancer patients, more strenuous effort than one-time depletion may be required to overcome immune regulatory mechanisms before effective antitumor immunity can be induced, with increasing risk of autoimmunity from each new regimen. Our results showed that even genetically resistant BALB/c mice became more susceptible to experimental autoimmune thyroiditis once Treg were removed, particularly during tumor regression. Patients expressing high-risk *HLA* haplotypes will require close monitoring of their autoreactivity to self-antigens when their immune regulatory mechanisms are modulated. In a pilot study to test Her-2 peptide vaccine with Flt3 ligand administered systemically as an adjuvant, 2 of 15 subjects developed elevated thyroid stimulating hormone with symptoms of grade 2 hypothyroidism, indicating thyroid destruction. Thyroid hormone replacement therapy was required at the conclusion of the study (22). Significantly elevated levels of antibody to thyroglobulin and thyroid peroxidase were detected in one patient, demonstrating autoimmunity to thyroid antigens. In another study, 14 patients with metastatic melanoma received gp100 peptide vaccines along with mAb to CTLA-4, which is expressed on regulatory and activated T cells (23). In six patients, grade III/IV autoimmune manifestations were observed, including dermatitis, enterocolitis, hepatitis, and hypophysitis. The three patients with objective cancer regression all developed severe autoimmune symptoms requiring treatment. Because of the grade III/IV autoimmune toxicity in ≥3 patients, accrual intended for 21 patients ceased after 14 patients were enrolled. Therefore, immunomodulating reagents which can amplify antitumor immunity in a profound manner can trigger significant autoimmunity to self-antigens. For patients with genetic predisposition, the risk of autoimmunity may be overwhelming. With the sensitive *in vitro* assays to monitor immune reactivity to self-antigens, it may be possible to detect the onset of autoimmunity during cancer immunotherapy before clinical symptoms and counter measures may be taken in a timely fashion.

Acknowledgments

Received 3/21/2005; revised 6/2/2005; accepted 7/11/2005.

Grant support: NIH grants CA 76340 (W-Z. Wei) and DK45960 (Y.M. Kong), grant W81XWH-04-1-0546 from the Department of Defense (W-Z. Wei), and grant from St. John Hospital and Medical Center (Y.M. Kong).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942–9.
2. Woo EY, Yeh H, Chu CS, et al. Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. J Immunol 2002;168:4272–6.
3. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol 2004;173:1444–53.
4. Wei WZ, Morris GP, Kong YM. Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells. Cancer Immunol Immunother 2004;53:73–8.
5. Jones E, Dahm-Vicker M, Simon AK, et al. Depletion of CD25+ regulatory cells results in suppression of melanoma growth and induction of autoreactivity in mice. Cancer Immun 2002;2:1–12.
6. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated Antigen 4 plays an essential role in the function of CD25+CD4+ regulatory cells that control intestinal inflammation. J Exp Med 2000;192: 295–302.
7. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S, Aavik E. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol 2002;3:135–42.
8. Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 2000;12:431–40.
9. Nakamura K, Kitani A, Striber W. Cell contact-dependent immunosuppression by CD4+ CD25+ regulatory T cells is mediated by cell surface-bound transforming growth factor β. J Exp Med 2001;194: 629–44.
10. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057–61.
11. Tanchot C, Vasseur F, Pontoux C, Garcia C, Sarukhan A. Immune regulation by self-reactive T cells is antigen specific. J Immunol 2004;172:4285–91.
12. Sakaguchi S. Control of immune responses by naturally arising CD4+ regulatory T cells that express toll-like receptors. J Exp Med 2003;197:397–401.
13. Gallimore A, Sakaguchi S. Regulation of tumour immunity by CD25+ T cells. Immunol 2002;107:5–9.
14. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–12.
15. Yu D, Hung MC. Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. Oncogene 2000;19: 6115–21.
16. Tzahar E, Yarden Y. The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. Biochim Biophys Acta 1998;1377:M25–37.
17. Baxevanis CN, Sotiropoulou PA, Sotiriadou NN, Papamichail M. Immunobiology of HER-2/neu oncoprotein and its potential application in cancer immunotherapy. Cancer Immunol Immunother 2004; 53:166–75.
18. Gullick WJ, Love SB, Wright C, et al. c-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br J Cancer 1991;63:434–8.
19. Menard S, Pupa SM, Campiglio M, Tagliabue E. Biologic and therapeutic role of Her-2 in cancer. Oncogene 2003;22:6570–8.
20. Piechocki MP, Ho YS, Pilon S, Wei WZ. Human ErbB-2 (Her-2) transgenic mice: a model system for testing Her-2 based vaccines. J Immunol 2003;171:5787–94.
21. Sutmuller RPM, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25+ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 2001;194:823–32.
22. McNeel DG, Knutson KL, Schiffman K, Davis DR, Caron D, Disis ML. Pilot study of an HLA-A2 peptide vaccine using flt3 ligand as a systemic vaccine adjuvant. J Clin Immunol 2003;23:62–72.
23. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003;100:8372–7.
24. Morris GP, Chen L, Kong YM. CD137 signaling interferes with activation and function of CD4+CD25+ regulatory T cells in induced tolerance to experimental autoimmune thyroiditis. Cell Immunol 2003;226:20–9.
25. Huang W, Kukes GD. Hashimoto's thyroiditis: an organ-specific autoimmune disease—pathogenesis and recent developments. Lab Invest 1999;79:1175–80.
26. Canonica GW, Cosulich ME, Croci R, et al. Thyroglobulin-induced T-cell *in vitro* proliferation in Hashimoto's thyroiditis: identification of the responsive subset and effect of monoclonal antibodies directed to Ia antigens. Clin Immunol Immunopathol 1984;32:132–41.
27. Kong YM, Lomo LC, Motte RW, et al. HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice: definitive association with HLA-DRB1*0301 (DR3) gene. J Exp Med 1996;184: 1167–72.
28. Vladutiu AO, Rose NR. Autoimmune murine thyroiditis: relation to histocompatibility (H-2) type. Science 1971;174:1137–9.
29. Esquivel PS, Rose NR, Kong YM. Induction of autoimmunity in good and poor responder mice with mouse thyroglobulin and lipopolysaccharide. J Exp Med 1977;145:1250–63.
30. ElRehewy M, Kong YM, Giraldo AA, Rose NR. Syngeneic thyroglobulin is immunogenic in good responder mice. Eur J Immunol 1981;11:146–51.
31. Kong YM, Okayasu I, Giraldo AA, et al. Tolerance to thyroglobulin by activating suppressor mechanisms. Ann N Y Acad Sci 1982;392:191–209.
32. Mahoney KH, Miller BE, Heppner GH. FACS quantitation of leucine aminopeptidase and acid phosphatase on tumor associated macrophages from metastatic and nonmetastatic mouse mammary tumors. J Leukocyte Biol 1985;38:573–85.
33. Rovero S, Amici A, Di Carlo E, et al. Inhibition of carcinogenesis by DNA vaccination. J Immunol 2000; 165:5133–42.
34. Boggio K, Nicoletti G, Di Carlo E, et al. Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. J Exp Med 1998;188:589–96.
35. Piechocki MP, Pilon S, Wei WZ. Quantitative measurement of anti-ErbB-2 antibody by flow cytometry and ELISA. J Immunol Methods 2002;259:33–42.
36. Kong YM. Experimental autoimmune thyroiditis in the mouse. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology. New York: John Wiley & Sons, Inc.; 1996. p. 15.7.1–15.7.16.
37. Kong YM, David CS, Giraldo AA, Elrehewy M, Rose NR. Regulation of autoimmune response to mouse thyroglobulin: influence of H-2D-end genes. J Immunol 1979;123:15–8.
38. Zhang W, Flynn JC, Kong YM. IL-12 prevents tolerance induction with mouse thyroglobulin by priming pathogenic T cells in experimental autoimmune thyroiditis: role of IFN-γ and the costimulatory molecules CD40L and CD28. Cell Immunol 2001;208:52–61.
39. Medina D, DeOme KB. Effects of various oncogenic agents on the tumor-producing capabilities of the D series of BALB/c mammary nodule outgrowth lines. J Nat Cancer Inst 1970;45:353–63.
40. Conaway DH, Giraldo AA, David CS, Kong YM. *In situ* kinetic analysis of thyroid lymphocyte infiltrate in mice developing experimental autoimmune thyroiditis. Clin Immunol Immunopathol 1989;53:346–53.
41. Curcio C, Di Carlo E, Clynes R, et al. Nonredundant roles of antibody, cytokines and perforin for the immune eradication of established Her-2/neu carcinomas. J Clin Invest 2003;111:1161–70.
42. Pilon SA, Kelly C, Wei WZ. Broadening of epitope recognition during immune rejection of ErbB-2-positive tumor prevents growth of ErbB-2-negative tumor. J Immunol 2003;170:1202–8.
43. Lindencrona JA, Preiss S, Kammertoens T, et al. CD4+ T cell-mediated HER-2/neu-specific tumor rejection in the absence of B cells. Int J Cancer 2004;109:259–64.

Available online at www.sciencedirect.com
ScienceDirect
Cellular Immunology 240 (2006) 96–106
Cellular Immunology
www.elsevier.com/locate/ycimm

Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice ☆

Jennifer Jacob [a,b], Olga Radkevich [a,c], Guido Forni [d], John Zielinski [a], David Shim [a], Richard F. Jones [e], Wei-Zen Wei [a,b,c,*]

[a] *Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, USA*
[b] *Department of Immunology and Microbiology, Wayne State University, Detroit, MI 48201, USA*
[c] *School of Medicine, Wayne State University, Detroit, MI 48201, USA*
[d] *Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy*
[e] *Department of Urology, Upstate Medical University, Syracuse, NY 13210, USA*

Received 18 May 2006; accepted 5 July 2006
Available online 22 August 2006

Abstract

To assess the efficacy of self versus heterologous ErbB-2 vaccines, the reactivity to human and rat ErbB-2 (Her-2 and neu, respectively) DNA vaccines were tested in normal, Her-2 or neu transgenic mice. When immunized with either Her-2 or neu DNA, normal BALB/c and C57BL/6 mice produced cross-reactive T cells, but only antigen specific antibodies. In Her-2 Tg mice, weak to no anti-Her-2 response was induced by either self Her-2 or heterologous neu DNA, demonstrating profound tolerance to Her-2 and the inability to induce anti-Her-2 immunity with either vaccine. In NeuT mice, vaccination with self neu but not heterologous Her-2 DNA induced anti-neu antibodies and delayed spontaneous tumorigenesis. Both neu and Her-2 DNA induced anti-neu T cell response, but depletion of CD8 T cells did not change the delay in tumorigenesis. Therefore, in NeuT mice, both self and heterologous DNA activated anti-neu T cells, although T cell response did not reach sufficient level to suppress spontaneous tumorigenesis. Rather, induction of anti-neu antibodies by self neu DNA is associated with the delay in spontaneous tumor growth. Overall, NeuT mice were more responsive to DNA vaccination than Her-2 Tg mice and this may be associated with the continuous production of neu by the 10 mammary glands undergoing tumor progression.

Keywords: Tumor vaccine; DNA vaccine; Her-2; Neu; Transgenic mice

1. Introduction

Her-2/neu[1] over-expression, which occurs in about 25% of breast cancers [1], is associated with poor prognosis [2], making Her-2/neu an important target of cancer therapy and active vaccination. Several forms of Her-2/neu based vaccines are under active investigation, such as Her-2 DNA developed in our lab [3,4], T cell reactive peptides [5,6], tumor cells expressing GM-CSF [7] or MHC II transactivator [8], dendritic cells loaded with apoptotic tumor cells [9], etc. Although encouraging results are observed in both experimental and clinical

☆ This study was supported by NIH grant CA 76340, T32-CA009531 (pre-doctoral training for Olga Radkevich) and W81XWH-04-1-0546 from the Department of Defense.
* Corresponding author. Fax: +1 313 832 4537.
E-mail address: weiw@karmanos.org (W-Z. Wei).

[1] *Abbreviations used:* APC, antigen presenting cell; 3T3/EKB, 3T3 cells transfected to express Her-2, K^d and CD80; 3T3/NKB, 3T3 cells transfected to express neu, K^d and CD80; DNA, deoxyribonucleic acid; DMEM, Dulbecco's modified Eagle's medium; E2, human ErbB-2 (Her-2); ELISPOT, enzyme linked immuno spot; FITC, fluorescein isothiocyanate; PE, phycoerythrin; GM-CSF, granulocyte–monocyte colony stimulating factor; Her-2, human ErbB-2; Her-2 Tg, Her-2 transgenic mice; MHC, major histocompatibility complex; neu, rat ErbB-2; NeuT, BALB NeuT mice; PBL, peripheral blood leukocytes; TM, transmembrane domain; TRP, tyrosinase-related protein; WAP, whey acidic protein promoter.

0008-8749/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellimm.2006.07.002

studies, further improvement of the vaccine regimen is still warranted.

Transgenic mice expressing either human Her-2 [10] or rat neu [11–13] have been established. BALB NeuT mice (NeuT) express a transforming rat neu in their mammary glands and females develop spontaneous mammary tumors around 17 weeks of age [11]. Her-2 transgenic mice (Her-2 Tg) express human Her-2 in the mammary gland and cerebellum under the control of whey acidic protein (WAP) promoter [10]. Unlike NeuT mice, Her-2 Tg mice do not develop spontaneous mammary tumors. Tolerance in Her-2 Tg mice was previously reported by our group [10]. Only 30% of Her-2 Tg mice rejected tumors after five time immunizations with DNA encoding a secreted form of Her-2 together with pGM-CSF, whereas all non-transgenic littermates rejected tumors. The tolerant status and immune reactivity to Her-2/neu vaccine in different model systems have not been directly compared and seemingly conflicting results in the literature can be problematic in designing further studies.

Immunization with cross-reactive microbial antigen has been shown to induce autoreactive antibodies or T cells, resulting in autoimmune diseases [14]. Heterologous antigens are commonly used to induce autoimmune diseases in the animal models. In the murine model of multiple sclerosis, immunization of mice or rats with bovine or guinea pig myelin basic protein leads to demyelination, resembling disease in humans [15,16]. Collagen-induced arthritis, the murine model of rheumatoid arthritis, can be induced by bovine, porcine, or human collagen, generating complement-fixing autoantibodies [17]. Autoantibodies are generated in experimental autoimmune myasthenia gravis following immunization with the *Torpedo californica* acetylcholine receptor antigen [18].

Vaccination with heterologous tumor-associated antigens has been tested by immunizing mice with human melanoma associated antigen gp100. Mice developed T cell response to self gp100 with significant anti-tumor activity [19]. Similarly, immunization with DNA encoding human tyrosinase-related protein (TRP), but not the self protein, induced both antibody and T cell responses to self TRP, leading to protection from B16F10 tumor growth [20].

In this study, we measured Her-2 and neu reactivity to DNA vaccination in normal, Her-2 Tg and NeuT mice. NeuT mice were much more responsive to neu DNA vaccine than Her-2 Tg mice to Her-2 DNA vaccine. When NeuT mice were immunized with self neu, but not heterologous Her-2 DNA, there was a delay in spontaneous tumorigenesis which was independent of CD8 T cells.

2. Materials and methods

2.1. Mice and cell lines

BALB/c and C57BL/6 (6–8 week old) female mice were purchased from Charles River Laboratory (Frederick, MD). Heterozygous NeuT mice, which express a transforming neu under the control of the mouse mammary tumor virus promoter were maintained by breeding with BALB/c mice [11,12]. Heterozygous Her-2 Tg, which express the full-length Her-2 under the whey acidic protein (WAP) promoter were maintained by breeding with C57BL/6 mice [10]. Transgene positive mice were identified by PCR. All animal procedures were performed in accordance with the regulation of Wayne State University, Division of Laboratory Animal Resources, following the protocols approved by the Animal Investigation Committee.

All cell lines were maintained *in vitro* in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated cosmic calf serum (Hyclone, Logan, UT), 5% heat-inactivated fetal calf serum (Sigma, St. Louis, MO), 10% NCTC 109 medium (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. All tissue culture reagents were purchased from Invitrogen (Gaithersburg, MD) unless otherwise specified.

D2F2 is a mouse mammary tumor cell line derived from a spontaneous mammary tumor that arose in a BALB/c hyperplastic alveolar nodule line D2 [21]. D2F2 cells were co-transfected with pRSV2/neo and either pCMV/neu, which encodes wild-type rat *neu*, or pCMV/E2, which encodes wild-type Her-2 [3]. Stable clones of D2F2/neu or D2F2/E2 were established and the expression of neu or Her-2 protein on the cell surface was verified by flow cytometry. Transfected cell lines were maintained in medium containing 0.8 mg/ml G418 (Geneticin, Invitrogen). The TUBO line was cloned from a spontaneous mammary tumor in a NeuT female and the cells express neu protein [22]. TUBO cells grow progressively in normal BALB/c mice and give rise to tumors which are histologically similar to those seen in NeuT mice [23].

Antigen presenting cells (APC) 3T3/NKB and 3T3/EKB were generated as previously described [24]. Briefly, BALB/c NIH 3T3 cells were transfected with K^d, B7.1, and Her-2 (EKB) or neu (NKB). Stable clones were selected, and maintained in supplemented DMEM (as above) with the addition of 0.8 mg/ml neomycin and 0.8 mg/ml zeocin. TC-1/E2 and TC-1/neu were generated by transfecting C57BL/6 TC-1 cells (generously provided by Dr. T.C. Wu, The Johns Hopkins University, Baltimore, MD) with pMSCV/puro and pCMV5/neu or pCMV5/E2. Stable clones were selected, and maintained in supplemented DMEM with 7.5 μg/ml puromycin. TC-1 is a tumor cell line derived by transforming lung epithelial cells with human papilloma virus-16 E6, E7 and ras oncogene [25]. Expression of neu or Her-2, K^d or K^b and B7.1 was measured by flow cytometry (Fig. 1E).

2.2. Peptides

Peptide corresponding to K^d restricted, dominant neu epitope, N63 (TYVPANASL) [26] was synthesized by New England Peptide, Inc. (Gardner, MA) and dominant Her-2 epitope, E63 (TYLPTNASL) by Quality Controlled

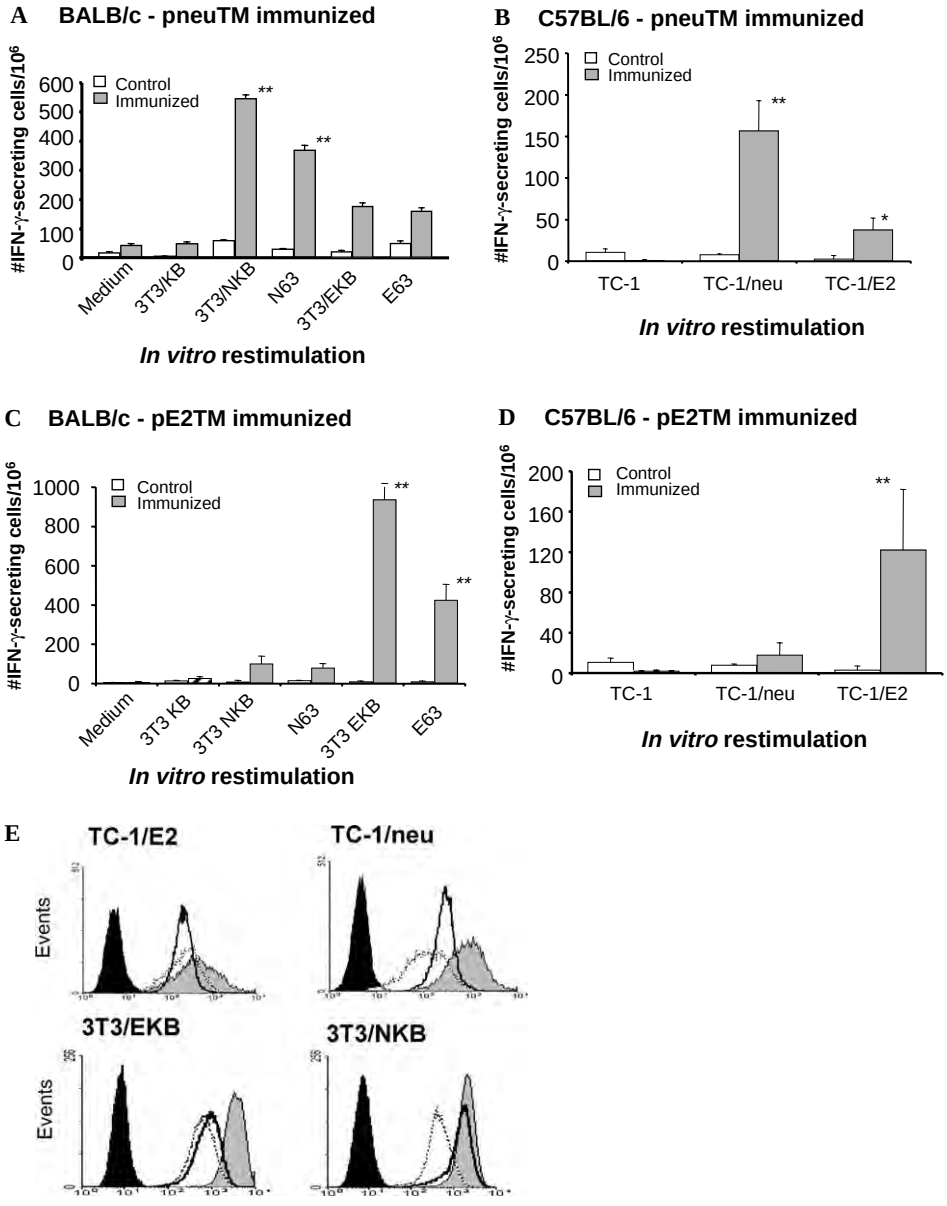

Fig. 1. Immunization of BALB/c or C57BL/6 mice with neu or Her-2 DNA induced cross-reactive T cells. BALB/c mice were electro-vaccinated once with pneuTM (A) or pE2TM (C). C57BL/6 mice were electro-vaccinated twice, with pneuTM (B) or pE2TM (D). Spleen cells from immunized BALB/c mice were cultured with 3T3/NKB, 3T3/EKB, or specific peptides. Peripheral blood cells from immunized C57BL/6 mice were cultured in the presence of TC-1/neu or TC-1/E2 cells. The ratio of spleen cells or peripheral blood cells to APC was 10:1. Peptides were added at 100 μg/ml. The results were expressed as number of spots per 10^6 spleen cells or PBL and analyzed by student's t test. $^{**}p < 0.005$, $^{*}p < 0.05$. (E) Expression of Her-2 or neu (grey shaded), K^b or K^d (dotted line) and B7.1 (black line) on antigen presenting cells was measured by flow cytometry. Isotype control was shown in black shade.

Biochemicals (Hopkinton, MA). Peptides were used for *in vitro* culture at a concentration of 20 μg/ml.

2.3. DNA immunization and tumor challenge

pcDNA 3.1 was purchased from Invitrogen (Carlsbad, CA). Construction of pcDNA-neuTM plasmid encoding the extracellular and transmembrane domains of rat neu was previously described [22]. pE2TM was generated by PCR amplification of full-length Her-2 with a 3′ primer engineered to create a stop codon at the end of the transmembrane domain. This created a fragment of Her-2 which included the extracellular and transmembrane domains. This E2TM fragment was cloned into the pCMV5 vector using HindIII and XbaI. The plasmid pEFBos-GM-CSF encoding murine granulocyte–monocyte colony stimulating factor (GM-CSF) was provided by Dr. N. Nishisaki at Osaka University, Osaka, Japan. Mice were injected in the quadriceps muscle with pEFBos-GM-CSF combined with pE2TM or pneuTM as previously described [3]. In most experiments, DNA injection was followed immediately by square wave electroporation at the injection site using a BTX830 (BTX Harvard Apparatus, Holliston, MA). A tweezer electrode was used to deliver eight pulses at 100 V for 20 ms. In experiments which

monitor spontaneous tumorigenesis, DNA was injected i.m. without electroporation.

In some of the mice, CD8 T cells were depleted by injections of 0.5 mg anti-CD8 mAb (clone 2.43), twice per week for 7 weeks.

To measure tumor growth, mice were challenged subcutaneously with 2×10^5 cells in the flank. Tumor growth was monitored by weekly palpation, and mice were sacrificed when any one dimension of the tumor reached 15 mm. Tumor volume was calculated by $(X^2 \times Y)/2$. X and Y represent the short and long dimension, respectively, of the tumor. Comparison of the survival curves was analyzed by the Log rank test. To measure spontaneous tumorigenesis, the size of individual tumors was measured and the total volume of all tumors in one mouse was combined and presented as a function of age.

2.4. Measurement of anti-neu and anti-Her-2 antibody by flow cytometry

For measurement of anti-neu antibody, 3T3/NKB cells were incubated with serially diluted immune mouse sera as we previously reported [27]. Fluorescein (FITC) or phycoerythrin (PE) conjugated goat-anti-mouse antibody directed to the γ-chain of mouse IgG (Jackson ImmunoResearch, West Grove, PA) were used to detect bound antibody. The c-erbB2/c-neu mAb (Ab4, clone 7.16.4; Calbiochem, San Diego, CA), which recognizes an extracellular domain of neu protein, was used as the positive control (Calbiochem, San Diego, CA). A standard curve was established using serially diluted Ab4 and the concentration of anti-neu antibody in the test sera was calculated by regression analysis as we described [27]. Normal mouse serum or isotype matched mAb was the negative control. Flow cytometric analysis was performed with a FACS Calibur (Becton Dickinson, San Jose, CA). To measure anti-Her-2 antibody, Her-2 overexpressing SKOV3 cells were incubated with immune mouse sera, followed by PE-goat-anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). The standard curve for Her-2 was generated using c-erbB2 mAb (Ab5, clone TA-1; Calbiochem, San Diego, CA) and analyzed as described above. Differences in antibody concentration were analyzed by the student's t test.

2.5. Measurement of T cell response by ELISPOT Assay

Neu and Her-2-reactive T cells were measured by ELISPOT assay. Spleen cells or peripheral blood lymphocytes (PBL) were suspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. $3–4 \times 10^5$ cells were added to each well of a 96-well HTS IP plates (Millipore, Bedford, MA) which were pre-coated with 2.5 μg/ml rat anti-mouse IFN-γ (IgG1, clone R4-6A2) and incubated for 48 h at 37 °C in 5% CO_2. To measure T cell response in BALB/c or NeuT mice, the engineered APCs, 3T3/NKB or 3T3/EKB were also added to the wells. The control 3T3/KB cells expressed H2-K^d and B7.1. To measure T cell response in C57BL/6 or Her-2 Tg mice, TC-1/E2 or TC-1/neu cells were added as APC. TC-1 cells were used as control. The ratio of spleen cells to APC was 10:1. Following a 48 h incubation, cells were removed and 2 μg/ml biotinylated rat anti-mouse-IFN-γ (IgG1, clone XMG 1.2) was added. All antibodies were purchased from BD/Pharmingen. Plates were incubated for another 12 h at 4 °C, then washed to remove unbound antibody. Bound antibody was detected by incubating the plates with 0.9 μg/ml avidin-horseradish peroxidase (Pharmingen) for 2 h at room temperature. The substrate 3-amino-9-ethylcarbazole (AEC; Pharmingen) in 0.1 M acetic acid and 0.003% hydrogen peroxide was added and the plate was incubated for 3–5 min. AEC solution was discarded and the plates were washed 6 times with water. The visualized cytokine spots were enumerated with the ImmunoSpot analyzer (CTL, Cleveland, OH) and the results expressed as number of cytokine producing cells per 10^6 spleen cells. Data were analyzed using the student's t test

3. Results

3.1. Neu and Her-2 cross-reactive T cells are induced by DNA immunization

To measure the immune cross-reactivity between Her-2 and neu, BALB/c and C57BL/6 mice were immunized 2 times with 50 μg of plasmid DNA encoding the extracellular and transmembrane domains of Her-2 (pE2TM) or neu (pneuTM) in combination with 50 μg of pGM-CSF. T cell response was measured 1–2 weeks following the last immunization using IFN-γ ELISPOT assay. In BALB/c mice, splenocytes were pooled from three mice, while in C57BL/c mice, peripheral blood lymphocytes were pooled from 3 to 6 mice. Anti-Her-2/neu T cell reactivity in splenocytes is comparable or somewhat higher than that in PBL of the same mice(unpublished results). Neu-reactive T cell response was measured after incubation with the 3T3/NKB (BALB/c background) or TC-1/neu cells (C57BL/6 background). Her-2 reactive T cells were measured after incubation with 3T3/EKB or TC-1/E2 cells. Expression of Her-2 or neu, K^d or K^b and B7.1 by the APC was demonstrated by flow cytometry (Fig. 1E). In BALB/c mice, 546 ± 40 and 370 ± 53 IFN-γ-secreting cells per million were detected after *in vitro* stimulation with 3T3/NKB cells and neu peptide N63 [26], respectively, demonstrating significant T cell response to the neu protein and that N63 was the dominant epitope (Fig. 1A). When cultured with 3T3/EKB or Her-2 peptide, E63, there were 160 ± 37 and 146 ± 32 responding cells, respectively, indicating significant cross-reactivity of anti-neu T cells with Her-2 epitopes (Fig. 1A). Control 3T3/KB cells which expressed only K^d and CD80, did not activate T cells. Therefore, in wild type BALB/c mice, T cells activated by pneuTM cross-reacted with Her-2 epitopes. In C57BL/6 mice immunized with pneuTM, there was a similar trend, where immunization resulted in 157 ± 36 neu-responsive cells and 38 ± 14 Her-2-responsive cells,

indicating cross-reactivity of anti-neu T cells with Her-2 epitopes (Fig. 1B).

In the converse experiment, BALB/c or C57BL/6 mice were immunized with pE2TM (Figs. 1C and D). After a 48 h incubation with APC or peptides, over 900 spots per million cells were detected when immune splenocytes were stimulated with 3T3/EKB (938 ± 81 cells) (Fig. 1C). Immune splenocytes also recognized Her-2 peptide E63 (426 ± 80 cells). Significant, but greatly reduced, response at 111 ± 60 and 33 ± 20 spots were detected when splenocytes were stimulated with either 3T3/NKB or N63, respectively (Fig. 1C), showing cross-reactivity. PBL from C57BL/6 mice immunized with pE2TM were re-stimulated with TC-1/E2 or TC-1/neu and showed significant anti-Her-2 response at 122 ± 60 cells per million with a low level anti-neu response at 18 ± 12 cells per million (Fig. 1D). Therefore, in BALB/c and C57BL/6 mice, where neu and Her-2 are foreign antigens, high levels of T cell response were generated to cognate antigens with cross-reactivity to the non-cognate antigens.

3.2. Antibodies induced by Her-2 and neu DNA recognized only cognate antigen

To determine whether Her-2/neu immunization also generated cross-reactive antibodies, sera were collected 2 weeks after the last immunization. Neu and Her-2 binding antibodies were measured by flow cytometry, as we previously described [27]. Although high levels of antibodies to the cognate neu (64 ± 15 μg/ml) and Her-2 (118 ± 48 μg/ml) were detected in BALB/c mice, no cross-reactivity was detected (Fig. 2A). When sera from immunized C57BL/6 mice were analyzed (Fig. 2B), the same specificity was observed where antibodies only recognized cognate antigen, showing that DNA vaccine induced only specific non-cross-reactive antibody.

3.3. Immunization with pE2TM or pneuTM protected BALB/c mice from tumors expressing either cognate or non-cognate antigen

To assess the level of protection from tumor growth, BALB/c mice were immunized twice with either pE2TM or pneuTM, admixed with pGM-CSF, and challenged with D2F2 tumor cells expressing either Her-2 (D2F2/E2) or neu (D2F2/neu). In mice immunized with cognate DNA (immunized with pneuTM and challenged with D2F2/neu, or immunized with pE2TM and challenged with D2F2/E2), all tumors were rejected (Fig. 3). Also, 4 of 7 mice immunized with pE2TM rejected D2F2/neu tumors (57%; Fig. 3A) and 5 of 6 mice immunized with pneuTM rejected D2F2/E2 tumors (83%; Fig. 3B). Since immunization with pneuTM and pE2TM induced cross-reactive T cells, but not antibodies, rejection of tumor cells transfected with non-cognate

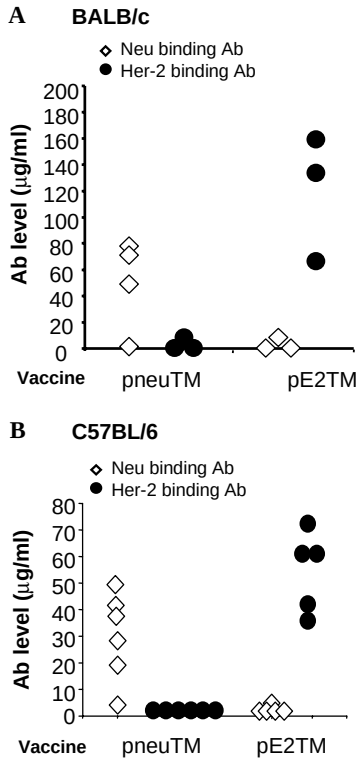

Fig. 2. Antibodies induced by pneuTM or pE2TM immunization in BALB/c (A) and C57BL/6 (B) mice did not cross-react. Mice were electrovaccinated twice, two weeks apart, with either pE2TM or pneuTM admixed with pGM-CSF. Sera were collected 2 week after the final immunization. Antibody binding to Her-2 or neu was measured by flow cytometry and antibody concentration was calculated by regression analysis as described in Section 2.

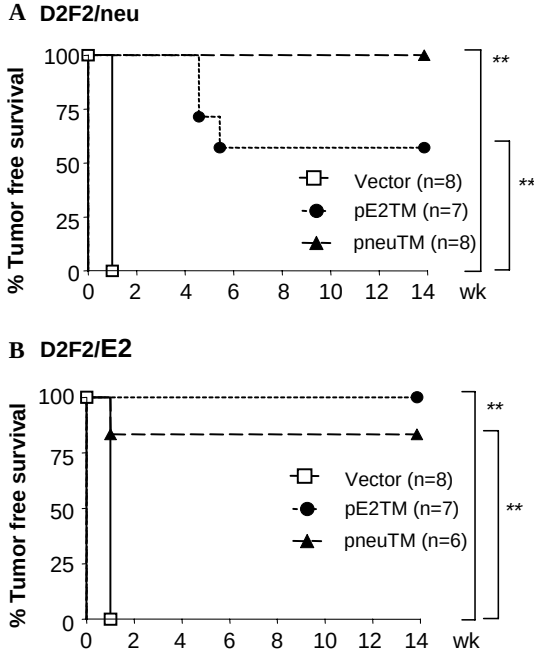

Fig. 3. Inhibition of tumor growth in BALB/c mice by vaccination with pE2TM or pneuTM. Mice were immunized twice i.m. 2 weeks apart with either pE2TM or pneuTM admixed with pGM-CSF. At 2 weeks after the final immunization, mice were challenged with D2F2/neu (A) or D2F2/E2 (B). Tumor growth was monitored by weekly palpation and the results expressed as % tumor free mice. There were 6–8 mice in each group. Results were analyzed by Log rank test, $^{**}p < 0.005$.

neu or Her-2 may be mediated primarily by cross-reactive T cells. This is consistent with our previous report that rejection of transfected D2F2 tumor was mediated primarily by T cells [28,29].

When mice were sacrificed at week 14, after they rejected tumors, sera were collected and the antibody profiles were measured again (Fig. 4). Mice immunized with pneuTM and rejected D2F2/neu produced antibodies to neu, which recognized Her-2 either weakly or not at all (Fig. 4A). Mice which rejected D2F2/E2 after pE2TM immunization produced antibody to Her-2 which did not cross-react with neu (Fig. 4B). Therefore, exposure to Her-2 or neu presented as DNA vaccine or tumor resulted in little or no cross- reactive antibody. When mice immunized with neu DNA were challenged with D2F2/E2, anti-Her-2 antibodies were induced by the challenging tumor (Fig. 4A). Similarly, mice immunized with Her-2 DNA and challenged with D2F2/neu produced low-level anti-neu antibody (Fig. 4B). Therefore, exposure to tumor cells was sufficient to activate humoral responses, which may contribute in part to tumor rejection in mice immunized with non-cognate antigen (Fig. 3).

3.4. Vaccination of Her-2 or neu transgenic mice with self or heterologous DNA

We tested whether heterologous DNA immunization can activate anti-neu or Her-2 in mice which express either neu or Her-2 transgene. NeuT mice were subjected to four rounds of electro-vaccination using self pneuTM in combination with pGM-CSF. Splenocytes were prepared 2 weeks after the final immunization. IFN-γ-producing cells measured after incubation with 3T3/NKB ranged from 220 to 407 per million (Fig. 5A), demonstrating the feasibility of inducing significant T cell response by DNA vaccination encoding the self antigen. Although anti-neu T cells were induced, T cells which recognized the dominant epitope, N63, were not found, indicating deletion of N63 reactive T cells during thymic selection (Fig. 5A). Unlike in BALB/c mice, neu reactive T cells did not recognize Her-2, suggesting a reduced T cell repertoire in NeuT mice.

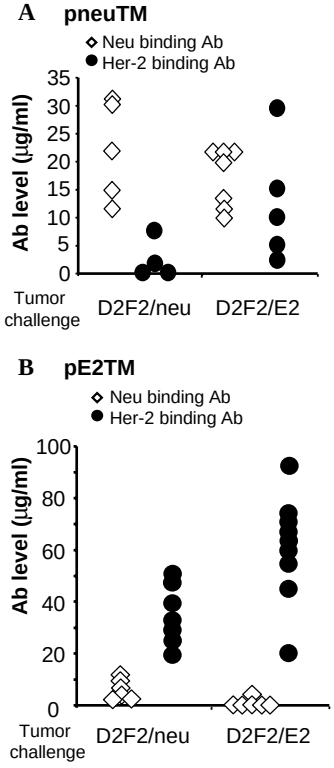

Fig. 4. Antibodies induced by DNA immunization and tumor rejection. Sera were collected from mice which were immunized with pneuTM (A) or pE2TM (B) and later rejected D2F2 tumors expressing either Her-2 or neu as described in Fig. 3 legend. Neu and Her-2 binding antibodies were measured as described in Section 2.

When immunized with heterologous pE2TM, splenocytes reacted strongly to 3T3/EKB or E63, as expected (Fig. 5B). In addition, significant cross-reactivity to neu was evident by the detection of 400–900 per million IFN-γ-producing neu-reactive cells following stimulation with 3T3/NKB (Fig. 5B). One of the four mice recognized N63, in addition to E63. Thus, immunization of NeuT mice with pE2TM encoding the heterologous Her-2 induced significant neu reactive T cells. Immunization with self or heterologous vaccine was also tested in Her-2 Tg mice, which are tolerant to Her-2. In these mice, 4 times immunization with Her-2 (pE2TM) did not result in any Her-2 nor neu reactive T cells when measured by ELISPOT following stimulation with TC-1/E2 (Fig. 5D), demonstrating profound tolerance to Her-2. When Her-2 Tg mice were immunized with pneuTM, 134 ± 99 IFN-γ-secreting neu-reactive T cells were generated. However, they did not recognize Her-2 (Fig. 5C), thus heterologous neu DNA vaccination was not sufficient to induce anti-Her-2 response in Her-2 Tg mice.

Therefore, repeated immunization with autologous DNA in NeuT, but not Her-2 Tg mice induced specific T cell response, indicating a weaker reactivity to Her-2 in Her-2 Tg mice. Consistent with the milder T cell tolerance in NeuT mice, T cell response to neu was also induced by both autologous and heterologous Her-2 DNA vaccination.

3.5. Antibodies to Her-2 and neu recognized only cognate antigen in transgenic mice

To determine whether Her-2 or neu tolerant mice generated cross-reactive antibodies upon vaccination, immune sera in NeuT and Her-2 Tg mice were analyzed. In NeuT mice, two of three mice produced anti-neu antibodies following three-time immunizations with pneuTM which did not bind Her-2 (Fig. 6A). Anti-neu antibody level (0–25 μg/ml) was lower than that induced by a single immunization in BALB/c mice (40–80 μg/ml), consistent with the moderate tolerant state of NeuT mice. Immunization with pE2TM encoding heterologous Her-2 induced over 60 μg/ml anti-Her-2 antibodies, but they did not cross-react with neu (Fig. 6A). Therefore, in both BALB/c and NeuT mice, antibodies induced by DNA vaccination only recognized cognate antigen. In Her-2 Tg mice, immunization with

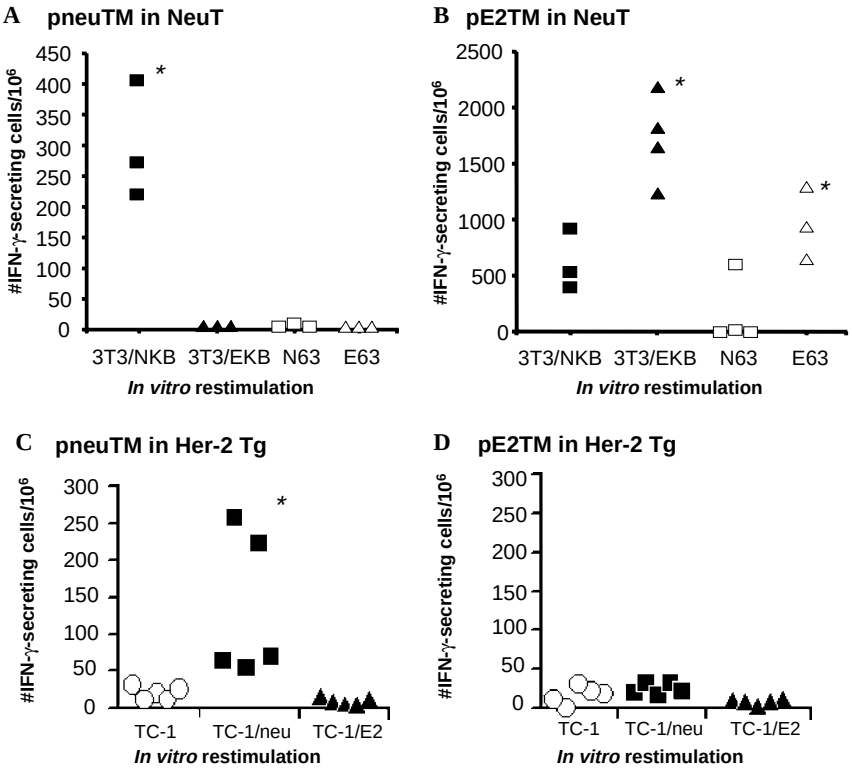

Fig. 5. T cell response in Her-2 or neu transgenic mice immunized with either heterologous or self DNA. NeuT mice were electro-vaccinated 4 times with self pneuTM (A) or heterologous pE2TM (B) DNA and pGM-CSF. Spleen cells were prepared 2 weeks after the final electro-vaccination and cultured with 3T3/NKB (filled squares) or 3T3/EKB (filled triangles) Cels, and N63 (open squares) or E63 (open triangles) peptides before analysis of IFN-γ production by ELISPOT assay. Her-2 Tg mice were electro-vaccinated 4 times with heterologous pneuTM (C) or self pE2TM (D). Spleen cells were prepared 10 days after the 4th vaccination and cultured in the presence of TC-1 (open circles), TC-1/neu (filled squares) or TC-1/E2 (filled triangles) cells. Results were analyzed by student's t test, $^{*}p < 0.01$.

pE2TM encoding self Her-2 resulted in very low-levels of Her-2 antibodies (2 ± 2 μg/ml; Fig. 6B) and immunization with pneuTM resulted in 51 ± 8 μg/ml anti-neu antibody, which did not recognize Her-2 (Fig. 6B), demonstrating that antibodies induced by DNA vaccines in wild type or Her-2/neu transgenic mice were specific to the cognate antigen, without detectable cross-reactivity.

3.6. Spontaneous mammary tumor development was delayed by self pneuTM, but not heterologous pE2TM immunization

NeuT female mice develop spontaneous mammary tumors between 17 and 21 weeks of age, with 10 tumors arising in a single mouse. To determine the effect of DNA immunization on spontaneous tumorigenesis, NeuT females were injected i.m. with either pneuTM or pE2TM admixed with pGM-CSF, without electroporation, when they were 6, 8, 10 and 12 weeks of age. The size of individual tumors was measured with a caliper and the total volume of all tumors in one mouse was calculated and presented as a function of age (Fig. 7). All mice injected with vector DNA developed spontaneous tumors before 120 days of age. Immunization with pneuTM significantly delayed spontaneous tumor formation, and 9 of 10 mice did not have palpable tumors until 150–280 days of age ($p = 0.0001$, Fig. 7B). Once the tumors appeared, the growth rate was comparable to those of control mice. In the six mice immunized with pE2TM, spontaneous tumors arose between 120 and 140 day of age, not significantly different than in mice receiving control vector. Therefore, self pneuTM, but not heterologous pE2TM immunization delayed spontaneous tumorigenesis in NeuT mice.

It has been suggested that antibody to neu is critical in the control of spontaneous tumorigenesis [30]. Sera were collected on day 147 or about 7 weeks after the last immunization. Anti-neu antibody was detected in 7 of 10 pneuTM immunized mice, but not vector or pE2TM immunized mice (Fig. 8). The antibody levels were plotted against the number of days required for the total tumor volume to reach 600 mm^3. pE2TM immunized NeuT mice did not produce anti-neu antibodies and all mice developed tumors before they were 120 days old. In pneuTM immunized mice, development of anti-neu antibody was associated with a delay in tumor formation (Fig. 8).

Immunization of NeuT mice with pE2TM induced neu reactive T cells (Fig. 5B), but did not protect against tumorigenesis (Fig. 7C). To test if T cells contribute to tumor growth inhibition, NeuT female mice immunized at 6, 8, 10, and 12 weeks of age with pneuTM were depleted of CD8 T cells between 13 and 20 weeks of age by specific mAb every

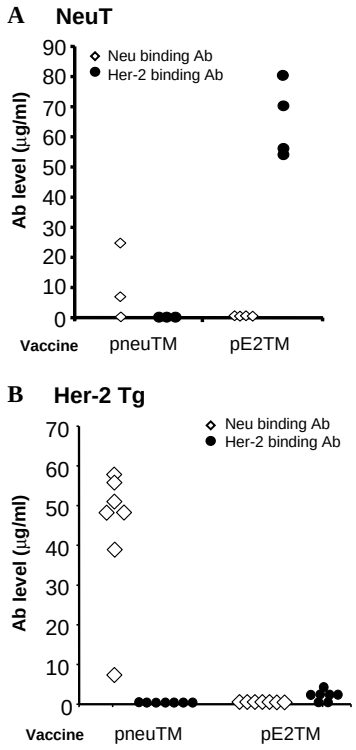

Fig. 6. Antibodies induced by DNA immunization in NeuT (A) and Her-2 Tg (B) mice did not cross-react. Mice were electro-vaccinated 3–4 times, two weeks apart, with either pE2TM or pneuTM, admixed with pGM-CSF. Sera were collected 2 weeks after the final immunization. Antibody binding to Her-2 or neu was measured as described in Section 2.

3–4 days. Spontaneous tumorigenesis was delayed in CD8 T cell depleted and vaccinated mice (Fig. 9A) when compared with untreated mice (Fig. 7A), but was similar to that in immunized mice with intact T cells (Fig. 7B). When analyzed at 18 weeks of age, the majority of mice immunized with pneuTM and depleted of CD8 T cells maintained anti-neu antibodies (Fig. 9B). These results support the role of vaccine-induced antibodies in suppressing spontaneous tumors in NeuT mice.

4. Discussion

When compared side-by-side, Her-2 Tg mice were much less responsive to Her-2 than NeuT mice to neu as measured by their humoral and T cell responses. Although the C57BL/6 background of Her-2 Tg mice and BALB/c background of NeuT mice may contribute to their reactivity, the difference in their antigen load warrants consideration. In NeuT mice, the antigen neu is continuously produced by the 10 mammary glands undergoing tumor progression. This rich reservoir of neu is a constant *in situ* antigen boost once immune reactivity is triggered by the active vaccination. In Her-2 Tg mice, a modest level of recombinant Her-2 is expressed in the cerebellum and the pregnant or lactating mammary glands with little expression in the virgin glands [10]. These mice do not develop tumors, thus they do not experience the same level of Her-2/neu antigen as in

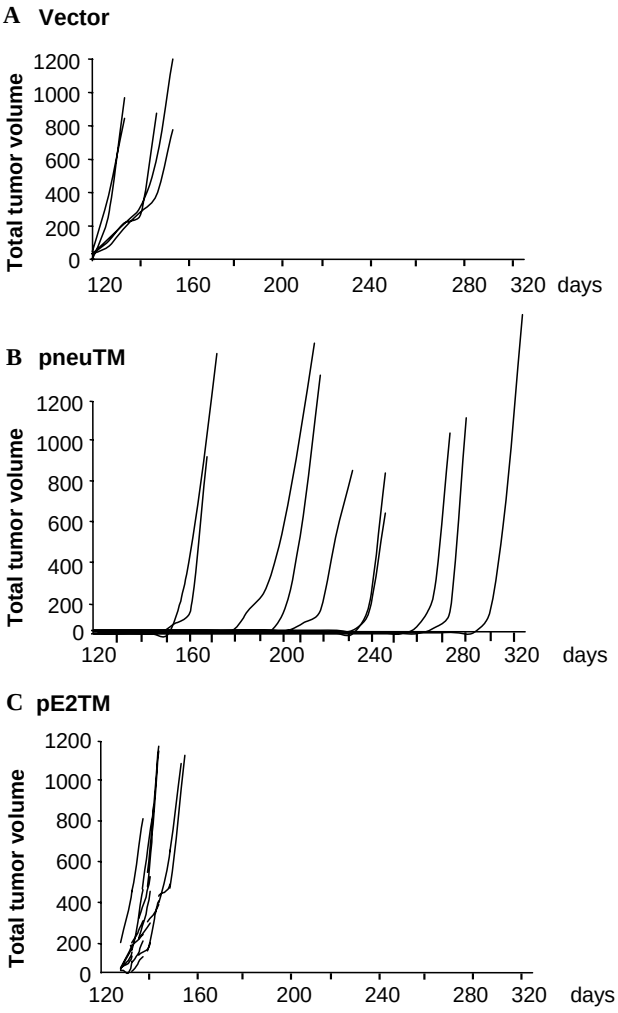

Fig. 7. Development of spontaneous tumors in NeuT female mice after immunization with pE2TM or pneuTM. Virgin females were immunized i.m. 4 times, 2 weeks apart, with pGM-CSF and control vector (A), pneuTM (B), or pE2TM (C), starting at 6 weeks of age. There were up to 10 spontaneous mammary tumors developing in each mouse. Each tumor was measured and total tumor volume of each mouse was calculated and plotted against their age in days.

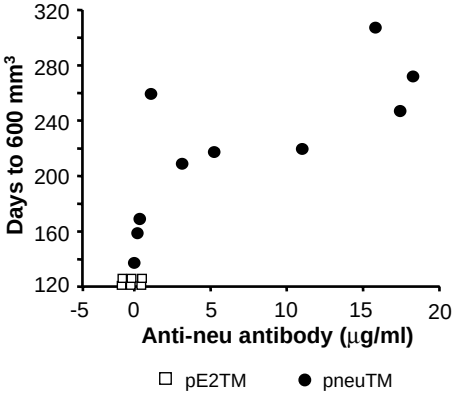

Fig. 8. Anti-neu antibody and spontaneous tumorigenesis in NeuT female mice. Anti-neu antibody levels in pneuTM (filled circles) or pE2TM (open squares) immunized mice were plotted against age in days when a total tumor volume of 600 mm^3 was reached.

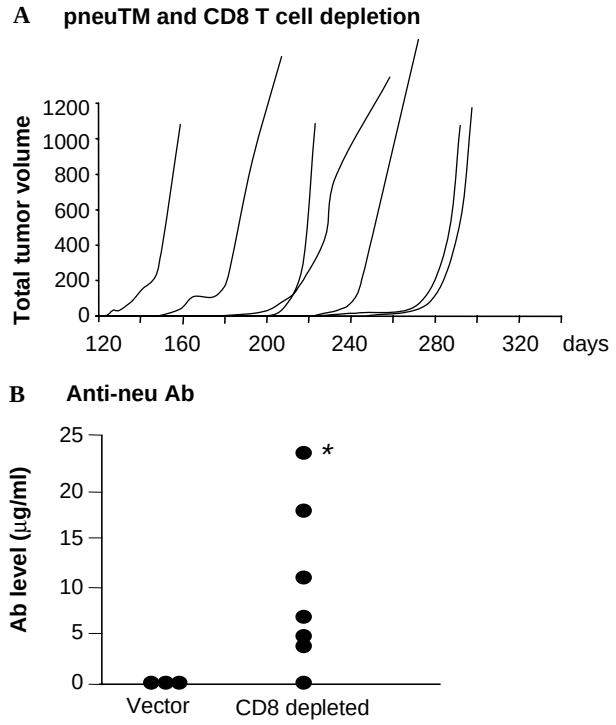

Fig. 9. Depletion of CD8 T cells following immunization with pneuTM did not abolish delay of spontaneous tumorigenesis in NeuT mice. Female NeuT mice were immunized at 6, 8, 10, and 12 weeks of age and depleted of CD8 T cells (A) by anti-CD8 antibodies every 3–4 days, between 13 and 20 weeks of age, as described in Section 2. Growth of spontaneous tumors was also measured as described in Section 2. (B) Antibody to neu was measured by flow cytometry. Vector immunized mice were the controls. The results were analyzed by student's t test, $^{*}p < 0.05$.

NeuT mice. Breast cancer patients in disease remission would not experience the level of Her-2 stimulation as in NeuT mice either. Therefore, the tolerant state in Her-2 Tg mice may mimic that in patients. NeuT mice with their spontaneous tumorigenesis are valuable for analyzing the role of humoral versus cellular immunity in neu-induced tumor progression.

When comparing immune reactivity to self versus heterologous DNA vaccines, Her-2 Tg mice showed weak to no anti-Her-2 response after self or heterologous DNA vaccination, consistent with their profound tolerance to this antigen. By depleting regulatory T cells before vaccination, a significant level of anti-Her-2 antibodies can be achieved with autologous vaccines (our unpublished results). In NeuT mice, both neu and Her-2 DNA induced anti-neu T cells, but this was not essential to tumor suppression because depletion of CD8 T cells did not change the delay in tumorigenesis although T cells may be effective in suppressing tumorigenesis if induced at higher level. Anti-neu antibodies induced by self neu but not heterologous Her-2 DNA is associated with a delay in spontaneous tumorigenesis. These findings are consistent with the efficacy of anti-Her-2 monoclonal antibodies observed in the clinics. Induction of anti-Her-2 antibodies by the autologous, rather than heterologous Her-2/neu and the efficacy of antibodies in suppressing tumorigenesis should be considered when developing clinical Her-2 vaccines.

The primary role of anti-neu antibody in NeuT spontaneous tumorigenesis was also observed by Park et al. who immunized NeuT mice with an adenoviral vector encoding neu [30]. In their study, anti-neu antibodies were induced by the adenovial vaccine encoding autologous neu and a significant delay in spontaneous tumorigenesis was observed. Using B cell deficient mice, immunization with the adenoviral vaccine was not protective against a neu^{+} tumor, supporting anti-neu antibody as the primary effector in neu-induced tumors.

BALB/c mice immunized with pneuTM exhibited cross-reactive T cells which recognized both Her-2 and neu and immunized mice rejected D2F2 tumors transfected with either Her-2 or neu. In NeuT mice, pneuTM immunization induced T cells reactive to 3T3/NKB but not the dominant neu epitope, N63 which was identified in BALB/c mice, indicating a reduced T cell repertoire (Fig. 5A). With this limited repertoire, cross-reactivity to Her-2 was also lost. Consistent with this finding, Reilly et al. reported the recognition of sub-dominant, rather than dominant epitopes by the immunized FVB *neuN* transgenic mice [31]. Bos et al. also described the recognition of sub-dominant CEA epitopes in CEA transgenic mice as a result of CEA expression in thymic epithelial cells [32]. Interestingly, immunization of NeuT mice with a distant heterologous Her-2 DNA induced T cells which recognized not only Her-2, but also neu antigen (Fig. 5B). Perhaps a broader and more potent activation of the residual neu reactive T cells can be induced by the distant Her-2 homologue [33], resulting in cross-reactivity to neu, while reactivity induced with self-neu is blunted with no demonstrable cross-reactivity to Her-2.

There have been a number of one-sided studies on the vaccination efficacy of heterologous Her-2 vaccines in neu transgenic mice. Immunization of NeuT mice with an adenoviral vector encoding Her-2 induced low level cross-reactive antibodies to neu and delayed tumor formation [26]. The adenoviral vector may have functioned as an adjuvant to induce a broader spectrum of antibodies than naked DNA, thus rendering an anti-tumor effect. T cell response to the foreign Her-2 was observed as expected, but the reactivity to neu was not shown and its effect on tumorigenesis could not be evaluated. In FVB *neuN* mice, immunization with Her-2 DNA every month for 10 months delayed spontaneous tumorigenesis, reduced tumor foci, and reduced tumor positive glands [13]. T cell response to Her-2 or neu was not measured either. Ten time DNA vaccinations may induce some T cell responses to exert anti-tumor effect, but this can only be speculated. Recently, Tegerstedt et al. [34] reported that when NeuT mice were immunized with a single dose of virus-like particle coated with heterologous Her-2, a significant number of T cells were generated to the foreign Her-2 antigen. Spontaneous tumorigenesis was delayed up to a year in 80% of NeuT mice. Since the immune response to neu was not measured either, the

mechanism was difficult to assess. Taking into consideration our data and these reported results, we suggest that T cell response can be induced by either self or heterologous vaccine, but the level necessary for suppressing spontaneous tumorigenesis may be very high. On the other hand, spontaneous tumors are suppressed by antibodies which can be induced by autologous DNA vaccines. The exquisite sensitivity of NeuT tumors to anti-neu antibodies may reflect their dependence on neu-mediated signals for their survival and proliferation.

Immunization with heterologous antigen has been effective in the treatment of experimental melanoma in mice, using human gp100 or TRP-2 as the vaccine [19,20]. The efficacy of self or heterologous antigen as a cancer vaccine will be determined by the immunogenicity of the self vaccine and the sensitivity of tumor cells to humoral or T cell response. With Her-2 antigen, induction of antibodies is highly advantageous and achievable, whereas antibodies to intracellular antigens, such as gp100 or TRP-2 will not be effective. T cells may be effective against either intracellular or membrane bound antigens and induction of sufficient level of T cell response in highly tolerant hosts remains a challenge.

The different tolerance status of Her-2 Tg and NeuT mice should be considered when using them in Her-2 immunotherapy studies. The design of clinical Her-2 vaccines should take into consideration that antibody response may be effective and is induced more effectively by autologous than heterologous DNA. T cell response will be required for controlling antibody resistant tumors and continues to be an important goal in cancer vaccine development.

Acknowledgments

The authors wish to thank Andi Cani for his excellent technical assistance and Bonnie Jewell and Serene Lane for their expert care of the experimental animals.

References

[1] D.J. Slamon, W. Godolphin, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Levin, S.G. Stuart, J. Udove, A. Ullrich, Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer, Science 244 (1989) 707–712.

[2] S. Sjogren, M. Inganas, A. Lindgren, L. Holmberg, J. Bergh, Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers, J. Clin. Oncol. 16 (1998) 462–469.

[3] W.Z. Wei, W.P. Shi, A. Galy, D. Lichlyter, S. Hernandez, B. Groner, L. Heilbrun, R.F. Jones, Protection against mammary tumor growth by vaccination with full-length, modified human ErbB-2 DNA, Int. J. Cancer 81 (1999) 748–754.

[4] R. Kiessling, W.Z. Wei, F. Herrmann, J.A. Lindencrona, A. Choudhury, K. Kono, B. Seliger, Cellular immunity to the Her-2/neu protooncogene, Adv. Cancer Res. 85 (2002) 101–144.

[5] M.L. Disis, T.A. Gooley, K. Rinn, D. Davis, M. Piepkorn, M.A. Cheever, K.L. Knutson, K. Schiffman, Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines, J. Clin. Oncol. 20 (2002) 2624–2632.

[6] D.G. McNeel, K.L. Knutson, K. Schiffman, D.R. Davis, D. Caron, M.L. Disis, Pilot study of an HLA-A2 peptide vaccine using flt3 ligand as a systemic vaccine adjuvant, J. Clin. Immunol. 23 (2003) 62–72.

[7] S. Murata, B.H. Ladle, P.S. Kim, E.R. Lutz, M.E. Wolpoe, S.E. Ivie, H.M. Smith, T.D. Armstrong, L.A. Emens, E.M. Jaffee, R.T. Reilly, OX40 costimulation synergizes with GM-CSF whole-cell vaccination to overcome established $CD8^+$ T cell tolerance to an endogenous tumor antigen, J. Immunol. 176 (2006) 974–983.

[8] J.A. Thompson, S.K. Dissanayake, B.R. Ksander, K.L. Knutson, M.L. Disis, S. Ostrand-Rosenberg, Tumor cells transduced with the MHC class II Transactivator and CD80 activate tumor-specific $CD4^+$ T cells whether or not they are silenced for invariant chain, Cancer Res. 66 (2006) 1147–1154.

[9] C. Cuadros, A.L. Dominguez, P.L. Lollini, M. Croft, R.S. Mittler, P. Borgstrom, J. Lustgarten, Vaccination with dendritic cells pulsed with apoptotic tumors in combination with anti-OX40 and anti-4-1BB monoclonal antibodies induces T cell-mediated protective immunity in Her-2/neu transgenic mice, Int. J. Cancer 116 (2005) 934–943.

[10] M.P. Piechocki, Y.S. Ho, S. Pilon, W.Z. Wei, Human ErbB-2 (Her-2) transgenic mice: a model system for testing Her-2 based vaccines, J. Immunol. 171 (2003) 5787–5794.

[11] F. Lucchini, M.G. Sacco, N. Hu, A. Villa, J. Brown, L. Cesano, L. Mangiarini, G. Rindi, S. Kindl, F. Sessa, Early and multifocal tumors in breast, salivary, harderian and epididymal tissues developed in MMTV-Neu transgenic mice, Cancer Lett. 64 (1992) 203–209.

[12] K. Boggio, G. Nicoletti, C.E. Di, F. Cavallo, L. Landuzzi, C. Melani, M. Giovarelli, I. Rossi, P. Nanni, G.C. De, P. Bouchard, S. Wolf, A. Modesti, P. Musiani, P.L. Lollini, M.P. Colombo, G. Forni, Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice, J. Exp. Med. 188 (1998) 589–596.

[13] S.M. Pupa, M. Iezzi, C.E. Di, A. Invernizzi, F. Cavallo, R. Meazza, A. Comes, S. Ferrini, P. Musiani, S. Menard, Inhibition of mammary carcinoma development in HER-2/neu transgenic mice through induction of autoimmunity by xenogeneic DNA vaccination, Cancer Res. 65 (2005) 1071–1078.

[14] M.B. Oldstone, Molecular mimicry, microbial infection, and autoimmune disease: evolution of the concept, Curr. Top. Microbiol. Immunol. 296 (2005) 1–17.

[15] S.D. Miller, W.J. Karpus, The immunopathogenesis and regulation of T-cell-mediated demyelinating diseases, Immunol. Today 15 (1994) 356–361.

[16] R.H. Swanborg, J.A. Stepaniak, Experimental autoimmune encephalomyelitis in the rat, in: J.E. Coligan, A.M. Kruisbeek, D.H. Margulies (Eds.), Current Protocols in Immunology, John Wiley, New York, 1996, pp. 15.2.1–15.2.14.

[17] L.K. Myers, E.F. Rosloniec, M.A. Cremer, A.H. Kang, Collagen-induced arthritis, an animal model of autoimmunity, Life Sci. 61 (1997) 1861–1878.

[18] P. Christadoss, M. Poussin, C. Deng, Animal models of myasthenia gravis, Clin. Immunol. 94 (2000) 75–87.

[19] W.B. Bowne, R. Srinivasan, J.D. Wolchok, W.G. Hawkins, N.E. Blachere, R. Dyall, J.J. Lewis, A.N. Houghton, Coupling and uncoupling of tumor immunity and autoimmunity, J. Exp. Med. 190 (1999) 1717–1722.

[20] J.S. Gold, C.R. Ferrone, J.A. Guevara-Patino, W.G. Hawkins, R. Dyall, M.E. Engelhorn, J.D. Wolchok, J.J. Lewis, A.N. Houghton, A single heteroclitic epitope determines cancer immunity after xenogeneic DNA immunization against a tumor differentiation antigen, J. Immunol. 170 (2003) 5188–5194.

[21] K.H. Mahoney, B.E. Miller, G.H. Heppner, FACS quantitation of leucine aminopeptidase and acid phosphatase on tumor-associated macrophages from metastatic and nonmetastatic mouse mammary tumors, J. Leukoc. Biol. 38 (1985) 573–585.

[22] S. Rovero, A. Amici, E.D. Carlo, R. Bei, P. Nanni, E. Quaglino, P. Porcedda, K. Boggio, A. Smorlesi, P.L. Lollini, L. Landuzzi, M.P. Colombo, M. Giovarelli, P. Musiani, G. Forni, DNA vaccination against rat her-2/Neu p185 more effectively inhibits carcinogenesis

than transplantable carcinomas in transgenic BALB/c mice, J. Immunol. 165 (2000) 5133–5142.
[23] P. Nanni, S.M. Pupa, G. Nicoletti, G.C. De, L. Landuzzi, I. Rossi, A. Astolfi, C. Ricci, V.R. De, A.M. Invernizzi, C.E. Di, P. Musiani, G. Forni, S. Menard, P.L. Lollini, p185(neu) protein is required for tumor and anchorage-independent growth, not for cell proliferation of transgenic mammary carcinoma, Int. J. Cancer 87 (2000) 186–194.
[24] W.Z. Wei, J.B. Jacob, J.F. Zielinski, J.C. Flynn, K.D. Shim, G. Alsharabi, A.A. Giraldo, Y.C. Kong, Concurrent induction of antitumor immunity and autoimmune thyroiditis in $CD4^+$ $CD25^+$ regulatory T cell-depleted mice, Cancer Res. 65 (2005) 8471–8478.
[25] K.Y. Lin, F.G. Guarnieri, K.F. Staveley-O'Carroll, H.I. Levitsky, J.T. August, D.M. Pardoll, T.C. Wu, Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen, Cancer Res. 56 (1996) 21–26.
[26] P. Gallo, S. Dharmapuri, M. Nuzzo, D. Maldini, M. Iezzi, F. Cavallo, P. Musiani, G. Forni, P. Monaci, Xenogeneic immunization in mice using HER2 DNA delivered by an adenoviral vector, Int. J. Cancer 113 (2005) 67–77.
[27] M.P. Piechocki, S.A. Pilon, W.Z. Wei, Quantitative measurement of anti-ErbB-2 antibody by flow cytometry and ELISA, J. Immunol. Methods 259 (2002) 33–42.
[28] M.P. Piechocki, S.A. Pilon, W.Z. Wei, Complementary antitumor immunity induced by plasmid DNA encoding secreted and cytoplasmic human ErbB-2, J. Immunol. 167 (2001) 3367–3374.
[29] S.A. Pilon, C. Kelly, W.Z. Wei, Broadening of epitope recognition during immune rejection of ErbB-2-positive tumor prevents growth of ErbB-2-negative tumor, J. Immunol. 170 (2003) 1202–1208.
[30] J.M. Park, M. Terabe, Y. Sakai, J. Munasinghe, G. Forni, J.C. Morris, J.A. Berzofsky, Early role of $CD4^+$ Th1 cells and antibodies in HER-2 adenovirus vaccine protection against autochthonous mammary carcinomas, J. Immunol. 174 (2005) 4228–4236.
[31] R.T. Reilly, M.B. Gottlieb, A.M. Ercolini, J.P. Machiels, C.E. Kane, F.I. Okoye, W.J. Muller, K.H. Dixon, E.M. Jaffee, HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice, Cancer Res. 60 (2000) 3569–3576.
[32] R. Bos, D.S. van, H.T. van, P. Kaaijk, R. Taubert, B. Kyewski, L. Klein, C.J. Melief, R. Offringa, Expression of a natural tumor antigen by thymic epithelial cells impairs the tumor-protective $CD4^+$ T-cell repertoire, Cancer Res. 65 (2005) 6443–6449.
[33] Y. Nagata, R. Furugen, A. Hiasa, H. Ikeda, N. Ohta, K. Furukawa, H. Nakamura, K. Furukawa, T. Kanematsu, H. Shiku, Peptides derived from a wild-type murine proto-oncogene c-erbB-2/HER2/neu can induce CTL and tumor suppression in syngeneic hosts, J. Immunol. 159 (1997) 1336–1343.
[34] K. Tegerstedt, J.A. Lindencrona, C. Curcio, K. Andreasson, C. Tullus, G. Forni, T. Dalianis, R. Kiessling, T. Ramqvist, A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors, Cancer Res. 65 (2005) 5953–5957.

Research Article

Control of Her-2 Tumor Immunity and Thyroid Autoimmunity by MHC and Regulatory T Cells

Jennifer B. Jacob,[1,2] Yi-chi M. Kong,[1,2] Chady Meroueh,[4] Daniel P. Snower,[4] Chella S. David,[5] Ye-Shih Ho,[3] and Wei-Zen Wei[1,2]

[1]Karmanos Cancer Institute, [2]Department of Immunology and Microbiology, School of Medicine, and [3]Institute of Environmental Health, Wayne State University; [4]Department of Pathology, St. John Hospital and Medical Center, Detroit, Michigan; and [5]Department of Immunology, Mayo Clinic College of Medicine, Rochester, Minnesota

Abstract

Immune reactivity to self-antigens in both cancer and autoimmune diseases can be enhanced by systemic immune modulation, posing a challenge in cancer immunotherapy. To distinguish the genetic and immunoregulation of tumor immunity versus autoimmunity, immune responses to human ErbB-2 (Her-2) and mouse thyroglobulin (mTg) were tested in transgenic mice expressing Her-2 that is overexpressed in several cancers, and HLA-DRB1*0301 (DR3) that is associated with susceptibility to several human autoimmune diseases, as well as experimental autoimmune thyroiditis (EAT). To induce Her-2 response, mice were electrovaccinated with pE2TM and pGM-CSF encoding the extracellular and transmembrane domains of Her-2 and the murine granulocyte macrophage colony-stimulating factor, respectively. To induce EAT, mice received mTg i.v. with or without lipopolysaccharide. Depletion of regulatory T cells (Treg) with anti-CD25 monoclonal antibody enhanced immune reactivity to Her-2 as well as mTg, showing control of both Her-2 and mTg responses by Treg. On mTg immunization, Her-2xDR3 and B6xDR3 mice expressing $H2^b$xDR3 haplotype developed more profound mTg response and thyroid pathology than Her-2 or B6 mice that expressed the EAT-resistant $H2^b$ haplotype. In Her-2xDR3 mice, the response to mTg was further amplified when mice were also immunized with pE2TM and pGM-CSF. On the contrary, Her-2 reactivity was comparable whether mice expressed DR3 or not. Therefore, induction of Her-2 immunity was independent of DR3 but development of EAT was dictated by this allele, whereas Tregs control the responses to both self-antigens. These results warrant close monitoring of autoimmunity during cancer immunotherapy, particularly in patients with susceptible MHC class II alleles. [Cancer Res 2007;67(14):1–8]

Introduction

Thymus-derived $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells (Treg; refs. 1–4), which also express glucocorticoid-induced tumor necrosis factor receptor family-related gene (5) and Toll-like receptors 4, 5, 7, and 8 (6), are potent suppressors of antitumor immunity (7–10). The evidence is convincing from mice harboring a *Foxp3* knock-in allele, *Foxp3gfp* or *Foxp3rfp*, which encodes a fluorescent fusion protein, that cells expressing Foxp3 are regulatory cells of a distinct lineage (3, 11, 12). We and others have shown that removal of $CD4^+CD25^{hi}$ Treg resulted in tumor regression and greater antitumor immunity (7, 8, 10, 13), indicating that Treg depletion can be a powerful regimen in cancer immunotherapy.

Modulation of the immune system to amplify antitumor immunity may, however, be accompanied by the induction of autoimmunity. Autoimmune symptoms or antibodies to self-antigens, such as thyroid antigens, occurred in 26% of melanoma patients undergoing IFN-α treatment, and this autoimmunity was associated with statistically significant improvements in both relapse-free and overall survival (14). In another trial testing a Her-2 peptide vaccine combined with systemic Flt-3 ligand, 2 of 15 subjects developed autoimmune hypothyroidism with increased levels of thyroid-stimulating hormone and autoantibodies to thyroid antigens (15), further indicating the induction of autoimmune symptoms by systemic immune activation.

Consistent with the observation in cancer patients undergoing immunotherapy, the most prevalent autoimmune manifestations in humans is thyroiditis, with 45% of women and 20% of men in the United States showing focal thyroiditis at routine autopsy, although only 1% of women and 0.5% of men exhibit clinical symptoms. Focal thyroiditis is strongly correlated with circulating antibodies to thyroglobulin and thyroid peroxidase (16). The disease is characterized by mononuclear cell infiltration and destruction of the thyroid, elevation of thyroid-stimulating hormone, and decrease of thyroid hormones (T3 and T4). Autoantibodies and T-cell responses to thyroglobulin and thyroid peroxidase (17, 18) are clinically sensitive indicators of autoreactivity.

Autoimmunity induced by immune modulation is, however, not just a positive indicator of responsiveness but can be a life-threatening disorder. In patients with metastatic melanoma who received gp100 peptide vaccines along with an antagonistic monoclonal antibody (mAb) to CTLA-4 (19–21), grade III/IV autoimmune manifestations were observed, including hypothyroidism, dermatitis, enterocolitis, hepatitis, and hypophysitis. The three patients in this study with objective cancer regression all developed severe autoimmune symptoms requiring intervention. Patients with autoimmune hypophysitis manifested hypocortisolism and low testosterone, requiring prolonged replacement therapy with steroids, thyroid hormone, and testosterone. Hence, the balance between cancer immunotherapy and autoimmunity becomes a critical issue.

The primary determining factor in many autoimmune diseases is the MHC class II allele (22). For example, human *HLA-DRB1*0301* (DR3) or murine $H2^k$ confers susceptibility to autoimmune thyroiditis (23), whereas murine $H2^d$ or $H2^b$ is associated with resistance (24). In this study, experimental autoimmune thyroiditis (EAT), the murine model of Hashimoto's thyroiditis, is used to assess autoimmunity. EAT is induced with mouse thyroglobulin

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Wei-Zen Wei, Karmanos Cancer Institute, Wayne State University, 110 East Warren Avenue, Detroit, MI 48201. Phone: 313-833-0715; Fax: 313-831-7518; E-mail: weiw@karmanos.org.

doi:10.1158/0008-5472.CAN-06-4755

(mTg), the 660-kDa storage protein for iodinated thyroid hormone. To simulate humans with circulating thyroid antigens and experiencing additional triggering factors, EAT is induced by injecting mTg with bacterial lipopolysaccharide (LPS). Alternatively, mTg is given repeatedly without adjuvant (25, 26) such that, in EAT-resistant BALB/c mice, repeated i.v. injection with mTg induces modest levels of humoral and cell-mediated immunity (7).

To simulate human tolerance to the tumor-associated antigen Her-2, Her-2 transgenic mice were generated by using the whey acid protein (WAP) promoter to drive the wild-type (WT) human *ErbB-2* gene and they exhibit profound immune tolerance to Her-2 (27). The EAT-susceptible human DR3 allele was introduced by mating HLA-DR3 transgenic mice with Her-2 transgenic mice. We show concurrent induction of autoreactivity to self-Her-2 and self-mTg in Treg-depleted Her-2 and Her-2xDR3 mice and that expression of DR3 dictates the severity of thyroid pathology without significantly affecting Her-2 immunity.

Materials and Methods

Mice. All animal procedures were conducted in accordance with accredited institution guidelines and the USPHS Policy on Humane Care and Use of Laboratory Animals.[6] C57BL/6 (B6) female mice were purchased from Charles River Laboratory. Heterozygous Her-2 mice, which express the full-length, WT human ErbB-2 (Her-2) under the WAP promoter, were maintained by breeding with normal B6 mice as we previously reported (27). Transgene-positive mice were identified by PCR. HLA-DR3 mice were generated by introducing the *HLA-DRA/DRB1*0301* transgene into class II–negative Ab^0 mice and backcrossed to B10 mice, resulting in DR3 transgenic Ab^0/B10 mice (23, 28). B6xDR3 F1 (B6xDR3) mice were produced by mating HLA-DR3 males with C57BL/6 females. Expression of DR3 by the peripheral blood leukocytes (PBL) was verified by flow cytometry using mouse mAb L243 to human HLA-DR (BD Biosciences). Her-2xDR3 F1 (Her-2xDR3) mice were produced by mating HLA-DR3 males with Her-2 females. Expression of Her-2 was determined by PCR as in Her-2 mice (27).

Cell lines. All tissue culture reagents were purchased from Invitrogen unless otherwise specified. Cell lines were maintained *in vitro* in DMEM supplemented with 5% heat-inactivated cosmic calf serum (HyClone), 5% heat-inactivated fetal bovine serum (Sigma), 10% NCTC-109 medium, 2 mmol/L L-glutamine, 0.1 mmol/L MEM nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Generation of Her-2–expressing EL4/E2 cells was described previously (29), and the cells were maintained in supplemented DMEM with 0.8 mg/mL G418 (geneticin, Sigma). TC-1/E2 was generated by transfecting C57BL/6 TC-1 cells (generously provided by Dr. T.C. Wu, The Johns Hopkins University, Baltimore, MD) with pMSCV/puro and pCMV5/E2 (30). TC-1 was a tumor cell line derived by transforming lung epithelial cells with human papillomavirus-16 E6, E7, and ras oncogene (31). Stable TC-1/E2 clones were selected and maintained in supplemented DMEM with 7.5 μg/mL puromycin.

Depletion of T-cell subsets *in vivo*. Rat hybridoma lines PC61 producing rat anti-mouse CD25, GK1.5 producing anti-CD4, and 2.43 producing anti-CD8 mAb (American Type Culture Collection) were propagated in severe combined immunodeficient mice. To deplete $CD25^{hi}$, CD4, or CD8 T cells, mice were injected i.p. with 1 mg anti-CD25, 0.5 mg anti-CD4, or 0.5 mg anti-CD8 mAb at the indicated times. Depletion of $CD4^+CD25^{hi}$, CD4, or CD8 T cells was verified by flow cytometry.

DNA immunization and tumor challenge. pE2TM was generated by PCR amplification of full-length Her-2 with a 3′ primer engineered to create a stop codon at the end of the transmembrane domain (29). This fragment of Her-2 encoding the extracellular and transmembrane domains was cloned into pCMV5 using *Hin*dIII and *Xba*I. pEFBos-GM-CSF encoding murine granulocyte macrophage colony-stimulating factor (GM-CSF) was provided by Dr. N. Nishisaki (Osaka University, Osaka, Japan). Mice were injected in the quadriceps muscle with plasmid DNA as described previously (32). DNA injection was followed immediately by square wave electroporation at the injection site using a BTX830 (BTX Harvard Apparatus). A tweezer electrode was used to deliver eight pulses at 100 V for 25 ms/pulse.

To measure tumor growth, mice were challenged s.c. with 2×10^5 EL4/E2 cells in the flank. Tumor growth was monitored by weekly palpation, and mice were sacrificed when any one dimension of the tumor reached 15 mm. Tumor volume was calculated by the following equation: $(X^2 \times Y) / 2$. X and and Y represented the short and long dimension, respectively, of the tumor. Differences in tumor incidence were analyzed by the log-rank test.

Measurement of anti-Her-2 antibody by flow cytometry. For measurement of anti-Her-2 antibody, human ovarian cancer cell line SKOV-3 was incubated with serially diluted immune mouse sera as we previously reported (33). Phycoerythrin-conjugated goat anti-mouse antibody directed to the γ-chain of mouse IgG (Jackson ImmunoResearch) was used to detect bound antibody using flow cytometry. Normal mouse serum or isotype-matched mAb was the negative control. A standard curve for Her-2 binding was generated using c-erbB2 mAb (Ab5, clone TA-1; Calbiochem). Antibody concentrations in the test sera were calculated based on the standard curve. The isotype of bound antibody was measured with FITC-goat anti-mouse IgG1 or IgG2c (Caltag). Results are shown as mean channel fluorescence intensity. Flow cytometric analysis was done with a FACSCalibur (Becton Dickinson). Differences in antibody concentration were analyzed by the Student's t test.

Immunization with mTg. mTg was prepared from frozen thyroids by fractionation on a Sephadex G-200 column as we described previously (34, 35) and diluted in nonpyrogenic saline before use. The presence of LPS was measured by *Limulus* amebocyte assay (Associates of Cape Cod; ref. 26). A 40 μg dose of mTg contained <0.5 ng of LPS.

Mice were injected i.v. with 40 μg mTg followed in 3 h with 20 μg *Salmonella enteritidis* LPS. The injections were repeated in 7 days. Alternatively, 40 μg mTg was injected i.v. on 4 successive days with 3 days of rest (26). This treatment was repeated for 4 weeks.

Measurement of anti-mTg antibody. Anti-mTg antibody titers were determined by ELISA as described previously (36). Briefly, Immulon I microtiter plates (Dynatech Laboratories, Inc.) were coated with mTg at 1 μg/well and serially diluted test sera were added. After washing, bound antibody was detected with alkaline phosphatase–labeled goat anti-mouse IgG and enzyme substrate. The isotype of bound antibody was determined with alkaline phosphatase–labeled goat α-mouse IgG1 or IgG2c (Southern Biotech). Sera pooled from EAT-susceptible CBA/J mice immunized with mTg and LPS were used as the control at a designated concentration of 10,000 units/mL. A standard curve of mTg binding was generated with serially diluted control sera, and relative concentrations of test sera were determined based on this standard curve.

Measurement of IFN-γ–secreting T cells by enzyme-linked immunospot assay. Her-2–reactive T cells were measured by enzyme-linked immunospot (ELISPOT) assay. Spleen cells or PBLs were suspended in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. A total of 3×10^5 to 4×10^5 cells was added to each well of a 96-well HTS IP plates (Millipore), which were precoated with 2.5 μg/mL rat anti-mouse IFN-γ (IgG1, clone R4-6A2), and cells were incubated for 48 h at 37°C in 5% CO_2. TC-1/E2 cells were added as antigen-presenting cells (APC). TC-1 cells were used as control. The ratio of spleen cells to APCs was 10:1. Following a 48-h incubation, cells were removed and 2 μg/mL biotinylated rat anti-mouse IFN-γ (IgG1, clone XMG 1.2) was added. All antibodies were purchased from BD PharMingen. Plates were incubated for another 12 h at 4°C and then washed to remove unbound antibody. Bound antibody was detected by incubating the plates with 0.9 μg/mL avidin-horseradish peroxidase (BD PharMingen) for 2 h at room temperature. The substrate 3-amino-9-ethylcarbazole (AEC; BD PharMingen) in 0.1 mol/L acetic acid and 0.003% hydrogen peroxide was added, and the plate was incubated for 3 to 5 min. AEC solution was discarded and the plates were washed six

[6] http://grants.nih.gov/grants/olaw/olaw.htm#pol

times with water. The visualized cytokine spots were enumerated with the ImmunoSpot analyzer (CTL Analyzers), and the results were expressed as the number of cytokine-producing cells per 10^6 cells. Data were analyzed using the Student's *t* test.

mTg-reactive T cells were measured with a two-step ELISPOT assay. Spleen cells were incubated with 80 μg/mL mTg in 96-well tissue culture plates for 3 days before the content of the wells was transferred to HTS IP plates and further incubated for 24 h. The detection and enumeration of cytokine spots were done as described.

Histologic evaluation of EAT. Thyroid specimens were sectioned vertically through both lobes, and 50 to 60 histologic sections were prepared from 10- to 15-step levels. The extent of mononuclear cell infiltration was scored based on the pathology index scale of 0 to 4 and presented as percentage thyroid infiltration: 0, no infiltration; 0.5, >0% to 10% thyroid infiltration consisting of perivascular foci without follicular destruction; 1.0, >10% to 20% thyroid infiltration with follicular destruction; 2.0, >20% to 40% diffuse thyroid infiltration; 3.0, >40% to 80% thyroid destruction; and 4.0, >80% to 100% thyroid destruction (35). The sections were scored without knowledge of the groups. Statistical differences were analyzed by the nonparametric Mann-Whitney *U* test.

Results

Induction of anti-Her-2 immunity in $H2^b$ versus $H2^b$xDR3 mice. Because expression of DR3 has been associated with immune reactivity to several self-antigens, the immune reactivity to self-Her-2 was tested in either Her-2 transgenic mice in the $H2^b$ background or Her-2xDR3 mice in the $H2^b$xDR3 background. B6 ($H2^b$) and B6xDR3 ($H2^b$xDR3) mice were used as controls. Mice were electrovaccinated four times, every 2 weeks, with pE2TM encoding the extracellular and transmembrane domains of Her-2. Equal amounts of pGM-CSF were also given in the same DNA mixture. Sera were collected 2 weeks after each immunization, and anti-Her-2 antibody was analyzed by flow cytometry as we previously reported (33). After the fourth immunization, B6 and B6xDR3 mice produced 88 ± 33 and 76 ± 30 μg/mL Her-2–specific antibodies, respectively, with no significant difference whether mice expressed DR3 (Fig. 1*A*). In tolerant Her-2 or Her-2xDR3 mice, less than 3 μg/mL of antibody were detected, regardless of DR3 status. These results verified the profound tolerance to Her-2 in Her-2 and Her-2xDR3 mice and that expression of DR3 did not increase their anti-Her-2 antibody response. F1

To test if Her-2 response was controlled by Treg (7, 8, 29), mice received anti-CD25 mAb 1 week before the first DNA electrovaccination and then they were boosted thrice every 2 weeks. Reduction in $CD4^+CD25^{hi}$ cells in the peripheral blood was verified by flow cytometry. Compared with mice carrying intact Treg (Fig. 1*A*), antibody levels were elevated in all four strains of mice immunized after Treg depletion (Fig. 1*B*). In B6 mice, antibody levels increased from 88 ± 33 to 173 ± 46 μg/mL ($P < 0.01$) after four vaccinations, and in B6xDR3 mice, the levels increased from 76 ± 30 to 163 ± 76 μg/mL ($P < 0.005$). Her-2 and Her-2xDR3 mice, which did not otherwise respond to DNA vaccination, produced 28 ± 27 and 29 ± 24 μg/mL antibodies, respectively, when immunized four times following Treg depletion. Therefore, Treg suppressed Her-2 reactivity whether Her-2 was a foreign or self-antigen and DR3 status did not change the outcome of the vaccination.

To measure T-cell response, PBLs from each group of six to eight mice were pooled after the third immunization and incubated for 48 h with the engineered APCs TC-1/E2. TC-1 cells expressed H2-K^b/D^b and B7.1 constitutively and were stably transfected with Her-2 to establish TC-1/E2. B6 and B6xDR3 mice produced 150 ± 17 and 134 ± 21/10^6 of IFN-γ–secreting Her-2–reactive T cells, respectively (Fig. 1*C*). After Treg depletion, the level increased to 487 ± 22 and 432 ± 30 spots, respectively ($P < 0.001$; Fig. 1*D*). In Her-2–tolerant mice, T-cell response was not detected even after Treg depletion and four immunizations (data not shown), again showing the profound tolerance in these mice.

Activation of both CD4 and CD8 T cells by Her-2 DNA vaccination. Her-2–reactive T cells were further analyzed in B6

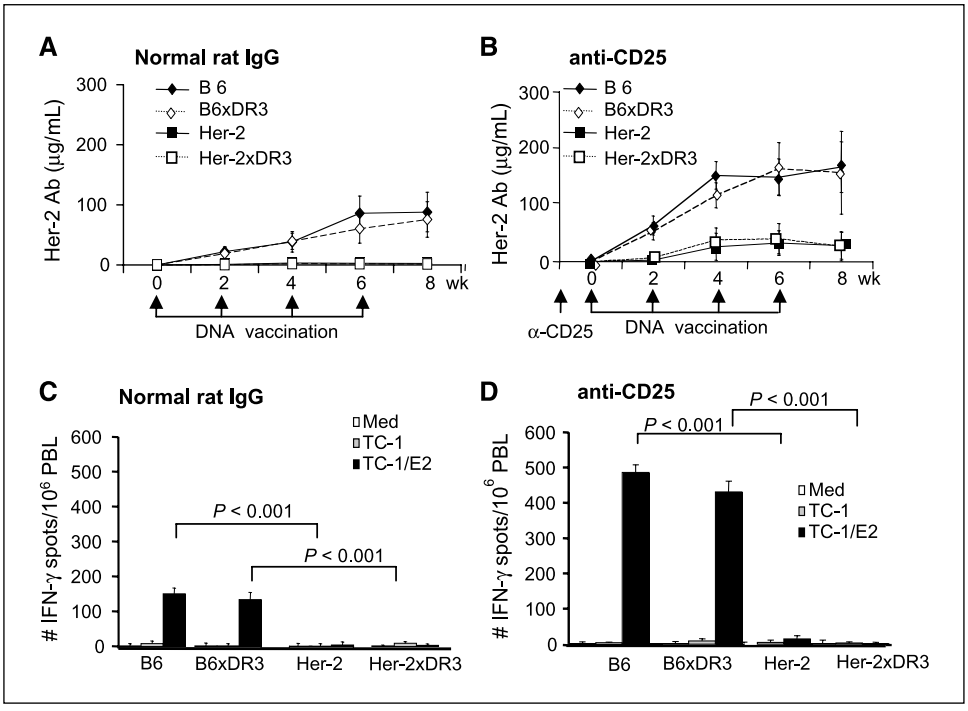

Figure 1. Anti-Her-2 immunity induced by pE2TM and pGM-CSF electrovaccination. B6, B6xDR3, Her-2, and Her-2xDR3 mice were electrovaccinated four times using pE2TM and pGM-CSF 1 wk after they received normal rat Ig (*A*) or anti-CD25 mAb (*B*). *A* and *B,* sera were collected 2 wks after each vaccination and antibody (*Ab*) levels were determined using flow cytometry and regression analysis. PBLs were collected following the third vaccination and incubated with TC-1 or TC-1/E2 cells for 48 h, and the number of IFN-γ–secreting T cells was enumerated. Mice received either normal rat Ig (*C*) or anti-CD25 (*D*) before vaccination. There were six to eight mice per group. *C* and *D, columns,* mean number of spots; *bars,* SD.

mice, which were depleted of Treg and vaccinated with pE2TM and pGM-CSF. After the first and second vaccination, 674 ± 37 and 761 ± 20 per million, respectively, Her-2–specific, IFN-γ–producing cells were detected in the spleen (Supplementary Fig. S1). Depletion of both CD4 and CD8 T-cell populations before the assay removed most, if not all, IFN-γ–producing T cells, showing that T cells are the primary responders. Depletion of either CD4 or CD8 T cells alone resulted in partial reduction as expected. Cells incubated with the control APC, TC-1, produced <10 spots/10^6 cells (data not shown).

To further verify the subtype of reactive T cells, mAb Y3P to IA^b, the only MHC II expressed by B6 mice, was added to CD8 T-cell–depleted spleen cells during *in vitro* stimulation and IFN-γ–producing cells were reduced by ~80% (data not shown). Conversely, when a mixture of mAb 28-14-8S to Db and B8-24-3 to K^b was added to CD4 T-cell–depleted spleen cells during culture, ~70% reduction in IFN-γ spots was observed (data not shown). Therefore, CD4 and CD8 T cells were the major IFN-γ–producing cells that responded to Her-2 DNA vaccination.

To further define CD4 T cells that responded to Her-2 DNA vaccination, we measured the level of anti-Her-2 IgG1 and IgG2c in the sera of B6, B6xDR3, Her-2, and Her-2xDR3 mice that were described in Fig. 1. Results from sera collected after the second vaccination are shown in Supplementary Fig. S2. Both IgG1 and IgG2c were induced in B6 and B6xDR3 mice, showing the activation of both Th1 and Th2 in nontolerant mice. Very little antibody was induced in Her-2 or Her-2xDR3 mice, consistent with their profound tolerance. After Treg depletion, Her-2–reactive IgG1 and IgG2c levels were both detected. Therefore, both Th1 and Th2 were activated in tolerant and nontolerant mice by DNA electrovaccination. Direct comparison of IgG1 versus IgG2c levels would not be informative because different secondary antibody must be used to measure the two IgG isotypes.

Tumor rejection in Treg-depleted, DNA-vaccinated mice. To measure antitumor activity, mice were depleted of Treg, electrovaccinated with pE2TM and pGM-CSF, and then inoculated s.c. with EL4/E2 cells 2 weeks after the last immunization. EL4/E2 tumors developed and progressed in B6 and Her-2 mice receiving blank vectors and they were sacrificed 4 weeks after tumor cell inoculation due to their tumor burden (Fig. 2*A*). In 13 of 16 (82%) B6 mice that received vaccinations twice and in 3 of 7 (43%) Her-2 mice that received vaccinations four times, tumors were rejected or regressed after transient growth, showing the activity of Her-2 vaccination. These results further verified immune tolerance in Her-2 mice because Her-2 mice received two more vaccinations than B6 mice to reach half the level of protection (Fig. 2*A*). In B6xDR3 and Her-2xDR3 mice, 92% (12 of 13) and 63% (5 of 8) of the mice eventually rejected their tumors after two- and four-time vaccinations, respectively (Fig. 2*B*). In one of nine control B6xDR3 mice that received blank vector, the tumor regressed spontaneously. Anti-Her-2 antibody level was measured at 3 weeks after tumor inoculation and elevated levels were found in vaccinated mice when compared with nonvaccinated mice (Fig. 2*C* and *D*). As expected, Her-2 and Her-2xDR3 mice produced lower levels of antibodies than Her-2–negative mice. Taken together, DR3 expression did not have a significant effect on tumor growth or antibody response.

Immune reactivity to self-thyroglobulin in $H2^b$ versus $H2^b$xDR3 mice. Induction of EAT was tested in B6, B6xDR3, Her-2, and Her-2xDR3 mice with a standard regimen (i.e., i.v. injection twice, 7 days apart, with 40 μg mTg followed in 3 h with

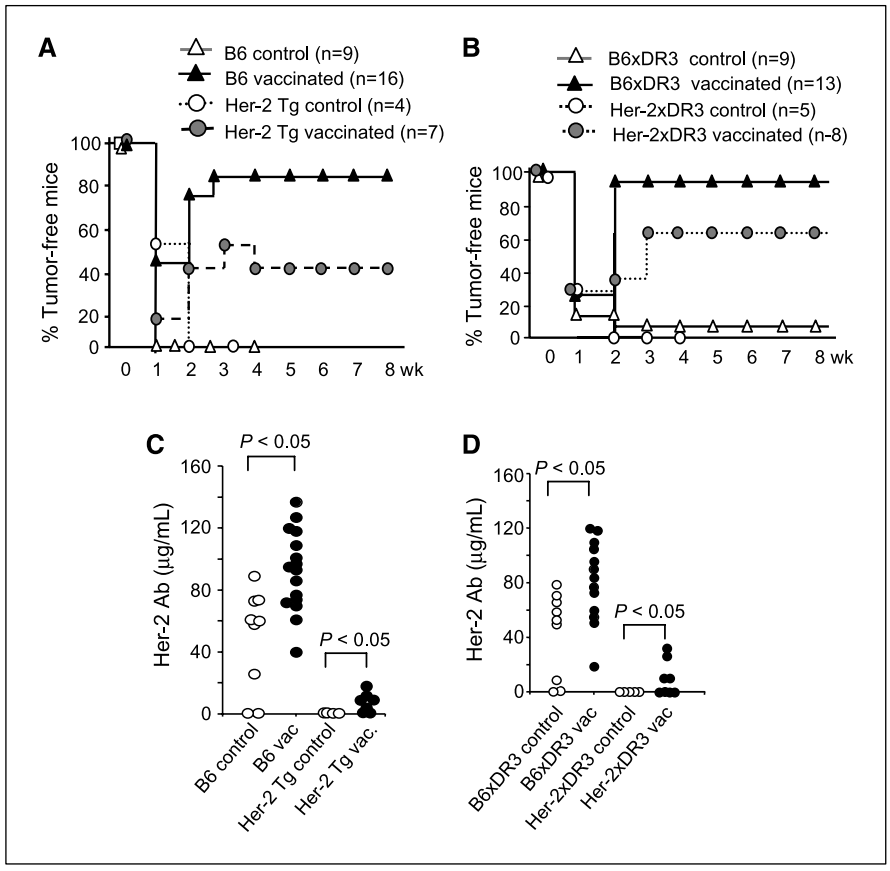

Figure 2. Tumor rejection after Her-2 DNA electrovaccination. Mice were challenged with 2×10^5 EL4/E2 cells s.c. after Treg depletion and electrovaccination and tumor growth was monitored. Mice received control pCMV5 or pE2TM and pGM-CSF. Percentages of tumor-free mice in B6 and Her-2 mice (*A*) or in B6xDR3 and Her-2xDR3 mice (*B*). Antibodies to Her-2 in B6 and Her-2 mice (*C*) or in B6xDR3 and Her-2xDR3 mice (*D*) were measured 3 wks following tumor inoculation.

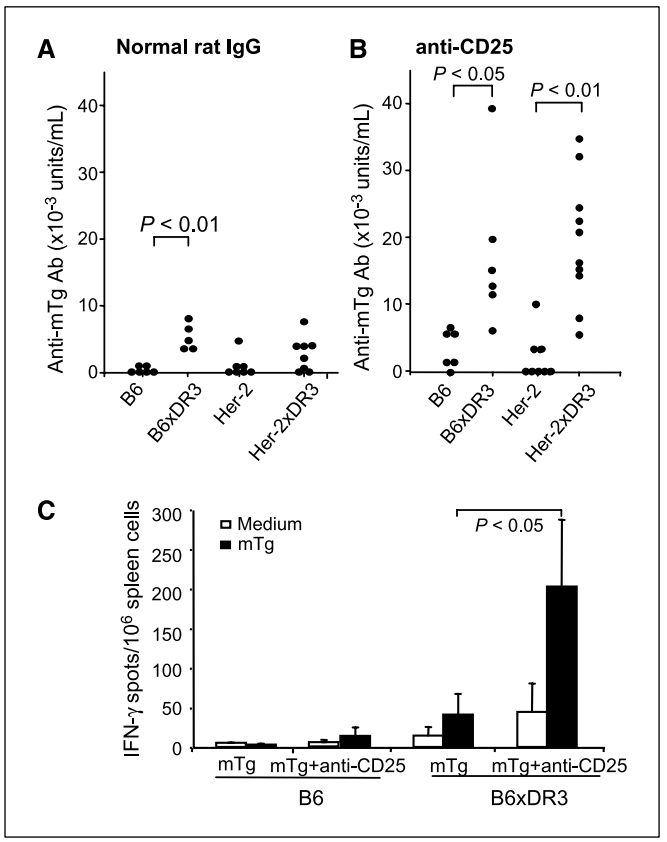

Figure 3. Antibody and T-cell responses to mTg with or without Treg depletion. Mice received two injections, 1 wk apart, of 40 μg mTg and 20 μg LPS with (*B*) or without (*A*) Treg depletion 1 week before mTg and LPS immunization. At 3 wks following the last mTg injection, sera were collected. The levels of anti-mTg antibodies measured by ELISA were expressed as units/mL as described in Materials and Methods. *C,* at 3 wks following the final injection of mTg and LPS, spleen cells from individual were collected and incubated with mTg in a two-step IFN-γ ELISPOT assay. There were five to nine mice in each group. *Columns,* mean spot numbers from individual mice; *bars,* SD.

20 μg LPS). Some mice also received anti-CD25 mAb 10 and 7 days before the first mTg injection to deplete Treg. At 3 weeks after the second injection with mTg and LPS, anti-mTg antibodies were measured by ELISA, and their relative concentrations were calculated by regression analysis based on pooled mTg-reactive control sera. B6 and Her-2 mice produced little to no anti-mTg antibodies except in one Her-2 mouse, whereas B6xDR3 and Her-2xDR3 mice produced 5 ± 2 and $3 \pm 3 \times 10^3$ units/mL, with significant difference observed between B6xDR3 and B6 mice (Fig. 3*A*). This higher reactivity in $DR3^+$ mice became prominent when Tregs were depleted before mTg injection (Fig. 3*B*), with 4 ± 3, 18 ± 12, 2 ± 4, and $20 \pm 9 \times 10^3$ units/mL detected in B6, B6xDR3, Her-2, and Her-2xDR3 mice, respectively. T-cell response was measured with a 3-day, two-step IFN-γ ELISPOT assay (Fig. 3*C*). The number of IFN-γ–producing T cells from each mouse was measured individually, and the mean and SD in each test group are shown. B6xDR3 mice produced more mTg-reactive T cells ($41 \pm 26/10^6$) than B6 mice ($3 \pm 1/10^6$). The number of mTg-reactive T cells from B6xDR3 was further amplified by Treg depletion to reach $203 \pm 74/10^6$ cells. Consistent with HLA-DR3 being a susceptible allele for EAT induction (23), its expression remains a dominant determinant of both anti-mTg antibody and T-cell responses.

The extent of thyroid infiltration and destruction by mononuclear cells were also analyzed 3 weeks after the second injection of mTg and LPS. B6 and Her-2 mice bore the EAT-resistant haplotype and exhibited weak EAT (Fig. 4*A*). Both B6 and Her-2 mice displayed mild pathology and thyroid infiltration (Fig. 4*C*). In DR3-expressing B6xDR3 and Her-2xDR3 mice, thyroid destruction was more extensive. After Treg depletion, more severe EAT was observed in all test strains and up to 80% of the thyroids in some B6xDR3 or Her-2xDR3 mice were involved (Fig. 4*B* and *C*). These results showed the determining effects of both DR3 and Treg on EAT development.

Balance between anti-Her-2 and anti-mTg immunity with Treg depletion. Because immune responses to both Her-2 and mTg were elevated after Treg depletion, we measured the immunity to Her-2 and mTg in mice exposed to both self-antigens. Her-2 and Her-2xDR3 mice received anti-CD25 mAb before they were injected i.v. with mTg, four times each week for 4 weeks, without LPS. We have previously shown that repeated treatment with mTg without LPS induced weak to moderate EAT in ~50% of susceptible mice

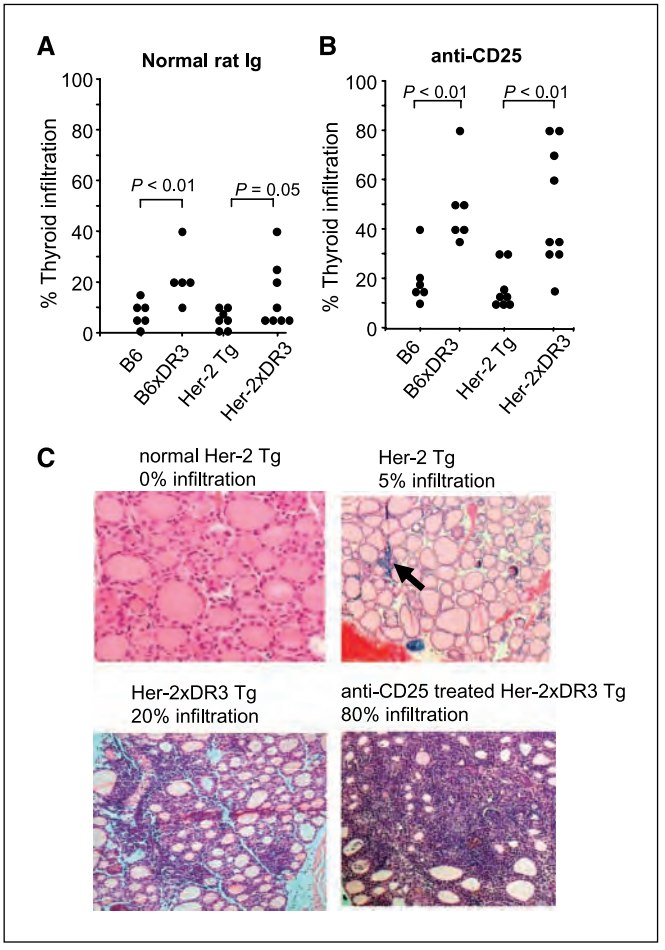

Figure 4. Induction of autoimmune thyroiditis. At 3 wks following the second injection of mTg and LPS, thyroid infiltration in mice with (*B*) or without (*A*) Treg depletion was determined by pathology score as described in Materials and Methods. *C,* representative H&E sections of normal and infiltrated thyroids. *Top left,* normal thyroid with no infiltration; *top right,* Her-2 mice with 5% infiltration (*arrow*) without follicular destruction; *lower left,* Her-2xDR3 mice with 20% infiltration showing follicular destruction of the thyroid; *lower right,* Her-2xDR3 mice depleted of Treg with 80% infiltration showing extensive destruction of the thyroid. There were five to nine mice in each group.

(26). LPS was omitted to avoid complication on immune reactivity to Her-2. At 14, 28, and 42 days after anti-CD25 mAb treatment, mice were immunized with pE2TM and pGM-CSF (Fig. 5). Humoral and cellular immune responses were measured at week 8. As expected, Her-2 mice with intact Treg did not produce anti-Her-2 antibodies (Fig. 5*A*). With Treg depletion, Her-2 and Her-2xDR3 mice immunized with both Her-2 DNA and mTg produced low levels of Her-2 antibodies averaging 12 ± 17 and 10 ± 11 μg/mL (Fig. 5*A*). Both IgG1 and IgG2c were detected in Her-2 immune sera, although at low levels (Supplementary Fig. S3). IFN-γ–producing T cells were also analyzed. Her-2 mice did not have detectable T-cell response. After Treg depletion, 38 ± 3 and 50 ± 1 spots/10^6 cells were observed in Her-2 and Her-2xDR3 mice, which received Her-2 DNA and mTg (Fig. 5*B*). Thus, reactivity to the Her-2 vaccination regimen in tolerant hosts was enhanced by Treg depletion in a DR3-independent fashion.

Antibody and T-cell responses to mTg were measured in Her-2 and Her-2xDR3 mice with or without Treg depletion. In Her-2 mice, little to no antibodies to mTg were detected even after Treg depletion (Fig. 5*C*). In mice also vaccinated with pE2TM and pGM-CSF, low level of mTg antibody at 11 ± 5 units/mL was detected. In Her-2xDR3 mice, repeated mTg injections resulted in 10 ± 5 units/mL of antibodies, which increased after Treg depletion, with some mice reaching >70 units/mL, whether mice also received Her-2 DNA vaccination. Both IgG1 and IgG2c were detected in mTg immune sera (Supplementary Fig. S3).

mTg T-cell response of individual mice was measured by the two-step ELISPOT assay using splenocytes. Her-2 mice showed little to no T-cell response to mTg whether Tregs were depleted or not (Fig. 5*D*). Her-2xDR3 mice generated 21 ± 7/10^6 of IFN-γ–secreting T cells after mTg injection. T-cell response increased to 84 ± 17/10^6 when mice were depleted of Treg cells and 189 ± 21 when mice were also vaccinated with Her-2 and GM-CSF DNA (Fig. 5*D*). Moreover, two of the six mice in the latter group developed 10% and 15% thyroid infiltration (data not shown). Thyroiditis was not detected in mice from the other groups. Therefore, Treg depletion enabled mice expressing HLA-DR3 to develop autoimmune thyroiditis when exposed simultaneously to a regimen of Her-2 vaccination with repeated mTg injections.

One might question whether bidirectional synergy between Her-2 and mTg immunity observed in Her-2–tolerant mice (Fig. 5) also occurs in nontolerant mice. Using a similar scheme as outlined in Fig. 5, WT B6 mice were depleted of Treg and vaccinated with Her-2 and GM-CSF DNA followed by 16 i.v. mTg injections. The single Her-2 DNA electrovaccination resulted in sustained Her-2 antibody production with 21 ± 5 μg/mL detected 6 weeks after vaccination (data not shown). Additional immunization with mTg, however, did not increase Her-2 response. Conversely, 16 i.v. injections with mTg induced modest level of mTg antibody as shown in Fig. 5. Additional Her-2 vaccination did not enhance mTg response either (data not shown). Therefore, we did not observe bidirectional synergy in WT mice. Chronic exposure to both tumor

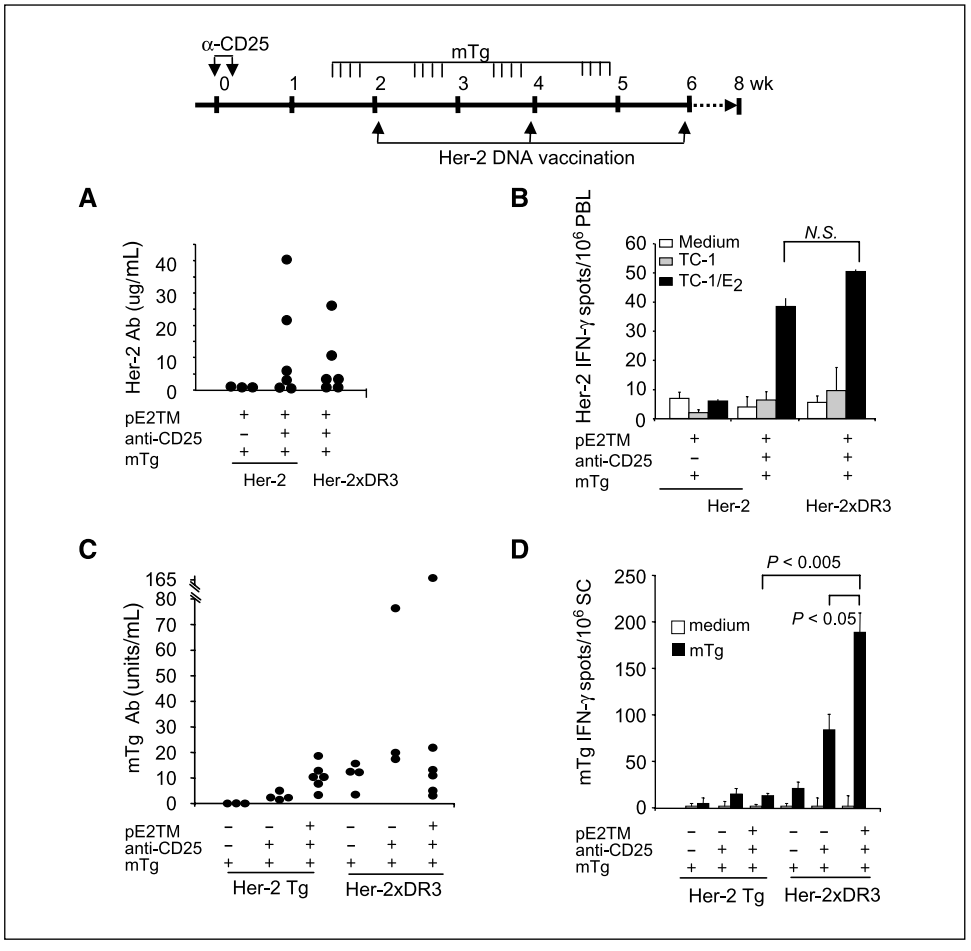

Figure 5. Concurrent immune responses to Her-2 and mTg. Her-2 and Her-2xDR3 mice received anti-CD25 mAb on days 0 and 2. mTg was given four times per week for 4 wks beginning on day 9. Mice were electrovaccinated thrice with pE2TM and pGM-CSF every 2 wks starting at day 14. Sera and splenocytes were collected at week 8. *A,* Her-2 antibodies were measured by flow cytometry and regression analysis. *B,* Her-2 T-cell response was analyzed by IFN-γ ELISPOT assay. *N.S.,* not significant. *C,* mTg antibodies were determined by ELISA. *D,* mTg T-cell response was measured by a two-step ELISPOT. There were three to six mice in each group.

and self-antigens in the tolerant mice may be important in the mutual amplification of immune reactivity to Her-2 and mTg.

Discussion

Expression of HLA-DR3 has minimal effect on anti-Her-2 or antitumor immunity in mice of the B6 background. Although one cannot rule out the possibility that other MHC class II alleles differ in their presentation of Her-2, DR3 status had little effect on Her-2 reactivity.

On the other hand, autoimmune disease to self-antigens is often associated with particular MHC class II alleles. Examples of such associations are systemic lupus erythematosus with expression of both HLA-DR2 and HLA-DR3 (37), multiple sclerosis with HLA-DR2 (38, 39), rheumatoid arthritis with HLA-DR4 (40, 41), type I diabetes with HLA-DR3 and HLA-DR4 (40, 42), and Hashimoto's thyroiditis and Graves' disease with HLA-DR3 (22, 40). Thus, MHC class II alleles, such as DR2, DR3, and DR4, are major determinants in the manifestation of several autoimmune diseases, presenting antigenic epitopes to peripheral T cells, which have escaped thymic scrutiny. In the current study, more severe EAT was induced in B6xDR3 and Her-2xDR3 mice than in B6 and Her-2 mice. During cancer immunotherapy, it would be important to closely monitor autoimmune indicators in patients with particular MHC class II alleles.

The weak anti-Her-2 immunity in Her-2 and Her-2xDR3 mice was augmented after $CD4^+CD25^{hi}$ Treg depletion such that elevated levels of anti-Her-2 IgG were observed. Although few IFN-γ–producing T cells were detected, T-cell help was indicated because anti-Her-2 IgG, including IgG1 and IgG2c, were induced, and tumor growth was inhibited. Further, Her-2 and Her-2xDR3 mice depleted of Treg and immunized with both pE2TM/pGM-CSF and mTg exhibited a modest increase in Her-2 T-cell response (Fig. 5*B*) compared with Treg depletion and pE2TM/pGM-CSF vaccination alone (Fig. 1*D*), suggesting increased Her-2 immunity by concurrent immune response to mTg. Therefore, Her-2–reactive T cells are under Treg regulation and can be amplified with adequate immune stimulation. Although modest, the detection of any T-cell response in Her-2–tolerant mice is significant. We have reported the profound tolerance to Her-2 in our human Her-2 Tg mice with very little antibody and no T-cell response induced by Her-2 DNA vaccination (30). The results in the current article are particularly important because Her-2 T-cell response was induced, for the first time, in Her-2 or Her-2xDR3 mice by immunization with both Her-2 and mTg after their Tregs were depleted. Several T-cell modulating reagents, such as anti-CTLA-4 and anti-4-1BB, may further amplify Her-2 T-cell response. We have also shown that anti-4-1BB enhanced EAT induction (43).

The combination of Treg depletion with active vaccination is undergoing clinical testing. In a phase I trial of metastatic renal cell carcinoma, the patients who received DAB389-interleukin-2 (Ontak, denileukin diftitox, Ligand Pharmaceuticals) before tumor RNA-transfected dendritic cell vaccines generated an enhanced Th1 responses (9), indicating the potential benefit of Treg depletion in cancer patients. Alternative agents for Treg deletion have been described, such as LMB-2, a fusion protein consisting of a single-chain Fv fragment of anti-human CD25 mAb with a truncated form of the bacterial *Pseudomonas* exotoxin A (44). Some of these reagents may be used alone or in combination with anti-CD25 mAb to remove Treg.

Anti-Her-2 antibodies reached a plateau after several immunizations whether Tregs were depleted or not (Fig. 1*A* and *B*). This may indicate the exhaustion of effector T and/or B cells or recovery of Treg to exert their suppressive effect because anti-CD25 mAb was given only before the first vaccination. It may be possible to further enhance anti-Her-2 immunity by repeated depletion of Treg with alternative agents.

Anti-mTg T-cell response in DR3-positive mice was significantly higher than that in DR3-negative mice, and this reactivity was further amplified when mice were also immunized with pE2TM and pGM-CSF and depleted of Treg. It will be important to monitor both arms of reactivity during cancer immunotherapy in association with MHC class II alleles. Although autoimmunity is sometimes considered a positive indicator in cancer immunotherapy (14), the autoimmune symptoms are unpredictable and must be managed. Moreover, every additional immunostimulatory regimen may potentiate autoimmune disease development. Thyroid dysfunction in susceptible individuals is often observed because of its prevalence and represents but one example of the possible complications.

Mutual amplification of Her-2 and mTg immunity observed in Her-2 and Her-2xDR3 mice was not observed in WT B6 mice. We and others have previously reported the pivotal role of CD4 T cells in the induction of EAT and the later contribution of CD8 T cells in thyroid destruction (45–47). Repeated activation of effector T-cell subsets by Her-2 and GM-CSF DNA vaccination in a Treg-depleted environment, coupled with an EAT susceptibility allele, may favor the activation of mTg-reactive CD4 T cells and EAT induction. Further elucidation of the mechanisms intertwining tumor rejection and autoimmune tissue damage will greatly expedite the development of effective vaccination regimen while minimizing or circumventing autoimmune complications.

Acknowledgments

Received 12/27/2006; revised 4/27/2007; accepted 5/11/2007.

Grant support: NIH grants CA76340, CA125680 (W-Z. Wei), and DK45960 (Y.M. Kong); Department of Defense grant W81XWH-04-1-0546 (W-Z. Wei); and St. John Hospital and Medical Center (Y.M. Kong).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank David Shim and Andi Cani for their technical assistance.

References

1. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem 2001;276:37672–9.
2. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057–61.
3. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. Nat Immunol 2003;4:330–6.
4. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in $CD4^+CD25^+$ T regulatory cells. Nat Immunol 2003;4:337–42.
5. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol 2002;3:135–42.
6. Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med 2003;197:403–11.
7. Wei WZ, Jacob JB, Zielinski JF, et al. Concurrent induction of antitumor immunity and autoimmune thyroiditis in $CD4^+$ $CD25^+$ regulatory T cell-depleted mice. Cancer Res 2005;65:8471–8.
8. Wei WZ, Morris GP, Kong YM. Anti-tumor immunity

and autoimmunity: a balancing act of regulatory T cells. Cancer Immunol Immunother 2004;53:73–8.

9. Dannull J, Su Z, Rizzieri D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 2005;115:3623–33.

10. Knutson KL, Dang Y, Lu H, et al. IL-2 immunotoxin therapy modulates tumor-associated regulatory T cells and leads to lasting immune-mediated rejection of breast cancers in neu-transgenic mice. J Immunol 2006; 177:84–91.

11. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 2005;22:329–41.

12. Wan YY, Flavell RA. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl Acad Sci U S A 2005;102:5126–31.

13. Sakaguchi S. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005;6: 345–52.

14. Gogas H, Ioannovich J, Dafni U, et al. Prognostic significance of autoimmunity during treatment of melanoma with interferon. N Engl J Med 2006;354: 709–18.

15. McNeel DG, Knutson KL, Schiffman K, Davis DR, Caron D, Disis ML. Pilot study of an HLA-A2 peptide vaccine using flt3 ligand as a systemic vaccine adjuvant. J Clin Immunol 2003;23:62–72.

16. Takamatsu J, Yoshida S, Yokozawa T, et al. Correlation of antithyroglobulin and antithyroid-peroxidase antibody profiles with clinical and ultrasound characteristics of chronic thyroiditis. Thyroid 1998;8:1101–6.

17. Huang W, Kukes GD. Hashimoto's thyroiditis: an organ-specific autoimmune disease—pathogenesis and recent developments. Lab Invest 1999;79:1175–80.

18. Canonica GW, Cosulich ME, Croci R, et al. Thyroglobulin-induced T-cell *in vitro* proliferation in Hashimoto's thyroiditis: identification of the responsive subset and effect of monoclonal antibodies directed to Ia antigens. Clin Immunol Immunopathol 1984;32:132–41.

19. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003;100:8372–7.

20. Blansfield JA, Beck KE, Tran K, et al. Cytotoxic T-lymphocyte-associated antigen-4 blockage can induce autoimmune hypophysitis in patients with metastatic melanoma and renal cancer. J Immunother 2005;28: 593–8.

21. Maker AV, Attia P, Rosenberg SA. Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. J Immunol 2005;175:7746–54.

22. Vaidya B, Kendall-Taylor P, Pearce SHS. Genetics of endocrine disease: the genetics of autoimmune thyroid disease. J Clin Endocrinol Metab 2002;87:5385–97.

23. Kong YM, Lomo LC, Motte RW, et al. HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice: definitive association with HLA-DRB1*0301 (DR3) gene. J Exp Med 1996;184: 1167–72.

24. Vladutiu AO, Rose NR. Autoimmune murine thyroiditis: relation to histocompatibility (H-2) type. Science 1971;174:1137–9.

25. Esquivel PS, Rose NR, Kong YM. Induction of autoimmunity in good and poor responder mice with mouse thyroglobulin and lipopolysaccharide. J Exp Med 1977;145:1250–63.

26. Elrehewy M, Kong YM, Giraldo AA, Rose NR. Syngeneic thyroglobulin is immunogenic in good responder mice. Eur J Immunol 1981;11:146–51.

27. Piechocki MP, Ho YS, Pilon S, Wei WZ. Human ErbB-2 (Her-2) transgenic mice: a model system for testing Her-2 based vaccines. J Immunol 2003;171:5787–94.

28. Strauss G, Vignali DA, Schonrich G, Hammerling GJ. Negative and positive selection by HLA-DR3(DRw17) molecules in transgenic mice. Immunogenetics 1994;40: 104–8.

29. Miller F, Jones RF, Jacob J, Kong YM, Wei WZ. From breast cancer immunobiology to her-2 DNA vaccine and autoimmune sequelae. Breast Dis 2004;20:43–51.

30. Jacob J, Radkevich O, Forni G, et al. Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. Cell Immunol 2006;240: 96–106.

31. Lin KY, Guarnieri FG, Stavely-O'Carroll KF, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 1996;56:21–36.

32. Wei WZ, Shi WP, Galy A, et al. Protection against mammary tumor growth by vaccination with full-length, modified human ErbB-2 DNA. Int J Cancer 1999;81:748–54.

33. Piechocki MP, Pilon SA, Wei WZ. Quantitative measurement of anti-ErbB-2 antibody by flow cytometry and ELISA. J Immunol Methods 2002;259:33–42.

34. Kong YM, David CS, Giraldo AA, Elrehewy M, Rose NR. Regulation of autoimmune response to mouse thyroglobulin: influence of H-2D-end genes. J Immunol 1979;123:15–8.

35. Kong YM. Experimental autoimmune thyroiditis in the mouse. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology. New York: John Wiley & Sons, Inc.; 1996.

36. Zhang W, Flynn JC, Kong YM. IL-12 prevents tolerance induction with mouse thyroglobulin by priming pathogenic T cells in experimental autoimmune thyroiditis: role of IFN-γ and the costimulatory molecules CD40l and CD28. Cell Immunol 2001;208:52–61.

37. Harley JB, Moser KL, Gaffney PM, Behrens TW. The genetics of human systemic lupus erythematosus. Curr Opin Immunol 1998;10:690–6.

38. Gregersen JW, Holmes S, Fugger L. Humanized animal models for autoimmune diseases. Tissue Antigens 2004;63:383–94.

39. Dyment DA, Ebers GC, Sadovnick AD. Genetics of multiple sclerosis. Lancet Neurol 2004;3:104–10.

40. Taneja V, David CS. HLA class II transgenic mice as models of human diseases. Immunol Rev 1999;169: 67–79.

41. de Vries RR, Huizinga TW, Toes RE. Redefining the HLA and RA association: to be or not to be anti-CCP positive. J Autoimmun 2005;25 Suppl:21–5.

42. Aly TA, Ide A, Jahromi MM, et al. Extreme genetic risk for type 1A diabetes. Proc Natl Acad Sci U S A 2006; 103:14074–9.

43. Morris GP, Chen L, Kong YM. CD137 signaling interferes with activation and function of $CD4^+CD25^+$ regulatory T cells in induced tolerance to experimental autoimmune thyroiditis. Cell Immunol 2003;226:20–9.

44. Attia P, Powell DJ, Jr., Maker AV, Kreitman RJ, Pastan I, Rosenberg SA. Selective elimination of human regulatory T lymphocytes *in vitro* with the recombinant immunotoxin LMB-2. J Immunother 2006;29:208–14.

45. Sugihara S, Izumi Y, Yoshioka T, et al. Autoimmune thyroiditis induced in mice depleted of particular T cell subsets. I. Requirement of Lyt-1 dull L3T4 bright normal T cells for the induction of thyroiditis. J Immunol 1988; 141:105–13.

46. Flynn JC, Conaway DH, Cobbold S, Waldmann H, Kong YM. Depletion of $L3T4^+$ and $Lyt\text{-}2^+$ cells by rat monoclonal antibodies alters the development of adoptively transferred experimental autoimmune thyroiditis. Cell Immunol 1989;122:377–90.

47. Kong YM, Waldmann H, Cobbold S, Giraldo AA, Fuller BE, Simon LL. Pathogenic mechanisms in murine autoimmune thyroiditis: short- and long-term effects of *in vivo* depletion of $CD4^+$ and $CD8^+$ cells. Clin Exp Immunol 1989;77:428–33.