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 Wei1,2

1Karmanos Cancer Institute, 2Department of Immunology and Microbiology, School of Medicine, and 3Institute of Environmental Health, Wayne State University; 4Department of Pathology, St. John Hospital and Medical Center, Detroit, Michigan; and 5Department 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 immune regulation 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 immunoreactivity to Her-2 as well as mTg, showing control of both Her-2 and mTg responses by Treg. When immunized with mTg, Her-2xDR3 and B6xDR3 mice expressing H2b x DR3 haplotype developed more profound mTg response and thyroid pathology than Her-2 or B6 mice that expressed the EAT-resistant H2a 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):7020-7]

Introduction

Thymus-derived CD4+CD25hiFoxp3+ 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+CD25hi 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 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 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.

The primary determining factor in many autoimmune diseases is the MHC class II allele (22). For example, human HLA-DRB1*0301 (DR3) or murine H2d confers susceptibility to autoimmune thyroiditis (23), whereas murine H2a or H2b 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.
(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. 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-DR3/DBB1*T0301 transgene into class II–negative Ab2 mice and backcrossed to B10 mice, resulting in DR3 transgenic Ab2/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 female mice. Presence of Her-2 gene 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 fetal bovine serum (Sigma), 10% NCTC-109 medium, 2 mM L-glutamine, 0.1 mM 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 mM/L 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+CD4−, 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 CD25+CD4−, CD4, or CD8 T cells was verified by flow cytometry.

DNA immunization and tumor challenge. pE2TM was generated by PCR amplification of full-length transmembrane 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 HindIII and XhoI. pE2TM-GM-CSF encoding murine granulocyte macrophage colony-stimulating factor (GM-CSF) was provided by Dr. N. Nishikawa (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 electroperoration 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 $2 \times 10^5$ 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 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 concentrations 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 μg of LPS.

Mice were injected i.v. with 40 μg mTg followed in 3 h with 20 μg Salmonella enteritis 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 1 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 anti-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 mM/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. A total of $3 \times 10^5$ to $4 \times 10^5$ 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-aminio-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
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.

mAb-reactive T cells were measured with a two-step ELISPOT assay. Spleen cells were incubated with 80 μg/mI mAb in 96-well tissue culture plates for 3 days before the content of the wells was transferred to HTS 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 H2b versus H2bxD3 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 H2b background or Her-2xD3 mice in the H2bxD3 background. B6 (H2b) and B6xD3 (H2bxD3) mice were used as controls. Mice were electroporated 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 B6xD3 mice produced 88 ± 33 and 76 ± 30 μg/mL Her-2–specific antibodies, respectively, with no significant difference whether mice expressed DR3 (Fig. 1A). In tolerant Her-2 or Her-2xD3 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-2xD3 mice and that expression of DR3 did not increase their anti-Her-2 antibody response.

To test if Her-2 response was controlled by Treg (7, 8, 29), mice received anti-CD25 mAb 1 week before the first DNA electro-vaccination and then they were boosted thrice every 2 weeks. Reduction in CD4+CD25+h T cells in the peripheral blood was verified by flow cytometry. Compared with mice carrying intact Treg (Fig. 1A), antibody levels were elevated in all four strains of mice immunized after Treg depletion (Fig. 1B). In B6 mice, antibody levels increased from 88 ± 33 to 173 ± 46 μg/mL (P < 0.01) after four vaccinations, and in B6xD3 mice, the levels increased from 76 ± 30 to 163 ± 76 μg/mL (P < 0.005). Her-2 and Her-2xD3 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-Kb/Db and B7.1 constitutively and were stably transfected with 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 B6xD3 mice produced 88 ± 33 and 76 ± 30 μg/mL Her-2–specific antibodies, respectively, with no significant difference whether mice expressed DR3 (Fig. 1A). In tolerant Her-2 or Her-2xD3 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-2xD3 mice and that expression of DR3 did not increase their anti-Her-2 antibody response.

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**Activation of both CD4 and CD8 T cells by Her-2 DNA vaccination.** Her-2–reactive T cells were further analyzed in B6 mice.
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/10⁶ cells (data not shown).

To further verify the subtype of reactive T cells, mAb Y3P to IAγ, 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 Kβ 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 mice, with lower levels of antibodies in Her-2 and 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 electro-vaccination. 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, electro-vaccinated 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. 2A). 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. 2A). 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. 2B). 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. 2C 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 H2b versus H2b×DR3 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 × 10⁵ 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) were monitored weekly. 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.
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 5F2 and 3F3/C2103 units/mL, with significant difference observed between B6xDR3 and B6 mice (Fig. 3A). This higher reactivity in DR3+ mice became prominent when Tregs were depleted before mTg injection (Fig. 3B), with 4F3, 18F12, 2F4, and 20F9/C2103 units/mL detected in B6, B6xDR3, Her-2, and Her-2xDR3 mice, respectively. T-cell response was measured with a three-day, two-step IFN-γ ELISPOT assay (Fig. 3C). 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 ± 36/106) than B6 mice (3 ± 1/106). The number of mTg-reactive T cells from B6xDR3 was further amplified by Treg depletion to reach 203 ± 47/106 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. 4A). Both B6 and Her-2 mice displayed mild pathology and thyroid infiltration (Fig. 4C). 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. 4B 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 (26). LPS was omitted to avoid complication on immune reactivity.

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 ± 3 × 10^{3} units/mL, with significant difference observed between B6xDR3 and B6 mice (Fig. 3A). This higher reactivity in DR3+ mice became prominent when Tregs were depleted before mTg injection (Fig. 3B), with 4 ± 3, 18 ± 4, 2 ± 4, and 20 ± 9 × 10^{3} units/mL detected in B6, B6xDR3, Her-2, and Her-2xDR3 mice, respectively. T-cell response was measured with a three-day, two-step IFN-γ ELISPOT assay (Fig. 3C). 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 ± 36/10^{6}) than B6 mice (3 ± 1/10^{6}). The number of mTg-reactive T cells from B6xDR3 was further amplified by Treg depletion to reach 203 ± 47/10^{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.

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to Her-2. At 14, 28, and 42 days after anti-CD25 mAb treatment, mice were immunized 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.

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.

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. 5C). 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. 5D). Her-2xDR3 mice generated 21 ± 7/106 of IFN-γ–secreting T cells after mTg injection. T-cell response increased to 84 ± 17/106 when mice were depleted of Treg cells and 189 ± 21 when mice were also vaccinated with Her-2 and GM-CSF DNA (Fig. 5D). 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. 5C. 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 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 thyroditis 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+ 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. 5B) compared with Treg depletion and pE2TM/pGM-CSF vaccination alone (Fig. 1D), 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-IBB, may further amplify Her-2 T-cell response. We have also shown that anti-4-IBB enhanced EaT induction (43).

The combination of Treg depletion with active vaccination is undergoing clinical testing. In a phase I trial of metastatic renal carcinoma, the patients who received DAB389-interleukin-2 (Ontak, denileukin difitox, Ligand Pharmaceuticals) before tumor RNA-transfected dendritic cell vaccines generated an enhanced Th1 response (9), indicating the potential benefit of Treg depletion in cancer patients. Alternative agents for Treg depletion 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. 1A 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 after 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 regimens while minimizing or circumventing autoimmune complications.

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