Enhanced Autoimmunity Associated with Induction of Tumor Immunity in Thyroiditis-Susceptible Mice

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Background: Immunotherapeutic modalities to bolster tumor immunity by targeting specific sites of the immune network often result in immune dysregulation with adverse autoimmune sequelae. To understand the relative risk for opportunistic autoimmune disorders, we studied established breast cancer models in mice resistant to experimental autoimmune thyroiditis (EAT). EAT is a murine model of Hashimoto's thyroiditis, an autoimmune syndrome with established MHC class II control of susceptibility. The highly prevalent Hashimoto's thyroiditis is a prominent autoimmune sequela in immunotherapy, and its relative ease of diagnosis and treatment could serve as an early indicator of immune dysfunction. Here, we examined EAT-susceptible mice as a combined model for induction of tumor immunity and EAT under the umbrella of disrupted regulatory T cell (Treg) function.

Methods: Tumor immunity was evaluated in female CBA/J mice after depleting Tregs by intravenous administration of CD25 monoclonal antibody and/or immunizing with irradiated mammary adenocarcinoma cell line A22E-j before challenge; the role of T cell subsets was determined by injecting CD4 and/or CD8 antibodies after tumor immunity induction. Tumor growth was monitored 3 × /week by palpation. Subsequent EAT was induced by mouse thyroglobulin (mTg) injections (4 daily doses/week over 4 weeks). For some experiments, EAT was induced before establishing tumor immunity by injecting mTg + interleukin-1, 7 days apart. EAT was evaluated by mTg antibodies and thyroid infiltration.

Results: Strong resistance to tumor challenge after Treg depletion and immunization with irradiated tumor cells required participation of both CD4⁺ and CD8⁺ T cells. This immunity was not altered by induction of mild thyroiditis with our protocol of Treg depletion and adjuvant-free, soluble mTg injections. However, the increased incidence of mild thyroiditis can be directly related to Treg depletion needed to achieve strong tumor immunity. Moreover, when a subclinical, mild thyroiditis was induced with soluble mTg and low doses of interleukin-1, to simulate pre-existing autoimmunity in patients subjected to cancer immunotherapy, mononuclear infiltration into the thyroid was enhanced.

Conclusions: Our current findings indicate that genetic predisposition to autoimmune disease could enhance autoimmunity during induction of tumor immunity in thyroiditis-susceptible mice. Thus, *HLA* genotyping of cancer patients should be part of any risk assessment.

Introduction

MANIPULATING AND TARGETING the immune network to boost tumor immunity has often resulted in undesirable autoimmune manifestations. Immunotherapeutic strategies, including the use of systemic immunomodulators (e.g., interferon- α and interferon- β) and monoclonal antibodies (mAbs) (e.g., anti-CTLA-4 and anti-CD52) directed to T and/or B cells plus other leukocytes, have led to immune dys-

regulation in both cancer and autoimmune disease patients and frequently triggered opportunistic autoimmune disorders, be they primary or secondary (1,2). For example, among the adverse immune responses in 139 metastatic melanoma patients given repeated doses of a CTLA-4 mAb (ipilimumab) and a peptide vaccine, 45% were classified as grade I/II and 36% as grade III/IV, which included enterocolitis and hypophysitis with multiple endocrine complications (3). In a trial with another CTLA-4 mAb (tremelimumab), also in

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conjunction with a peptide vaccine, adverse effects included pituitary or adrenal gland dysfunction, thyroid disease, and treatment-related deaths (4). The high prevalence of autoimmune thyroid disease in the general population (5) may be one major reason for its prominence among various clinical trials and systemic therapy (1). Data from routine necropsy of Caucasians in the United States and the United Kingdom show 45% of women and 20% of men with focal thyroiditis (6,7). Moreover, a national survey reported that 4.6% of the U.S. population suffered from hypothyroidism—4.3% with subclinical (mild hypothyroidism) and 0.3% with clinical symptoms (8). It is thus not surprising that, in a clinical trial of Flt3 ligand as a systemic peptide vaccine adjuvant in prostate cancer patients, 2 of 15 patients developed elevated levels of thyrotropin (TSH) with hypothyroidism-like symptoms. Their pretreatment sera revealed antibodies (Abs) to thyroid antigens (9), indicating exacerbation of a pre-existing subclinical condition. A third patient showed an elevated TSH level without symptoms.

Both peptide vaccines used in the clinical trials with CTLA-4 mAbs (3,4) and Flt3 ligand (9) were HLA-A2-restricted. Hence, the patients were selected for the *HLA* class I allele (A2) without regard to their *HLA* class II allele. Since susceptibility to nearly all autoimmune diseases is associated with class II genes, the relative risk of which autoimmune diseases would arise after cancer immunotherapy could not be assessed or realistically correlated with its outcome. However, the wide prevalence of thyroid autoimmunity indicates that it may be a suitable indicator of autoimmune sequelae.

Recently, we undertook to combine breast cancer vaccination models with experimental autoimmune thyroiditis (EAT) to probe the balance between the two in a therapeutic design that disrupted regulatory T cell (Treg) function. We selected mouse strains with known MHC-encoded resistance to EAT, and tolerance to a tumor antigen, as it might exist in cancer patients, due to the presence of a transgene *Her-2/rat neu* (a family member of human epidermal growth factor receptor). EAT was induced with repeated injections of mouse thyroglobulin (mTg) without adjuvant to simulate physiologic release of circulatory mTg (10), and prior Treg depletion, which has been shown to increase thyroiditis incidence and severity (11,12). In an EAT-resistant BALB/c (*H2^d*) strain, Treg depletion enhanced tumor regression and facilitated mild EAT induction (13). In *neu*-tolerant BALB/c mice, implanted *neu*⁺ tumors also regressed after Treg depletion and DNA vaccination (14). In *Her-2* tolerant, EAT-resistant C57BL/6 mice also harboring the *HLA-DRB1*03:01* transgene to encode EAT susceptibility (15), the (*H2^b*-*Her-2xDR3*)F₁ mice withstood tumor challenge again only after Treg depletion and *Her-2* DNA vaccination (16). Although the presence of the DR3 allele in F₁ mice increased the autoimmune response, it was moderated by the EAT-resistant *A^b* allele, consistent with EAT-resistant DQ8 (*HLA-DQA1*03:01/DQB1*03:02*) transgene downregulation of DR3-mediated thyroiditis (17). In all three combined models, immune stimuli from concurrent tumor regression and EAT development had a detectable, mutually enhancing effect.

In the current study, the combination model was developed in EAT-susceptible CBA/J (*H2^k*) mice, using an immunogenic tumor variant, A22E-j, from a spontaneous mammary adenocarcinoma cell line (18,19), as presented in a preliminary report (20). The characterization of this model in providing strong tumor immunity and enhancing EAT development is described.

Materials and Methods

Mice

Female CBA/J mice (*H2^k*) were purchased at 6 weeks of age from Harlan Sprague-Dawley (via C. Reeder; NIH, Frederick, MD). Mice were kept on acidified, chlorinated water and used at 8–12 weeks of age. All procedures are in compliance with the Care and Use of Animals guidelines as reviewed by Wayne State University.

Depletion and monitor of T cell subsets in vivo

To monitor T cell subset depletion, peripheral blood leukocytes (PBL) were prepared from heparinized blood (0.5 mL) obtained from the tail artery for fluorescence-activated cell sorter (FACS) analysis. After water lysis to remove red blood cells, PBL were resuspended in FACS buffer [1% bovine serum albumin, 5% normal rabbit serum, and 0.1% sodium azide in 1× phosphate buffered saline (PBS)] and labeled with appropriate mAbs as described below (21). Typically, 20,000 events/sample were acquired uncompensated on a FACScan flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR) (12).

To deplete Tregs, a rat anti-mouse CD25 mAb (rat IgG_{1a}) was used. PC61 hybridoma cells (22) were propagated and sent to Harlan Bioproducts (Harlan Bioproducts for Science, Indianapolis, IN) for injection into athymic nude mice. CD25 mAb concentration in the resultant ascites fluid was determined by enzyme-linked immunosorbent assay with an anti-rat Ig₁ mAb (clone B46-5, mouse IgG₁; BD Biosciences). CD4⁺CD25⁺ T cells were depleted with two doses of 0.5 mg mAb, intravenously (i.v.) 4 days apart, before immunization as per experimental protocol and depletion in PBL was assessed by FACS 6–8 days after the second dose (12). For double labeling, rat anti-mouse CD25 mAb conjugated with phycoerythrin (PE) (7D4, rat IgM; Southern Biotech, Birmingham, AL) and rat anti-mouse CD4 mAb conjugated with fluorescein isothiocyanate (FITC) (GK1.5, rat IgG_{2b}; eBioscience, San Diego, CA) were used. A representative Treg depletion of ~89% is depicted in Supplementary Figure S1 (Supplementary Data are available online at www.liebertonline.com/thy).

For CD4⁺ and CD8⁺ T cell depletion, rat anti-mouse CD4 mAb (640 µg YTS 191.1* + YTA 3.1*, rat IgG_{2b}) and rat anti-mouse CD8 mAb (320 µg YTS 169.4*, rat IgG_{2b}) were injected i.v. 4 days apart (21), after antitumor vaccination as indicated in each protocol. Mice were bled 6 days after the second dose of mAb to verify depletion in PBL using FACS. Depletion of CD4⁺ and CD8⁺ T cells was determined by using different Abs for labeling. Incubation with mAb YTS 177.9.6* (rat IgG_{2a}) against murine CD4 was followed by biotinylated secondary mAb RG7/1.30 (murine IgG_{2b}; BD Biosciences) against rat IgG_{2a} and Streptavidin-PE (BD Biosciences). Incubation with mAb YTS 105.18* (rat IgG_{2a}) to murine CD8 was followed by biotinylated RG7/1.30 and Streptavidin-PE. A T cell receptor mAb conjugated with PE (H57-597, hamster IgG; BD Biosciences) was used to measure total T cell number.

*Monoclonal antibodies were kindly provided by Dr. H. Waldmann and are available as follows: YTS 191.1 & YTA 3.1 (Santa Cruz Biotech, Santa Cruz, CA); YTS 169.4 & YTS 177.9.6 (Abcam, Cambridge, United Kingdom); YTS 105.18 (University of Cambridge Enterprise, Cambridge, United Kingdom).

Induction and assay of EAT

mTg was prepared from frozen mouse thyroids (Mayo Clinic, Rochester, MN; Harlan Bioproducts for Science; or Pel-Freez LLC, Rogers, AR) by fractionation of thyroid extracts on a Sephadex G-200 column (23). The presence of endotoxin was checked by the *Limulus* amoebocyte assay (Associates of Cape Cod, Woods Hole, MA) (10) (a 40 μ g dose contained <1 ng or <0.25 EU of endotoxin).

EAT was induced by immunizing mice i.v. in one of two ways: (a) 40 μ g of mTg in 0.1 mL followed 3 hours later with varying doses of interleukin-1 (IL-1) (5000, 10,000, or 20,000 U) (eBioscience) in 0.2 mL nonpyrogenic PBS, and injections were repeated 7 days later (24); or (b) repeated injections of 20 or 40 μ g mTg, 16 injections over 4 weeks (4 daily injections/week at the first 4 days). In some experiments, CD4⁺CD25⁺ T cells were depleted with two doses of 0.5 mg CD25 mAb before immunization 4 days apart, to enhance the responses to mTg (12). To assess thyroid pathology, the thyroids with intact trachea were sectioned vertically through both thyroid lobes (50–60 sections from 10–15 step levels) and stained with hematoxylin and eosin. Mononuclear cell infiltration was scored (blinded scorer) on an index of 0–4.0: 0, normal thyroid; 0.5, small interstitial foci of infiltration involving >0–10% of the thyroid; 1.0, follicular destruction with >10–20% involvement; 2.0, >20–40% involvement; 3.0, >40–80% involvement; and 4.0, >80% involvement (23). Serum mTg Abs were measured by enzyme-linked immunosorbent assay, using plate-bound mTg (1 μ g/well in Immulon II microtiter plates) and alkaline phosphatase-labeled goat anti-mouse IgG (Sigma, St. Louis, MO) (23). The optical density (OD)_{405nm} values were corrected for nonspecific binding by subtracting the OD of normal mouse serum.

Induction and assay of tumor immunity

A mouse mammary adenocarcinoma cell line, A22E-j, was derived from the tumor line SP1, which originated as a spontaneous tumor in a retired female CBA/J breeder. Further subcloning of SP1 resulted in the highly immunogenic/MHC class I⁺ line, designated A22E-j (18,19). After *in vitro* expansion in supplemented RPMI with 10% fetal calf serum, tumor cells were γ -irradiated (γ -tumor cells) with 10,000 rads (¹³⁷Cs-Gammacell 40; Atomic Energy of Canada, Ltd., Ottawa, Canada). Untreated mice or mice depleted of Tregs received 2 injections subcutaneously of 4 $\times$ 10⁶ γ -tumor cells in the left inguinal region, 7 days apart. For challenge, mice were inoculated subcutaneously with 1 $\times$ 10⁵ live tumor cells in the right inguinal region. Tumor growth was monitored 3 $\times$ /week by palpation; once tumor dimensions were measurable, calipers were used. Mice were euthanized when any tumor dimension reached 20 mm or because of ulcerated tumor (20). Tumor volume was calculated as follows: [longer tumor dimension $\times$ (shorter tumor dimension)²]/2.

Serum tumor Abs were determined by FACS analysis. A22E-j tumor cells were grown to 85–90% confluency, dislodged with 1 $\times$ PBS–0.02 M tetra-ethylenediamine acetic acid, washed twice with the medium, and dispensed into a 96-well plate (5 $\times$ 10⁵/well). The cells were then washed twice and resuspended in FACS buffer. Serum samples at 1:10 or 1:20 (for test sera) or 1:20 (for the positive control) were added to each well (50 μ L) for 30 minutes at 4°C and washed. AffiniPure F(ab')₂ fragment from goat anti-mouse IgG, specific for

the Fc γ fragment and conjugated to either FITC or PE (Jackson ImmunoResearch Laboratories, West Grove, PA), was added and incubated for 30 minutes at 4°C. For positive controls, supernatant (50 μ L/well) containing either anti-mouse H2D^k mAb (15–5–5S, murine IgG2a; ATCC, Manassas, VA) or anti-mouse H2K^k mAb (16–3–22S, murine IgG2a; ATCC) was used for MHC class I staining (80–90% positive). In addition, a positive antiserum from a previous experiment was also used. For negative control, supernatant (50 μ L/well) containing anti-mouse IA^d mAb (MK-D6, murine IgG2a; ATCC) was used. Labeled tumor cells were resuspended in FACS buffer, and ~20,000 events/sample were acquired uncompensated in a FACScan flow cytometer; data were analyzed with FlowJo software. The Ab levels are shown as mean fluorescence intensity of FITC or PE signals after subtraction of background staining. In early experiments, background staining was determined by labeling with the FITC-conjugated secondary Ab alone. Later, the assay sensitivity was improved with a PE-conjugated secondary Ab, and subtracting background staining (normal serum + PE-conjugated secondary Ab).

Statistical analysis

Histologic data and tumor Ab results were analyzed nonparametrically with the Mann–Whitney *U*-test. *p*-Values <0.05 were considered statistically significant.

Results

Induction of tumor immunity in CBA/J mice requires both Treg depletion and immunization with irradiated tumor cells

The A22E-j tumor subline was selected for its stable tumorigenic and immunogenic properties and the subcutaneous dose of 1 $\times$ 10⁵ usually killed 100% of CBA/J mice in 5–7 weeks (20). After Treg depletion, two doses of 4 $\times$ 10⁶ ¹³⁷Cs-irradiated tumor (γ -tumor) cells on days –7 and 0, before tumor challenge on day 0 (Fig. 1A, protocol), induced strong protective immunity. Both Treg depletion and γ -tumor cell immunization were required for 100% survival, while all untreated control mice succumbed, and, in those given anti-CD25 or γ -tumor cell treatment separately, death was delayed by only 1–2 weeks (Fig. 1B). Prolonged survival in some mice corresponded with the slower rate of tumor growth as shown by tumor volume (Fig. 1C). Up to the last day of euthanization (day 49), significant tumor-binding Abs were observed only in the protected group (Fig. 1D).

Role of CD4⁺ and CD8⁺ T cell subsets in tumor immunity

To examine the role of T-cell subsets in protection against tumor challenge after antitumor induction with Treg depletion and γ -tumor cells, CD4⁺ and/or CD8⁺ T cells were depleted with two doses of mAbs to CD4 or CD8, 4 days apart, beginning 14 days after the second γ -tumor cell dose (21). The CD4⁺ and CD8⁺ T cell depletion was verified by analyzing the PBL 6 days later, as shown in Supplementary Figure S2. The lethal tumor challenge was given 1 day later. All mice given tumor alone developed tumors, whereas antitumor immunization prevented tumor growth in the immunized control group. Mice depleted of CD4⁺ or CD8⁺ T cells were

fully resistant to lethal tumor challenge 25 days later, indicating that by day 14 postimmunization, strong resistance had developed (data not shown). However, mice depleted of both $CD4^+$ and $CD8^+$ T cells succumbed about 2 weeks sooner than the tumor control group, as the immune cells were evidently depleted rapidly. We next reduced the time interval for depletion of T cells to 7 days after the second dose of γ -tumor cells (Fig. 2A). We also examined the role of tumor Abs at three different time points: first, 7 days after the first γ -tumor cell dose (day -18); second, 6 days after depletion of $CD4^+$ and/or $CD8^+$ T cells (day -1); and third, after tumor inoculation (day 21) (Fig. 2B). Tumor-binding Ab concentrations 7 days after the first γ -tumor dose were comparable in all groups given the depletion regimen, but were greatly reduced, compared to immunized controls, 6 days later (day -1) when $CD4^+$ and/or $CD8^+$ T cells had been depleted. By day 21 after tumor challenge, however, Ab levels in all depleted groups were increased and were comparable to the nondepletion controls. As shown above, all mice in the tumor control group grew tumors, and all immunized mice resisted challenge (Fig. 2C). When both $CD4^+$ and $CD8^+$ T cells were depleted, the mice succumbed at an accelerated rate. However, 3 of 8 mice with $CD4^+$ T cell depletion did not survive, while 1 of 8 mice with $CD8^+$ T cell depletion alone succumbed to tumor challenge. The data suggest that, while Abs play little role in resistance, the protective mechanisms require both $CD4^+$ and $CD8^+$ T cell engagement to fully suppress tumor growth and are fully established by 14 days, but not 7 days, after the immunizing regimen.

Induction of tumor immunity can exacerbate adjuvant-free EAT induction due to prior Treg depletion

We have used an adjuvant-free EAT induction regimen where only soluble mTg was repeatedly injected to simulate the physiologic release of mTg into the circulation (10), and Treg depletion increased thyroiditis incidence and severity (11,12) in the combined models of tumor immunity and EAT induction in EAT-resistant strains (13,15,16). Accordingly, tumor immunity was induced with Treg depletion and γ -tumor cells in EAT-susceptible CBA/J mice and challenged on day 0, followed by 40 μ g mTg i.v. on days 0–24 (16 $\times$ over 4 weeks) (Fig. 3A). We then assessed the

FIG. 1. Induction of tumor immunity requires both Treg depletion and immunization with irradiated tumor cells. **(A)** Experimental protocol. Mice ($n=8$ /group) were either untreated (tumor control) or depleted of Tregs with CD25 mAb and/or immunized with ^{137}Cs -irradiated tumor (γ -tumor) cells before challenge with 1×10^5 live tumor cells. Mice were bled and euthanized on day 49, or sooner, if one tumor dimension reached 20 mm or the tumor became ulcerated. **(B)** Tumor growth was monitored 3 $\times$ weekly, and percent survival is shown. **(C)** The rate of tumor growth was measured in dimensions with calipers and presented as tumor volume ($\dagger$ denotes euthanasia due to ulcerated tumor). **(D)** Tumor-binding Ab concentrations of individual mice at time of euthanasia were measured by fluorescence intensity using the FITC-conjugated secondary Ab. Positive tumor-binding Ab control value=14 at 1:20 dilution. Ab, antibody; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; Treg, regulatory T cell.

FIG. 2. Tumor immunity is not well established by 7 days, but still requires both CD4⁺ and CD8⁺ T cells. (A) Experimental protocol. Tumor immunity was induced in mice ($n=8$ /group) by Treg depletion and γ -tumor cell immunization, followed by CD4⁺ and/or CD8⁺ T cell depletion 7 days later. On day 0, live tumor cells were injected. Mice were bled on days -18, -1, and 21 and on day 49 at euthanization or sooner if necessary. (Dotted lines demarcate additions to Fig. 1.) (B) Tumor-binding Ab concentrations were measured by fluorescence intensity using the PE-conjugated secondary Ab at three different time points before euthanization: (a) 7 days after the first γ -tumor cell dose (day -18); (b) 6 days after depletion of CD4⁺ and/or CD8⁺ T cells (day -1); and (c) 21 days after tumor inoculation (day 21). (C) Tumor growth was monitored 3 $\times$ weekly, and percent survival is shown. PE, phycoerythrin.

influence of inducing EAT on tumor incidence and percent survival on day 35. While tumor control mice all succumbed, tumor immunity provided 100% protection (Fig. 3B). No effect was observed on inhibition of tumor growth by the presence of moderate EAT development ($p=0.4$). On

FIG. 3. Induction of tumor immunity exacerbates adjuvant-free EAT induction due to prior Treg depletion. (A) Experimental protocol. Mice ($n=6$ /group) were subjected to Treg depletion, γ -tumor cell immunization, live tumor challenge on day 0, and repeated 40 μ g mTg injections as shown, or Treg depletion and mTg injections only. They were bled and euthanized on day 35 post-tumor inoculation or sooner if necessary. (Dotted lines demarcate additions to Fig. 1.) (B) Tumor growth was monitored 3 $\times$ weekly, and percent survival is shown. (C) Thyroids were harvested on day 35 post-tumor inoculation, and percent thyroid infiltration is presented for individual mice. EAT, experimental autoimmune thyroiditis; mTg, mouse thyroglobulin.

the other hand, induction of tumor immunity required Treg depletion (Fig. 1), and thyroiditis was significantly enhanced when Treg depletion was followed by repeated mTg doses to simulate physiologic release of circulatory mTg, compared to mTg doses alone ($p=0.03$; Fig. 3C). The

histopathological data of 5%, 15%, 20%, and 35% thyroid infiltration from this experiment are shown in Supplementary Figure S3.

Establishing pre-existing autoimmune status by priming with mTg and varying doses of IL-1 as adjuvant

In Figure 3, we show that Treg depletion to aid induction of tumor immunity could lead to enhanced EAT development. Another clinical scenario could involve cancer patients with ongoing or subclinical autoimmune conditions being subjected to immunostimulation, as shown by elevated TSH levels and hypothyroidism in prostate cancer patients given peptide vaccine and Flt3 ligand adjuvant (9). To mimic ongoing autoimmunity, we primed mice with 40 μ g mTg and various doses of IL-1. IL-1 is one of the inflammatory cytokines released when bacterial lipopolysaccharide is administered and high doses can substitute for lipopolysaccharide in EAT induction (24). Initially, mice were immunized with mTg and 20,000 U IL-1 followed by Treg depletion and repeated doses of mTg. At euthanization, 35 days after the first soluble mTg dose, thyroid infiltration was observed in mice with additional mTg doses, but had only marginally significant pathology compared to mice treated with mTg+20,000 U IL-1 (data not shown).

We next titrated the IL-1 dose to reduce the degree of pre-priming toward subclinical condition. We also lowered the soluble mTg doses to 20 μ g. Mice were given mTg and either 10,000 U or 5000 U IL-1, followed by Treg depletion and repeated injections of mTg (Fig. 4A). To ascertain that priming against mTg had occurred, mice were bled 7 days after the second dose of mTg and 10,000 U or 5000 U IL-1, and tested for mTg Abs. The mice showed measurable mTg Ab titers (Fig. 4B, day -15), which increased at 11 days after the last dose of repeated mTg injections (day 35), with higher levels in the 10,000 U IL-1-treated group. Similarly, thyroiditis was more severe in the 10,000 U IL-1-treated group than in the 5000 U IL-1-treated group (Fig. 4C). There was no significant difference between the group primed with mTg and 10,000 U IL-1 followed by Treg depletion and repeated mTg injections and the group primed with mTg and 10,000 U IL-1 alone. There was also no significant difference between the group treated with mTg and the lower dose of 5000 U IL-1, followed by Treg depletion and repeated mTg injections, and the group given mTg and 5000 U IL-1 only. However, when both 10,000 U and 5000 U IL-1 pretreated groups were compared with mice without the pretreatment, thyroid pathology was significantly greater ($p=0.002$ and $p=0.047$, respectively). Since thyroiditis induced by mTg with 10,000 U IL-1 was more severe than with 5000 U IL-1, exacerbation was more difficult to discern. In contrast, while thyroiditis induced with mTg and 5000 U IL-1 treatment alone was not greatly different from Treg depletion and repeated mTg injections, the combination did show a significant difference. Thus, the data suggest that mTg+5000 U IL-1 could potentially represent a subclinical condition.

Effect of combining prepriming by mTg and 10,000 U IL-1 with tumor immunity induction

We first tested prepriming with mTg and 10,000 U IL-1, followed by induction of tumor immunity with Treg deple-

FIG. 4. Thyroiditis induced with mTg and either 5000 or 10,000 U IL-1 represents mild to moderate pre-existing autoimmunity, and only mild thyroiditis is enhanced by subsequent Treg depletion and repeated mTg doses. **(A)** Experimental protocol. Mice (6/group) were primed with 40 μ g mTg and either 10,000 or 5000 U IL-1 followed by Treg depletion and/or additional doses of 20 μ g mTg. Mice were bled and euthanized on day 35. (Dotted lines demarcate additions to Fig. 3.) **(B)** Mice were bled on days -15 and 35, and mTg Ab levels were measured by enzyme-linked immunosorbent assay. The bars represent the mean Ab level of all mice in the group. **(C)** Thyroids were harvested on day 35, and percent thyroid infiltration is presented for individual mice. IL-1, interleukin-1.

tion and γ -tumor cell immunization. Repeated mTg doses were also initiated on day 0 of lethal tumor challenge (Fig. 5A). Whereas control mice given live tumor cells succumbed to the tumor, induced tumor immunity protected 100% of the mice as before (Fig. 5B). All mice with the additional pre-priming by mTg and 10,000 U IL-1 and mTg doses also

FIG. 5. Moderate ongoing EAT is not enhanced by induced tumor immunity and additional doses of mTg and vice versa. **(A)** Experimental protocol. Mice (6–8/group) were either unimmunized or immunized with 40 µg mTg and 10,000 U IL-1, followed by induction of tumor immunity, lethal tumor challenge, and repeated 40 µg mTg doses. Mice were bled and euthanized on day 35 or sooner if necessary. (Dotted lines demarcate additions to Fig. 4.) **(B)** Tumor growth was monitored 3×weekly, and percent survival is shown. **(C)** Thyroids were harvested on day 35, and percent thyroid infiltration is presented for individual mice ($n=8$).

survived. Tumor-binding Ab concentrations determined at euthanization on day 35 were similarly high in mice with induced tumor immunity and in mice preprimed with mTg and 10,000 U IL-1 and repeated mTg injections (data not shown). On day 35, the extent of thyroid infiltration was also unchanged by the presence of tumor growth inhibition and additional mTg injections (Fig. 5C).

FIG. 6. Mild thyroiditis is enhanced when combined with induction of tumor immunity and additional doses of mTg without affecting tumor immunity. **(A)** Experimental protocol. Mice (6–8/group) were primed with 40 µg mTg and 5000 U IL-1, followed with or without antitumor induction, tumor challenge, and/or additional doses of 20 µg mTg. They were euthanized on day 35 or sooner if necessary. **(B)** Tumor growth was monitored 3×weekly, and percent survival is shown. **(C)** Thyroids were harvested on day 35, and percent thyroid infiltration is presented for individual mice.

Mild thyroiditis induced with mTg and 5000 U IL-1 is enhanced when combined with tumor immunity induction and repeated mTg doses

The above experiment was repeated with the lower 5000 U IL-1 dose since, as shown in Figure 4C, it represents a milder, subclinical condition (Fig. 6A). In all mTg + 5000 U IL-1-treated

groups, priming was confirmed by mTg Ab production 7 days later. Again, the percent survival remained 100% in the presence of ongoing EAT (Fig. 6B). Thyroiditis in the group primed with mTg and 5000 U IL-1 along with Treg depletion and repeated mTg doses was significantly higher than in the group treated only with Treg depletion and mTg injections ($p=0.01$) (Fig. 6C). Thyroiditis induced with mTg and 5000 U IL-1 with Treg depletion and repeated mTg doses was also significantly higher than in mice primed with mTg and 5000 U IL-1 only ($p=0.04$). Tumor growth inhibition in mice with induced tumor immunity did not further affect or increase the autoimmune response. Thus, although induced tumor immunity was not compromised, thyroiditis severity could be exacerbated by the tumor immunity induction regimen involving Treg depletion and the simulation of physiologic release of mTg.

Discussion

From recent studies of three models combining breast cancer vaccination and EAT development on EAT-resistant backgrounds, we have learned that, similar to self-tolerance to mTg in EAT, tolerance to Her-2/neu antigen in Her-2/neu-transgenic mice can be broken by Treg depletion followed by immunization with Her-2 DNA + pGM-CSF, the murine granulocyte macrophage colony-stimulating factor (14,16). Moreover, the resultant tumor regression can add to the immune stimuli to augment EAT development in a resistant strain (14). In F₁ mice co-expressing H2A^b and HLA-DR3, induction of Her-2 immunity is independent of DR3, but the extent of EAT development corresponds to the susceptibility allele DR3 (16). To understand the extent of MHC class II gene influence on autoimmunity risk during immunotherapy, the CBA/J strain, a well-characterized, EAT-susceptible strain, was selected.

After testing a CBA/J tumor immunogenic variant, an anti-tumor immunity model that could be incorporated into our adjuvant-free protocol of EAT induction was obtained (20). We determined that strong tumor immunity against lethal challenge to this tumor line, A22E-j, required both Treg depletion and immunization with γ -tumor cells (Fig. 1). This strong immunity was established at 14 days, but incomplete at 7 days, postimmunization, as shown by depletion of CD4⁺ and/or CD8⁺ T cells (Fig. 2). These experiments also suggested that protection could not be attributed solely to tumor Abs, since all mice succumbed after both CD4⁺ and CD8⁺ T cell depletion, even more rapidly than tumor controls, despite the presence of tumor Ab levels equivalent to immunized mice with no depletion. When soluble mTg was repeatedly administered, the immune response to tumor was not reduced, nor any enhancement of tumor immunity discernible since 100% of the immunized mice were already protected (Fig. 3B). As to EAT development, the mTg injections during tumor regression, which might have provided immune stimuli as seen with EAT-resistant mice (14), did not lead to changes in thyroid infiltration (Fig. 3C). However, as we reported previously (12), mTg doses simulating normal physiologic fluctuation of circulatory mTg levels usually result only in low incidence and mild thyroid infiltration even in susceptible strain unless there is prior Treg depletion. Thus, Treg depletion associated with induction of tumor immunity did increase EAT incidence and extent of infiltration after repeated soluble mTg doses ($p=0.03$; Fig. 3C).

If indeed the protocol to boost tumor immunity could lead to an increased autoimmune response, what if an autoimmune condition were to exist before vaccine therapy as seen in a clinical trial with Flt3 ligand (9)? To address the question whether the extent of pre-existing autoimmune condition influenced the autoimmune sequelae, we titrated the dose of IL-1 as adjuvant to control the extent of pre-existing EAT following a previous protocol (24). When the IL-1 dose was reduced from 20,000 U to 10,000 or 5000 U and given with mTg, priming to mTg was verified by the production of mTg Abs (Fig. 4B). The extent of thyroid infiltration was greater in the mTg/10,000 U IL-1-treated group than in the mTg/5000 U IL-1-treated group, as one might expect (Fig. 4C). After additional Treg depletion and repeated mTg doses, a difference, although less significant than the 10,000 U group, was seen with the combination of mTg/5000 U IL-1 pretreatment followed by Treg depletion + soluble mTg. Thus, pretreatment with mTg/5000 U IL-1 could potentially represent a subclinical (mild thyroiditis) condition.

The selection of the 5000 U IL-1 dose over the 10,000 U IL-1 dose for further study of subclinical condition appeared warranted, when we examined the data from incorporating induction of tumor immunity with prepriming with mTg/10,000 U IL-1 to assess changes in tumor immunity and extent of thyroid infiltration. No effect on either tumor immunity or thyroiditis was observed. Additional repeated mTg doses also had no effect on the extent of protective immunity or EAT development (Fig. 5B, C). Apparently, the maximum for each parameter had been reached. In contrast, when prepriming was carried out with mTg/5000 U IL-1 and verified by mTg Ab production 7 days later, tumor immunity remained strong and unchanged, but the mild thyroiditis was significantly augmented by Treg depletion and repeated mTg doses (Fig. 6B, C).

Our findings in this EAT-susceptible strain show that perturbation of Treg function is the primary reason for EAT augmentation and is unrelated to the immune response to tumor immunization involving both CD4⁺ and CD8⁺ T cell expansion with attendant cytokine release. Tumor immunity and EAT development appear to proceed independently. Also, where prior EAT development had occurred as with 10,000–20,000 U IL-1, further Treg depletion and soluble mTg treatment as could be expected from physiologic release of mTg into the circulation had no discernible effect. This observation is in line with normal Treg function, where it is most efficient in preventing the activation of autoreactive T cells, rather than inhibiting an ongoing autoimmune response (12,25). Moreover, we have shown that Tregs influence susceptibility but do not supersede MHC class II restriction (26). In susceptible strain, no additional stimuli are necessary to contribute to EAT development other than repeated mTg doses (10,11), unlike concurrent induction of tumor immunity and EAT in EAT-resistant strains, where tumor regression could provide further immune stimuli (14,16). In clinical trials, treatment with systemic adjuvant enhanced autoimmune thyroiditis (9), and treatment with anti-CTLA-4 and anti-CD52 led to adverse autoimmune manifestations (3,4). These agents decreased Treg function either by depletion or by interference, or both (1). Since the HLA class II status of the patients was unknown, its specific contribution to the observed autoimmunity could not be correlated with the resulting risk or the severity. Clearly, both thyroiditis-susceptibility and thyroiditis-

resistance alleles were present, as were the additional stimuli from responses to tumor peptide vaccination.

In conclusion, targeting specific sites of the immune system either to boost cancer immunity or inhibit severe autoimmune symptoms has led to further immune dysregulation and severe autoimmune sequelae in both sets of patients. Particularly risky immunotherapeutic treatments are those perturbing the Treg network. Moreover, not only are MHC class II genes involved in Treg ontogeny and selection, but also their role lies in specific autoantigen presentation. With more and more immunotherapeutic mAbs, including anti-CTLA-4, receiving approval from the Food & Drug Administration, potential autoimmune complications should receive greater scrutiny.

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Author Disclosure Statement

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