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Autoimmune thyroiditis as an indicator of autoimmune sequelae during cancer immunotherapy

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Abstract

Improving cancer immunotherapy by targeting T cell network also triggers autoimmunity. We disrupted regulatory T cell (Treg) function to probe the balance between breast cancer vaccination and autoimmune thyroiditis (EAT) in four models, with particular attention to MHC-associated susceptibility, EAT induction with mouse thyroglobulin (mTg) without adjuvant, and tolerance to Her-2/neu in transgenic mice. 1) In EAT-resistant BALB/c mice, Treg depletion enhanced tumor regression, and facilitated mild thyroiditis induction. 2) In Her-2 tolerant C57BL/6 mice expressing HLA-DR3, an EAT-susceptibility allele, Her-2 DNA vaccinations must follow Treg depletion for (Her-2xDR3)F₁ mice to resist tumor challenge; thyroiditis incidence was moderated by the EAT-resistant IAb allele. 3) In neu tolerant, EAT-resistant BALB/c mice, implanted neu⁺ tumor also regressed only after Treg depletion and DNA vaccinations. Tumor immunity was long-term, providing protection from spontaneous tumorigenesis. In all three, immune stimuli from concurrent tumor regression and EAT development have a noticeable, mutually augmenting effect. 4) In Treg-depleted, EAT-susceptible CBA/J mice, strong tumor protection was established by immunization with a cell vaccine. mTg injections led to greater thyroiditis incidence and severity. Combination models with MHC class II diversity should facilitate autoimmunity risk assessment and management while generating tumor immunity.

Keywords

Immunotherapy and autoimmunity; Autoimmunity risk; Regulatory T cell; Her-2/neu tolerance; Thyroiditis indicator; Autoimmune sequelae

1. Cancer immunotherapy trials reveal autoimmune complications

To improve the effectiveness of cancer immunotherapy and incorporate recent understanding of the immune system, specific targeting of the T cell network has become prominent in clinical trials. Depletion or blockade of regulatory T cell (Treg) function or activation of effector T cells by immunomodulating agents can enhance anti-tumor immunity, but the same regimen

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also triggers autoimmunity. A recent review summarized the responses of 139 metastatic melanoma patients who were treated with repeated doses of a CTL-associated antigen 4 (CTLA-4) monoclonal antibody (ipilimumab) in conjunction with a peptide vaccine [1]. Immune-related adverse events (IRAE) included grade 1/2 dermatitis with pruritis, and the more problematic grade 3/4 enterocolitis and hypophysitis, resulting in intervention or cessation of therapy. Autoimmune hypophysitis with multi-organ endocrine disorders was documented in an earlier trial including patients with metastatic melanoma and renal cancer [2]. Of the 139 patients, grade 3/4 IRAE were observed in 28% (14 of 50) of patients with an objective response, but no anti-tumor response in the remaining 72%. Of 36 patients experiencing grade 1/2 IRAE, only 8 displayed a response [1]. The total response rate of 17% included 3 patients with complete response and more severe IRAE, and 20 with partial response. Thus, IRAE were judged to be associated with the probability of improved anti-tumor response. Yet, of those 62% (86 of 139) with mild to severe IRAE, 74% (64 of 86) showed no objective improvement.

In other trials using systemic immunomodulators, interferon (IFN)- α -2b treatment of melanoma patients resulted in 26% developing anti-thyroid and other autoantibodies [3]. A review of earlier IFN- α therapy trials of breast cancer and carcinoid tumor patients showed up to 30% autoimmune thyroid disease in patients with or without pre-existing thyroid autoantibodies [4]. In a recent review of 134 IFN- α -treated melanoma patients, 15% developed autoimmune thyroiditis [5]. In prostate cancer patients treated with Flt3-ligand adjuvant for a peptide vaccine from a family member of human epidermal growth factor receptor (Her-2/rat neu), 2 of 15 developed elevated levels of thyroid-stimulating hormone (TSH) and antibodies to thyroid antigens [6]. The high frequency of autoimmune thyroid disease undoubtedly relates to the prevalence of Hashimoto's thyroiditis and Graves' disease in the general population. Among US Caucasians, 45% of women and 20% of men show focal thyroiditis at routine autopsy, and 1% of women and 0.05% of men exhibit clinical symptoms [7,8]. While new and revised regimens are being initiated and pondered [9], the high autoimmune manifestations clearly necessitate better risk assessment and establishment of assay criteria, besides close monitoring and rapid initiation of intervention. In using an HLA-A2-restricted peptide as vaccine, both melanoma and prostate cancer clinical trials enrolled HLA-A*0201 patients. Attention to the HLA composition of each patient, taking into account the HLA class II gene association with susceptibility to autoimmune diseases and their prevalence in the general population, should be an important part of risk assessment and management.

2. Criteria for combination models for experimental autoimmune thyroiditis (EAT) and breast cancer

Recently, we undertook to combine tumor vaccine and autoimmune thyroiditis models to study the balance between the two during therapy that targets Treg function. We selected mouse strains with known susceptibility or resistance alleles to EAT and breast cancer models in which DNA vaccines have known efficacy. In each separate experimental system, Treg influence is either well established, as with EAT induction in both susceptible and resistant strains, or can be studied to enhance DNA vaccine efficacy, as seen in wild type mice or in mice transgenic for Her-2/neu breast cancer antigen, as described in the following sections.

Suppressor T cell (now known as Treg) influence on the autoimmune response was first observed in the mid-70s, when neonatal thymectomy with or without irradiation in mice [10] and rats [11] led to spontaneous thyroiditis, indicating the presence of autoreactive T cells. Indeed, suppression can be overcome when a genetically susceptible mouse strain is immunized with mouse thyroglobulin (mTg), either with LPS as adjuvant [12] or alone in repeated doses [13] simulating normally circulating Tg in the host. EAT became an established

autoimmune disease model for Hashimoto's thyroiditis, sharing the same major autoantigen, Tg, and pathogenic mechanisms involving thyroid destruction by infiltrating CD4⁺, CD8⁺ T cells and macrophages [12]. EAT also compares favorably to Hashimoto's thyroiditis for the predominant influence of genetic factors, since susceptible MHC class II genes are associated with both EAT (*H2A^k*) and Hashimoto's thyroiditis (DR3) [12,14]. Environmental factors, such as increased iodine intake, exacerbates autoimmune thyroid disease in mice [15,16], although the epidemiological evidence in humans is less clear [7].

To offset such predisposing factors in mice, self tolerance can be strengthened by mTg pretreatment to prevent EAT induction, possibly due to the activation of Tregs [17], which were subsequently identified to be CD5⁺ [18] and later CD4⁺, as mAbs became more discriminatory [19]. The most recent Treg surface marker is CD25; CD25 mAb enables *in vitro* separation as well as *in vivo* depletion of CD4⁺CD25⁺ Tregs and permits functional studies in EAT [20].

Removal of natural CD4⁺CD25⁺ Tregs enables EAT-resistant strains to develop EAT following mTg immunization with or without adjuvant [21,22]. Moreover, in EAT-susceptible strains, removal of naturally existing CD4⁺CD25⁺ Tregs or Tregs expanded and/or differentiated following mTg pretreatment abrogates tolerance and exacerbates the severity of mTg-induced thyroiditis [20,23,24]. Antigen-specificity of Treg activation has been demonstrated with endogenously released mTg by TSH infusion in an osmotic pump, resulting in tolerance strengthened to withstand EAT induction by mTg and LPS adjuvant [25]. These Tregs also exhibit the specific Foxp3⁺ marker, but this intracellular marker is unsuitable for isolation or depletion of CD4⁺CD25⁺ Tregs [24].

The studies in both susceptible and resistant strains reveal that, while CD4⁺CD25⁺ Tregs influence susceptibility, they do not supersede MHC class II restriction [23]. This finding indicates that inserting EAT induction into tumor models on different MHC backgrounds, in conjunction with Treg depletion, would not alter greatly the genetic restriction on thyroiditis development. Moreover, several immunomodulators used to enhance tumor immunity have been examined in EAT tolerance; administration of anti-CD137 [20], anti-glucocorticoid-induced tumor necrosis factor receptor GITR [26], or anti-CTLA-4 [24] with tolerogenic mTg interferes with tolerance induction to EAT.

As to the role of Treg influence on tumor immunity, we [27] and others [28] have shown that depleting CD4⁺CD25⁺ Tregs from tumor-bearing mice resulted in the regression of certain mouse tumors, suggesting a potential immunotherapeutic tool in boosting tumor immunity. Thus, models combining tumor vaccination and EAT under the umbrella of Treg depletion should enable an assessment of the balance between autoimmunity and tumor immunity and whether MHC class II genes play a role in this balance.

3. Concurrent induction of EAT and anti-tumor immunity in EAT-resistant strain

We first used an established breast cancer model in an EAT-resistant (*H2A^d*) strain [22]. The TUBO cell line was derived from a spontaneous mammary tumor from a BALB rat neu transgenic (NeuT) mouse and grows progressively in BALB/c mice. CD25 mAb given at the time of tumor inoculation depleted Tregs and enabled tumor immunity to develop, resulting in tumor regression. Treg depletion also facilitated mild thyroiditis induction with mTg and LPS. To simulate physiologic exposure to circulatory antigen, mTg was given i.v. 16x, 4x/wk over 4 wks. In Treg-depleted mice with regressing tumor, mTg antibody level and IFN- γ production were increased, and 43% developed mild thyroiditis (5–10% thyroid involvement). Interestingly, immune responses to neu or mTg were greater than in control mice given mTg

or tumor separately, suggesting that, while tumor immunity may have been enhanced during autoimmune stimuli, autoimmunity may also have been augmented even with an EAT-resistant MHC haplotype.

One cytokine recently associated with chronic inflammation in autoimmune disease models of multiple sclerosis [29] and arthritis [30] is IL-17, which is produced by a CD4⁺ effector T cell subset distinct from the Th1 and Th2 lineage [31]. Since immunity to TUBO tumors and EAT is primarily Th1-dependent, we determined the relative contribution of IL-17-producing cells in TUBO regression and EAT development after Treg depletion [32]. Using separate groups of BALB/c female mice, TUBO cells were inoculated with CD25 mAb 1 day apart, and EAT induction with mTg/LPS followed Treg depletion by 7 days; control mice were not given CD25 mAb. Severe thyroiditis (Fig. 1A) or tumor regression (Fig. 1C) was observed only in Treg-depleted mice, where significant IFN- γ -producing T cells were also observed (Fig. 1B, 1D). However, IL-17-producing T cells were detectable only in mice with thyroiditis (Fig. 1B). Mice receiving LPS injections without mTg had no measurable IL-17 response (data not shown). Whether IL-17 production contributed to augmented anti-neu antibody and T cell response after concurrent induction of tumor regression and autoimmune thyroiditis [22] is unknown, nor is it known the stimulus provided in these mice to enhance the autoimmune response.

4. Concurrent induction of EAT and anti-tumor immunity in (HLA-DR3xC57BL/6)F₁ mice tolerant to Her-2

We next produced an F₁ strain by mating EAT-resistant B6 (H2A^b) mice with HLA-DR3 transgenic mice to introduce an EAT susceptibility allele for mTg [14]. Both wild type B6 mice and Her-2 transgenic B6 mice were used as a parental strain, and the resultant F₁ mice expressed both A^b and DR3 [33]. Furthermore, the Her-2xDR3 mice were tolerant to both mTg and Her-2, simulating human conditions. Indeed, to induce tumor rejection in Her-2xDR3 mice, Treg depletion alone was insufficient and must be followed by 3x electroporation of Her-2 DNA vaccine + pGM-CSF. Tumor rejection was similar in Her-2 transgenic mice expressing either A^b or A^b/DR3. After repeated mTg doses without LPS, initiated 7 days post-Treg depletion, the presence of DR3 resulted in 33% of mice showing thyroiditis, but the incidence and severity were reduced in Her-2xDR3 mice, as expected in a cross between EAT-resistant A^b and -susceptible DR3 haplotype [33]. Thus, induction of Her-2 immunity is independent of DR3 expression, but EAT induction is enhanced by the presence of DR3. As in wild type BALB/c mice (Sect. 3), we noted a modest increase in IFN- γ -producing T cell responses to Her-2 and mTg, when both Her-2 vaccine and mTg were given concurrently to Treg-depleted mice, compared to mice under separate Her-2 vaccine and mTg regimens. Thus, each regimen provided additional stimuli to the response of the other. In these experiments, tumor regression was not assessed in mice given concurrent immunization. It is unknown if tumor regression would further increase the response to EAT induction in this model.

5. Concurrent induction of EAT and anti-tumor immunity in NeuT BALB/c mice tolerant to neu

In Sect. 3, we described earlier data wherein neu⁺ TUBO tumors underwent regression after Treg depletion [22]. Since Her-2 tolerant B6 mice with or without DR3 transgene required vaccination in addition to Treg depletion to induce tumor regression (Sect. 4) [33], we recently re-examined BALB/c mice made tolerant to neu to determine if Her-2/neu tolerant mice in a different strain likewise required both Treg depletion and subsequent neu DNA vaccination to induce tumor immunity, and if concurrent EAT induction with repeated doses of mTg affected the response to both "self" antigens [34]. Also, since untreated NeuT females normally develop

spontaneous tumors at 17–19 weeks, protection from spontaneous tumorigenesis after inducing tumor immunity could also be assessed. Indeed, only Treg depletion followed by neu DNA vaccination (3x biweekly pneuTM + pGM-CSF and electroporation) abrogated tolerance to neu and resulted in complete regression of implanted neu⁺ TUBO tumors [34]. Furthermore, long-term protection from spontaneous tumorigenesis was observed in 58% of mice. To assess the impact of tumor regression on EAT development in this EAT-resistant strain, mTg injections with or without LPS were initiated later, about 3–4 wks after Treg depletion when Tregs were slowly re-emerging from the thymus. Nevertheless, there were significant increases in IFN- γ -producing T cell responses to both neu and mTg in tumor-regressing mice. When LPS adjuvant was used, all 7 mice developed thyroiditis up to 60% thyroid destruction, whereas only 2 of 4 mice not given tumor challenge developed moderate thyroiditis. Thus, concurrent induction of EAT and tumor immunity with tumor regression after Treg depletion can provide additional stimuli to each regimen, and any tumor immunity-enhancing protocol must seriously consider the extent of developing autoimmune sequelae.

6. Development of combined model of tumor immunity and EAT in EAT-susceptible CBA strain

In the three combination models described above, the MHC haplotypes were either EAT-resistant or F₁ strain harboring a resistance allele. The risk in patients susceptible to autoimmune diseases can only be studied in a susceptible host where thyroiditis would be more severe and further compounded by targeting the T cell compartment. We thus began developing a tumor protection model in the CBA (H2^k) strain, using an immunogenic variant, A22E-j, with enhanced class I expression, which had been derived following 5-azacytidine treatment of a CBA spontaneous mammary adenocarcinoma line, SP1 [35,36]. Fig. 2A illustrates the 100% tumor-free finding in Treg-depleted mice after vaccination with two doses of irradiated A22E-j cells and challenge as shown by the protocol. Treatment with irradiated tumor cells or CD25 mAb (not shown) separately did not produce strong immunity to challenges. When we rechallenged the protected mice on day 68, 100% of the mice were again resistant to challenge, indicating strong memory to a tumor antigen (data not shown). In a subsequent experiment, repeated mTg doses were given without LPS to simulate the physiologic presence of circulatory Tg in all individuals (Fig. 2B). The majority (67–75%) of mice developed moderate thyroid destruction regardless of the presence of protected tumor immunity (in 100% mice receiving tumor challenge). In addition to the reported role of CD8⁺ cells in protection [37], preliminary data suggest that both CD4 and CD8 effector T cell subsets contribute to the protective mechanisms in this model. This new combination immunotherapy model shows promise and requires further characterization.

7. Concluding remarks

In probing potential autoimmune sequelae during cancer immunotherapy simulating human conditions, we have examined four combination models using both EAT-resistant and -susceptible strains with attention to mTg immunization protocol without adjuvant. In transgenic, resistant strains made tolerant to Her-2/neu, protection requires Treg depletion plus repeated vaccinations. Additional stimuli occasioned by tumor regression and EAT development appear to augment both T cell responses, warranting intensive monitoring of IRAE during immunotherapy. The use of the EAT-susceptible model will aid the study of exacerbation of pre-existing subclinical conditions due to the high prevalence of autoimmune thyroid disease, as already observed [6].

Take-home messages

- Cancer immunotherapy targeting T cell network also triggers autoimmune sequelae.
- Combination models for breast cancer and autoimmune thyroiditis with MHC class II diversity enable a study of balancing the two and ultimately tilting it toward tumor immunity.
- Additional stimuli during tumor regression and thyroiditis development augment T cell responses, warranting establishment of assay criteria to monitor IRAE (immune-related adverse events).

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Fig. 1.

IL-17 secretion is associated with thyroiditis but not tumor regression in EAT-resistant BALB/c female mice. Mice were depleted of CD25⁺ Tregs 7 days prior to immunization 2x with 40 μ g mTg followed in 3 hrs by 20 μ g LPS, i.v. (A) Thyroid pathology was determined 28 days later. (B) mTg-specific T cells producing either IFN- γ or IL-17 were assayed by ELISPOT. In another group, mice were inoculated on day 0 with neu⁺ TUBO tumor cells; CD25 mAb was given on day 1. (C) All mice grew tumors, but tumors regressed only in mice depleted of Tregs. (D) Mice with tumor regression were assayed for IFN- γ or IL-17 response to neu by ELISPOT, following restimulation with neu⁺ 3T3NKB and neu⁻ control 3T3KB cells.

Fig. 2.

Depletion of CD25⁺ Tregs combined with irradiated tumor cell immunization protects mice from tumor challenge and enhanced the incidence and severity of mTg-induced thyroiditis in an EAT-susceptible strain. (A) Each female CBA/J mouse was given 1 mg CD25 mAb on days -18 and -14 followed by $2-3 \times 10^6$ irradiated tumor cells (A22E-j) on days -7 and 0 before challenge with 1×10^5 live tumor cells on day 0, as shown (6 mice/group). (B) Soluble mTg without adjuvant was given 16x during tumor rejection in vaccinated mice as shown. Thyroid pathology for individual mice was determined on day 79–86.