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**EXAMINING AUTOIMMUNE SEQUELAE DURING CANCER IMMUNOTHERAPY
IN A COMBINED AUTOIMMUNE THYROIDITIS AND TUMOR MOUSE MODEL**

by

SURESH CHANDRA KARI

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

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DOCTOR OF PHILOSOPHY

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**MAJOR: IMMUNOLOGY AND
MICROBIOLOGY**

Approved by:

Advisor

Date

DEDICATION

īśvarah paramah krsnah

sac-cid-ānanda-vigrahaḥ

anādir ādir govindah

sarva-kāraṇa-kāraṇam

The above verse is in Sanskrit from Brahma Samhita. It translates as follows;

“Krsna who is known as Govinda is the Supreme Godhead. He has an eternal blissful spiritual body. He is the origin of all. He has no other origin and He is the prime cause of all causes”.

I am eternally grateful and indebted to my parents Sitaramachandra Rao and Aruna Lakshmi and brother Mahesh Chandra for making me capable enough to stand where I am today.

I would like to thank my wife Nivedita for all her support and love.

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CHAPTER 1

INTRODUCTION

I. Experimental autoimmune thyroiditis

Experimental autoimmune thyroiditis (EAT) is an animal model of Hashimoto's thyroiditis (HT), an organ-specific, T cell-mediated autoimmune disorder of humans (1). The prevalence rate of this hypothyroid syndrome is 8/1000 in the US population (2). Graves' disease or hyperthyroidism is also widely prevalent in the US population. Hypothyroidism is more common in women compared to men (3). In a study conducted in USA, subclinical thyroiditis was observed in 4.6% of the population (4,5). As is evident from the data, HT affects a substantial number of individuals in the population and, many of whom are not even diagnosed as having the disease (6-8). It is therefore important to study the disease in order to understand the various predisposing factors and also the mechanism of disease development. Rose *et al.* (9-11) immunized dogs, rabbits and guinea pigs with homologous and heterologous thyroid extracts in complete Freund's adjuvant, which resulted in thyroiditis and autoantibody production. Later work in our laboratory with self antigen, mouse thyroglobulin (mTg) in mice, by immunization alone without adjuvant demonstrated the existence of autoreactive T cells which could be primed and activated to produce autoimmune thyroiditis (12). Some earlier models of EAT also included the use of rat and chicken (13). Because of the ease of manipulation and the wide range of strains and reagents, making it easier to study the immunogenetics and pathogenic mechanisms of the disease, the murine model has become the most important.

The major histocompatibility complex (MHC) plays an important role in defining susceptibility to autoimmune disorders. The peptide-binding groove of MHC molecules contains variable regions and this genetic polymorphism determines the specificity and affinity of peptide

binding and T cell recognition. For HT, the association with human leukocyte antigen (*HLA*) complex has been studied in patient populations and genetic susceptibility has been associated with the *HLA-DRA/DRB1*03:01* (HLA-DR3) allele, *HLA-DRB1*04:05* (HLA-DR4), *HLA-DRB1*11/*12* (HLA-DR5) in Caucasians. The mouse *H2* class II genes are very similar to the *HLA* class II region in defining susceptibility in multiple autoimmune disorders (14). In particular, murine EAT has been very valuable in pinpointing the *HLA* class II association in HT using transgenic mice (15-17). For example, the introduction of *HLA-DR* and *DQ* genes into mouse MHC class II knockout mice demonstrated that HLA-DR3 is the susceptibility allele for EAT (18) and that *HLADQ*03:01/DQB1*03:02* (HLA-DQ8) molecules can downmodulate DR3-mediated thyroiditis (19).

For murine EAT, susceptibility has been mapped to the *H2 k* and *s* haplotypes, while the *b* and *d* haplotypes are resistant (20). The *k* haplotype strain CBA/J is used in our lab. In the CBA/J strain, females are somewhat more susceptible to the disease compared to males. In the *H2* complex, susceptibility has been narrowed to the class II subregion, especially the *IA* subregion (21).

The disease is induced in mice by immunization with 40 µg of mTg intravenously (iv), followed by 20 µg of *Salmonella enteritidis* lipopolysaccharide (LPS) 3 hrs later. These injections are repeated after 7 days and EAT is usually evaluated 3 weeks later (22). After immunization, the readout for EAT is T cell proliferation to mTg *in vitro*, mTg autoantibody levels and thyroid pathology. The self-reactive T cells, both CD4⁺ and CD8⁺, as well as macrophages, traffic to the thyroid and cause the destruction of the follicles. The extent of thyroid infiltration is determined by examining histologic sections. Since LPS induces the production of IL-1 *in vivo* (23), immunomodulatory IL-1 cytokine can also be used as a T cell

adjuvant to induce EAT. This protocol involves using 20 μg mTg followed 3 hrs later with varying doses of IL-1 (24). The injections are repeated after 7 days and the mice sacrificed 3 weeks later to examine thyroid pathology. In our EAT model, repeated injections of 40 μg mTg can also induce autoimmunity without the need for adjuvant in a susceptible strain (12). The induced disease with repeated injections of soluble self antigen can be exacerbated by depletion of $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ regulatory T cells (Tregs) prior to the induction process (25).

The kinetics of mononuclear cell infiltration into the thyroid were investigated by immunochemical analysis (26). The ratio of CD4^+ and CD8^+ T cells increased from 2.4 on day 21 to 3.0 on day 42. The experiment therefore clearly indicated the early importance of CD4^+ T cells which recruited macrophages for thyroid infiltration and destruction. The CD8^+ T cells that followed are also involved in thyroid pathology as evidenced by their cytotoxic destruction *in vitro* of thyroid monolayer (27).

In spite of having susceptible *HLA* alleles and autoreactive T cells, not all susceptible humans and animals develop autoimmunity. This self resistance against the manifestation of autoimmunity is important in control of onset of disease in susceptible individuals as well as in individuals where the disease process has already started.

II. Tolerance in EAT

Natural self resistance against autoimmunity is called tolerance. Tolerance helps in discriminating between self and non-self. It is achieved by two processes: 1) Central tolerance- where high affinity autoreactive T cells are eliminated in the thymus; and 2) peripheral tolerance- where autoreactive T cells against self antigens are actively suppressed (28-30). Central tolerance not only involves deletion of autoreactive T cells but also the selection of Tregs. The Treg population helps in maintaining T cell tolerance in autoreactive T cells which have escaped

central tolerance, and contributes towards peripheral tolerance (28,31). It was earlier believed that natural tolerance to EAT in genetically susceptible, nonimmunized mice was due to sequestration of antigen or antigen which is not released into the circulation in the host. As a result the autoreactive T cells could not react as they did not see the antigen. In experiments shown by Kong *et al.* in 1982 (32), this notion was not sustainable. They also challenged that autoimmunity is not due to lack of suppressor T cells (Tregs) as these cells exist in susceptible mice. Subsequent studies showed that several protocols can strengthen tolerance against induction of EAT: 1) Bovine thyroid-stimulating hormone (TSH) infusion via mini-osmotic pumps placed in the peritoneal cavity (33,34); 2) high dose tolerance with two doses of 100 µg of deaggregated mTg (dmTg), 7 days apart; 3) daily low dose of 10 µg mTg for 10 days (33); and 4) LPS, which interferes with the kinetics of mTg clearance, given 24 hrs before mTg (34). Tolerance induction by all four protocols was shown to correlate with raised, circulatory mTg for 2-3 days (33,34).

The cells mediating tolerance were first identified as Thy-1⁺ T cells (32). With the advances of monoclonal antibody (mAb) technology, rat IgG2b monoclonal antibodies (mAbs) against mouse L3T4⁺ (CD4⁺) and Lyt2⁺ (CD8⁺) T cells were used in depletion studies (35). The data showed that CD4⁺ T cells were responsible for the induced tolerance with mTg and tolerance was abrogated when these cells were depleted. Thus tolerance mechanisms involve active suppression that requires the constant presence of Tregs. Other experiments, subtolerogenic doses of dmTg along with gamma-irradiated spleen cells (γSC) taken from mTg-primed mice were found to inhibit the induction of EAT (36). It was interesting to note that this synergistic suppression was effective only when dmTg was given before γSC, suggesting that suppression was dependent on initial activation of regulatory CD4⁺ T cells. The difference

between using dmTg alone (32) and adding γ SC was the importance of CD8⁺ Tregs in the second protocol (36), as CD4⁺ Tregs were activated first in order to suppress EAT induction. As to the duration of tolerance, this was tested by challenging with mTg and LPS at intervals after pretreatment with dmTg, two doses 7 days apart. Significant decrease in mTg antibody titers and thyroid infiltration was observed beginning 10 days to 94 days after pretreatment (37). The duration of tolerance was similar when either dmTg or TSH infusion was used for tolerance induction (37).

The contribution of cytokines to the tolerant status was next examined. Human recombinant IL-1 β (IL-1 β), a pro-inflammatory cytokine, given at 4000U. 3 hrs after the injection of mTg, inhibited the induction of tolerance and the animals developed EAT upon challenge with mTg plus LPS (24). However, the timing of IL-1 β administration was important in inhibiting the establishment of tolerance. IL-1 β given 1 or 2 days after dmTg pretreatment failed to inhibit tolerance induction to EAT. Similar to the use of LPS in the challenge, IL-1 β was unable to abrogate suppression in animals already tolerized with dmTg. Next, the role of IL-12 was examined (38). IL-12 along with dmTg primed the autoreactive T cells and converted tolerogenic dmTg into an immunogen. As was shown with IL-1, the timing of IL-12 injection along with dmTg was important. On the other hand, IL-4 and IL-10 were found to have minimal or no role in EAT tolerance induction (39).

Sakaguchi *et al.* (40) investigated the mechanisms of peripheral tolerance and found that it was mediated by CD4⁺CD25⁺ Tregs in naive mice. Thus CD25 (IL-2R α chain) becomes an additional marker for Tregs. This Treg population constitutes about 10% of the peripheral CD4⁺ cells. When the CD4⁺ population was depleted of CD4⁺CD25⁺ Tregs and transferred into BALB/c athymic nude mice, spontaneous multiorgan autoimmunity developed. Thus, as we

discussed earlier (32,35), peripheral tolerance is actively maintained in the periphery by Tregs which keep the self-reactive clones of T cells in check. Subsequently, Hori *et al.*, (41) examined scurf mutant mice that express a multiorgan lymphoproliferative disorder. It was found that a forkhead transcription factor Foxp3 was lacking, and that this gene was expressed more in the CD4⁺CD25⁺ Treg population. To date, Foxp3 is the most specific marker available for Tregs.

To define the importance of CD4⁺CD25⁺ Tregs in induced tolerance to EAT, Morris *et al.*(42) depleted CD4⁺CD25⁺ Tregs with CD25 mAb and abrogated the established tolerance, again demonstrating its importance in EAT. Moreover, tolerance cannot be induced without the presence of this Treg subset (25).

Tolerance mechanisms in EAT were studied in recent years with the aid of the CD25 marker for Treg isolation. Several costimulatory molecules were examined. CD137 is a member of the tumor-necrosis factor receptor superfamily and is found on activated B and T cells, activated NK cells, DCs (43,44). CD137 is known to interact with 4-1BB ligand and is involved in lymphocyte survival, costimulation and enhancement of effector function. Morris *et al.* (42) observed that anti-CD137 given 2 hrs prior to the injection of dmTg inhibited the induction of tolerance. CD137 mAb was found to partially abrogate established tolerance *in vivo* in mice. The effect on established tolerance was also demonstrated *in vitro*, when CD137 mAb inhibited the suppression by CD4⁺CD25⁺ Tregs. The cell target of CD137 mAb appeared to be mTg-primed T cells, where the anti-CD137 agonistic role increased the proliferative response.

Glucocorticoid-induced tumor necrosis factor receptor (GITR) was next examined for its immunomodulatory role on Tregs and tolerance (45). Injection of GITR mAb also interfered with tolerance induction by dmTg and converted its role from a tolerogen to an immunogen, since thyroiditis resulted without subsequent EAT induction. This conversion was reminiscent of

IL-1 and IL-12 action in providing a priming stimulus (24,46). For comparative purposes, in EAT, IL-1 and anti-GITR are the stronger autoimmune stimuli than IL-12 and anti-CD137.

In a similar manner, administration of CTLA-4 mAb with dmTg interfered with tolerance induction, indicating the important role of CTLA-4 as a marker for Treg function. However, once the mTg autoreactive T cells had been primed, the presence of CTLA-4 mAb was no longer effective; it did not inhibit the suppressive action of Tregs *in vitro* on mTg-primed T cells (25).

III. Clinical problem: increased autoimmunity while boosting anti-tumor immune response

Combination of surgery, radiation and chemotherapy are the current treatment strategies for tumor (47). Since these treatment modalities are non-specific and impose many side effects along with recurrences due to incomplete removal of tumor, there has been increased focus on immunotherapeutic strategies targeting the tumor or immune network. Tumor immunotherapy provides either passive or active immunity against tumors (48). Active immunotherapy involves harnessing the immune system by vaccination with tumor-associated antigens, or just tumor antigens, to induce or expand antigen-specific T cells (47). Passive immunotherapy includes many immunologic agents made outside of the host. Some sources of tumor antigens are: products of oncogenic viruses and products of oncogenes or mutated oncosuppressors (49). Tumor antigens are shared antigens between cancer cells and normal cells and may differ in terms of expression (50).

Tumors are known to suppress and evade the immune response using various immunosuppressive factors such as TGF- β , adenosine, prostaglandins E2, gangliosides (50,51). The immunosuppressive effect may be directed toward a tumor-specific response or it might be local or systemic immunosuppression. Tumors are also known to actively recruit and accumulate

Tregs or more specifically induced Tregs (iTregs) (52-54). This generalized immunosuppression or iTreg activation is one of the many reasons responsible for lack of good immune response against tumors (55,56).

As we discussed in the previous sections, autoimmune thyroid disease in animals can be generated by breaking tolerance using various protocols such as self antigen, heterologous antigen (15), or DNA (57), with or without adjuvant and Th1 cytokines to prime T cells. In a similar fashion, antigen-specific, anti-tumor responses can be generated by breaking tolerance using tumor antigens and Th1 cytokines (58), or heterologous antigens or DNA (59-61), whole-cell vaccines (62), peptide vaccines (63,64) and recombinant protein vaccines (55,56). These anti-tumor immune responses can be further enhanced by using immunomodulators. Some of them are CTLA-4 mAbs (65), anti-vascular endothelial growth factor mAbs to block growth of blood vessels (66) and heat shock protein antibodies (67,68).

Antigen-presenting cells (APCs) such as DCs capture tumor as well as self antigens during anti-tumor immune responses, leading to priming of autoreactive T cells and autoimmune sequelae associated with tumor immunotherapy (69). Immunotherapy must deal with many obstacles: immune tolerance to tumor antigens, weak antigens, and immune evasion mechanisms employed by growing tumors (70,71). Tolerance may be broken during an infection or tissue damage providing costimulatory signals and cytokines to autoreactive T cell expansion, increasing undesirable sequelae during tumor immunotherapy.

Autoimmune thyroiditis was reported in patients treated with IL-2 and lymphokine-activated killer cells for metastatic melanoma (72). Interestingly, 5 of the 7 patients having hypothyroidism had tumor regression, whereas only 5 of the 27 patients who were euthyroid had evidence of tumor regression. The authors hypothesized that treatment regimen

might be causing hypothyroidism by exacerbating pre-existing autoimmune thyroid condition and is associated with favorable anti-tumor response.

There are cellular antigens overexpressed by tumors (73). One example is MART-1 in melanoma (74). Any vaccination regimen targeting MART-1 could potentially lead to autoimmune responses against melanocytes leading to vitiligo (75). In another report, treating melanoma patients with vaccine and low dose IL-2 led to autoimmune toxicities such as vitiligo, diabetes and autoimmune thyroiditis (76). Patients with head and neck cancers undergoing radiation and chemotherapy also developed autoimmune thyroiditis or subclinical thyroiditis (77). Subclinical hypothyroidism was evident by high TSH level but normal T4 levels which was termed as compensated thyroid. The authors surmised that chemotherapy was sensitizing the thyroid gland to radiation leading to destruction and subsequent thyroiditis.

Blansfield *et al.* (78) reported autoimmunity in melanoma patients treated with mAb against CTLA-4. CTLA-4 mAb prevents the negative costimulatory signal to be delivered to the activated T cells. Anti-CTLA-4 also inhibits the functioning of Tregs which have higher expression of CTLA-4 on their surface than the activated T cells (25). Therefore using this antibody not only will enhance the anti-tumor immune response but will also lead to generalized autoimmunity. The autoimmune manifestations, including diabetes, hypophysitis, hypothyroidism, were reported in 25% of the patients. Tuve *et al.* (79) used a tumor cell line TC-1, which was transfected to stably express CTLA-4 antibody, to examine TC-1 tumor progression in mice. In immunocompetent mice given CTLA-4 transfected TC-1 tumors, majority of the tumors regressed completely, whereas tumors grew in SCID mice and CD8 knockout mice. The anti-tumor immune response was shown to correlate with IFN- γ -secreting CD8⁺ T cells. The blockade by CTLA-4 was also combined with anti-CD25 Treg depletion and

was found to enhance the tumor regression synergistically. There was complete absence of accompanying autoimmunity with intratumoral CTLA-4 blockade with or without systemic Treg depletion.

Interferon- α as a systemic modulator has been used to treat hepatitis C virus infection, hairy cell leukemia and Kaposi's sarcoma (80). The treatment regimen is known to initiate or exacerbate HT, type 1 diabetes, Graves' disease and systemic lupus erythematosus. In a study on hepatitis C virus infection, the prevalence of patients with hypothyroidism increased from 6.1% to 17.2% (81). Female gender and the presence of thyroid peroxidase (TPO) antibodies were considered to be risk factors in developing autoimmune hypothyroidism and could also lead to exacerbation of clinical hypothyroidism or of subclinical hypothyroidism (82,83). Autoimmune disorders, such as psoriasis, type 1 diabetes and systemic lupus erythematosus have also been shown to arise during treatment of conditions such as renal cell carcinoma, bladder carcinoma, Kaposi's sarcoma with interferon α (84). In an article by McNeel *et al.* (85), 2 out of the 15 subjects treated with E75 immunodominant HLA-A2- restricted epitope from HER-2/neu tumor antigen as a vaccine and Flt-3 ligand as an adjuvant for prostate cancer developed elevated TSH levels and hypothyroidism. Examining retrospectively, high anti-thyroid antibody titer was found in at least one patient who developed hypothyroidism later which is consistent with the clinical picture of pre-existing subclinical autoimmune hypothyroidism.

Human epidermal growth factor receptor-2 (HER-2) is a marker overexpressed or amplified in approximately 20% of breast cancer (86). Since HER-2 was an attractive target for treating breast cancers, trastuzumab or Herceptin, a humanized mAb against HER-2, was developed with encouraging results (86). HER-2 is also expressed by heart muscles cells (71) and cardiac dysfunction was observed in 13% of the patients being treated with paclitaxel, an

antiproliferative agent, and trastuzumab compared to 3-7% in patients treated with trastuzumab alone (86,87).

The reasons for autoimmune toxicities were also discussed in many articles. Some authors have argued that autoimmune side effects are due to increased antibody levels from triggering autoreactive B cell clones leading to autoimmunity (88). One article suggests that the serum levels of B cell-activating factor be assessed as risk factor before initiation of interferon therapy which might affect the development of thyroid autoimmune disorders (89,90). It was suggested that long term treatment with IFN- α leads to increased thyroid autoimmunity and this phenomenon was reversible after withdrawal (90).

It is clear from these clinical reports that immunomodulation with cytokines, stimulatory or blocking antibodies can have a broad effect on the immune system leading to different types of sequelae, particularly autoimmune responses. Of these autoimmune side effects, thyroiditis seems not to be associated with a particular type of therapy during many trials. One reason could be that the vast population is afflicted from thyroiditis but is unaware of it (4.3% subclinical hypothyroidism, according to national health and nutrition examination survey III) (5). The disease was also higher in females than in males and more in whites compared to blacks (3). It may also be correlated with the levels of autoantibodies both to thyroglobulin and thyroid peroxidase, and more so with thyroid peroxidase antibodies.

IV. Autoimmunity-tumor models

In recent years, there have been efforts made to understand the mechanisms involved in the interplay between tumor immunity and autoimmunity. Our lab and others are developing combined models of tumor and autoimmunity in order to mimic the clinical scenarios and understand the underlying mechanisms.

Early studies in 1980 (91) in a methylcholanthrene-induced fibrosarcoma model in BALB/c x C57BL/6F1 mice demonstrated that Tregs were derived from the thymus. The immunity against the tumor was generated by injection of endotoxin (*Salmonella enteritidis* lipopolysaccharide B) (92) and the cells from such animals were used to treat syngeneic recipients. The transfer of primed spleen cells against the tumor caused regression in the recipients which were devoid of T cells (thymectomized and whole body irradiated). The tumor bearing hosts however acquired a state of immunological tolerance which was dependent on the growth of the tumor. Excision of tumor however led to the emergence of tumor-specific immune response which prevented further growth.

It is now acknowledged that Tregs can inhibit priming of the immune system against tumor (93). Depletion of Tregs followed by vaccination against a poorly immunogenic B16 melanoma was shown to prime the immune system and induce a CD8⁺ T cell immune response.

The B16 melanoma in mice has also been used as a model of tumor and autoimmunity. In this model, the immune response is against melanoma-associated antigens and a robust T cell-mediated immune response can also react with melanocytes leading to destruction and vitiligo (94,95). Since B16 tumor is poorly immunogenic, immunostimulatory regimens such as, altered peptide vaccination with IL-2 could aid the overcoming of tolerance (95).

CD8⁺ T cells are important in rejection against melanoma as demonstrated in CD4 and CD8 knockout mice in C57BL/6 mice (96). Residual tumor cells in the host were important to achieve concomitant tumor immunity which aids the rejection of secondary tumors at distant sites. While it is known that CD8⁺ T cells play a protective role in the destruction of melanoma, the role of CD4⁺ T cells is unclear. Some groups claimed that, in order to prime CD8⁺ T cells, CD4⁺ T cells were not required and priming could take place in complete absence of CD4⁺ T

cells as evidenced by depletion studies (94,97). Others showed the importance of CD4⁺ T cells in the long-term protection (98).

Although a shared antigen as melanocyte in different organs is one part of the clinical picture, the other part is the completely unrelated autoimmune side effects during tumor immunotherapy. Our autoimmunity and tumor models represent the steps taken to address this other question and to find out the reasons therein.

In order to study the effect of EAT on established tumor models, the first combined EAT-tumor model was developed in BALB/c (*H2^d*) mice (99). The tumor cell line used was TUBO which was derived from a spontaneous mouse mammary tumor in BALB/c NeuT transgenic mice (100). Treg depletion within 7 days of tumor inoculation led to increased anti-tumor immune response and regression of tumors, as TUBO is sufficiently immunogenic to stimulate the immune system. Protection was found to be long term in the Treg-depleted animals, as the mice rejected a second tumor challenge even at week 14. Treg depletion also increased anti-mTg immune response in animals injected with tumor followed by repeated injections of mTg (40 µg) without LPS. This model had one drawback as it was not in an EAT-susceptible strain.

In another series of experiments, HER-2xDR3 and HER-2 transgenic mice were used to study the regulation of tumor immunity and autoimmunity (101). These mice were generated to simulate the clinical scenarios of HER-2 tolerant tumor patients with an HT-susceptible allele, HLA-DR3 (101). The HER-2 tolerant mice harbored the human *erB-2* gene (102) and HER-2 immunity was induced with HER-2 DNA vaccine in the presence of granulocyte monocytes colony stimulating factor. It was found that anti-HER-2 immunity in HER-2 and HER-2xDR3 mice was increased after Treg depletion. While autoimmune responses against mTg were also increased in HER-2 and HER-2xDR3 transgenic mice, anti-mTg T cell immune response was

higher in DR3 transgenic mice, as expected. HER-2 immune responses were therefore independent of HLA-DR3, whereas Tregs controlled the immune response to both antigens (mTg and HER-2). This model is technically cumbersome, because HER-2 transgenic mice are heterozygous and when mated with DR3 transgenic mice, the F1 animals would carry both DR3 and $H2^b$ genes.

In a third EAT-tumor model, BALB/c NeuT female mice which spontaneously develop mammary tumors were used to examine the influence of tumor regression on mTg immunity (103). There was significant enhancement of autoimmunity including thyroid pathology in mice undergoing tumor regression. Thus, autoimmunity risk might be associated with increased stimulus during vigorous anti-tumor immune responses.

These tumor-autoimmunity models provide us with a basic idea about the potential problems which can be encountered in cancer patients suffering with tumors. These models, though important in identifying the potential interplay between tumor immunity and autoimmunity, focus more on the possible use of autoimmunity as an indicator for robust anti-tumor immune response, as well as potential undesirable side effects. They can also be used to measure the increase in anti-tumor immune response due to autoimmunity in a synergistic or additive manner. However, these models do not focus on autoimmune responses linked to particular MHC molecules or the role of Tregs in controlling peripheral tolerance, since EAT-susceptible strain may render stronger autoimmune responses. We therefore searched for a combined model of tumor and autoimmunity in an EAT-susceptible strain.

V. EAT-tumor model in CBA/J ($H2^k$) strain

Study in (DR3x $H2^b$) F1 mice has shown that MHC class II haplotype has little or no effect on tumor immunity but it has an effect on EAT induction (101). In EAT-susceptible strains,

depletion of Tregs has been shown to enhance autoimmune responses and replenishing Tregs reduces thyroid pathology (104). Similarly it has been shown that removing Tregs enhances the anti-tumor immune responses in EAT-tumor models (99).

Searching for a suitable tumor in EAT-susceptible CBA/J mice led us to a tumor line reported by Elliot *et al.* (105). This tumor arose spontaneously in a female retired CBA/J breeder. The histology and the ultrastructural morphology were similar to mammary ductal carcinoma. This cell line was designated as SP1 and was grown *in vitro* in cell cultures. After treatment with DNA alkylating agents, a subline A22E-j was found to have a relatively high expression of MHC class I ($H2^k$) genes and is more immunogenic than the parent line. This cell line was cultured and tested *in vivo* for both tumorigenic and immunogenic properties and was used as a stock tumor line for our tumor-EAT model.

An initial report on the establishment of an anti-tumor induction model involves depleting Tregs followed by vaccination with irradiated tumor cells 7 days apart (106). Subsequent challenge with live tumor cells subcutaneously demonstrated 100% survival rate. For this dissertation, the anti-tumor induction model has been combined with the EAT model where the disease is induced by repeated injections of mTg (12).

As discussed previously, autoimmune thyroid disorders appear frequently during immunotherapy protocols treating tumors or viral diseases. One of the reasons is the high prevalence of a population with focal thyroiditis (3,107), existing as subclinical autoimmune thyroiditis. As discussed previously, the study by McNeel *et al.* (85) demonstrated the potential pitfalls of tumor immunotherapy administered with a systemic adjuvant which could enhance pre-existing subclinical autoimmune hypothyroidism. Those patients with high antibody levels against thyroglobulin had subclinical thyroiditis which was enhanced by the use of a systemic

adjuvant. We tried to create similar clinical scenarios in our mouse model of thyroiditis and tumor.

Since in the clinical trials the end points are regression and patient survival, little effort has been made forward understanding the genetics of the responses or potential side effects such as severe autoimmune sequelae. To understand the basis of increased autoimmunity and assess potential risk, we have attempted in our experimental designs to generate various clinical scenarios in our mouse model and sought answers to some of the questions.

CHAPTER 2

MATERIALS AND METHODS

Mice. CBA/J mice (females) were purchased at 6 weeks of age from Harlan Sprague-Dawley (Frederick, MD, via C. Reeder, NIH). Mice were kept on acidified, chlorinated water before being used at 8-12 weeks of age.

Preparation of peripheral blood leukocytes (PBL) for T cell subset analysis. The procedure briefly involved collecting 0.5 ml of heparinized blood from each mouse from the tail artery. The red blood cells were lysed with distilled water at room temperature, 4.5 ml/tube for 10 seconds, after which the reaction was stopped by adding 0.5 ml of 10x phosphate buffered saline (PBS). It was followed by adding 5 ml of 1x Hanks' balanced salt solution and washing for 10 min by centrifugation at 1000 rpm (120xg). The process was repeated twice.

mAbs and T cell subset depletion. For the depletion of Tregs *in vivo*, rat anti-mouse CD25 mAb was used. PC61 hybridoma cells (derived from parent cell lines: P3X63Ag8.653 (murine myeloma cell line) and OFA rat spleen cells; ATCC, Manassas, VA) (108) were propagated in the lab, frozen and sent to Harlan Bioproducts (Harlan Bioproducts for Science, Indianapolis, IN) where hybridoma cells were injected into athymic nude mice and the resultant ascites fluid served as the source of CD25 mAb (rat IgG1_λ). PC61 concentration was determined with ELISA anti-rat Ig_λ mAb (clone B46-5, mouse IgG1, BD Biosciences, San Jose, CA). CD4⁺CD25⁺ T cells were depleted with two doses of 0.5 mg mAb iv 4 days apart prior to immunization as per experimental protocol and the depletion was assessed by fluorescent activated cell sorter (FACS) 6 or 7 days after the second dose (104). While CD25 is a marker for Tregs, the most specific

marker for Tregs is Foxp3 (41,109). In the EAT model, it has been shown previously that CD4⁺CD25⁺Foxp3⁺ Tregs are responsible for peripheral tolerance, the removal of which abrogates tolerance (25).

For FACS analysis, the PBL preparation was resuspended in FACS buffer (1% bovine serum albumin, 5% normal rabbit serum, 0.1% sodium azide in 1x PBS) and labeled with appropriate mAbs as described below.

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 IgG2b, eBioscience, San Diego, CA) were used. The cells were then fixed and permeabilized as per manufacturer's instructions prior to labeling with Foxp3 mAb conjugated with cychrome 5.5 (FKJ-16S, rat IgG2a, eBioscience) to verify depletion of CD4⁺CD25⁺Foxp3⁺ cells (usually 70-90% of the Tregs are depleted) (25).

For CD4 and CD8 T cell depletion, rat anti-mouse CD4 mAb (640 mg YTS191.1 + YTA 3.1, rat IgG2b) and rat anti-mouse CD8 mAb (320 mg YTS 169.4, rat IgG2b), kindly supplied by Dr. H. Waldmann (Univ. Oxford, U.K.), were injected iv 4 days apart (110), after anti-tumor vaccination at the times indicated in each protocol. Mice were bled 6-7 days after the second dose of mAb to assess the depletion in PBL using FACS. In order to monitor the accurate depletion of CD4⁺ and CD8⁺ T cells by FACS analysis, different antibodies were used for labeling. For CD4⁺ T cells, mAb YTS 177.9.6 (rat IgG2a, kindly supplied by Dr. H. Waldmann) against murine CD4 at 1:50 dilution was followed by biotinylated secondary mAb RG7/1.30 (murine IgG2b, BD Biosciences) against rat IgG2a at 1:100 dilution and Streptavidin-PE (BD Biosciences) at 1:400 dilution. For CD8⁺ T cells, mAb YTS 105.18 (rat IgG2a, kindly supplied by Dr. H. Waldmann) at 1:50 dilution was followed by biotinylated RG7/1.30 at 1:100 dilution

and Streptavidin-PE at 1:400 dilution. Labeling with T cell receptor (TCR) mAb conjugated with PE (H57-597, hamster IgG anti-mouse TCR) at 1:80 dilution, was performed to measure total T cell number and assess depletion of different subsets of T cells.

Usually, 20,000 events/sample tube were acquired uncompensated on FACScan flow cytometer (BD, San Jose, CA) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR) (104).

Induction and assay of EAT.

Thyroglobulin. mTg was obtained from frozen mouse thyroids (Mayo Clinic, Rochester, MN or Harlan Bioproducts for Science, or Pel-Freez LLC., Rogers, AR), fractionated on a Sephadex G-200 column as previously described (22). Presence of LPS was checked in mTg by the *Limulus* amoebocyte assay (Associates of Cape Cod, Woods Hole, MA) (a 40 µg dose contained <1ng of LPS). Aliquots were stored frozen at 2 mg/ml and diluted for use in nonpyrogenic PBS.

Immunizations. EAT induction was carried out injection of 40 µg mTg in 0.1 ml iv followed 3 hrs later by 20 µg LPS (trichloroacetic acid precipitated and kindly supplied by Dr. C. Jeffries) in 0.1 ml on days 0, 7. Immunization or priming with mTg was also performed iv by injection of 40 µg of mTg followed 3 hrs later with varying doses of IL-1, 5,000, 10,000 or 20,000U in 0.2 ml nonpyrogenic PBS, 7 days apart. IL-1 was purchased from eBioscience at 0.5 mg/ml which contained 1×10^4 units/µg. Immunization with mTg was also performed without adjuvant by repeated injections of 20 or 40 µg mTg, 16 injections over 4 weeks (4 daily injections/week at the first 4 days each week). In some experiments, CD4⁺CD25⁺ T cells were depleted with two doses of 0.5 mg CD25 mAb prior to immunization 4 days apart, to enhance the responses to mTg (104).

Proliferative response to mTg. Spleen cells (6×10^5 cells/well) were cultured in RPMI plus 1% normal mouse serum (supplemented with 25 mM HEPES buffer, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 2 mM L-glutamine, 50 μM β -mercaptoethanol) for 5 days, at 37°C under 5% CO₂ with or without 40 $\mu\text{g/ml}$ mTg in flat or round bottom 96-well tissue culture plates (Falcon, USA). Cultured cells were pulsed with 1.0 $\mu\text{Ci/well}$ [³H] thymidine 18 hrs before harvesting onto glass fiber filter paper (Tomtech Mach3Man Cell harvester, LKB Wallac, Gaithersburg, MD). The incorporation of radioactive thymidine was assessed by a Microbeta Plus 1450 liquid scintillation counter (LKB Wallac) (22).

Determination of mTg antibody. Mice were bled from the tail artery and the resulting sera were stored at -20°C. mTg Abs were measured by ELISA, using plate-bound mTg (1 $\mu\text{g/well}$ in Immulon II microtiter plates) and alkaline phosphatase-labeled goat anti-mouse IgG (Sigma), as described previously (38). The OD_{405nm} values were corrected for nonspecific binding by subtracting the OD of NMS.

Evaluation of thyroiditis. Histologic examination of H&E stained thyroids was used to assess thyroid pathology. The thyroids with intact trachea were sectioned vertically through both thyroid lobes (50-60 sections from 10-15 step levels). Mononuclear cell infiltration was scored (double-blind) 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 (22).

Tumor model in EAT-susceptible CBA/J mice.

Tumor cell line, stock aliquots, and expansion for experimental use. A mouse mammary adenocarcinoma cell line A22E-j was used in our lab (105). It was derived from the tumor line, SP1, which originated as a spontaneous tumor in a retired female CBA/J breeder. The

SP1 cell is virtually negative for expressing MHC class I molecules and is very tumorigenic but not very immunogenic. Treatment of SP1 with 5-azacytidine (which inhibits DNA methylation and is known to activate some genes) resulted in a less tumorigenic and more immunogenic A22 cell line with increased MHC class I expression (111). The histology and the ultrastructural morphology were similar to mammary ductal carcinoma. A subclone of A22 tumor was resected after growth *in vivo* and was designated A22E. Further subcloning resulted in the highly immunogenic/MHC class I⁺ line, designated A22E-j (105,112). Both SP1 and A22E-j were kindly supplied by Dr. Bruce Elliot (Queen's University, Kingston, Ontario, Canada). When immunogenicity and tumorigenicity of A22E-j cell line appeared stabilized as reported earlier (106), the cell line was expanded twice and stored in aliquots in liquid nitrogen in supplemented RPMI containing ~40% fetal calf serum (FCS, Atlanta Biologicals, Lawrenceville, GA) and 10% dimethyl sulfoxide and used as stock for immunization, tumorigenesis and assay for tumor antibodies.

For each experimental use, the stock A22E-j aliquots were thawed, washed, and cultured in 75 cm² tissue culture flasks in supplemented RPMI with 10% FCS as follows. When the monolayer was 85-90% confluent, the cells were passaged. This involved removing the spent medium, adding 5 ml of pre-warmed (37°C) RPMI, and incubating for 5 min in the CO₂ incubator. After removal of medium, 5 ml of 0.125% trypsin was added, and the cells were again incubated for 5 min at 37°C in the CO₂ incubator, followed by washing twice at 1000 rpm (120xg) for 5 min. The cultured tumor cells were expanded 2-3 times to achieve the required cell numbers. No cells expanded for experimental use were returned to liquid nitrogen.

Induction of anti-tumor immunity with irradiated tumor cells. After *in vitro* expansion, tumor cells were γ -irradiated (γ -tumor cells) with 10,000 rads using a cesium source

(¹³⁷Cs-Gammacell 40, Atomic Energy of Canada, Ltd., Ottawa, Canada). Untreated mice or mice depleted of Tregs on days -14 and -10 with 0.5 mg CD25 mAb iv were immunized sc with 4x10⁶ γ -tumor cells in the left inguinal region on days -7, 0 (106). For challenge, mice were inoculated sc on day 0 with 1x10⁵ live tumor cells in the right inguinal region. Tumor growth was monitored 3x/week by palpation. Mice were sacrificed when any tumor dimension reached 20 mm or because of ulcerated tumor (106).

Tumor immunotherapy after tumor inoculation. Mice were first inoculated sc with varying tumor doses of A22E-j on day 0 in the right inguinal region followed by Treg depletion iv with CD25 mAb on days 0, 4. Mice were then immunized with 4x10⁶ γ -tumor cells sc on days 7, 14 in the left inguinal region. Tumor growth was monitored 3 times/week and mice were sacrificed when any tumor dimension reached 20 mm or because of ulceration.

Determination of tumor antibody. Sera were collected from mice with anti-tumor immunized and unimmunized mice and stored at -20°C. A22E-j tumor cells were grown in 75 cm² tissue culture flasks until 85-90% confluency. The cells were dislodged by first washing with 1x RPMI at 37°C in incubator followed by 5 min at room temperature using 1x PBS-0.02M ethylene diamine tetra-acetic acid (EDTA). The cells were then washed two times with complete medium (10% FCS) and dispensed into 96-well plate (5x10⁵/well). The cells were washed twice in FACS buffer and resuspended. Serum samples at 1:10 or 1:20 dilution were then added to each well (50 μ l) for 30 min at 4°C and washed. AffiniPure F(ab')₂ fragment from goat anti-mouse IgG conjugated to either FITC or PE (Jackson ImmunoResearch Laboratories, PA), specific for Fc γ fragment of mouse IgG, was added and incubated for 30 min at 4°C. For positive controls, supernatant (50 μ l/well) containing either anti-mouse H2D^k mAb (15-5-5S, murine IgG2a) or anti-mouse H2K^k mAb (16-3-22S, murine IgG2a), both kindly supplied by Dr. B.

Elliott, was used for MHC class I staining (80-90% of cells were positive, data not shown). A positive antiserum from a previous experiment was also used as positive control. For negative control, supernatant (50 μ l/well) containing anti-mouse IA^d mAb (MK-D6, murine IgG2a, ATCC) was used.

The labeled tumor cells were resuspended in FACS buffer processed through FACScan flow cytometer; usually 20,000 events/sample tube were acquired uncompensated and analyzed with FlowJo software. The antibody levels were shown as mean fluorescence intensity of FL1 (FITC) channel or FL2 (PE) channel. Early experiments with tumor antibody involved the use of FITC-conjugated secondary antibody, background was subtracted using secondary antibody alone. In order to improve sensitivity of the assay, brighter fluochrome, i.e. PE-conjugated secondary antibody, was used and non-specific binding (with normal serum) was subtracted from each serum sample before presenting the data.

Statistical analysis. Differences between groups of *in vitro* proliferation assays were analyzed using the unpaired Student's *t*-test. Histologic data and tumor antibody results were analyzed non-parametrically with the Mann-Whitney *U* test. P values <0.05 were considered statistically significant.

CHAPTER 3

RESULTS

I. Characterization of anti-tumor immunity model in CBA/J mice.

As reviewed recently, there are various strategies to induce anti-tumor immune responses (47). Inducing anti-tumor immune responses by various strategies however also leads to activation of autoreactive T cells leading to autoimmune sequelae (107). The importance of the T cell compartment in both autoimmunity and tumor immunity led us to design scenarios that assess the contribution of CD4⁺ and CD8⁺ T cells and the known role of Tregs in regulating these immune responses (25,99). In EAT, inhibiting Treg function leads to enhanced thyroiditis (25). Treg depletion therefore serves as a good way of manipulating the immune system and mimicking the impact of using immunotherapeutic or immunomodulatory regimens for treating various disorders including cancers. Since the immunogenetics of various cancers were not well known, we first examined combined autoimmunity and tumor models in EAT-resistant strains (103). While Tregs are important in regulation of autoimmune, and anti-tumor immune responses, only EAT has well-defined MHC class II-based susceptibility (12,20,113). For this reason, we decided to design a combined autoimmunity and tumor model in our EAT-susceptible strain, CBA/J mice.

A. Establishing tumor immunity induction protocol in CBA/J mice

Apart from maintaining peripheral self tolerance (40), Tregs masks the immune responses to tumor antigens and depletion of Tregs unmask the immune response leading to rejection of established tumors (114,115). Treg depletion is important in the early phase of tumor immunity which involves priming of immune cells against tumor antigens. Since we did not know what tumor antigens in the A22E-j line might induce immunity, we resorted to using intact γ -tumor

cells as vaccines (106,116-118). We immunized mice using γ -tumors cells with or without prior depletion of Tregs and compared the immunity induced (Fig. 1A). The mice inoculated with A22E-j tumor cells developed tumors starting on days 7-21. The groups treated with either Treg depletion alone or γ -tumor cells alone also showed a slightly delayed tumor growth but all eventually succumbed. The mice given Treg depletion followed by γ -tumor cells, the anti-tumor induction protocol, showed 100% protection (Fig. 1B) against the growth of tumors. In order to assess the role of antibodies in tumor protection, mice were bled at day 49, or earlier at the time of sacrifice due to tumor growth. The highest antibody level was observed in the fully protected group. Tumor-binding antibodies were analyzed by fluorescence intensity (Fig. 1C).

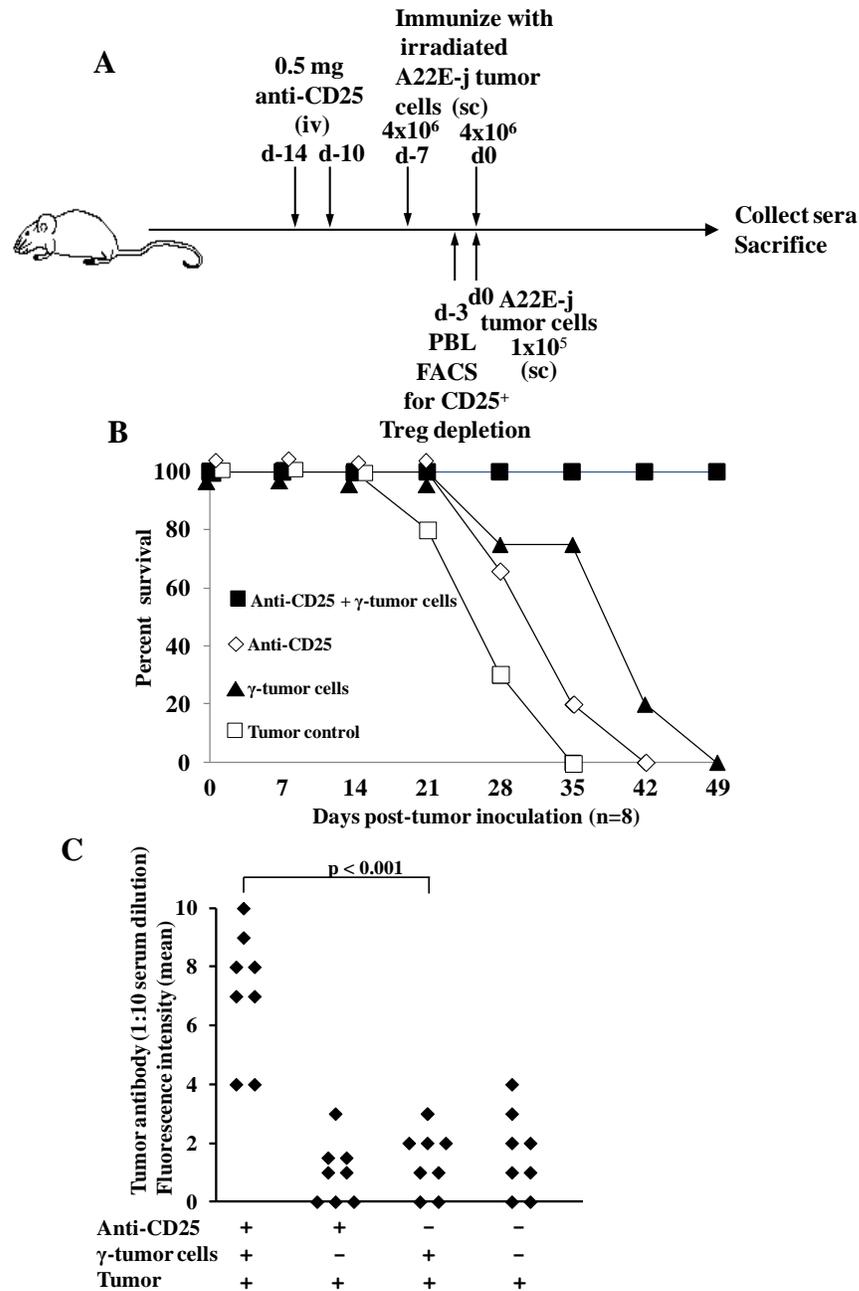


Figure 1. Induction of anti-tumor immunity requires both Treg depletion and anti-tumor immunization with irradiated tumor cells. **A.** Experimental protocol. On the days shown and in groups of 8, mice were either depleted of Tregs with anti-CD25 mAb and immunized with γ -tumor cells, depleted of Tregs alone, or immunized with γ -tumor cells alone. Treg depletion in PBL was verified 7 days after the second dose of CD25 mAb by FACS analysis. On day 0, all groups plus tumor control group were challenged with live tumor cells. Mice were bled and sacrificed on day 49, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor growth was monitored 3x weekly and percent survival is shown. **C.** Tumor-binding antibody concentrations of individual mice at time of sacrifice were measured by fluorescence intensity using the FITC-conjugated secondary antibody (see Materials and Methods).

B. Induced tumor immunity is protective against a second lethal challenge.

To examine the duration of induced tumor immunity, mice were depleted of Tregs and immunized with γ -tumor cells, followed by tumor challenge on day 0 (Fig. 2A). Mice were given a second lethal challenge of 1×10^5 tumors on day 28 or day 35. Tumor control groups were inoculated with tumors on day 0 and day 28 or 35. The animals were bled on day 28 and at the time of sacrifice after the second lethal challenge. All mice in the tumor control groups succumbed to the tumor. Mice with induced tumor immunity survived the first lethal dose, as well as the second lethal dose given on day 28 or day 35 (Fig. 2B). Tumor antibody concentrations were higher in mice with induced tumor immunity than tumor control groups and remained unchanged after rechallenge (Fig. 2C).

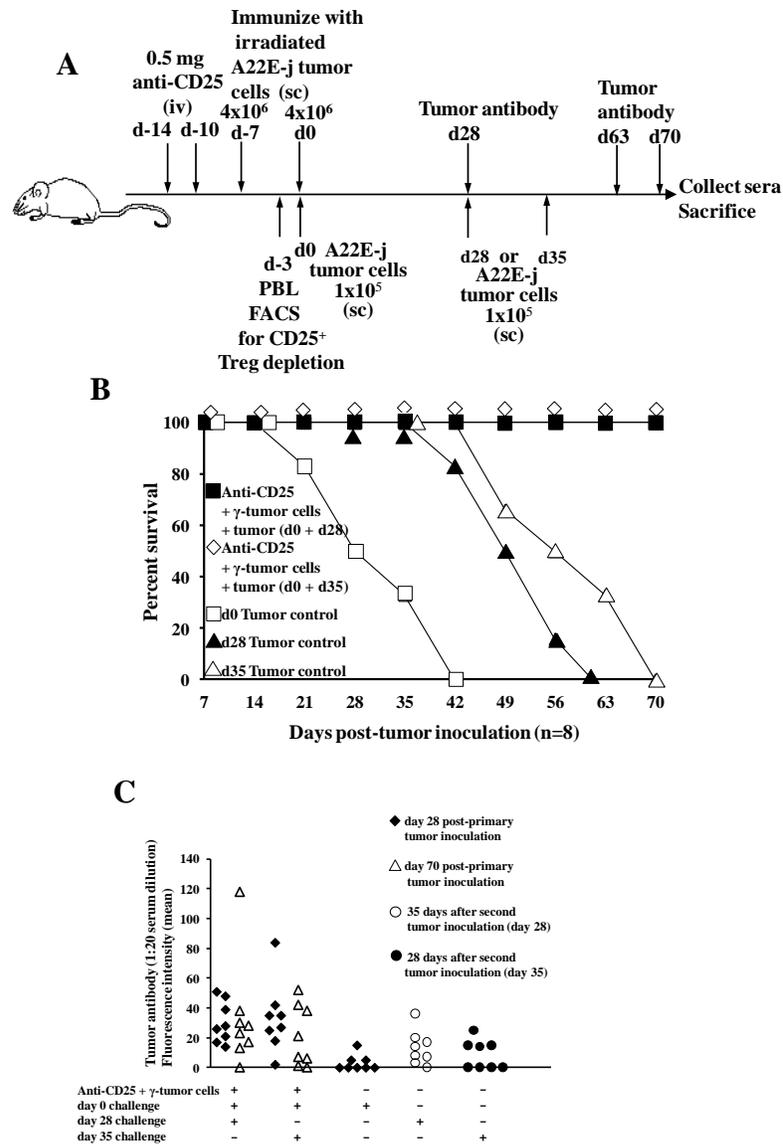


Figure 2. Induced tumor immunity is protective against a second lethal challenge at least 5 weeks later. **A.** Experimental protocol. On the days shown and in groups of 8, mice were depleted of Tregs with anti-CD25 mAb and immunized with γ -tumor cells. Treg depletion in PBL was verified 7 days after the second dose of CD25 mAb by FACS analysis. On day 0, all groups plus tumor control group were challenged with live tumor cells. Two additional groups were given a second challenge on day 28 or day 35. Mice were bled on day of sacrifice, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor growth was monitored 3x weekly and percent survival is shown. **C.** Tumor-binding antibody concentrations were measured by fluorescence intensity using the PE-conjugated secondary antibody. Sera were collected from mice after the first challenge (day 0) on day 28 and day 70. Mice given a second lethal challenge on day 28 or day 35 were bled, respectively, 35 days or 28 days later.

C. Role of CD4 and CD8 T cell subsets in tumor immunity

We examined the role of T cell subsets in protection against tumor challenge after anti-tumor induction. As shown in Fig. 3A, after Treg depletion followed by two doses of γ -tumor cells (the induction protocol for establishing anti-tumor immunity), CD4⁺ and/or CD8⁺ T cells were depleted with mAbs against CD4 or CD8 14 days after the second dose of γ -tumor cell immunization. The CD4⁺ and CD8⁺ T cell depletion was verified 6 days later (Fig. 3B). PBLs were stained with CD4-FITC and CD8-FITC mAb to assess depletion. TCR-PE staining was performed on all samples for comparison (data not shown). All mice given tumor alone developed tumors, whereas immunization prevented tumor growth in the immunized control group (Fig. 3C). The mice depleted of both CD4⁺ and CD8⁺ T cells did not develop any protection against tumors and, in fact, succumbed at a rapid rate. In contrast, mice depleted of CD4⁺ or CD8⁺ T cells alone were all protected from tumor growth. Comparing the tumor-binding antibody levels on day 56, the immunized group had higher antibody levels than the CD4-depleted group, while the CD8-depleted group had tumor-binding antibody levels comparable to the immunized control (Fig. 3D).

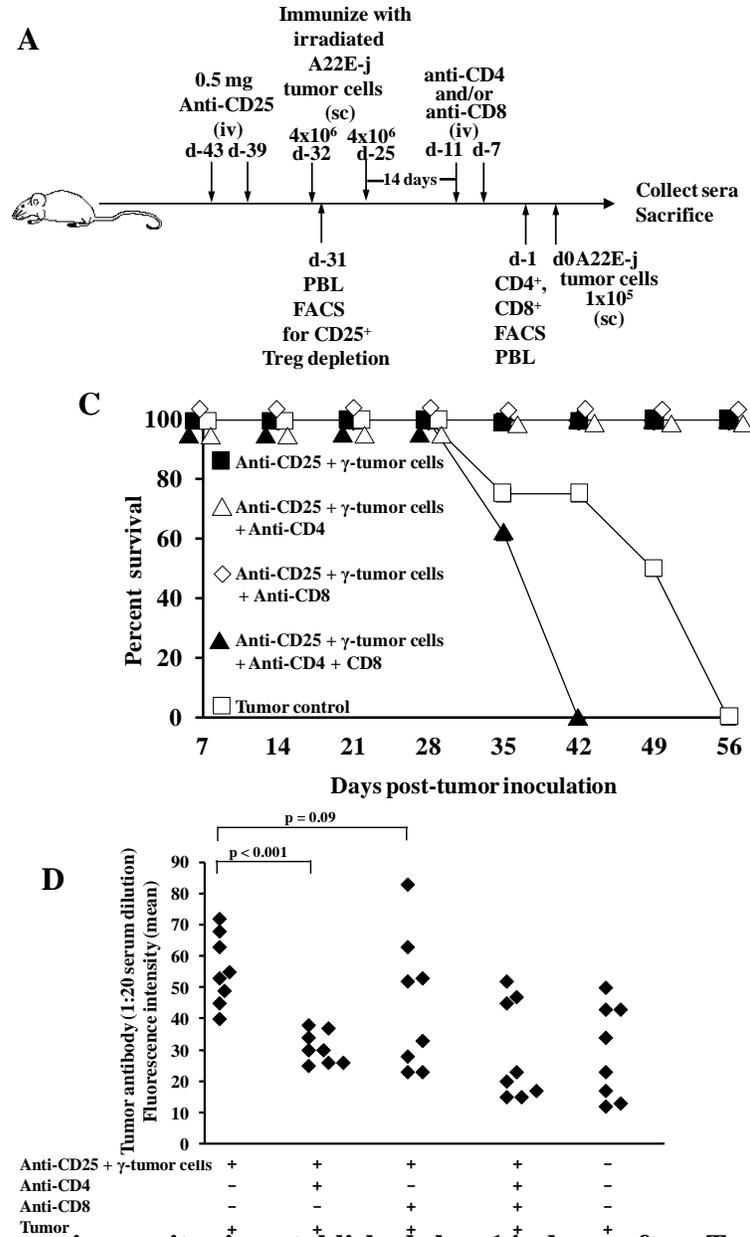
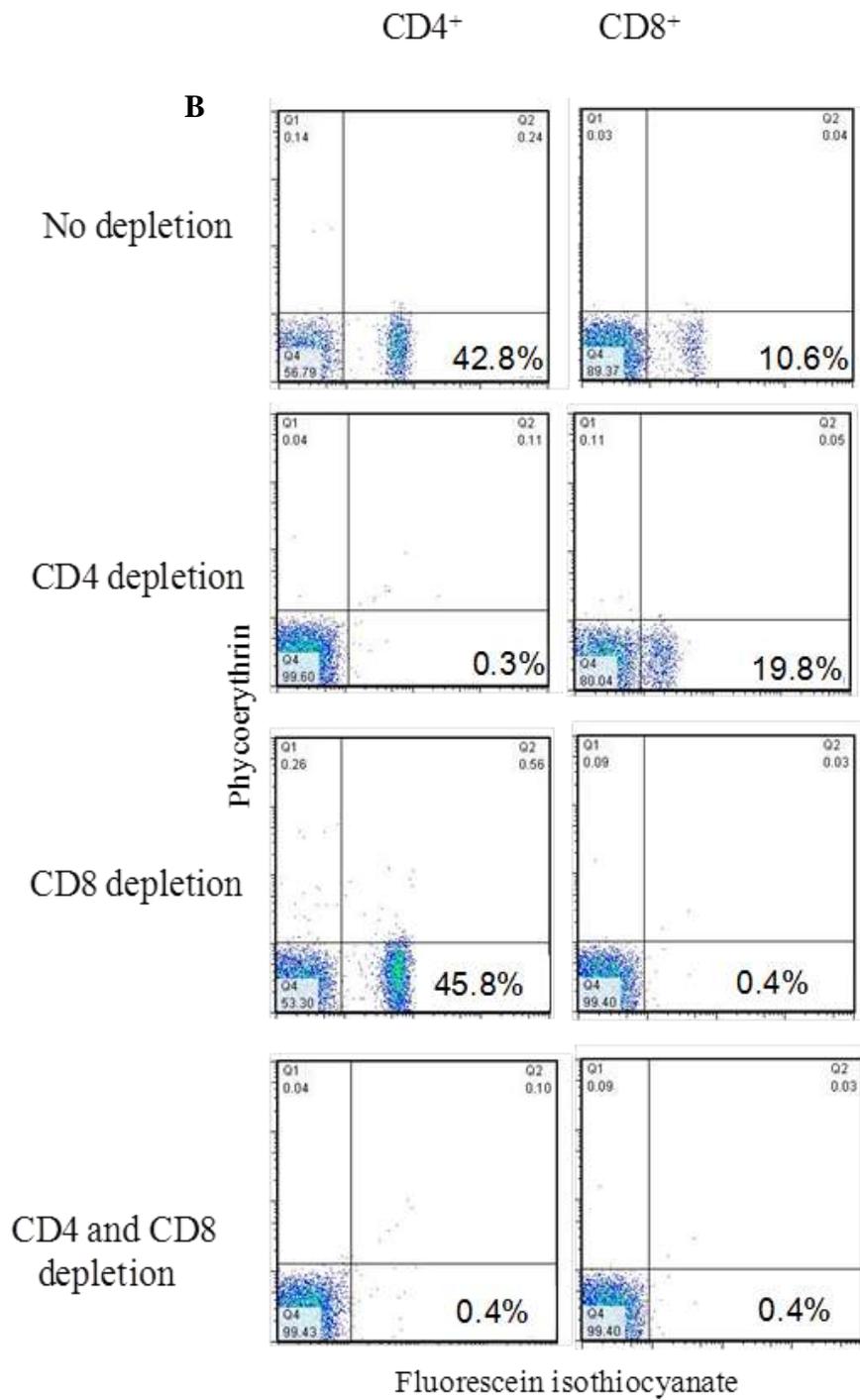


Figure 3. Anti-tumor immunity is established by 14 days after Treg depletion and immunization with irradiated tumor cells. **A.** Experimental protocol. Tumor immunity was induced by Treg depletion and γ -tumor cell immunization in groups of 8 as shown. $CD4^+$ and/or $CD8^+$ T cells were depleted with CD4 and/or CD8 mAbs 14 days after the second γ -tumor cell dose (days -11, -7). Depletion in PBL was verified 6 days later (day -1) by FACS analysis. On day 0, live tumor cells were injected. Mice were bled and sacrificed on day 56, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Depletion of CD4 and CD8 T cells was verified by FACS analysis of PBL with either CD4-FITC or CD8-FITC (2 mice/group). **C.** Tumor growth was monitored 3x weekly and percent survival is shown. **D.** Tumor-binding antibody concentrations were measured by fluorescence intensity using the PE-conjugated secondary antibody.

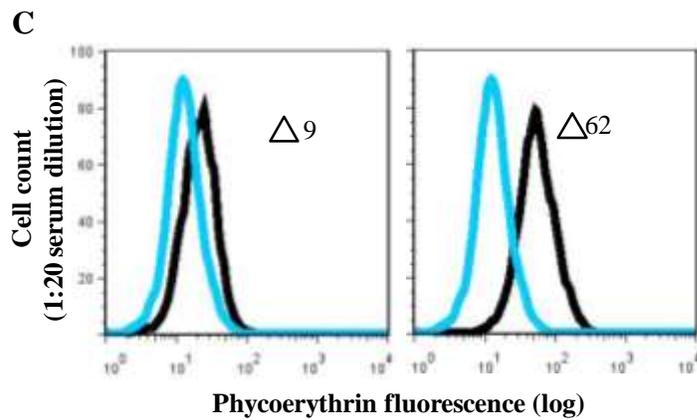
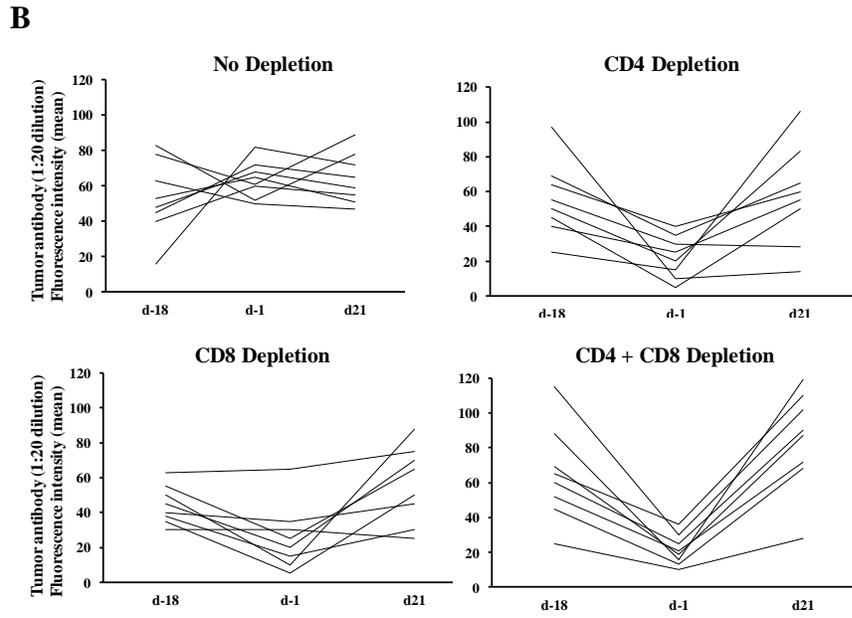
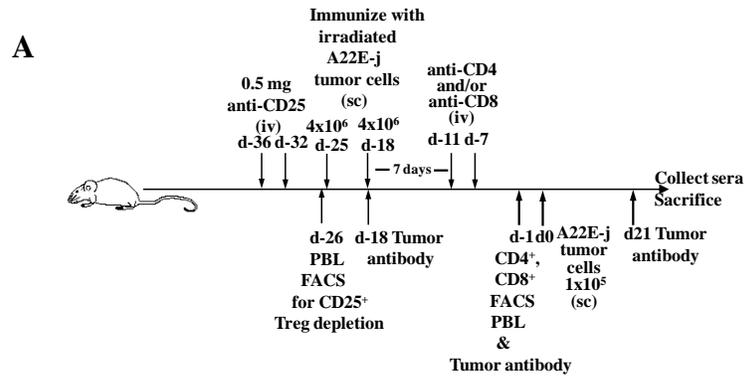
See Next Page for Fig. 3B



Since tumor immunity was already established 14 days prior to T cell depletion, we reduced the time interval for depletion of T cells. CD4⁺ and CD8⁺ T cells were depleted 7 days after the second dose of γ -tumor cells (Fig. 4A). In particular, we examined the role of tumor antibodies in protective mechanisms. Mice were bled at three different time points: one, 7 days after first γ -tumor cell dose (day -18), second, 6 days after depletion of CD4 and CD8 T cells (day -1) and third, after the tumor inoculation (day 21) (Fig. 4B). Tumor-binding antibody concentrations 7 days after the first γ -tumor dose were comparable in all the groups. Depletion resulted in a drop in antibody concentrations 6 days later (day -1) in all groups, compared to the immunized control. By day 21 after tumor challenge, however, antibody levels in all depleted groups were increased and were comparable to the no-depletion controls (Fig. 4B). A representative tumor-binding assay is depicted in Fig. 4C with the positive serum (1:20) in the right panel.

As with the earlier experiment, all mice in the control group grew tumors, whereas all mice in the immunized group were protected (Fig. 4D). Again, when both CD4⁺ and CD8⁺ T cells were depleted, the animals succumbed at a more rapid rate than the controls. However, with CD4⁺ T cell depletion alone, 3/8 mice did not survive. With CD8⁺ T cell depletion alone, one mouse succumbed to the tumor challenge. Taken together there is requirement for CD4 and CD8 T cells; however they play a major role in anti-tumor protection.

Taken together, the data from both experiments suggests that protective mechanisms require both CD4 and CD8 T cells to be fully established and continued after 14 days to prevent tumor growth (Fig. 3). But CD4 T cells appeared to exert their influence beyond the 7-day interval.



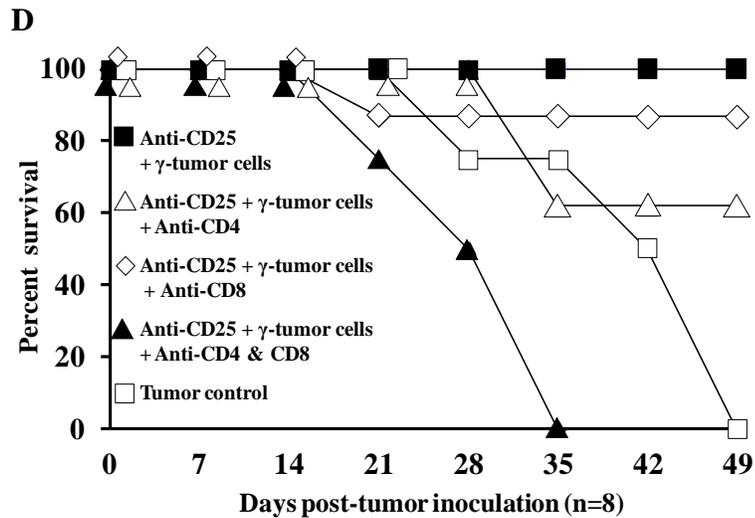


Figure 4. CD4 and CD8 T cell subsets both play an important role in anti-tumor immunity.

A. Experimental protocol. Tumor immunity was induced by Treg depletion and γ -tumor cell immunization in groups of 8 as shown. $CD4^+$ and/or $CD8^+$ T cells were depleted with CD4 and/or CD8 mAbs 7 days after the second γ -tumor cell dose (days -11,-7). Depletion of $CD4^+$ and/or $CD8^+$ T cells in PBL was verified 6 days later (day -1) by FACS analysis. On day 0, live tumor cells were injected. Mice were bled on days -18, -1 and 21 and on day 49 at sacrifice, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor-binding antibody concentrations were measured by fluorescence intensity using the PE-conjugated secondary antibody on three different time points prior to sacrifice: One, 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). **C.** Representative tumor antibody concentration is depicted as overlays of histogram of PE channel. The binding of the non-specific serum sample is shown in blue color with samples to be measured in black color (right panel, positive serum $\Delta 62$). **D.** Tumor growth was monitored 3x weekly and percent survival is shown.

II. Examining concurrent induction of tumor immunity and its effect on autoimmunity in a combined thyroiditis and tumor model.

A. Treg depletion and adjuvant-free model of EAT induction.

HT is an autoimmune disease which is modeled in mice using mTg as self antigen. The adjuvant-free model of EAT induction has also been described where it was found that repeated doses of soluble mTg could be used to induce moderate thyroiditis at about 50% incidence (12). It was found further that Treg depletion before repeated injections increased the incidence and enhanced severity (25). We repeated the experiment where Treg depletion was performed on days -14, -10 prior to EAT induction by repeated administration of mTg for 4 weeks (Fig. 5A). When we examined mTg antibody levels 35 days later at the time of sacrifice, the antibody levels were much higher from the group with Treg depletion and repeated injections, compared to the group with repeated mTg injections alone (Fig. 5B). Examination of thyroid pathology from a composite of three experiments showed an increase of incidence in thyroid infiltration in the group with prior Treg depletion compared to repeated mTg injections (54 vs. 36%) and increased thyroid destruction (50 vs. 5%) (Fig. 5C).

B. Combined EAT and anti-tumor induction model.

In clinical scenarios, we envision tumor patients who are susceptible to autoimmune thyroiditis being subjected to immunotherapy. The scenario could be a concurrent induction of thyroiditis and tumor immunity as we have reported in EAT-resistant mice undergoing tumor immunity induction after challenge (99,101,103). In EAT-susceptible CBA/J mice, we depleted mice of Tregs followed by anti-tumor immunization with γ -tumor cells. The mice were inoculated with live tumor cells on day 0, followed by repeated administration of mTg from day 0-24 and sacrificed on day 35 (Fig. 6A). We assessed the influence of inducing EAT on tumor incidence and percent survival. While control mice all succumbed to the tumor, anti-tumor immunity provided 100% protection (Fig. 6B). No effect was observed on inhibition of tumor growth by the presence of moderate EAT development ($p=0.4$). On the other hand, induction of tumor immunity required Treg depletion (See Fig. 1), and thyroiditis was significantly enhanced following repeated mTg doses to simulate physiologic release of circulatory mTg, compared to mTg doses alone ($p=0.01$) (Fig. 6C).

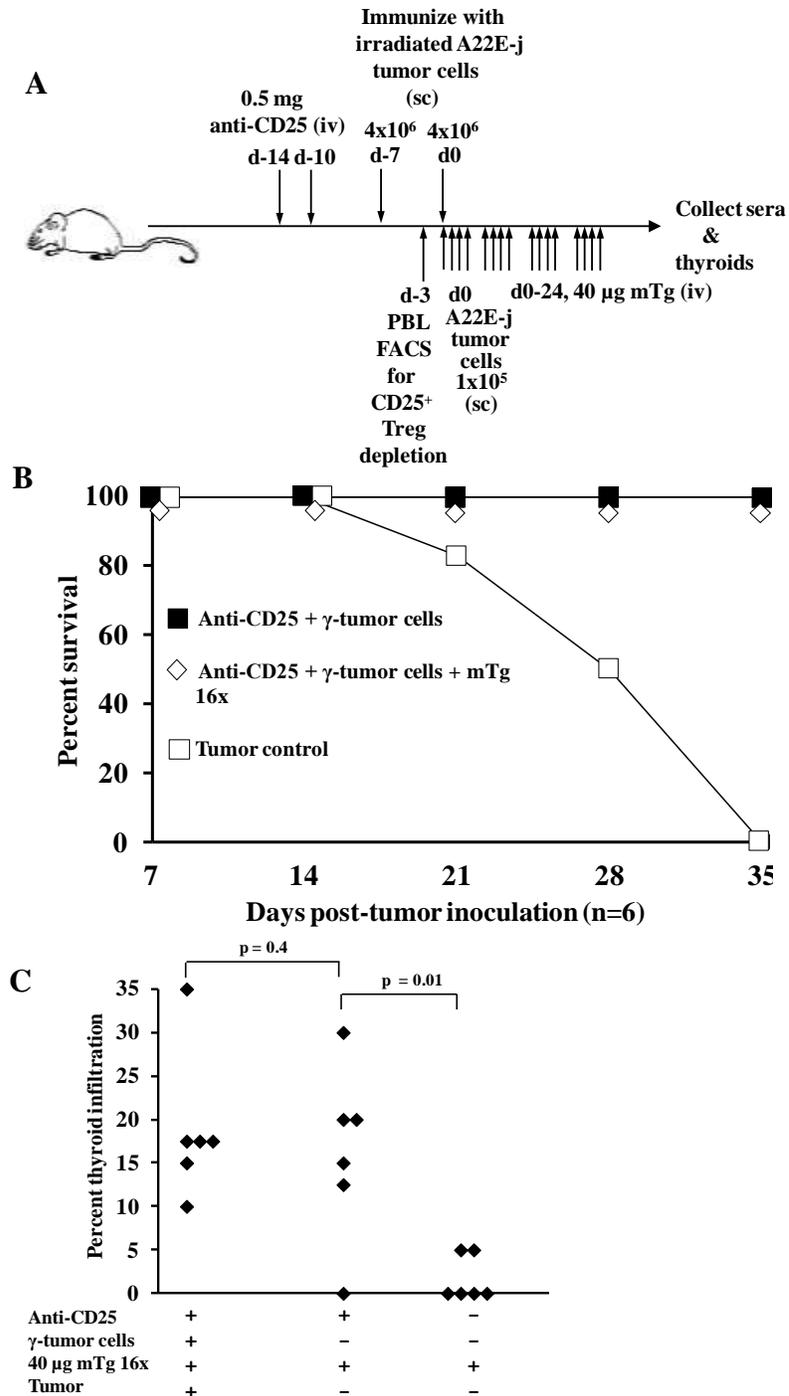


Figure 6. Induction of tumor immunity can exacerbate adjuvant-free EAT induction due to prior Treg depletion. **A.** Mice, in groups of 6, were subjected to either Treg depletion, γ -tumor cell immunization, live tumor challenge on day 0, and repeated mTg injections as shown, or Treg depletion and mTg injections only. They were bled and sacrificed on day 35 post-tumor inoculation, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor growth was monitored 3x 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.

III. Determining autoimmune sequelae based on the extent of autoimmunity prior to tumor immunity enhancement regimen.

Of the various clinical scenarios envisaged, one scenario is tumor patients with ongoing or subclinical autoimmune conditions being subjected to immunostimulation. One obvious concern is the exacerbation of ongoing autoimmune responses. McNeel *et al.* (85) reported elevated TSH levels and hypothyroidism in prostate cancer patients given peptide vaccine and Flt-3 ligand as an adjuvant. Examined retrospectively, a high anti-thyroid antibody titer was found in at least one patient who subsequently developed hypothyroidism, a clinical picture consistent with pre-existing subclinical autoimmune hypothyroidism. We proceeded to mimic the clinical scenarios in our model of thyroiditis and cancer.

A. Inducing EAT with various doses of mTg and IL-1

To mimic ongoing autoimmunity, we primed mice with 40 μ g of mTg and various doses of IL-1. IL-1 is one of the inflammatory cytokines released when LPS is used as T cell adjuvant and high doses can substitute for LPS in EAT induction (24). We began by immunizing mice with mTg and 20,000U IL-1, 7 days apart, on the days shown. Treg depletion and repeated doses of mTg followed (Fig. 7A). At sacrifice, 35 days after the first mTg dose, splenocytes were assayed for *in vitro* proliferative response to mTg. A strong response was observed from mice immunized with mTg and 20,000U IL-1, which was not enhanced by additional Treg depletion and mTg doses or mTg doses only (Fig. 7B). Mice with Treg depletion and repeated mTg injections or repeated injections of mTg alone showed little mTg-specific proliferation. The extent of thyroid infiltration of individual mice between the groups was compared (Fig. 7C). Although mice given mTg and 20,000U IL-1 followed by Treg depletion and repeated mTg injections had only marginally significant pathology over mTg and 20,000U of IL-1 only

($p=0.06$), these mice displayed significantly more severe thyroid destruction than mice with Treg depletion and repeated mTg doses ($p=0.007$). These data suggest that, in EAT-susceptible mice, pre-existing autoimmune condition could potentially be enhanced by the anti-tumor induction regimen requiring Treg depletion.

We next titrated the IL-1 dose to reduce the degree of pre-priming toward subclinical condition. Mice were given mTg and either 10,000U or 5,000U IL-1, followed by Treg depletion and repeated injections of mTg (Fig. 8A). To ascertain that priming against mTg had occurred, mice were bled 7 days after the second dose of mTg and 10,000U or 5,000U IL-1 and tested for mTg antibodies. The mice showed measurable mTg antibody titers (Fig. 8B, day -15) which increased at 11 days after the last dose of repeated mTg injections (Fig. 8B, day 35), with higher levels in the 10,000U IL-1-treated group. Similarly, thyroiditis was more severe in the 10,000U IL-1-treated than the 5,000U IL-1-treated group (Fig. 8C). There was no significant difference between the group primed with mTg and 10,000U IL-1 and the group primed with Treg depletion and repeated mTg injections and the group primed with mTg and 10,000U IL-1 alone ($p=0.4$). There was also no significant difference between groups treated with mTg and the lower dose of 5,000U IL-1, followed by Treg depletion and repeated mTg injections and the group given anti-CD25 and repeated mTg injections only ($p=0.3$). However, in both 10,000U and 5,000U IL-1 pre-treated groups, thyroid pathology was significantly greater than mice without pretreatment ($p=0.002$ and $p=0.04$ respectively). More importantly, 10,000U IL-1 alone treatment induced significantly higher pathology than that of Treg depletion and repeated mTg injections. However, thyroiditis induced with 5,000U IL-1 alone treatment was not different than Treg depletion and repeated mTg injections. These data suggest that mTg + 5,000U IL-1 could represent subclinical condition.

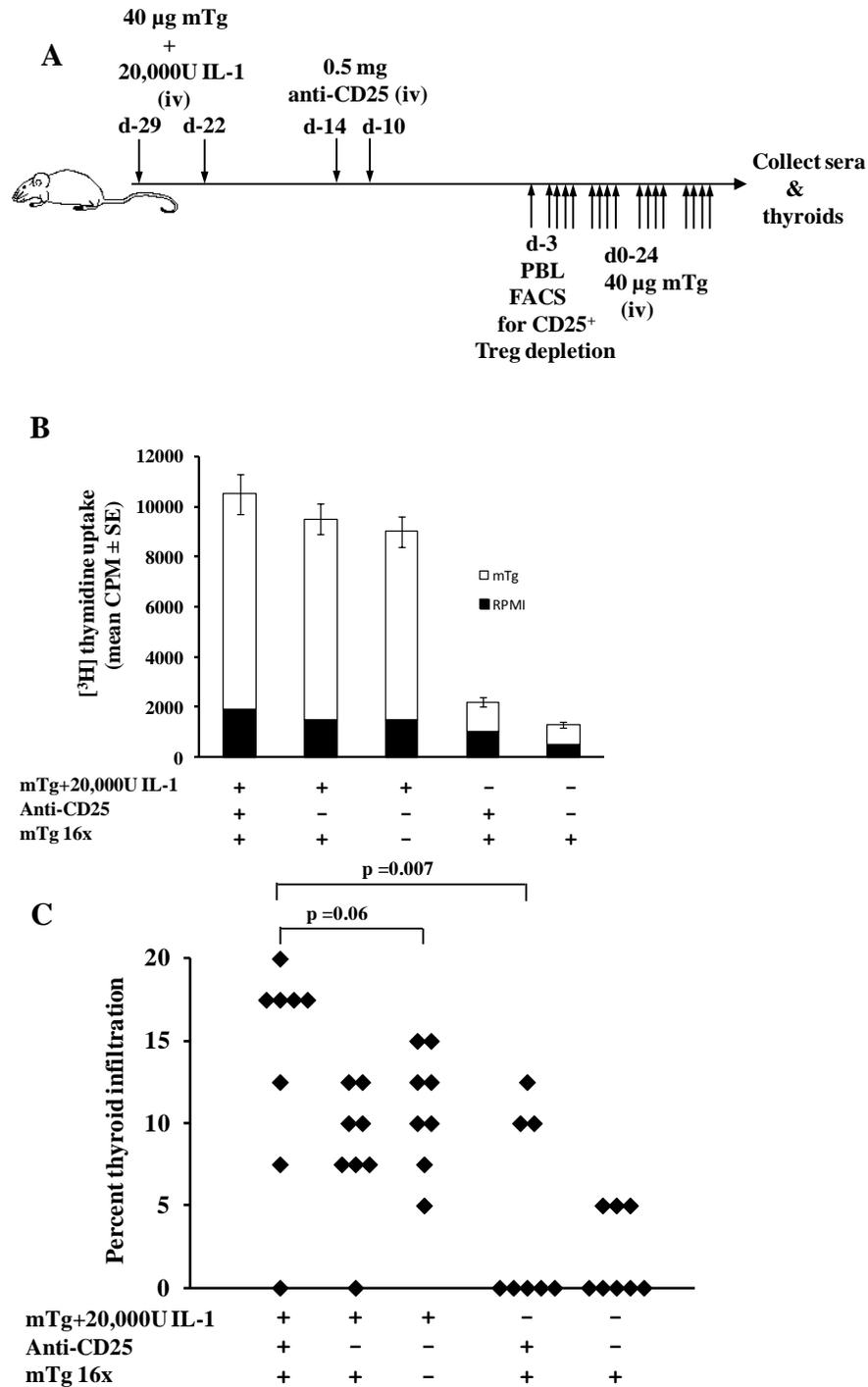


Figure 7. Thyroiditis induced with mTg and 20,000U IL-1 is not affected by subsequent Treg depletion and repeated mTg doses. **A.** Experimental protocol. Groups of 8 mice were either immunized with 40 µg mTg and 20,000U IL-1, followed by Treg depletion and/or additional doses of mTg on the days shown. Mice were bled and sacrificed 35 days after the first mTg dose. **B.** *In vitro* proliferative response to mTg was assessed by [³H] thymidine uptake of cultured spleen cells and shown with background CPM (see Materials and Methods). **C.** Thyroids were harvested and percent thyroid infiltration is shown for individual mice.

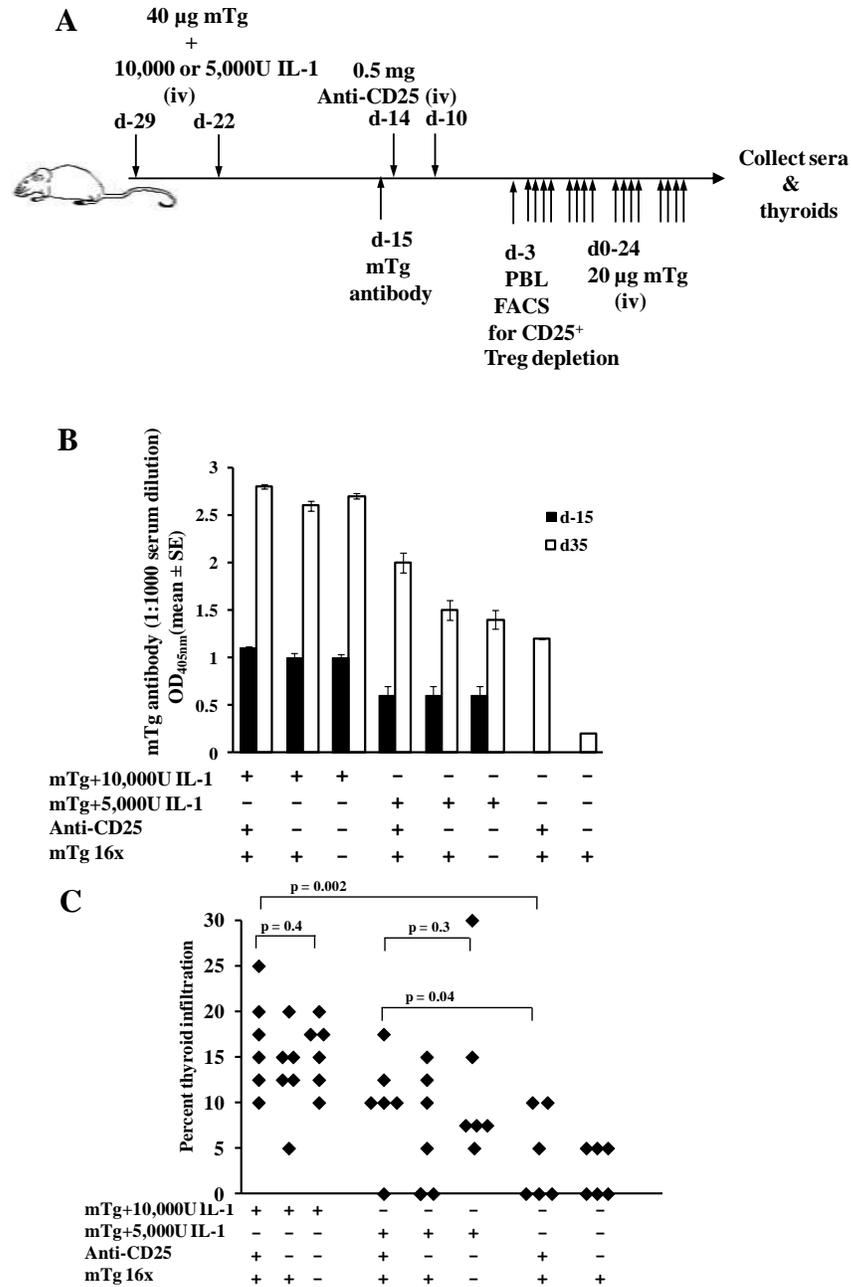


Figure 8. Thyroiditis induced with mTg and either 5,000U or 10,000U represents mild to moderate pre-existing autoimmunity which is not exacerbated by subsequent Treg depletion and repeated mTg doses. **A.** Experimental protocol. Mice, in groups of 6-8, were either primed with 40 µg mTg and either 10,000U or 5,000U IL-1 followed by Treg depletion and/or additional doses of mTg on the days shown. Mice were bled and sacrificed 35 days after the first repeated injection mTg dose. **B.** Mice were bled on days -15, 35 for serum and mTg antibody levels were measured by ELISA. The bars represents mean of the antibody level of all mice in the group (n=6). **C.** Thyroids were harvested and percent thyroid infiltration is presented for individual mice (n=6).

B. Combining pre-priming to mTg and 10,000U IL-1 with tumor immunity induction.

We first tested pre-priming with mTg and 10,000U IL-1, followed by induction of tumor immunity with Treg depletion and γ -tumor cell immunization (Fig. 9A). Whereas, control mice given live tumor cells succumbed to the tumor, induced tumor immunity protected 100% of the mice as before (Fig. 9B). All mice in the group pre-primed with mTg and 10,000U IL-1 also survived. To determine if tumor challenge would affect the extent of thyroid infiltration, mice primed with mTg and 10,000U IL-1 alone were killed 28 days after priming and compared with those sacrificed 35 days after tumor inoculation (day 63 after priming). Both groups developed mild to moderate thyroiditis, which was not affected by subsequent tumor inhibition (Fig. 9C).

To assess the role of physiologic release of mTg, we administered additional mTg doses. Mice were pre-primed, depleted of Tregs and given γ -tumor cells and live tumor challenge, followed by repeated mTg injections (Fig. 10A). The percent survival remained 100% and not affected by repeated mTg injections (Fig. 10B). Tumor-binding antibody concentrations determined at sacrifice on day 35 were similarly high in mice with induced tumor immunity and in mice pre-primed with mTg and 10,000U IL-1 and repeated mTg injections (Fig. 10C). On day 35, the extent of thyroid infiltration was also unchanged by the presence of tumor growth inhibition or additional mTg injections (Fig. 10D).

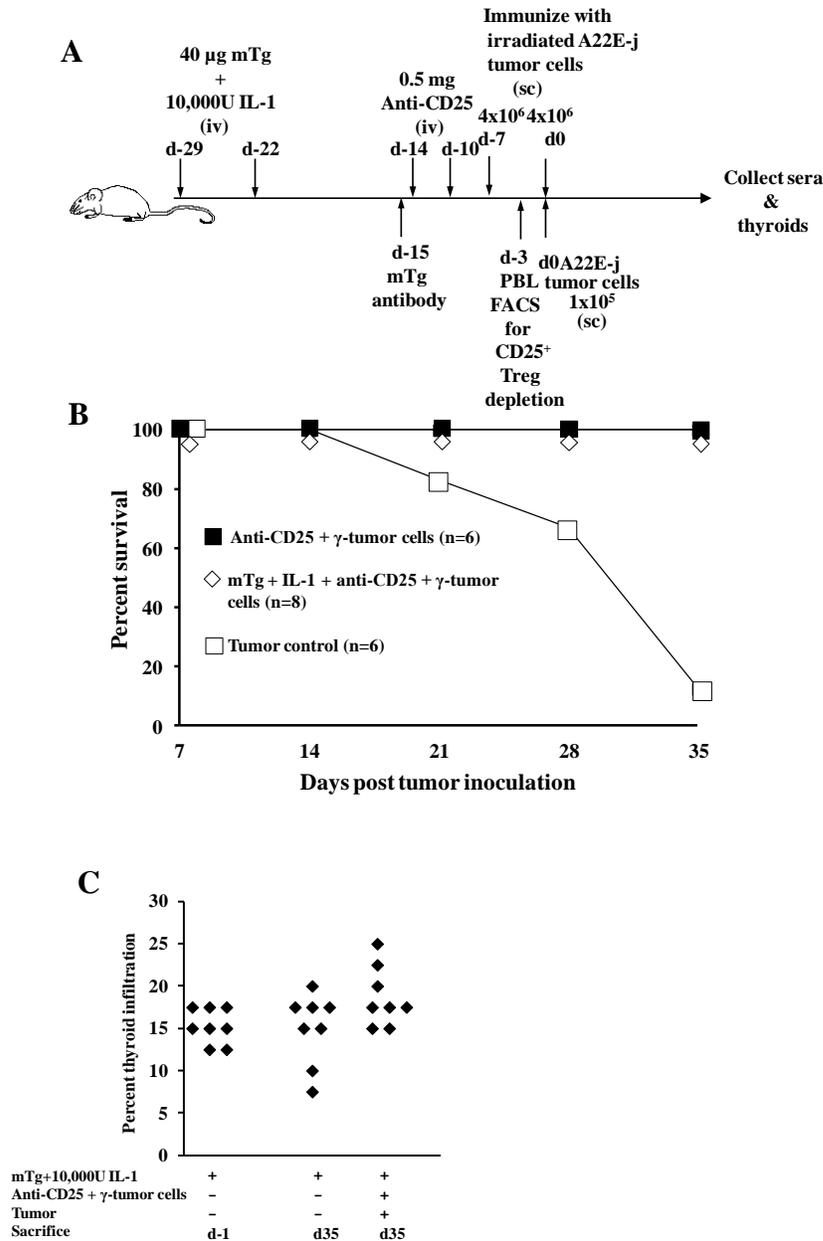


Figure 9. Induced anti-tumor immunity has no effect on ongoing EAT induced with mTg and 10,000U IL-1 and vice-versa. A. Experimental protocol. Groups of 8 mice were either unimmunized or immunized with 40 μ g mTg and 10,000U IL-1, followed by induction of tumor immunity and lethal tumor challenge. Some mice were sacrificed on day -1 prior to tumor challenge on day 0 to assess thyroiditis; otherwise, mice were sacrificed on day 35, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor growth was monitored 3x weekly and percent survival is shown. **C.** Thyroid pathology obtained on day 35 is presented as percent thyroid infiltration for individual mice (n=8).

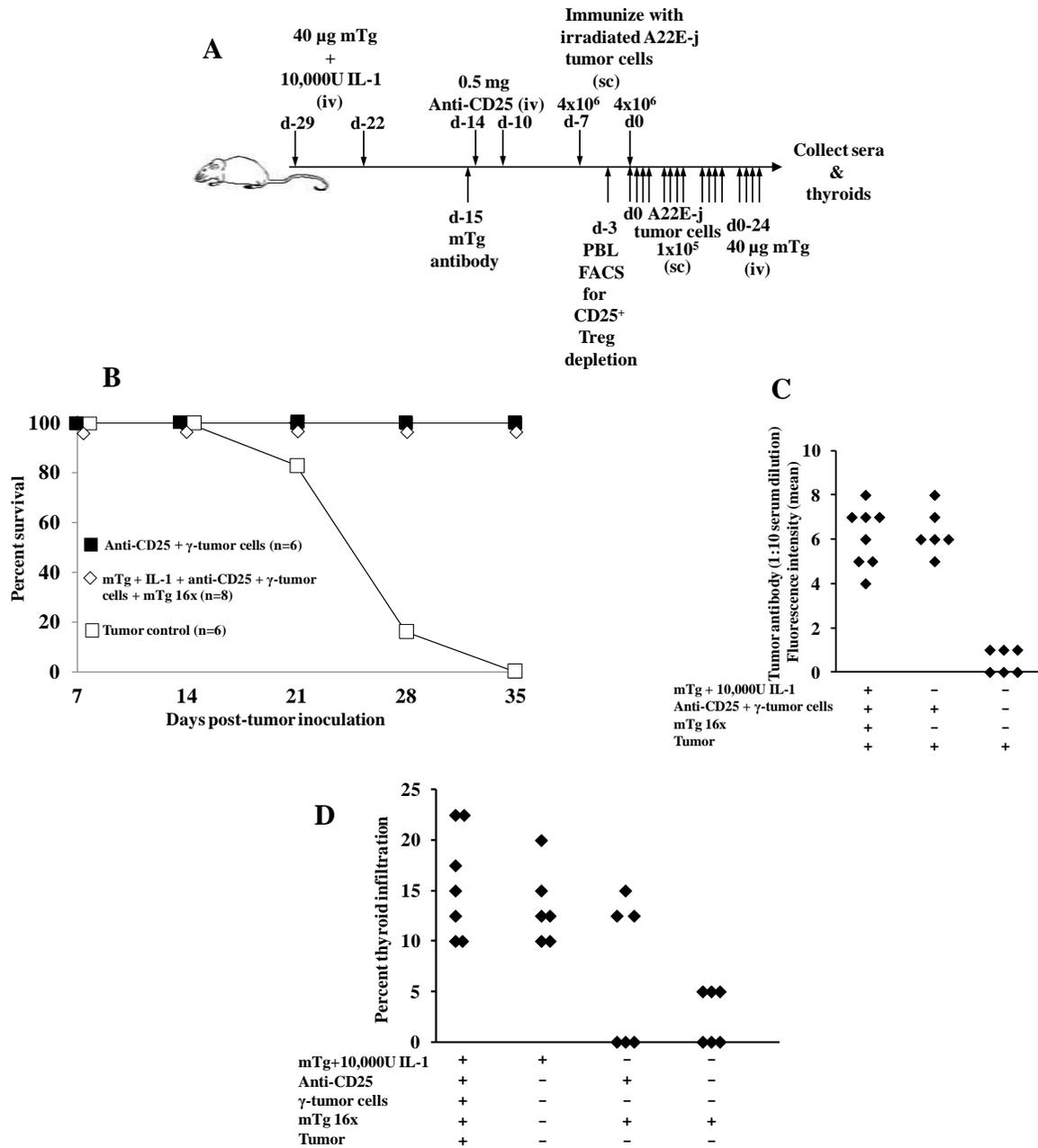


Figure 10. Additional doses of mTg coupled with induction of anti-tumor immunity does not affect ongoing EAT induced with mTg and 10,000U IL-1 and vice-versa. A. Experimental protocol. Mice, in groups of 6-8, were either unimmunized or immunized with mTg and 10,000U IL-1 followed by induction of anti-tumor immunity and lethal tumor challenge on day 0. On days 0-24, repeated mTg doses were also administered to one group. For tumor antibody assay, mice were bled and sacrificed on day 35 post-tumor inoculation, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor growth was monitored 3x weekly and percent survival is shown. **C.** Tumor-binding antibody concentrations were measured by fluorescence intensity using the FITC-conjugated secondary antibody (n=6-8). **D.** Thyroids were harvested on day 35 and percent thyroid infiltration is presented for individual mice (n=6-8).

C. Mild thyroiditis induced with mTg and 5,000U IL-1 is enhanced when combined with tumor immunity induction and repeated mTg doses.

The above experiment was repeated with the lower 5,000U IL-1 dose to represent a mild subclinical condition (Fig. 11A). Again, the percent survival remained 100% in the presence of ongoing EAT (Fig. 11B). Thyroiditis in the group primed with mTg and 5,000U IL-1 along with Treg depletion and repeated mTg doses was significantly higher than the thyroiditis in the group treated only with Treg depletion and mTg injections ($p=0.01$) (Fig. 11C). Thyroiditis induced with mTg and 5,000U IL-1 with Treg depletion and repeated mTg doses was also significantly higher than thyroiditis in mice primed with mTg and 5,000U IL-1 only ($p=0.04$). Tumor growth inhibition in mice with induced tumor immunity did not further affect nor increase the autoimmune response ($p=0.2$). 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.

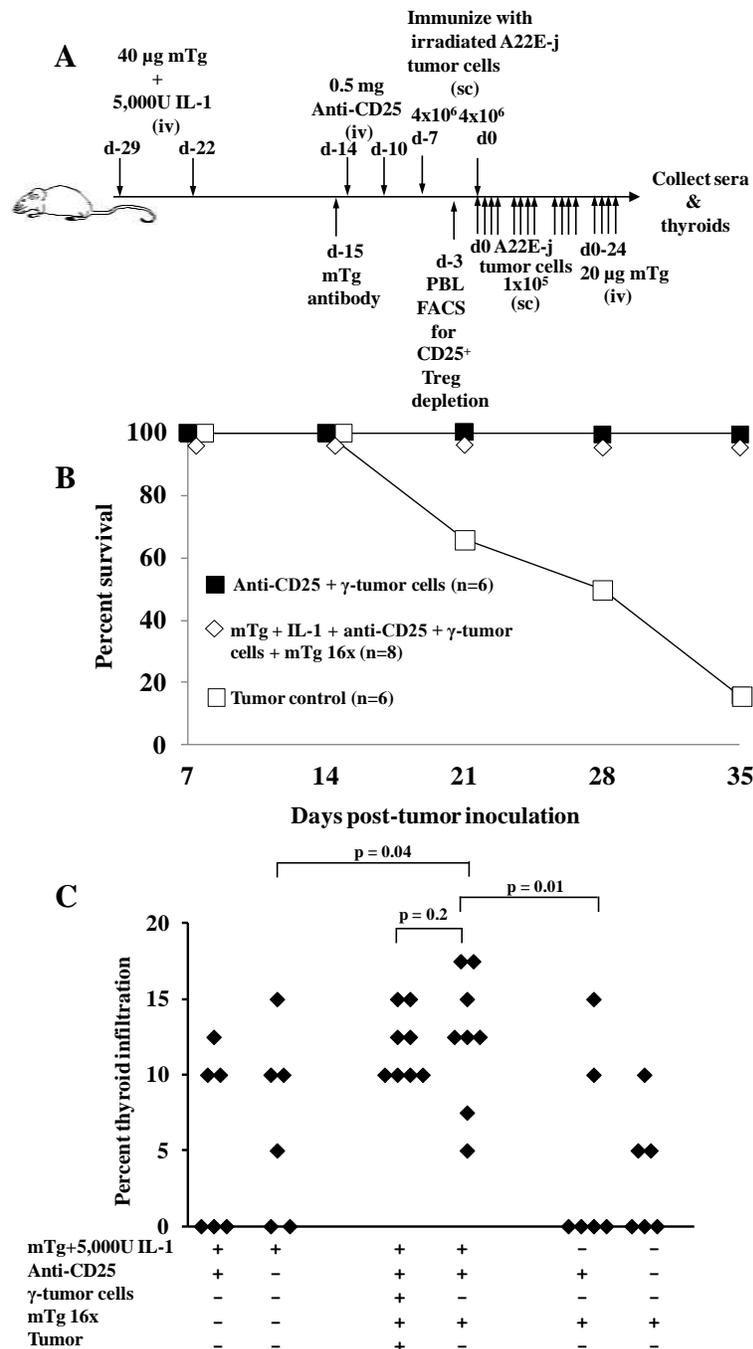


Figure 11. Mild thyroiditis induced with mTg and 5,000U IL-1 is enhanced when combined with induction of anti-tumor immunity and additional doses of mTg without affecting tumor immunity. **A.** Experimental protocol. Mice, in groups of 6-8, were primed with 40 μ g mTg and 5,000U IL-1, followed with or without anti-tumor induction and tumor challenge (day 0) and additional doses of mTg (days 0-24) as shown. They were sacrificed on day 35, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor growth was monitored 3x weekly and percent survival is shown. **C.** Thyroids were harvested and percent thyroid infiltration is presented for individual mice (n=6-8).

IV. Establishing an immunotherapeutic protocol to combat tumor growth in CBA/J mice.

Our tumor immunity induction protocol is a prophylactic model to inhibit tumor growth. Tumor regression can release various pro-inflammatory cytokines which could enhance the ongoing autoimmunity as was seen in the EAT-resistant strain (103). Since our tumor model was in an EAT-susceptible strain and the A22E-j tumor was fast-growing and lethal, it was of particular interest to determine: 1) If an immunotherapeutic model to suppress tumor development was feasible; and 2) if pre-existing autoimmunity could be influenced by the induced tumor inhibition.

A. Setting up the model to inhibit tumor growth.

We began with the lethal A22E-j tumor dose studied above and inoculated mice with 1×10^5 or half the dose of 5×10^4 on day 0. On the same day, we initiated the tumor immunity induction regimen. The mice were depleted of Tregs on days 0, 4 and immunized with γ -tumor cells on days 7,14 (the immunotherapy protocol) (Fig. 12A). Tumor growth was presented as tumor volume from individual mice. The immunotherapy group with Treg depletion and γ -tumor cell immunization did not protect mice from tumor growth with 1×10^5 tumor dose, nor did Treg depletion or γ -tumor cells alone (Fig. 12B). We calculated the percent survival from the mice with 1×10^5 tumor dose from a composite of two identical experiments, which reiterated that immunotherapy regimen was not effective for 1×10^5 tumor dose (Fig. 12C). Tumor antibody concentrations were evaluated on day 42 and showed that, despite high antibody levels in the Treg-depleted and immunized group (Fig. 12D), death was only delayed in 70% of the mice which eventually succumbed (Fig. 12C).

When the tumor dose was reduced by half to 5×10^4 (Fig. 12A), 3 of 6 animals were tumor-free with Treg depletion and γ -tumor cell treatment (Fig. 12E). In a repeat experiment, 4

of 6 mice were tumor-free. Anti-CD25 or γ -tumor cells alone did not protect the mice against 5×10^4 tumor dose in either experiment (Fig. 12E). Percent survival from a composite of these two experiments showed 58% protected of the mice in the Treg depletion and γ -tumor cell treatment group (Fig. 12F). Comparing the tumor antibody levels, the immunotherapy group had significantly higher antibody levels than the other groups (Fig. 12G), but clearly the tumor antibodies were not sufficient to suppress tumor growth.

We next repeated the use of 5×10^4 tumor dose and compared it with a dose reduced by half to 2.5×10^4 tumor dose. With the 5×10^4 tumor dose, we again observed 50% (4/8) of the mice protected against tumor growth by Treg depletion and γ -tumor cell treatment (Fig. 13B). One of the four protected mice had an early palpable tumor which then regressed. In a repeat experiment, 5 of 8 mice were tumor-free. Percent survival from a composite of these two experiments showed 44% survival with 5×10^4 tumor dose (Fig. 13C).

With the 2.5×10^4 tumor dose, none of the 8 mice treated with the immunotherapy regimen developed progressive tumors; 2 of the 8 developed palpable tumors which then regressed. The tumor control group showed a more retarded tumor growth, but all eventually succumbed, indicating that 2.5×10^4 cell dose is nevertheless lethal. In a repeat experiment, all 8/8 mice in the immunotherapy group again inhibited tumor development. Percent survival from these two experiments, showing the successful immunotherapeutic regimen, is presented in Fig. 13E.

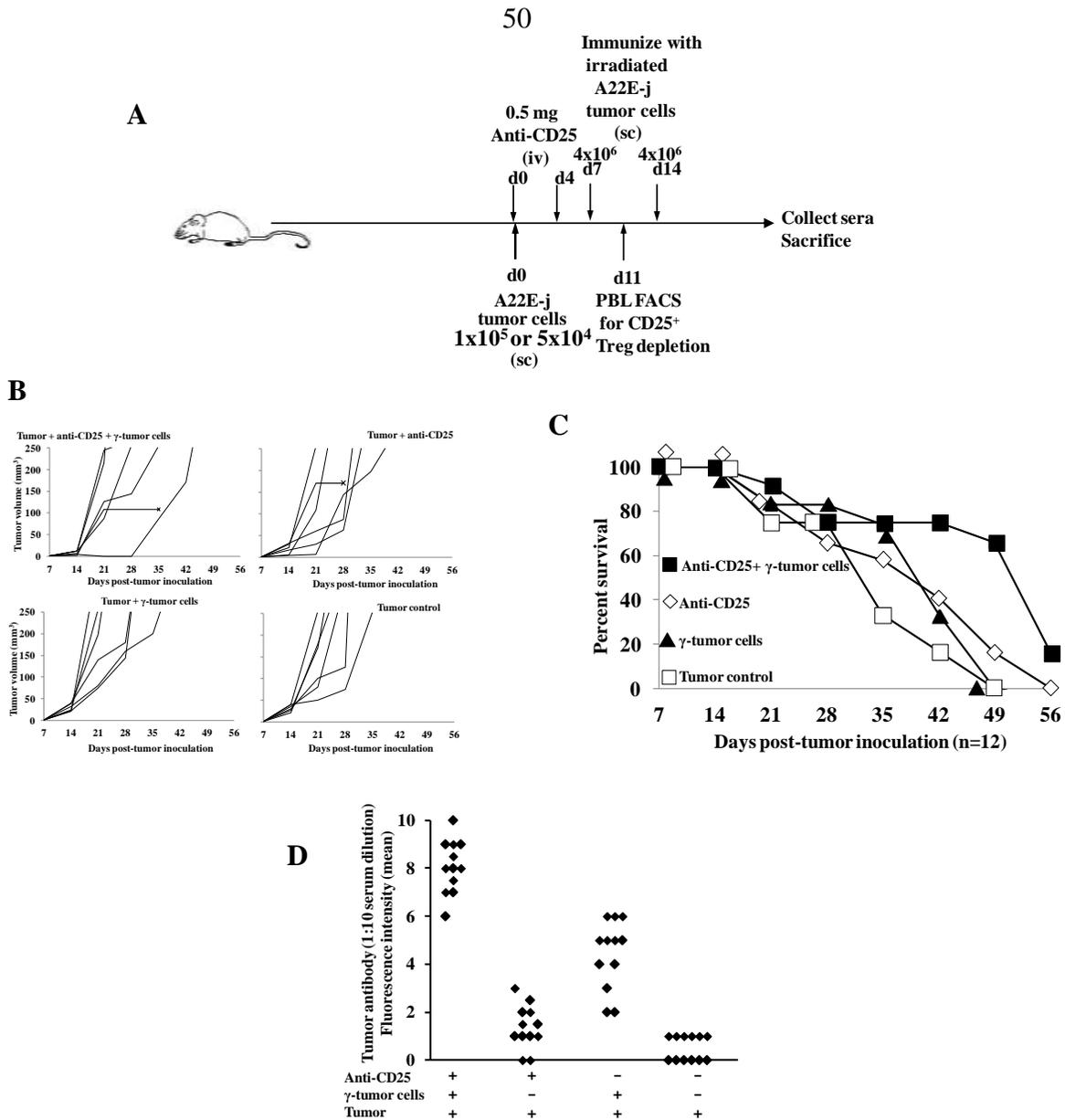
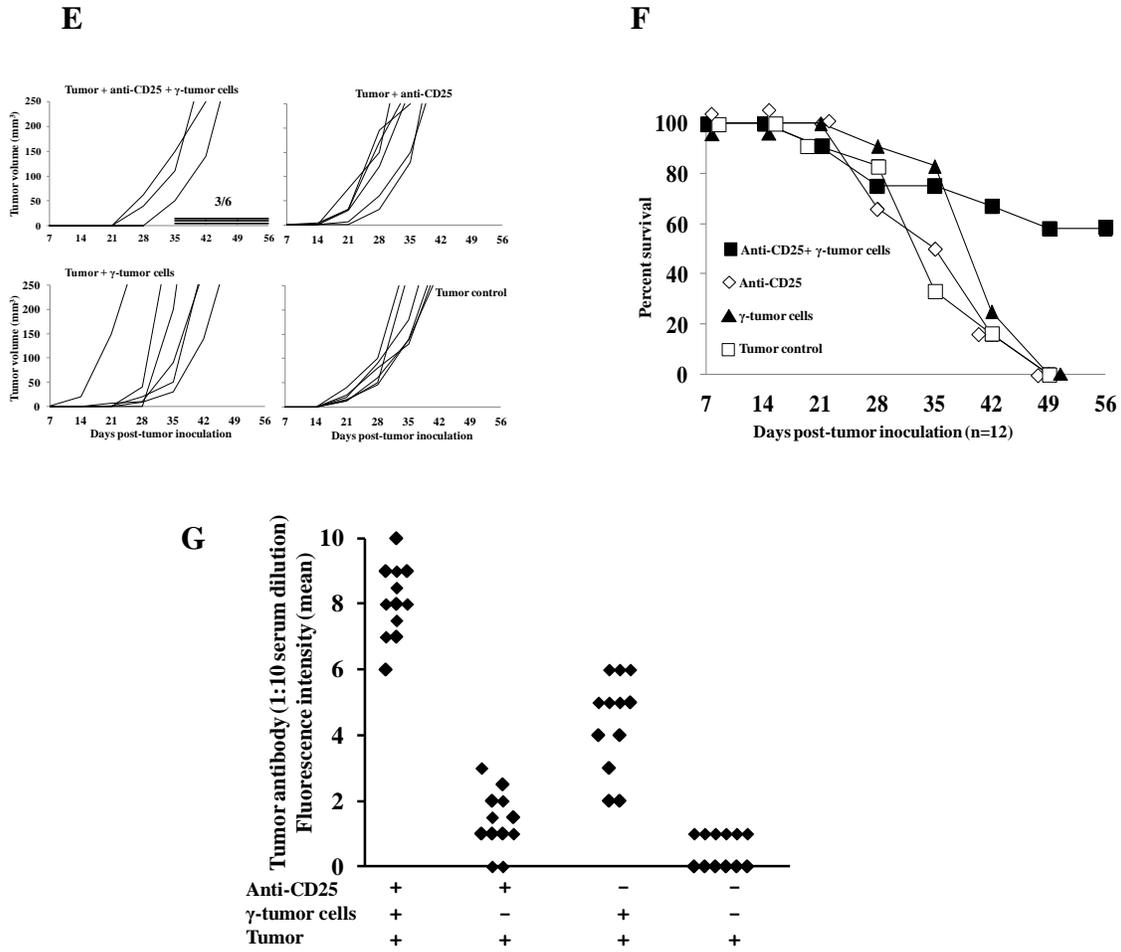


Figure 12. Induction of anti-tumor immunity at the same time as tumor inoculation affords no protection at 1×10^5 tumor dose, but partial protection at 5×10^4 tumor dose. A. Experimental protocol. In groups of 6, mice were inoculated with either 1×10^5 or 5×10^4 tumor cells on day 0. Mice were depleted of Tregs with CD25 mAb and immunized with γ -tumor cells, depleted of Tregs alone, or immunized with γ -tumor cells alone as shown. Mice were bled and sacrificed on day 56, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated.

For 1×10^5 tumor dose:

B. Kinetics of tumor growth is shown as tumor volume for individual mice from one experiment. **C.** Tumor growth was monitored 3x weekly and percent survival is shown from a composite of two experiments. **D.** Sera were collected at day 42 post-tumor inoculation and tumor-binding antibody concentrations were measured by fluorescence intensity using the FITC-conjugated second antibody from a composite of two experiments.



For 5×10^4 tumor dose:

E. Kinetics of tumor growth is shown as tumor volume for individual mice from one experiment. **F.** Tumor growth was monitored 3x weekly and percent survival is shown from a composite of two experiments. **G.** Sera were collected at day 42 and tumor-binding antibody concentrations were measured by fluorescence intensity using the FITC-conjugated secondary antibody from a composite of two experiments.

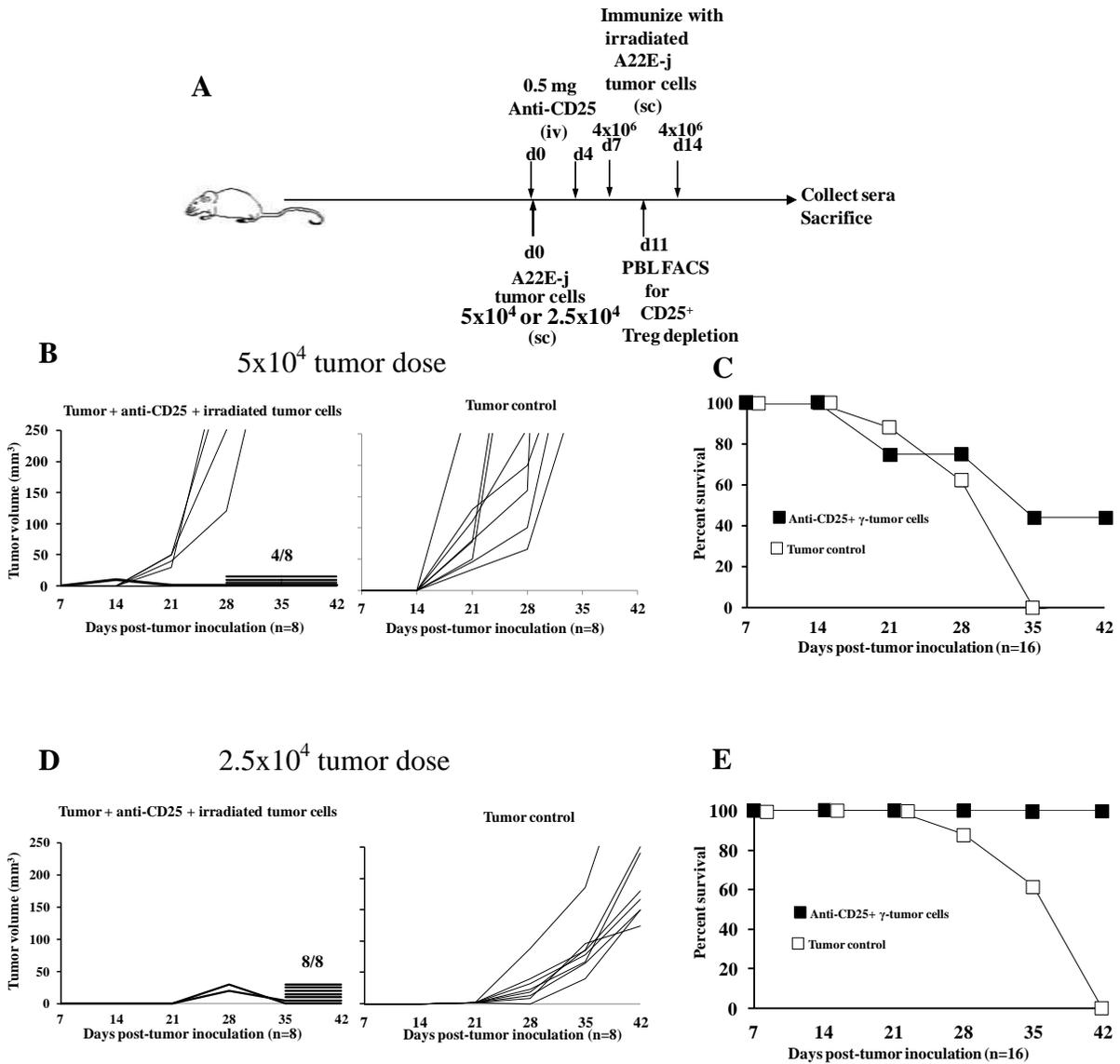


Figure 13. Induction of anti-tumor immunity at the same time as a lower but lethal tumor dose of 2.5×10^4 results in 100% protection. **A.** Experimental protocol. Mice, in groups of 8, were inoculated with either 5×10^4 or 2.5×10^4 tumor cells on day 0, and depleted of Tregs with CD25 mAb and immunized with γ -tumor cells as shown. Mice were bled and sacrificed on day 42, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B & C for 5×10^4 tumor dose, D & E for 2.5×10^4 tumor dose:** **B, D,** Kinetics of tumor growth is shown as tumor volume for individual mice from one experiment. **C, E.** Tumor growth was monitored 3x weekly and percent survival is shown from a composite of two experiments.

B. Protective mechanisms in the immunotherapeutic model.

We next examined the role of CD4 and CD8 T cell subsets, as well as tumor-binding antibodies, in this immunotherapeutic model using the 2.5×10^4 tumor cell dose. CD4 and/or CD8 T cells were depleted as before with two doses 4 days apart (days 17, 21), at 3 days after the second γ -tumor cell dose. Mice were bled on day 16 just prior to depletion and at sacrifice on day 42. Whereas the immunotherapy protocol initiated at the time of tumor inoculation again inhibited tumor development in 100% (16/16) of the mice, depletion of both CD4 and CD8 T cells resulted in only 20% (3/16) survival (Fig. 14B). Tumor-binding antibody levels showed little change from day 16 to day 42 in the no depletion group (Fig. 14C). In the CD4-depleted group, there was a reduction in antibody levels at day 42, and in the CD8-depleted group, antibody levels were somewhat higher. Since the antibody levels in the CD4- and CD8-depleted mice showed similar levels to the non-depleted group, these antibodies appeared to play a minor, if any, role in inhibiting tumor growth.

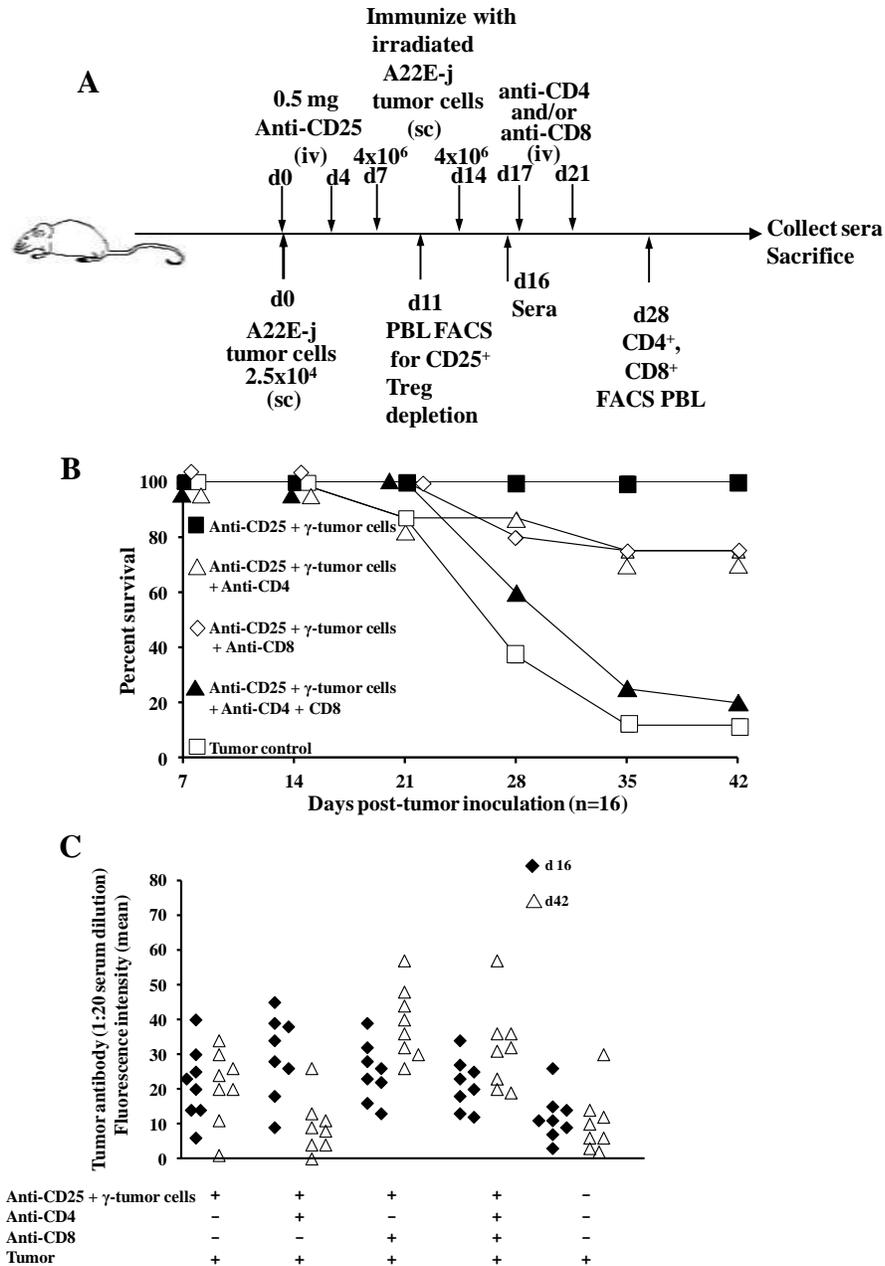


Figure 14. CD4 and CD8 T cell subsets both play an important role in tumor immunity induced along with tumor inoculation. A. Experimental protocol. Mice, in groups of 8, were inoculated with 2.5×10^4 tumor dose, followed by Treg depletion and γ -tumor cell immunization. Anti-CD4 and/or anti-CD8 depletion was carried out with two doses of mAbs, 4 days apart (days 17, 21), at 3 days after the second γ -tumor cell dose, as shown. Depletion was verified 7 days later by FACS analysis. Mice were bled on day 16 and at sacrifice on day 42, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor growth was monitored 3x weekly and percent survival is shown from a composite of two experiments. **C.** Tumor-binding antibody concentrations were measured by fluorescence intensity using the PE-conjugated secondary antibody. Data from day 16, prior to CD4 and CD8 T cell depletion, and day 42 from one experiment are presented (n=8).

C. Determining mutual effect of subclinical EAT and tumor immunotherapy regimen.

Mice were primed with mTg and 5,000U IL-1, followed by live tumor inoculation at the time of initiation of tumor immunity induction. First, we tested with 5×10^4 tumor dose (Fig. 15A). As shown previously in Fig. 12F and Fig. 13C, about 50-60% survival could be obtained by the immunotherapy regimen (Fig. 15B). Thus, little effect was observed due to pre-priming with mTg and 5,000U IL-1 and additional mTg doses. As before, thyroiditis induced with mTg and 5,000U IL-1 pre-priming and additional Treg depletion and mTg injections was significantly higher than that observed with Treg depletion and repeated injections of mTg ($p=0.02$) (Fig. 15C). On the other hand, thyroiditis induced with mTg and 5,000U IL-1 alone was not significantly different from the group with added Treg depletion and repeated mTg injections ($p=0.3$). Ongoing immunotherapy and tumor growth inhibition had no observable influence on EAT induction (Fig. 15C).

As previously observed (Fig. 13E), the tumor immunotherapy protocol afforded 100% protection to the 2.5×10^4 tumor dose. When combined with pre-priming (mTg and 5,000U IL-1) and added Treg depletion and mTg doses (Fig. 16A), no discernible influence was seen by the ongoing thyroiditis on tumor immune responses, which remained at 90% survival (Fig 16B). In the reverse, thyroiditis induced with mTg and 5,000U IL-1 with added immunotherapy regimen and repeated mTg injections was not significantly different from the group without the immunotherapy regimen (Fig 16C).

In conclusion, both the tumor doses when combined with mTg and 5,000U of IL-1 pre-priming did not have any adverse effect on the ongoing thyroiditis and vice-versa.

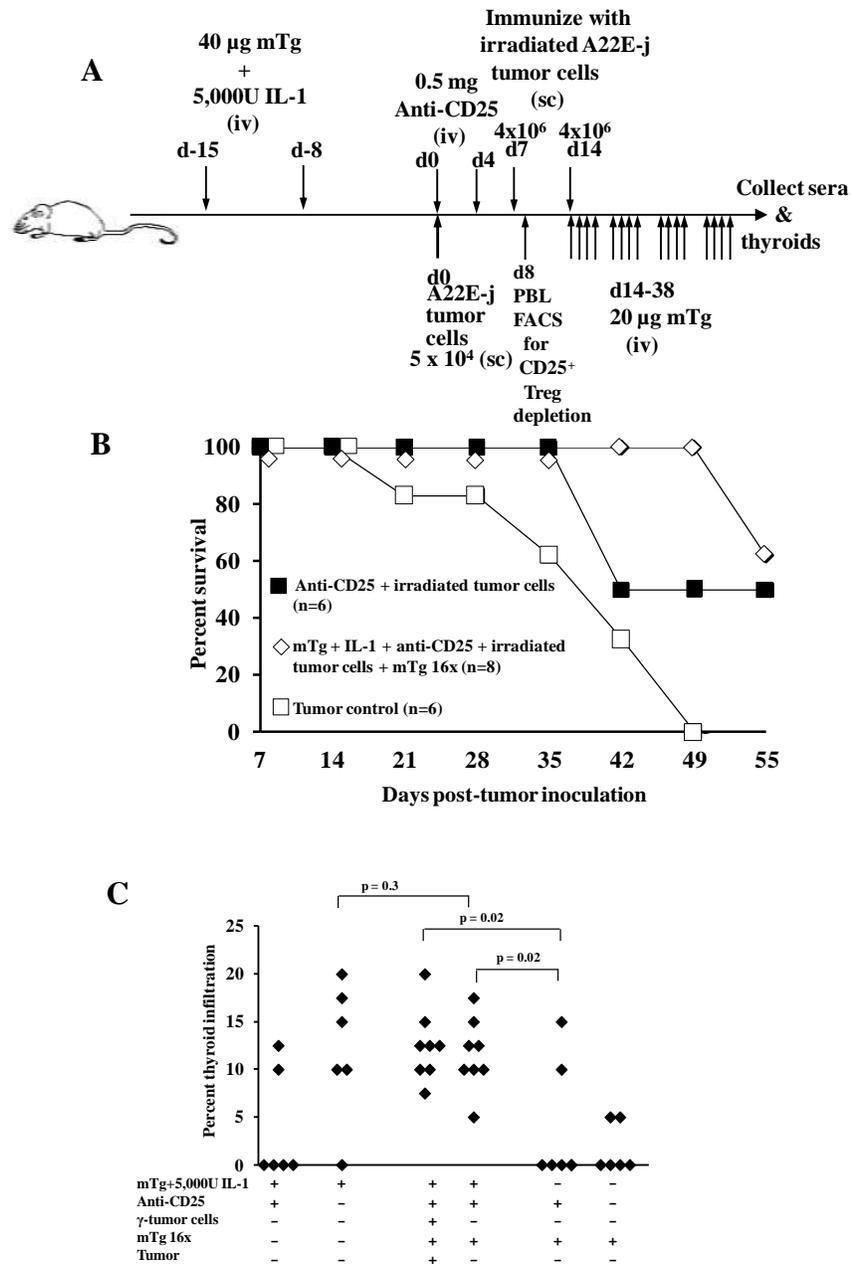


Figure 15. Partial protection afforded by induction of tumor immunity to 5×10^4 tumor inoculation is unaltered by ongoing EAT and additional mTg doses. **A.** Experimental protocol. Mice were primed with $40 \mu\text{g}$ mTg and $5,000\text{U}$ IL-1 only, or primed and given anti-CD25 and γ -tumor cell doses along with 2.5×10^4 tumor dose, followed by repeated mTg doses as shown. Control mice received anti-CD25 and repeated mTg doses following priming. **B.** Mice from tumor-injected groups were sacrificed on day 55, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. Tumor growth was monitored 3x weekly and percent survival is shown. **C.** Thyroids were obtained 41 days after the first mTg doses and percent thyroid infiltration is presented for individual mice ($n=6-8$).

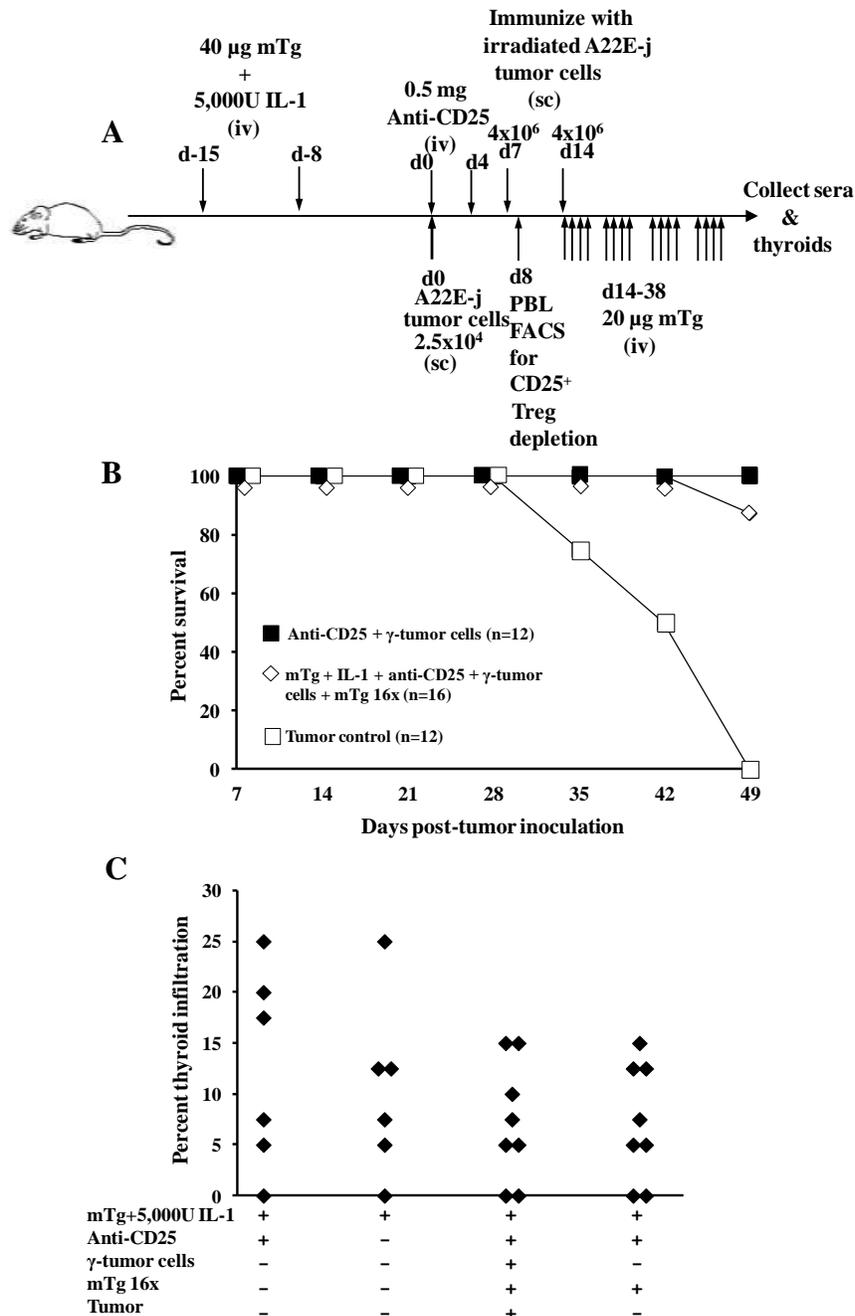


Figure 16. Protection afforded by induction of tumor immunity to 2.5×10^4 tumor inoculation is unaltered by ongoing EAT and additional mTg doses. **A.** Experimental protocol. Mice were primed with 40 µg mTg and 5,000U IL-1 only, or primed and given anti-CD25 and γ -tumor cell doses along with 2.5×10^4 tumor dose, followed by repeated mTg doses as shown. Control mice received anti-CD25 and repeated mTg doses following priming. Mice from tumor control group were sacrificed on day 49, or sooner, if one tumor dimension reached 20 mm or tumor became ulcerated. **B.** Tumor growth was monitored 3x weekly and percent survival is shown. **C.** Thyroids were harvested at day 49, 11 days after the repeated mTg doses and percent thyroid infiltration is presented for individual mice from one experiment (n=6-8).

CHAPTER 4

DISCUSSION

I. Characterization of anti-tumor immunity model in CBA/J mice

Our attempt to develop an anti-tumor induction model in EAT susceptible CBA/J mice was to simulate the clinical scenarios of patients with susceptible alleles being subjected to immunotherapy. Central tolerance mechanisms take care of many autoreactive T cells but there is always a pool of autoreactive T cells in the periphery which can be primed to react to self-antigens. These autoreactive T cells are kept in check by the Tregs, either natural or induced, breaking tolerance is the way forward to make immune system respond robustly against the tumor which sometimes leads to sequelae such as increased autoimmunity or enhancement of ongoing autoimmune responses (69).

We developed our anti-tumor induction model using Treg depletion prior to anti-tumor immunization with γ -tumor cells (Fig. 1A) and found that combined Treg depletion and anti-tumor immunization treatment was required for protection against lethal tumor challenge. This was also observed previously in an EAT-resistant strain, where Treg depletion coupled with DNA vaccine led to tumor rejection (103). Treg depletion can overcome the dominant tolerance mechanism exerted by Tregs on self-antigens, but has to be coupled with immunization to prime and activate autoreactive T cell pool. Along with the immunization, Treg elimination creates a beneficial host microenvironment by affecting host innate immune system (119).

The anti-tumor immunization that we employed was using irradiated whole tumor cells. Whole cell vaccines are attractive when the tumor antigens are not defined, tumor antigens on surface could be presented to T cells through the MHC class I and class II pathway, avoiding the problem of finding MHC restricted epitope (118,120). Vaccination with whole tumor cell

vaccines induce immune response, priming CD8⁺ and CD4⁺ T cells via cross-presentation or direct tumor-antigen presentation respectively, and by prolonged release of tumor associated antigens taken up by APCs for the activation of immune effector cells (121).

We also sought to understand the long term protection involved in anti-tumor induction model. It was found that mice withstood secondary tumor inoculation 4-5 weeks after the primary challenge demonstrating long-term memory response in our model. Our results are similar to previous models of autoimmunity and EAT where long-term protection was observed in mice in BALB/c mice and NeuT transgenic mice (99,103) where antibody and T cell responses were still detectable at week 60 after primary immunization (103). In our model, even though we do not have an assay to measure the T cell responses, experimental evidence suggests that T cells are playing a major role in anti-tumor immune responses (Fig. 3B and Fig. 4D) and maybe in the long term memory responses as well. The antibody levels were not elevated after secondary tumor inoculation, but this assay is inadequate to define its role in tumor protection.

We also attempted to define the protective mechanism involved and found involvement of CD4⁺ and CD8⁺ T cells. The immunity is T cell-mediated as evidenced by depletion of both CD4⁺ and CD8⁺ T cells leading to complete lack of development of protection. With the 14 days time interval between immunization and depletion of T cells, complete loss of protection was observed with combined CD4⁺ and CD8⁺ T cells depletion. However, there was no loss of protection observed with either CD4⁺ or CD8⁺ T cell depletion alone indicating that tumor protection was already well established by the time of depletion and taking out single component did not perturb the immune response. Since the importance of each T cell subset could not be discerned with the protocol, we performed the experiment with a shorter time period between immunization and depletion of T cells, i.e. a 7-day time interval. As with previous experiment,

depletion of both CD4⁺ and CD8⁺ T cells led to complete lack of development of protection. We can also surmise that both CD4⁺ and CD8⁺ T cells have a synergistic role in tumor protection. Similarly, tumor antibody levels were found to be elevated comparably in all groups at day 21, 28 days after depletion especially in the combined CD4 and CD8 group where there was complete lack of protection against tumor growth. This suggests that antibody has very little role to play in the protection. However, in the absence of any functional assay to further define its role, we speculate that antibody has a secondary role in the protective mechanism and the major role is T cell-mediated.

II. Examining concurrent induction of tumor immunity and its effect on autoimmunity in a combined thyroiditis and tumor model.

EAT is an induced disease model where one needs to break tolerance in order to prime autoreactive T cells against mTg. Breakdown of tolerance could be accomplished by the use of adjuvants or even by repeated administration of antigen alone without adjuvant. Adjuvant-free model of EAT induction leads to a weaker thyroiditis characterized by infiltration of thyroid in 50% of the mice and some destruction of thyroid follicles (Fig 5C) (12). But when the same protocol is repeated with prior depletion of Tregs which lowers the threshold for the mTg-specific autoreactive T cells to be primed and activated, there is increased incidence and severity of thyroiditis in susceptible strain (25). This shows that genetic component (MHC class II) and Tregs complement each other in defining susceptibility to autoimmune disorder. Since in the earlier section, we also learned that Treg depletion and anti-tumor immunization both are necessary and sufficient to induce a robust anti-tumor immune response, we combined the two protocols in order to observe any enhancements of immune responses either way and to simulate clinical scenarios of tumor patients undergoing immunotherapy (Fig. 6A). Adjuvant-free model

of EAT induction was used to evaluate less pronounced changes in susceptibility and also to simulate physiologic release of mTg in the host. Use of adjuvant was also avoided to prevent undue beneficial effect to the tumor immune responses. There was no adverse effect of tumor immunotherapy on EAT and vice-versa. Prior depletion of Tregs however enhanced thyroiditis induced with repeated injections of mTg emphasizing the importance of Tregs in maintaining peripheral tolerance in EAT model, and also in the anti-tumor induction model where prior Treg depletion helps in inducing anti-tumor immune response to reject tumor growth (Fig. 6B). Therefore, the results suggest that anti-tumor induction protocol is detrimental to autoimmune thyroiditis in our mouse model. However, examining the components involved, Treg depletion (tumor immunomodulation) and not γ -tumor cell treatment is responsible for the enhancement observed in EAT. This is in line with the clinical picture where tumor immunomodulation enhances autoimmunity; tumor vaccination regimen however may or may not influence autoimmunity. In our mouse model, both EAT and anti-tumor immune responses were not influenced by each other. However, in an combined model of EAT and tumor in BALB/c mice, we found synergistic enhancement of immune response against tumor (HER-2) and to mTg during tumor regression when there was a concurrent induction of tumor immunity and EAT (99). Similar mutual amplification was also observed in (HER-2xDR3) F1 transgenic mouse model of tumor immunity and EAT (101). It was hypothesized that it being a model of tumor regression, regressing tumor led to the release of inflammatory cytokines systemically, which not only enhanced thyroiditis but also anti-tumor immune responses. In our tumor induction model, there is no tumor regression, instead we are preventing the growth of the tumor. In clinical scenarios however, patients are subjected to systemic immunomodulators and other treatment modalities such as irradiation which can further enhance both anti-tumor and autoimmune responses.

III. Determining the autoimmune sequelae based on the extent of autoimmunity prior to tumor immunity enhancement regimen.

As we discussed previously, immunotherapy often brings up autoimmune sequelae or exacerbate ongoing autoimmunity in patients in clinical trials. In order to mimic it, we set up tumor immunotherapy model with ongoing thyroiditis to look for exacerbation.

We started with various doses of IL-1 to prime mice against mTg. IL-1 has been used here as a T cell adjuvant (24). Addition of exogenous IL-1 has been shown to act as adjuvant when administered alongside antigen in different animal models of autoimmunity (24,122). Apart from the adjuvant properties, IL-1 has been shown to break tolerance against self antigens by priming and expanding effector T cells (24,123) and by inhibition of CD4⁺CD25⁺ Tregs (122). IL-1 has a pleiotropic effect on many immune cells leading to proinflammatory environment locally and systemically, which promote autoimmune conditions. Since IL-1 was a weaker stimulus than LPS itself, we used it to prime mice against mTg to observe subtle changes of susceptibility and used it to define pre-existing or ongoing autoimmune condition to be further subjected to anti-tumor induction protocol.

Our priming experiments started by using 20,000U IL-1, which was the highest dose tested. mTg and 20,000U IL-1 treatment for priming induced thyroiditis in all mice (Fig 7C), which was found to be severe than that of Treg depletion and repeated mTg injections. Addition of Treg depletion and mTg repeated injections however did enhance the ongoing thyroiditis marginally. This gave us the first indication that ongoing autoimmunity could be enhanced further. Examining the results further, we decided to test lower doses of IL-1 in order to define various gradations of ongoing autoimmunity. We compared pre-priming of 10,000U with that of 5,000U IL-1. mTg and 10,000U IL-1 induced thyroiditis in all mice in the group (Fig 8C).

Addition of Treg depletion and repeated mTg injections (20 μ g) however did not significantly enhance ongoing thyroiditis. Similar results were also evident with mTg and 5,000U IL-1 pre-priming. However, the 5,000U IL-1 dose was found to be weaker compared to 10,000U IL-1 dose and was classified as being subclinical.

As we observed previously with tumor experiments, combined Treg depletion and γ -tumor cell treatment form anti-tumor induction model (Fig. 1-4). We have also observed that with various doses of IL-1 (20,000, 10,000 or 5,000) used for priming along with mTg, there was an indication of enhancement with addition of Treg depletion and repeated mTg injections. In order to assess the effect of tumor and anti-tumor induction protocol on ongoing thyroiditis, we combined the mTg and 10,000U IL-1 pre-priming with tumor immunity induction to mimic the clinical scenarios and also to observe the influence of autoimmunity and tumor protection vice-versa. The results indicated lack of any deleterious effect of tumor immunity on ongoing thyroiditis and vice-versa. We further added repeated mTg injections to mimic physiologic release of mTg in the host, however, no enhancement was observed with additional stimulation. mTg and 10,000U IL-1 pre-priming is therefore enough to break tolerance to mTg and induce a stronger thyroiditis compared to that induced with repeated mTg injections. Adding weaker restimulation to the ongoing autoimmunity did not enhance the thyroiditis further. Our tumor immunity induction is very strong and induces 100% protection all the time. As evident in our earlier results (Fig 3B and Fig 4B), the tumor immune responses are established early and last for the duration of the experiment. That is one of the reasons that we did not observe any influence of ongoing EAT on induced tumor protection. This result of ours may or may not be a true picture of clinical scenarios. In clinics, patients are subjected to many different regimens at the

same time and the perturbation of the immune system in one host might influence the ongoing or induced immune responses.

The pre-priming dose of 5,000U IL-1 induced mild thyroiditis in mice but inconsistently in the group. We wanted to explore this further and, as with 10,000U IL-1, combined the priming dose of 5,000U IL-1 with tumor immunity induction and added soluble mTg doses to simulate physiologic release of mTg. In our model of combined EAT and tumor protection, the anti-tumor immune responses and autoimmune thyroiditis were unchanged in the presence of each other or, there might be some influence which we were not able to observe because of the shortfall of the model. Ongoing thyroiditis induced with mTg and 5,000U IL-1 combined with Treg depletion and repeated mTg injections was significantly enhanced when combined with additional Treg depletion and repeated mTg injections. We can surmise from the results that individual components such as Treg depletion and repeated mTg injections when combined together can certainly exacerbate ongoing thyroiditis in certain conditions. This suggests that the priming dose of 10,000U IL-1 is too strong and therefore is enough to make all autoreactive T cells cross the threshold needed to be activated, a weaker stimulus such as additional mTg doses therefore was unnecessary as the pool of autoreactive T cells were already activated. In contrast, with 5,000U IL-1, the priming dose is not enough to make all autoreactive T cells cross the threshold to be activated. Additional stimulation with Treg depletion and repeated mTg injections helps the unprimed autoreactive T cells reach the threshold for priming leading to exacerbated thyroiditis. This could be one of the reasons why we observe such a wide range of fluctuations with 5,000U IL-1 dose. Similarly, our EAT induction protocol with repeated mTg injections is also variable in priming autoreactive T cells and induces weak thyroiditis in about 50% of mice in our susceptible strain. Adding Treg depletion lowers the threshold for activation of autoreactive T

cells and inducing thyroiditis. However, significant enhancement with the protocol was observed less frequently and is also one of the reasons for variability on the final exacerbation of thyroiditis. Combining everything together for interpretation makes it clear why some patients, and not all patients, undergoing immunotherapy could present autoimmune sequelae. Our work has demonstrated the importance of pre-existing subclinical condition which can be enhanced under favorable conditions to present clinical autoimmune disease.

In a melanoma brain tumor model, shared melanoma antigen also expressed by skin and brain tissue was used to prime the immune system (124). The tumor growth in brain was rejected by vaccination but did not lead to any autoimmune responses in the brain in spite of shared antigen. However, the autoimmune responses were observed in skin only when an inflammatory response was induced prior to anti-tumor vaccination. This is similar to our results with ongoing inflammation, only subclinical autoimmune condition was shown to be enhanced in our mouse model.

IV. Establishing an immunotherapeutic protocol to combat tumor growth in CBA/J mice.

As discussed previously in the concurrent model of EAT and anti-tumor induction, tumor regression influenced the outcome of thyroiditis in combined EAT and tumor model, in non-transgenic BALB NeuT female mice (103) with significant EAT enhancement observed only in mice undergoing tumor regression after HER-2 DNA vaccination (103). Since we observed this phenomenon in an EAT-resistant strain, we wanted to further explore the implications of tumor-growth prevention after tumor inoculation (therapeutic as opposed to prophylactic in earlier experiments) in an EAT-susceptible strain. For this reason, we set up immunotherapy regimen in CBA/J mice. We tested different doses of tumor cells for protection and first tested two doses, 1×10^5 and 5×10^4 tumor cells. With 1×10^5 dose, no protection was observed with immunotherapy

protocol (Fig 12C). This lethal dose is fast growing with palpable tumors as early as day 7 post-tumor inoculation (Fig. 12B). In previous experiments, our aim was to prevent the growth of the tumor after induction of anti-tumor immune responses. We also assessed from our T cell subset depletion studies that anti-tumor immune responses are induced as early as 7 days post-tumor inoculation. In spite of starting the immunotherapy regimen as early as day 0 with Treg depletion and γ -tumor cell treatment by day 7, we were not able to prevent the growth of the tumor. Treg depletion nor γ -tumor cell treatment alone helped in slowing down or protect mice from the tumor growth. With 5×10^4 tumor cells, 50% protection was achieved with immunotherapy protocol (Fig. 12F). This tumor dose, although lethal, did not grow as fast the previous dose. Mice started showing palpable tumors on day 14 post-tumor inoculation, although there was variability in terms of tumor growth (Fig. 12E). Our immunotherapy regimen therefore was able to inhibit tumor growth in at least 50% of mice in the group. The repeat experiment confirmed our results further. Treg depletion alone or γ -tumor cell treatment alone failed to protect mice from lethal challenge. We then reduced the challenge dose of tumor further to 2.5×10^4 and found 100% protection (Fig. 13E). With the present tumor dose, we found tumors growing later and becoming palpable from day 21 onwards (Fig. 13D), our immunotherapy regimen was therefore successful in preventing the growth of tumors at 2.5×10^4 tumor dose which is nevertheless lethal as well.

We also examined the role of antibody in the immunotherapy and found high antibody levels in mice treated with immunotherapy protocol with 1×10^5 , 5×10^4 tumor dose (Fig. 12D, 12G). Higher antibody levels against tumor however, did not translate into protection against tumor growth.

We further examined the role of CD4 and CD8 T cell subsets in immunotherapy protocol. We observed 75% of mice still protected when depleted of CD4 or CD8 T cells depletion alone (Fig. 14B). However with combined CD4 and CD8 T cell depletion, 80% of mice were not protected anymore. This result of ours is similar to the anti-tumor induction model (Fig. 4D) where combined CD4 and CD8 depletion led to loss of development of protection. We examined antibody in the immunotherapy model and found the antibody levels dropping after CD4 T cell depletion but not after CD8 T cell depletion as expected, because of the presence of CD4 T cells helping antibody production. However, there was no substantial drop in antibody levels in the group depleted of both CD4 and CD8 T cells. This is similar to what was observed in the anti-tumor induction model (Fig. 4C), where high antibody levels were found on day 21 in the group treated with anti-CD4 and anti-CD8, despite loss of protection in all mice. Our present study however was unable to define the protective mechanism further except showing the involvement of CD4 and CD8 T cells.

We used the mTg and the lowest dose of 5,000U IL-1 for pre-priming as subclinical pre-existing EAT in the immunotherapy model to look for exacerbation (Fig. 15A). We started the combined model with 5×10^4 tumor cell dose for challenge. Tumor immunotherapy had no adverse effect on ongoing thyroiditis induced with mTg and 5,000U IL-1 (Fig. 15C). As we discussed previously, 5,000U IL-1 as a priming dose is subclinical which can be enhanced with additional Treg depletion and repeated mTg injections. This pre-priming dose is however variable as we did not observe enhancement in Fig. 8C, but did in Fig. 11C. In the experiment with 5×10^4 tumor dose, no enhancement was observed with 5,000U IL-1 pre-priming dose.

Since 5×10^4 tumor dose affords only 50% protection, we used 2.5×10^4 tumor dose to set up the combined model of immunotherapy and thyroiditis (Fig. 16A). Tumor immune responses

were unchanged in the presence of ongoing thyroiditis (Fig. 16B). Thyroiditis with mTg and 5,000U IL-1 as the pre-priming dose did not show any exacerbation with the added immunotherapy regimen (Fig. 16C). This result is in contradiction to what was observed in the EAT-resistant strain where regressing tumor caused enhanced thyroiditis and vice-versa (103). In our EAT-susceptible strain, the tumor inoculum is very small and we saw very small palpable tumors regressing in few mice within the group (2 out of 8). This effect may not be enough to cause a system wide change in immune response leading to exacerbation of thyroiditis. In EAT-resistant strain, the DNA vaccine was robust and led to regression of tumors as big as 400 mm² (103). Similarly in the EAT-resistant strain, the DNA vaccine also had granulocyte monocyte colony stimulating factor as an adjuvant. That could explain some of the systemic immunomodulation as well.

Conclusion

In clinical scenarios, systemic immunomodulation or adjuvant therapy is used to boost immune responses even when antigens are not well defined (107). This emphasis on immunomodulation creates scenarios where large doses of immunomodulators are administered for prolonged time periods which leads to increased tumor-specific responses but also creates problems for the immune system in terms of sequelae such as autoimmunity (107). This dissertation has explored the interaction of induced anti-tumor immune responses and the induced autoimmune responses on each to mimic the clinical scenarios of cancer patients undergoing immunotherapy. Our work demonstrated that the immunotherapeutic protocols used could have detrimental effects on the subclinical autoimmune diseases whereas the full blown clinical disease was not exacerbated further. In our mouse model of concurrent induction of thyroiditis and tumor immunity, we found thyroiditis exacerbation with anti-tumor induction

regimen, however, anti-tumor immune responses were unchanged. Modeling the pre-existing autoimmune scenarios, we found that subclinical thyroiditis was enhanced by tumor immune responses as a consequence of Treg depletion and repeated mTg injections, while autoimmune thyroiditis and tumor immunity were not affected by each other. Finally, developing an immunotherapy regimen to prevent tumor growth, we found both autoimmune thyroiditis and anti-tumor immune responses not influenced by each other.

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ABSTRACT**EXAMINING AUTOIMMUNE SEQUELAE DURING CANCER IMMUNOTHERAPY IN A
COMBINED AUTOIMMUNE THYROIDITIS AND TUMOR MOUSE MODEL**

by

SURESH CHANDRA KARI

December 2012

Advisor: Dr. Yi-chi M. Kong**Major:** Immunology and Microbiology**Degree:** Doctor of Philosophy

Some clinical trials of immunotherapeutic agents against cancers have resulted in the development of autoimmune sequelae, including autoimmune thyroiditis. It has been suggested that the immunotherapy resulted in an alteration in the immunoregulatory mechanism(s). Using experimental autoimmune thyroiditis (EAT), a mouse model of Hashimoto's thyroiditis (HT), the importance of regulatory T cells (Tregs) has been well established; inhibition of Tregs leads to enhanced thyroiditis with repeated doses of mouse thyroglobulin (mTg) without adjuvant. To simulate cancer patients, we developed a combined EAT and tumor model in our EAT-susceptible mice (CBA/J). In first establishing the tumor model, we found Treg depletion and irradiated tumor cell immunization necessary for robust anti-tumor immunity. We examined the anti-tumor induction model further and found memory response where mice withstood secondary tumor challenge even 28 or 35 days after the primary challenge. Role of CD4 and CD8 T cells was elucidated and both T cell subsets were observed to mediate protection. Although it was found that the anti-tumor induction protocol induced tumor antibody, its role in the protection was unclear in our present studies.

The anti-tumor induction model was then combined with EAT induction with repeated injections of mouse thyroglobulin (mTg) without adjuvant. Although prior Treg depletion enhanced EAT and was necessary for good anti-tumor immunity induction, the concurrent induction of EAT and anti-tumor immunity had no observable influence on each other. To determine the influence of tumor immunotherapy on pre-existing autoimmune thyroiditis, we primed mice with mTg and various doses of interleukin-1 β (IL-1 β). We tested various doses of IL-1 β (20,000, 10,000 and 5,000U) and combined it with subsequent anti-tumor and EAT induction model. We found no mutual influence of either component (either tumor immune responses or EAT) on each other with various doses tested. However, we found enhanced thyroiditis in mice tested with the lowest dose of IL-1 β , i.e. 5,000U, which was enhanced with Treg depletion and repeated mTg injections. In order to examine the influence of regressing tumor on prior autoimmunity, we developed a tumor immunotherapy model and combined it with pre-existing EAT induced with mTg and 5,000U IL-1 β . Our results did not show any influence of regressing tumor on prior EAT with two doses of tumors tested.

Our studies helped in dissecting out the role of various components in enhancement of pre-existing autoimmune thyroiditis. Our studies suggest that immunomodulation for tumor immunotherapy could enhance thyroiditis. Similarly, pre-existing thyroiditis primed with an mTg and lower IL-1 β dose of 5,000U can be exacerbated as well.

AUTOBIOGRAPHICAL STATEMENT

SURESH CHANDRA KARI

EDUCATION:**Ph.d., 2012**

Wayne State University School of Medicine
Department of Immunology and Microbiology

Masters in Veterinary Microbiology, 2005

MVSc (Masters of Veterinary Science) in Microbiology,
College of Veterinary Sciences, Tirupati, India

Thesis: *Attenuation and Evaluation of Bluetongue Virus Serotype -2*

BVSc (Bachelors of Veterinary Science, DVM equivalent), 2002

College of Veterinary Sciences, Hyderabad, India

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