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Resetting the T Cell Repertoire in Prostate Cancer Bearing Host

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This is the first annual report on the grant "Resetting the T cell repertoire in prostate cancer bearing host". A major obstacle to effective anti-tumor immune response is immune tolerance to tumor antigens, mostly caused by the defective cancer-reactive T-cell repertoire and increased immune suppression by regulatory T cells. We proposed to reset the immune system of cancer-bearing host by rescuing cancer-reactive T cells and by eliminating the generation and survival of Treg. (1). To rescue cancer-reactive T cells by preventing clonal deletion of tumor-reactive T cells in the thymus. (2). To block the Treg production using anti-B7 antibodies and to optimize the immunotherapy of prostate cancer using antibodies and fusion proteins. In the past funding period, we have submitted two papers that summarized our results from specific aim 2 in modulating Treg production in anti-tumor immunity and related subject on the role of costimulatory molecule B7 on NKT cell development. We are in the process to finish the data analyses for specific aim 1.

Tumor immunology, costimulatory molecules, tumor antigens, immunotherapy.
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(4) Introduction

This is the second annual report on the grant “Resetting the T cell repertoire in prostate cancer bearing host”.

A major obstacle to effective anti-tumor immune response is immune tolerance to tumor antigens, mostly caused by the defective cancer-reactive T-cell repertoire and increased immune suppression by regulatory T cells. It has been realized that T cell development in thymus is a continuous process throughout life span and androgen blockade results in the complete regeneration of the thymic function and restoration of peripheral T cell phenotype and function, both in mice and in human. We have demonstrated in the transgenic mouse prostate cancer (TRAMP) model that T cells reactive to tissue specific tumor antigens are deleted in the thymus. Our preliminary studies further indicated that lymphototoxin (LT)-aire pathway likely controls the expression of tumor antigens and the clonal deletion of T cells specific for tumor antigens in the thymus. We have also shown that costimulatory molecules are required for the generation of regulatory T cells (Treg). We proposed to reset the immune system of cancer-bearing host by rescuing cancer-reactive T cells and by eliminating the generation and survival of Treg.

In our proposal, we have proposed: (1). To rescue cancer-reactive T cells by preventing clonal deletion of tumor-reactive T cells in the thymus. (2). To block the Treg production using anti-B7 antibodies and to optimize the immunotherapy of prostate cancer using antibodies and fusion proteins. In the past funding period, we have published two papers that summarized our results from specific aim 2 in modulating Treg production in anti-tumor immunity. We have submitted the third paper in the role of lymphotoxin and its receptor in T cell negative selection. We applied the lymphotoxin receptor beta IgG Fc fusion protein to TRAMP prostate cancer mice and achieved the cancer preventive effect. We are going to submit a new proposal to DOD to continue this line of work.
(5) Body of Annual Report

Task I. Rescuing cancer-reactive T cells by preventing clonal deletion of tumor-reactive T cells in the thymus. (Month 1-36). (In progress).

I-A. We will evaluate the contribution of aire and LT, the two major regulators of peripheral antigen expression in the thymus and of clonal deletion of tumor-reactive T cells in the thymus, using mice with targeted mutations of aire, lymphotoxin alpha, and lymphotoxin beta receptor. (Month 1-12) (Finished)
   a. We have obtained the aire and LT alpha knock out mice from our collaborator, Dr. Yang-Xin Fu at the University of Chicago. We have started to breed the TRAMP mice with mice with targeted mutations. Two generations of cross will be needed to generate TRAMP+ aire (-/-) and TRAMP+ LT alpha (-/-). We are in good progress in this step and expect to have the right genotypic mice within 3-4 months. (Month 1-4). (Finished)
   b. Analyze the mice at 8-12 weeks to obtain information on peripheral antigen expression profile from thymus by real time PCR. (Month 3-8). (Finished)
   c. Analyze the effects of targeted mutations of aire, LT alpha and LT beta receptor on the thymic expression of peripheral tissue antigens and tumor associated antigens. (Month 5-12). (Finished)

I-B. We will inhibit the expression of peripheral antigens in the thymus by using a soluble LT receptor that blocks membrane-bound LT and evaluate its effect in clonal deletion and mouse susceptibility to the spontaneously arisen prostate cancer in TRAMP mice (Month 1-36). (Finished).
   a. Backcross the three strains of knock out mice to B10.BR background at least 5 generations. We are currently in 4th generation in heterozygous state. We will backcross another generation (Month 1-6). (Finished)
   b. Breed the mice generated in Task I-B-a with T cell receptor transgenic mice TgB. Two generations of cross will be needed to generate TgB+ aire (-/-) and TgB+ LT alpha (-/-) mice. (Month 6-18). (In progress).
      We have generated TgB+ aire (-/-) mice in past year. However, an unexpected genetic makeup of the LT alpha gene prevented us in generating TgB+ LT alpha (-/-) mice. The purpose of the breeding is to replace the H-2b background of LT alpha (-/-) mice to H-2k for the B10.BR-TGB mice to present H-2k related SV40 large T antigen epitope. After several months of frustration, we realized that LT alpha gene is in the same chromosome as H-2 (MHC gene). We will not be able to obtain the mice we expected.
      We recently obtained the TCR-I transgenic mice that is in C57BL/6 background and recognize the H2-Dd-restricted SV40 large tumor antigen epitope I (residues 206-215) from Jackson Laboratory. We will breed this mice to LT alpha (-/-) mice to get TCR-I (+) LT alpha (-/-) mice.
   c. Generate TRAMP x TgB F1 mice that carry different target mutations. (Month 12-21). (Finished).
   d. To study the impact of LT-aire pathway mutations on the tumor antigen expression and clonal deletion of the tumor antigen specific T cells in the thymus. (Month 18-24). (In progress).
e. To study the function of rescued high avidity T cells in the TRAMP x TgB model. (Month 24-36). *(Finished)*.

f. To study the function of rescued high avidity T cells in the TRAMP model. (Month 24-36). *(Finished)*.

g. Using soluble LT beta receptor Ig fusion protein to treat TRAMP x TgB F1 mice to examine the effect of fusion protein on tumor antigen expression and T cell clonal deletion in the thymus. (Month 13-24). *(Finished)*.

h. Using soluble LT beta receptor Ig fusion protein to treat TRAMP mice to examine the effect of fusion protein on tumor antigen expression and T cell clonal deletion in the thymus. (Month 13-24). *(Finished)*.

i. Long term survival surveillance on the mice that treated with fusion protein, record the tumor incidence, tumor size, metastasis and autoimmune side effect. (Month 18-36). *(In progress)*.

I-C. We will explore the combination of androgen ablation and soluble LT beta receptor Ig fusion protein and evaluate the effect on thymic regeneration and rescuing tumor antigen specific T cells in older TRAMP mice (Month 1-36). *(In progress. We request modification)*.

a. Examine the effect of surgical castration combined with soluble LT beta receptor Ig fusion protein treatment in older wild type mice (Month 1-12). *(In progress)*.

b. Determine the thymic regeneration, thymic microenvironment and thymic T cell output in surgical castrated and soluble LT beta receptor Ig fusion protein treated older mice (Month 3-15). *(In progress)*.

c. Determine the peripheral T cell function by antigen immunization in surgical castrated and soluble LT beta receptor Ig fusion protein treated older mice (Month 3-15). *(In progress)*.

d. Examine the effect of surgical castration combined with soluble LT beta receptor Ig fusion protein treatment in tumor bearing 5-7 month old TRAMP mice (Month 9-24). *(In progress)*.

e. Determine the generation of Tag epitope IV specific T cells from surgical castrated and soluble LT beta receptor Ig fusion protein treated tumor bearing TRAMP mice (Month 10-24). *(In progress)*.

f. Long-term tumor incidence and tumor growth and metastasis observation in surgical castrated and soluble LT beta receptor Ig fusion protein treated TRAMP mice (Month 13-36). *(In progress)*.

The progress in this sub-aim is delayed due to the approval of animal surgical castration procedure. It was reviewed and considered to cause excessive pain to mice in surgical castration by DOD ACURO committee. It took me a long time for back and forth communication with ACURO. I eventually decided to substitute the castration surgery with medical castration using hormone ablation treatment with LHRH injection to inhibit androgen production. We will start this part of project very soon.

I-D. The impact of soluble LT beta receptor Ig fusion protein on autoimmune inflammation.
a. We will systemically examine the effect of soluble LT beta receptor Ig fusion protein in inducing autoimmune side effects. Wild type mice age week 4, 6, 8, 16, 24, 36 will be injected with soluble LT beta receptor Ig fusion protein and short-term (within two weeks) and long-term (three months) side effect will be studied. Histological sections will be prepared and the lymphocytic infiltration will be examined in skin, colon, stomach, heart, lung, liver and kidney. Immunofluorescence staining will be done on frozen section of kidney and skin tissue to detect IgG and C3 deposits. (Month 13-36). (Finished).

b. All the above mentioned (Aim I-B, I-C) mice that were treated with soluble LT beta receptor Ig fusion protein will be examined vigorously as described above. (Month 1-36). (Finished).

II. Transient blockade of Treg production and immunotherapy of prostate cancer (Month 1-36). (In progress).

II-A. We will develop the immunotherapy of prostate cancer by transient B7 blockade. We will identify an optimal blockade condition that reduces Treg development without blocking activation of effector T cells. (Month 9-24) (Finished)

a. To perform adaptive transfer experiments to examine the short-term and long-term effect of costimulatory blockade on Treg and the effect on the tumor antigen specific T cell activation. (Month 9-15). (Finished)

b. To study the activation, differentiation of tumor antigen specific T cells after the release of Treg blockade. (Month 11-17). (Finished)

c. To study the recruitment of T cells into prostate tissue (Month 12-24). (Finished).

The manuscript “B7 Blockade Re-Balances Regulatory T cells and Tumor-reactive T Cells for Prevention and Therapy of Prostate Cancer” was published and is attached as Appendix 1.

II-B. We will explore combination therapy targeting at regenerating thymic function, reducing Treg and rescuing cancer-reactive T cells. (Month 13-36). (In progress).

a. To study the impact of LT beta receptor Ig fusion protein on the development of Treg. (Month 13-18). (Finished).

b. To modify the combination therapy (surgical castrated, soluble LT beta receptor Ig fusion protein treatment, anti-B7 antibody treatment) in the TRAMP x TgB mouse model (Month 15-24). (In progress).

c. To modify the combination therapy (surgical castrated, soluble LT beta receptor Ig fusion protein treatment, anti-B7 antibody treatment) in the TRAMP mouse model (Month 25-36). (In progress).

The manuscript “Targeting Lymphotoxin-mediated Negative Selection to Prevent Prostate Cancer in Mice with Genetic Predisposition” was submitted for publication and is attached as Appendix 2.
(6) Key Research Accomplishments

- We have found that B7-1 and B7-2 are required for the production of regulatory T cells. Anti-B7 antibodies significantly reduced the number of Treg in the thymus and in the spleen. *(Appendix 3, paper published).*
- We have shown that anti-B7 antibody treatment in adult TRAMP mice prolonged the mice survival for more than 10 weeks.
- We have established that expression of tissue-specific antigen/tumor antigen in the thymus and clonal deletion of tumor-reactive T cells can be modulated in the TRAMP mice.
- We have established collaboration with Department of Radiology in University of Michigan to successfully measure prostate glands and prostate tumors with MRI.
- We demonstrated that temporary blockade of B7-1 and B7-2 reduced the number of regulatory T cells and conveyed considerable therapeutic effects in TRAMP mice with spontaneous prostate cancer. To our knowledge, this is the first time that the prostate cancer in the TRAMP mice can be effectively treated when the large tumors can be demonstrated.
- Mechanistically we showed that transient blockade of B7-1/2 resets the balance of Treg and cancer reactive T cells to confer prevention and therapy of prostate cancer. A second major advantage is that the data can be easily translated into human use as the drug that blocks B7-1 and B7-2 (FDA approved CTLA4Ig) has already been approved for the treatment of autoimmune diseases. It is possible to dramatically shorten the path of clinical development for the novel immunotherapy. *(Appendix 1, paper published)*
- We showed that the development of NKT cells are defect in the mice with targeted mutations of B7-1/2 and CD28. The percentage of TCRβ+NK1.1+ as well as TCRβ+α-Galcer/CD1d+ (iVα14 NKT) cells population are significantly reduced in the thymus, spleen and liver in the mice with targeted mutations of B7-1/2 and CD28.
- We have shown the mice with target mutation of costimulatory molecules have defect NKT cell function. B7 and CD28 deficient mice develop much less severe ConA induced hepatitis, which is known mediated by NKT cells. *(Paper published in PLoS ONE and attached in Annual report 2008 as Appendix 2).*
- We have shown that targeted mutation of the LTα gene efficiently rescued tumor-reactive T cells, drastically reduced cancer incidence and almost completely ablated metastasis.
- We have shown that, remarkably, short-term treatments with LTβRIg interrupted clonal deletion, reduced the size of primary cancer and completely prevented metastasis later in life, thus providing an easily translatable immune prevention for those with genetic predisposition to cancer.
(7) Reportable Outcomes:

Manuscripts:


(8) Conclusions:

In summary, in this funding period, we have demonstrated that a short-term anti-B7 blockade re-balances regulatory T cells and tumor-reactive T cells for prevention and therapy of prostate cancer.

It is generally agreed that immunotherapy is very inefficient for treatment of established tumors. This can be more challenging in transgenic tumor models where malignant tumor cells continue to arise due to transgenic expression of oncogenes. Our data demonstrated that even when administrated at a time when the TRAMP mice show more than three fold enlargement of prostate size, transient blockade of B7-1 and B7-2 dramatically reduced the rate of tumor growth. Thus, at eight weeks after initiation of the treatment, the prostate of the control Ig-treated expanded by five fold in volume. In contrast, those from anti-B7-treated mice expanded by less than two fold during the same period. When the palpable tumors were used as endpoint, the anti-B7 treatment at 25 weeks reduced tumor development by 7 weeks. Nevertheless, perhaps because of the continuous production of new cancer cells from the germline insertion of SV40 large T antigen and waning of antibodies, short term treatment did not completely eradicated the tumors. However, consider the relatively simplicity of the treatment, it may show greater efficacy when apply to human prostate cancer patients as the cancer growth rate is expected to be slower than oncogene transgenic mice such as TRAMP mice.

Identification of genetically susceptible individuals calls for preventive measures to minimize the life-long cancer risk of these high risk populations. Immune prevention is made necessary by the anticipated health thread but only possible by predictability of antigens. Lack of enough high affinity of T cells against tumor-associated antigens and unpredictability of tumor antigen make antigen-based immune prevention untenable for cancer. To address this issue, we explored a non-antigen-based cancer immune prevention using the TRAMP mice that spontaneously develop prostate cancer with 100% penetrance. We show that targeted mutation of the LTα gene efficiently rescued tumor-reactive T cells, drastically reduced cancer incidence and almost completely ablated metastasis. Remarkably, short-term treatments with LTβR Ig interrupted clonal deletion, reduced the size of primary cancer and completely prevented metastasis later in life, thus providing an easily translatable immune prevention for those with genetic predisposition to cancer.
(9) References:
None.
B7 Blockade Alters the Balance between Regulatory T Cells and Tumor-reactive T Cells for Immunotherapy of Cancer

Penghui Zhou, Xincheng Zheng, Huiming Zhang, Yang Liu, and Pan Zheng

Abstract

Purpose: In prostate cancer bearing host, regulatory T (Treg) cells restrain activity of tumor antigen specific T cells. Because B7:CD28 interactions are needed for both function of CD4+CD25+ Treg cells and CD8+ effective T cells, targeting this pathway may help to overcome the immunotherapy barriers.

Experimental Design: The anti B7 1/87 2 monoclonal antibodies were administered to a transgenic mouse model of prostate cancer (TRAMP) ectopically expressing SV40 large T antigen in different tumor development stages for prevention and therapy of prostate cancer. The treatment was also tested in treating transplanted MC38 colon adenocarcinoma in mice.

Results: Here, we showed that short term administration of anti B7 1/87 2 monoclonal antibodies in TRAMP mice leads to significant inhibited primary tumor growth and the size of metastatic lesions. The treatment is effective to inhibit MC38 colon cancer growth. Correspondingly, this treatment results in a transient reduction of Treg in both thymus and the periphery. In vivo cytotoxicity assay revealed T antigen specific CTL effectors in anti B7 treated but not control IgG treated TRAMP mice.

Conclusions: Transient blockade of B7 1/87 2 alters the balance between Treg and cancer reactive T cells to enhance cancer immunotherapy.

Many of tumor antigens identified thus far are self antigens (1-4) and may therefore trigger immune tolerance. Logically, mechanisms that mediate self tolerance may contribute to inadequacy of tumor immunity. The best characterized mechanism of self tolerance is clonal deletion (5, 6). In this context, we have shown that tumor antigen controlled by tissue specific promoter is also expressed in the thymus to trigger clonal deletion (7).

In addition to clonal deletion, CD4+CD25+ regulatory T (Treg) cells play a pivotal role in the maintenance of peripheral self tolerance (8-12). Accumulating evidence also support a role for Treg in restrained cancer immunity. Thus, cancer patients have elevated numbers of Treg cells in the blood of malignant effusions (13-15). Treg cells are also recruited and accumulated at tumor sites in animal models and in cancer patients (16-18). Correlation between the number of CD4+CD25+ Treg cells and clinical outcomes in some, although not all, cancer patients supported the hypothesis that Treg may suppress the effector function of tumor antigen specific T cells, allowing tumor growth in the presence of tumor antigen specific T cells (19, 20). Consistent with this concept, the removal of CD4+CD25+ Treg cells by an anti CD25 monoclonal antibody promoted rejection of transplanted tumor cells (21). However, this approach has shown little efficacy in animals with spontaneous tumors, which better reflect the challenge of cancer immunotherapy. In a recent study using a transgenic model of prostate dysplasia, anti CD25 monoclonal antibody (mAb) treatment at age 12 weeks caused only 25% reduction in the prostate mass at 20 weeks, although extended observation has not been carried out to document long term effect (22).

Alternatively, it is worth considering conditions that are selectively required for the generation and maintenance of Treg. CD28+ and B7 1/87 2- mice have markedly decreased numbers of CD4+CD25+ Treg cells in the thymus as well as in the periphery (23-25). Meanwhile, we and others have reported a significant role for B7:CD28 interaction in clonal deletion of some, although not necessarily all, self antigens (26, 27). As such, transient blockade of B7 1/87 2 may reduce Treg while increase the frequency of cancer reactive T cells, thus overcoming the two major barriers to effective cancer immunity.

Transgenic mouse model of prostate cancer (TRAMP) is a well established mouse model for prostate cancer with clearly defined progression of prostate cancer that resembles the human disease (28). Metastasis to periaortic lymph nodes and lungs can be detected frequently (29). By the time the mice are 24 to 30 weeks old, the prostate cancer becomes palpable in...
The abdomen. We have adopted the TRAMP mouse model to test our hypothesis while facing the challenge of treating established spontaneous tumors. We report here that transient blockade of B7 1/B7 2 with mAbs resulted in temporal deletion of Treg and rescue of cancer reactive T cells from clonal deletion. These effects associated with increased effector function of CTLs. Remarkably, the relatively simple treatment confers prevention and therapy of the spontaneous prostate cancer and transplant able colon cancer. Because recombinant protein that blocks B7 1 and B7 2 has already been approved for human use, the path for translating our observation into patient care is considerably shorter than most therapeutic approach.

Materials and Methods

Experimental animals. C57Bl/6 mice and TRAMP mice expressing the SV40 T antigen (TAg) controlled by rat probasin regulatory elements in the C57Bl/6 background were purchased from The Jackson Laboratory. The mice were bred at the animal facilities of the Ohio State University and the University of Michigan. All animal experimen tal procedures were reviewed and approved by The Ohio State University and University of Michigan Institutional Animal Care and Use Committees. Mice were typed for SV40 TAg by isolation of mouse tail genomic DNA. The PCR based screening assay was described previously (7). Transgenic mice expressing T cell receptor (TCR) specific for SV40 large TAg (TGB) have been described (30). Generation of TRAMP mice expressing TGB TCR (TGB-TRAMP) was also described (7).

Antibody treatment of the TRAMP mice. TRAMP mice were treated with anti B7 1 and anti B7 2 antibodies at two different stages. In the first regimen, 4 to 6 week old TRAMP mice were injected intraperitoneally with five injections of anti B7 1 (rat anti mouse CD80, clone 3A12; ref. 31) and anti B7 2 (hamster anti mouse CD86, clone GL 1; American Type Culture Collection; ref. 32) antibodies or control hamster/rat IgG (Sigma) at 100 μg/antibody/injection every other day. Long term prostate cancer incidence was recorded by physical examination. In the second regimen, 25 week old TRAMP male mice without palpable prostate cancer were treated intraperitoneally with the anti B7 or control IgG at 100 μg/antibody/injection for five injections every other day. The magnetic resonance imaging (MRI) examination was carried out before treatment and 8 weeks later at age 33 weeks. In a separate experiment, 25 week old TRAMP male mice were treated with one intraperitoneal injection of 1 mg anti CD25 (PC61) or control rat IgG (33). The efficiency of anti CD25 depletion was examined by flow cytometry with staining PBL using conjugated anti CD4, anti CD25 (clone 7D4; American Type Culture Collection), and anti Foxp3. The MRI examination was carried out before treatment and 5 weeks later at age 30 weeks. For long term prostate cancer incidence study, anti B7 and control CD25 treated mice were examined at least weekly for palpable tumor at lower abdomen and were euthanized when they either become moribund or with tumor size exceeding 5% of body weight.

Six to 8 week old TRAMP or TRAMP/TGB mice were sublethally irradiated (500 rad) on day 0 and the treatment started on day 1 with either anti B7 1/B7 2 mAbs (100 μg/each) or control rat/hamster IgG (100 μg/each) intraperitoneally. The mice were treated six times every other day. One week after the last treatment, the mice were sacrificed and the total thymocytes and splenocytes were harvested and stained with fluorochrome conjugated antibodies anti CD4 (RM4.5), anti CD8 (53.6.7), and anti V8.1b.8.2 (MR5 2; BD).

For transplantable tumor model, MC38 murine colon carcinoma cells were grown in RPMI with 5% fetal bovine serum and subcutaneously injected to male C57Bl/6 mice (5 x 105 per mouse). Ten days after injection, mice were divided evenly into two groups based on the tumor sizes and administered intraperitoneally with either anti B7 or control IgG three times every other day. Peripheral blood was collected at 0 and 6 days (0 day is the day before the administration of antibodies) and the splenocytes were collected at 14 days and stained with anti CD4, CD8, CD25, and Foxp3 antibodies (BD).

Proliferation of T cells to antigenic peptides. Total spleen cells (8 x 10^5 per well) from control immunoglobulin or anti B7 treated TRAMP x TGB (H2b cxk) F1 mice were cultured with the given concentrations of SV40 TAg K560 56S peptide or control HSV gB peptide in Click's Eagle's Hank's amino acid medium for 72 h. The proliferation of T cells was determined by incorporation of [3H]thymidine pulse (1 μCi/well) during the last 6 h of culture. The data presented are means of triplicates with variation from the means <15%.

Peptide synthesis. All peptides used were synthesized by Research Genetics. The peptides were dissolved in DMSO at a concentration of 10 mg/mL and diluted in PBS or culture medium before use. Peptides used were SV40 TAg K560 56S SEFLKRI (7) and HSV gB peptide gB498 505 SSIERAFIL (34).

Immunohistochemistry. Mouse organs were fixed with 10% buffered formalin. Tissue sections were stained with H&E and examined under a microscope. Frozen sections were prepared and stained with 2 μL antibodies specific for CD3 (2C11, hamster IgG), CD3+ foci were counted using x20 microscope visual fields.

In vivo cytotoxicity assay. Spleen cells from C57Bl/6 mice were pulsed with 10 μg/mL of either SV40 TAg K560 56S SEFLKRI or a control peptide HSV gB498 505 SSIERAFIL in the presence of either 0.5 or 5 mmol/L CFSE, respectively. After mixing at a 1:1 ratio, the labeled cells were injected intravenously into recipients and spleen cells were harvested 20 h later and analyzed by flow cytometry for the relative abundance of CFSElow (SV40 TAg peptide) and CFSEhi (HSV peptide) populations.

Detection of anti double stranded DNA. Anti DNA antibodies were measured by ELISA according to the published procedure (35).

MRI of prostate. The progression of prostate cancer in the TRAMP model was measured by MRI as described (36). Briefly, MRI experiments were done on a Varian system equipped with a 7.0 Tesla, 18.3 cm horizontal bore magnet (300 MHz proton frequency). For MRI examination, the mice will be anesthetized with sodium pentobarbital (70 mg/kg intraperitoneally) and maintained at 37 °C inside the magnet using a heated circulation water blanket, with pelvis motion (due to respiration) minimized by a small plastic support placed before
insertion into a 3 cm diameter quadrature birdcage coil (USA Instruments). Multislice images were acquired using a T1-weighted spin echo sequence (TR/TE = 880/13, field of view = 30 x 30 mm using a 128 x 128 matrix, slice thickness = 1.5 mm, and slice separation = 1.0 1.6 mm). Each set contained 9 to 25 slices and enough sets were obtained to provide contiguous image data of the prostate tumor. Prostate volume will be measured using the formula: $V = 4/3(D_1 + D_2)/4$, where $D_1$ and $D_2$ correspond to the longest and shortest (transverse and sagittal) diameters measured from the MRI image. The accuracy of this measurement was confirmed by comparing preneoplastic MRI volumes with postneoplastic actual prostate volumes in select cases.

**Results**

Anti-B7-1/B7-2 antibody treatment of young TRAMP mice reduced Treg cells in both the thymus and the periphery and delay development of prostate cancer. We and others have reported that targeted mutation of CD28 and B7 1/B7 2 abrogated generation of Treg cells (23). To test whether this pathway can be targeted for transient reduction of Treg, we treated C57Bl/6 mice with either anti B7 1/B7 2 mAbs or control IgG five times every other day. Thymi and spleens were harvested 8 days after the last injection. Cells were stained for flow cytometry analysis. This treatment did not affect either the total cellularity or the numbers of CD4 and CD8 T cells (Fig. 1A). However, the numbers of CD4+FoxP3+CD25+ cells were reduced by 50% in thymus and by 4 fold in the spleen (Fig. 1B). When gated on lymphocyte gate, all CD4+ T cells are CD3+ (Supplementary Fig. S1). Therefore, all FoxP3+CD25+ cells analyzed in this study are Treg. These data indicate that Treg cells can be significantly reduced in both the thymus and the spleen by anti B7 1/B7 2 antibodies.

To investigate whether anti B7 1/B7 2 antibody treatment delay the development of prostate cancer, 4 week old male TRAMP mice were treated with either control IgG or anti B7 1/ B7 2 antibodies and the incidence of cancer development was followed by physical examination. Using 50% of mice with palpable prostate cancer as a reference point, we observed that anti B7 delayed the tumor development by >14 weeks (Fig. 1C). Therefore, anti B7 treatment may be valuable for prevention of prostate cancer development.

Enhanced tumor specific cytotoxicity after anti-B7-1/B7-2 antibody treatment. To test tumor antigen specific immunity following anti B7 1/B7 2 treatment, we further investigated the tumor specific cytotoxicity by an in vitro killing assay. Six week old male TRAMP mice were injected intraperitoneally with anti B7 1/B7 2 mAbs or control IgG five times every other day. Two weeks after the first injection, they received an intravenous injection of a 1:1 mixture of SV40 TAg peptide pulsed (CFSE+) and control HSV gB peptide pulsed (CFSE-) spleen cells. The spleens were stained 20 h later and analyzed by flow cytometry. As shown in Fig. 2A, in mice treated with anti B7 antibodies, the SV40 TAg pulsed targets were preferentially eliminated, whereas the CFSE+ and CFSE- cells remained at the 1:1 ratio in control immunoglobulin treated mice. These data showed that anti B7 treatment enhanced CTL response against the SV40 large TAg without intentional immunization.

Anti-B7 antibodies rescued SV40 large T-specific T cells from clonal deletion in the TRAMP mice. Our previous studies have shown that SV40 large TAg is expressed in the thymic peripheral antigen expressing cells in the TRAMP mice and that such expression caused nearly complete deletion of transgenic T cells expressing a TCR specific for a SV40 large TAg peptide presented by H 2Kb (7). Moreover, we reported that perinatal blockade of B7 1 and B7 2 reduced clonal deletion of autoreactive T cells (26). To test whether the anti B7 treatment rescues SV40 TAg specific T cells from clonal deletion in the TRAMP mice, we produced TRAMP mice expressing the SV40 TAg specific TGB TCR and divided the double transgenic mice with either anti B7 mAbs or control IgG treatment groups.

As the mice recovered from irradiation, a new wave of bone marrow derived cells will differentiate into mature T cells in the thymus. This de novo process increases sensitivity of blocking studies (37). To study the effect of anti B7 treatment on newly formed T cells undergone thymic development and clonal deletion, we gave sublethal irradiation (500 rad) to TGB single transgenic and TRAMP/TGB double transgenic mice. At 1 week after six treatments, the thymic cellularity and mature CD8 T cells were measured by flow cytometry. As shown in Fig. 2B, due to clonal deletion, the numbers of reconstituted thymocytes were extremely low in the double transgenic TGB TRAMP mice compared with single transgenic TGB. Importantly, anti B7 treatment increased thymic cellularity by ~10 fold (Fig. 2B). A corresponding increase in the CD8 T cells expressing high levels of Vj88 transgenic TCR was observed in both spleen and thymus (Fig. 2C, left). When the spleen cells were analyzed for CD4/CD8 T cell ratios, it was clear that, perhaps due to clonal deletion, T cells in the control immunoglobulin treated mice have lost the predominance of CD8 subset due to expression of MHC class I restricted TCR. This is corrected to a large extent by anti B7 treatment (Fig. 2C, right). Thus, anti B7 treatment greatly reduced efficiency of clonal deletion. However, the numbers of transgenic T cells in the anti B7 treated TGB TRAMP mice were still much reduced in comparison with TGB mice, which showed that the rescue is only partial.

To test whether the T cells rescued by anti B7 treatment were responsive to tumor antigen, we stimulated spleen cells from control immunoglobulin or anti B7 treated mice with different concentration of the SV40 TAg peptide or control peptide from HSV peptide. As shown in Fig. 2D, anti B7 treated spleen cells underwent a significant proliferation to SV40 TAg peptide. Based on the dose response, the anti B7 treated spleen cells were at least 100 fold more responsive than the control immunoglobulin treated spleen cells, which corresponded to increased number of antigen specific T cells. Therefore, the anti B7 rescued T cells are functional. However, after in vitro stimulation, the rescued T cells showed poor cytotoxicity (data not shown), which suggests that the rescued T cells may be functionally impaired to some extent.

Anti-B7-1/B7-2 antibody treatment cause significant albeit transient reduction of Treg in mice with established prostate cancer. One of the most difficult challenges in cancer immunotherapy is the treatment of established solid tumors. It has been shown that microscopic lesion of prostate cancer can be observed in the TRAMP mice between ages 18 and 24 weeks (29). To confirm the development of tumor in the 25 week old TRAMP mice in our colony, we used the MRI to compare the size of the prostate at 25 weeks. As shown in Fig. 3A, all of the 12 TRAMP mice tested had considerably larger
prostate organ sizes compared with non-TRAMP littermate. Thus, essentially all of the 25 week old TRAMP mice developed cancer in the prostate.

To determine the effect of anti B7 antibodies for B7 1 and B7 2, we injected either control or anti B7 mAbs every other day for five times. The blood samples were collected at 0, 1, 2, or 6 weeks after antibody treatment and stained for either anti CD25 or anti Foxp3 in conjunction with anti CD4. As shown in Fig. 3B, in comparison with control immunoglobulin treated mice, significant reduction of Treg can be observed in the peripheral blood at 1 and 2 weeks after completion of the treatment. Interestingly, the number of Treg is restored to normal levels at 6 weeks after completion of the treatments. Thus, in mice bearing established prostate cancer, anti B7 1 and anti B7 2 antibodies caused a significant albeit transient reduction of Treg in tumor bearing mice.
Anti-B7 antibodies delayed growth of established prostate cancer without autoimmune side effects. To determine whether anti B7 antibodies can confer therapeutic effect in mice with established prostate cancer, we randomly divided 25 week old TRAMP mice into two groups and measured their tumor size before the treatment with either control immunoglobulin or anti B7 antibodies, starting at 25 weeks. After five injections, the mice were followed for the tumor progression by either palpation or MRI. As shown in Fig. 3C, at age 33 weeks (8 weeks after first treatment), in the control IgG treated group, the volume of prostate expanded by 2.5 to 9 fold with an average of 4.5 fold. In contrast, all but one anti B7 treated mice show <2 fold expansion of the prostate volume. Mann Whitney test indicate that the difference was statistically significant (P = 0.04). Because the tumors are not palpable at the beginning of the treatment, we also used the time when the mice developed palpable tumors as a second endpoint with larger sample size (12 mice for each group). As shown in Fig. 3D, even treated as late as age 25 weeks, the anti B7 antibodies delayed tumor development by ~7 weeks.

In the TRAMP model, lymph node metastasis has occurred at 25 weeks (29); we therefore tested the effect of anti B7 treatment on metastatic lesions in other organs, including lung, kidney, and liver. As shown in Fig. 4A, 3 of 6 mice in the control immunoglobulin treated group have substantially higher number of metastatic lesions in lung. In addition, massive metastatic lesions were found in kidney (1 of 6) and liver (2 of 6) (data not shown). Only one case of metastasis was observed in the anti B7 treated group, and the metastasis is limited to the lung. In addition, the metastatic lesions in the anti B7 treated group were substantially smaller than those found in the control immunoglobulin treated group (Fig. 4A).

Corresponding to reduced tumor growth, we have observed increased T cell infiltrating into tumors. Immunohistochemistry.
Fig. 3. Anti-B7-1/B7-2 mAb treatments of mice with established prostate cancer inhibited cancer progression. A, MRI measurement of prostate volumes of 25-week-old normal and TRAMP mice. Left, representative local images of male B6 and TRAMP mice. The prostate was identified with thick white outlines. Right, prostates sizes of 3 B6 and 12 TRAMP mice, all at age 25 wk. B and C, anti-B7 treatment initiated at 25-week-old TRAMP mice transiently depleted Treg. Male TRAMP mice were administered intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μg 3A12 and 100 μg GL-1) or control IgG (1:1 mixture of 100 μg hamster and 100 μg rat IgG) five times every other day. Peripheral blood was taken at 0, 1, 2, and 6 wk; 0 wk is the day before injection. Cells were stained for flow cytometry. Plots are gated on CD4⁺ cells. D, CD25⁺/FoxP3⁺ cell number started to reduce following the first week of treatment and almost recovered to normal levels 1 mo after the treatment was stopped. Data have been repeated two times, involving a total of 12 mice per group. D, MRI image of TRAMP mice at 25 and 33 wk (8 wk after starting treatments with either control immunoglobulin or anti-B7 mAbs). Summary data are ratio of prostate volumes at 33 versus 25 wk when the treatments started. D, Kaplan-Meier analysis for incidence of palpable tumors in TRAMP mice treated with either control immunoglobulin or anti-B7 antibodies at age 25 wk.
Fig. 4. Anti-B7 blockade in tumor-bearing mice reduces the number and size of metastatic lesions in the TRAMP mice and increases infiltration of T cells into tumors but does not cause autoimmunity. A, internal organs of mice from Fig. 3C were analyzed for metastatic lesions. Three sections of liver, lung, kidney, intestine, and heart, 30 μm apart, were examined double blind by a pathologist. A representative field of lung sections of control immunoglobulin-treated mice (3 of 6 mice analyzed have metastasis) and the only metastatic lesion in anti-B7 treated group are shown. Yellow arrows, metastatic lesions. In the control immunoglobulin-treated group, massive metastases were also observed in the liver (2 of 6) and kidney (1 of 6). B to D, mice from Fig. 3C were analyzed for infiltrating lymphocytes and autoimmune reactions. B, representative tumor sections stained with anti-CD3 mAb. C, fluorescence-activated cell sorting profiles showing representation of CD4 and CD8 T cells and the CD4⁺CD25⁺Foxp3⁺ T cells. Top left, profiles of mononuclear cells isolated from the tumor; bottom left, profiles from the gated CD4 T cells. Data are from pooled cells from 6 mice per group. Top right, frequencies of CD4 and CD8 T cells among mononuclear cells isolated from the prostate cancer; bottom right, ratio of Treg over CD4 or CD8 T cells. Mean ± SE (n = 6). D, serum anti-double-stranded DNA antibodies. Data are from an ELISA using 1:50 dilution of sera. Mean ± SE (n = 6).
staining revealed an increased numbers of T cell infiltration (Fig. 4B). Quantitative analysis by flow cytometry indicated that the frequency of T cells among the mononuclear cells from the collagenase treated prostate cancer tissue increased by 4.5 fold, with the majority of the T cells are of CD8 subsets (Fig. 4C). In both groups, higher percentage of CD4+ T cells expressed Foxp3 than what was found in the lymphoid organ (Fig. 4C, bottom left), similar to observations made by others (33). Nevertheless, the percentage of Treg is significantly lower in the anti B7 treated group. Moreover, the ratio of Treg over effector T cells were fully recovered to normal levels at 5 weeks after the treatment. Five weeks after anti CD25 treatment when mice reached age 30 weeks, two groups of TRAMP mice were reexamined by MRI. As shown in Fig. 6B, the prostate sizes were enlarged by 3 to 5 fold during the 5 week period due to the aggressive prostate cancer growth. Compared with the control group, the prostate sizes were increased by 2 to 3 fold in anti CD25 treated group (Fig. 6C). The significant difference revealed an effect of Treg depletion on tumor growth. However, this treatment is substantially less effective than transient B7 blockade (the average after/before treatment prostate size ratio in anti CD25
A cancer therapy: patients in the preclinical treatment group is 2.55 after 5 weeks compared with anti-B7 treatment average ratio is 1.72 after 8 weeks; Fig. 3).

**Discussion**

Traditionally, blockade of costimulatory molecules B71 and B72 has been explored for treatment of autoimmune diseases and transplant rejection (38). Recent studies that reveal a critical role for B71/B72 in the production and maintenance of Treg (23-25) and in clonal deletion of self-reactive (26) as well as cancer reactive T cells (7) suggest that this pathway may be targeted for overcoming the barrier of immune tolerance in cancer setting. The data described herein showed unexpected efficacy of this new approach.

We have chosen the TRAMP mice, which developed malignant transformation of prostate epithelial cells as early...
as 12 weeks to test this notion. Our data showed that a short term anti B7 blockade before the development of pathologic lesions delays the development of palpable tumor for ~14 weeks. These data show that a short term anti B7 treatment may prevent the development of prostate cancer among individuals with predisposition of prostate cancer.

It is generally agreed that immunotherapy is very inefficient for treatment of established tumors (39). This can be more challenging in transgenic tumor models where malignant tumor cells continue to arise due to transgenic expression of oncogenes. Our data showed that, even when administered at a time when the TRAMP mice show >3 fold enlargement of prostate size, transient blockade of B7 1 and B7 2 dramatically reduced the rate of tumor growth. Thus, at 8 weeks after initiation of the treatment, the prostate of the control immunoglobulin treated expanded by 5 fold in volume. In contrast, those from anti B7 treated mice expanded by <2 fold during the same period. When the palpable tumors were used as endpoint, the anti B7 treatment at 25 weeks reduced tumor development by 7 weeks. Nevertheless, perhaps because of the continuous production of new cancer cells from the germ line insertion of SV40 large TAg and waning of antibodies, short term treatment did not completely eradicated the tumors. Because the majority of tumors that developed in human have clonal origin, the malignant transformation is likely less frequent that what is observed in transgenic model of spontaneous tumors. Therefore, the relatively simple treatment may show greater efficacy. Given the broad function of B7 1 and B7 2 in host immune system, including T cell costimulation at both priming and effector phases, Treg generation and maintenance, and clonal deletion, it is unlikely that a single mechanism is responsible for the therapeutic efficacy reported herein.

First, we have shown significant albeit transient reduction of Treg in both thymus and the peripheral blood. Because the treatment with anti CD25 antibody also showed some efficacy in slowing prostate tumor growth in TRAMP mice, Treg depletion alone is sufficient to convey significant, although less marked, protection. It is worth noting that anti CD25 antibody depletes almost 95% of CD4+CD25+ cells in 6 days; however, 60% of CD4+Foxp3+ cells still remained in peripheral blood at the same time. Because the treated mice had more CD25 Foxp3+ cells than the untreated mice, anti CD25 ablated part of CD25 Foxp3+ cells and down regulation of CD25 on others. On the other hand, anti B7 treatment caused similar extent of reduction in the CD4 Foxp3+ cells regardless of their CD25 phenotype. It is unclear whether the different depletion profile contributed to different efficacy.

Interestingly, the number of Treg returns to normal levels at 6 weeks after reconstitution. It is therefore of interest why the antitumor effect appears to have lasted long after the frequency of Treg is restored. In this regard, it should be emphasized that in vivo Treg reconstitution is almost universal for all methods of Treg depletion, including antibody elimination and treatment of toxin targeting Treg that express the specific receptor for the toxin (40-42). In all cases, however, restoration of Treg did not prevent the immune response against antigen or pathogen. These studies suggested that numerical restoration of Treg is usually not accompanied by immune suppression of ongoing immune response and therefore made it plausible that temporary reduction of Treg can promote cancer immunity.

Second, in line of the function of B7 in clonal deletion of autoreactive T cells, including some tumor reactive T cells, it is possible that anti B7 treatment also rescues some tumor reactive T cells that are otherwise deleted. In this regard, we showed that transient blockade of B7 1 and B7 2 reduced the clonal deletion of SV40 T reactive CTL. Therefore, it is likely that anti B7 blockade may also increase the frequency of tumor reactive T cells. Taken together, by reducing the burden of Treg and increasing the frequency of cancer reactive T cells, B7 blockade resets the balance between regulatory burden and effector function. These two factors provide plausible explanation for the prevention described herein. Because the TGB mice do not survive long enough for us to study clonal deletion at 25 weeks, due to insertional mutation by TCR transgene (43), the effect of rescue of tumor reactive T cells in the therapy setting remains to be shown.

It is possible to argue that because the majority of cancer patients developed cancer late in their life when the thymic function has deteriorated, the rescue of TCR repertoire may be less relevant for cancer immunotherapy in humans. Nevertheless, we would like to point out that continuous production of T cells has been shown throughout the lifespan (44). Moreover, it is worth pointing out that hormone ablation is part of the standard therapy for prostate cancer. An unexpected benefit of this therapy is reinvigoration of thymic function (45). Therefore, it may be valuable to combine anti B7 blockade with hormone ablation in human prostate cancer treatment.

Finally, it is worth pointing out that blockade of B7 1 and B7 2 with their soluble receptor CILAR1g has been approved for therapy of autoimmune disease with little side effect (38). In this study, we showed that, despite the modulation of Treg and rescue of potentially self reactive T cells, anti B7 blockade does not trigger autoimmune side effect. The availability of a safe drug makes blockade of B7 1 and B7 2 an attractive approach for the cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Biological Sciences, Immunology

Targeting Lymphotoxin-mediated Negative Selection to Prevent Prostate Cancer in Mice with Genetic Predisposition

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Abstract

Identification of genetically susceptible individuals calls for preventive measures to minimize the life-long cancer risk of these high risk populations. Immune prevention is made necessary by the anticipated health thread but only possible by predictability of antigens. Lack of enough high affinity of T cells against tumor-associated antigens and unpredictability of tumor antigen make antigen-based immune prevention untenable for cancer. To address this issue, we explored a non-antigen-based cancer immune prevention using the TRAMP mice that spontaneously develop prostate cancer with 100% penetrance. We show that targeted mutation of the \(LT\alpha\) gene efficiently rescued tumor-reactive T cells, drastically reduced cancer incidence and almost completely ablated metastasis. Remarkably, short-term treatments with LT\(\beta\)RIg interrupted clonal deletion, reduced the size of primary cancer and completely prevented metastasis later in life, thus providing an easily translatable immune prevention for those with genetic predisposition to cancer.
Introduction

One of the most important advances in cancer research is the identification of individuals with increased susceptibility (1). Broadly speaking, genetic susceptibility can be conferred by inactive alleles of tumor suppressor gene or by hypermorphic alleles of oncogenes (2, 3). In extreme cases, inactivating mutations of tumor suppressor genes such as p53 (4), APC (5, 6) and BRCA1/2 (7-9) resulted in nearly 80% life-long cancer risk. The high penetrance of the high risk allele was responsible for their identification. It is estimated that about 1-5% of cancer cases are caused by dominant familial susceptibility alleles, yet the majority of the genes remains to be identified (10). In addition to the high penetrance cancer susceptibility alleles, recent genetic studies allow identification of numerous susceptibility loci that can be identified by genetic markers (11-16). It is anticipated that increasing numbers of individuals will be diagnosed with high cancer risk, which provides enormous opportunity for cancer prevention. Moreover, family history alone can serve as a powerful tool to identify individual with high risk. For example, a comprehensive studies involving more than 2 million nuclear families reveals that individuals with affected sib and at least one parent can have more than 30-fold higher risk of colon-rectal cancer (17).

Identification of genetically susceptible individuals calls for preventive measures to minimize the life-long cancer risk of these high risk populations, such as prophylactic surgery (18). Given vital importance of many organs,
prophylactic surgery is difficult to implement to those yet to develop cancer. Therefore, other preventive measures are badly needed. The notion of chemoprevention was first demonstrated more than 30 years ago (19). Its efficacy has been demonstrated in several large clinical trials (20-22). Generally speaking, the drug or nutritional supplement must be administrated repeatedly over the life time. Therefore, chemoprevention has a high burden of compliance and drug safety. On the other hand, thanks to immunological memory, immunity can last a life time without the stringent requirement of frequent boosting. Unfortunately, we are not aware of any attempt to use immune prevention to reduce both risk and mortality of cancer among the population with high genetic predisposition to cancer.

Immune prevention is made necessary by the anticipated health thread and possible by predictability of antigens carried by pathogens. The classic notion of immune prevention is based on immunization with antigens expressed by the pathogens. The power of immune prevention is best demonstrated by large scale of prevention of various infection diseases, including eradication of smallpox. However, adoption of immune prevention to cancer is limited by several factors. First, compared with pathogens, cancer antigens are poorly defined, unpredictable and more heterogeneous (23-25), which makes it considerably more difficult to design antigen-based vaccine for the purpose of prevention. Second, since cancers are derived from normal tissues, most of high affinity T cells reactive to such peripheral tissue antigens in the cancer cells have
been deleted (26). Lack of high affinity tumor-reactive T cells would in theory makes immune prevention difficult to attain.

Recent studies have demonstrated that clonal deletion of T-cell reactive to peripheral antigens depends on their expression in the thymic medullar epithelial cells (27, 28). Since tumors are comprised of malignantly transformed cells from normal tissues and therefore likely express tissue-specific antigens, it is of interest to determine whether these T cells can be rescued for the purpose of immune prevention. Since lymphotoxin α (LTα) gene play a major role in the development and function of medullar epithelial cells (29, 30), especially in the context of clonal deletion of peripheral antigen-reactive T cells, blocking this pathway may allow one to rescue tumor-reactive T cells to prevent the development of cancer. Using mice with targeted mutation of LTα (31), we reveal here a valuable target for rescuing prostate cancer-reactive T cells and for cancer immune prevention. More importantly, transient blockade of LTα significantly reduced the sizes of prostate cancer and eliminated cancer metastasis. To our knowledge, this is the first non-antigen-based immune prevention for cancer and it has a realistic chance to be translated into clinical care of those patients with high genetic risk for cancer.
Results

Targeted mutation of LTα limits clonal deletion of SV40 T antigen-specific T cells

One of our groups has recently demonstrated a critical role for LTα in clonal deletion of T cells specific for tissue-specific antigens (29). As a first test to determine whether this pathway can be explored for cancer immune prevention, we take a transgenic approach to determine whether this pathway can be explored for rescue of cancer-reactive T cells. We crossed the transgenic mice expressing TCR specific for SV40 large T antigen (Tag-I) (32) to the TRAMP mice expressing SV40 large T antigen under the control of probasin promoter (33), with the null mutation in none, one or two alleles of LTα gene. The development of the transgenic T cells was evaluated by flow cytometry.

As shown in Fig. 1a, in the Tag-I/TRAMP double transgenic mice, targeted mutation of one or both allele of the LTα gene resulted in significant increase in total thymic cellularity. A dramatic increase in % of DP and a significant decrease in DN% were observed among the transgenic TCR⁺ cells. Targeted mutation of both alleles of LTα eliminated the DN while expanded the DP and CD8 SP subsets (Fig. 1b&c). In addition, the numbers of transgenic T cells are greatly increased in the spleens of LTα-deficient mice (Fig. 1d, e). Remarkably, partial rescue was observed in the heterozygous mice (Fig. 1). Therefore, LTα play a critical role in clonal deletion of SV40-large T antigen-reactive T cells.
Targeted mutation of \(LT\alpha\) inhibits development of spontaneous prostate cancer

To test the role for \(LT\alpha\) in the onset of prostate cancer, measured the size of prostates at 30 weeks by magnet resonance imaging (MRI) (34). Representative images are shown in Fig. 2a, while the summary data are shown in Fig. 2b. These data demonstrated that the size of prostate was reduced by more than 3-fold in the TRAMP mice with either heterozygous or homologous deletion of \(LT\alpha\) (Fig. 2b, c). At 34 weeks, the three groups of mice were sacrificed for double blind histology analyses of the cancer development and metastasis. As shown in Fig. 2c, 100% WT mice developed malignant prostate cancer, with metastasis in 7/12 cases. Among them, one mouse had metastasis in kidney only, while six others had metastasis in the lung including two that also had metastasis in the liver. In mice with homozygous mutation, only 45% (5/11) mice developed malignant tumors. Remarkably, 4/11 mice had normal prostate morphology, while two others had prostate intraepithelial neoplasia (PIN). Only 1/11 mice had metastasis, in both liver and lung. A reduction of cancer incidence 13/16 was also observed in the heterozygous mice. Two heterozygous mice had completely normal prostate and one mouse had PIN. Moreover, only 1 in 16 heterozygous mice show lung metastasis. Since lymph node development is preserved in the heterozygous mice (data not shown), the major reduction of metastasis cannot be attributed to the lack of lymph nodes, which occurred in mice with homozygous \(Lt\alpha\) mutation (31). \(X^2\) analysis indicates a gene-dose
dependent reduction both in rate of malignancy (P=0.0071) and metastasis
(P=0.0023). Taken together, our data presented in Figure 1 and 2 demonstrated
that targeted mutations of \(LT_\alpha\) rescued tumor-reactive T cells and increased host
resistance to prostate cancer.

The administration of \(LT_\beta\)Rlglg rescues tumor-reactive T cells without
provoking autoimmune inflammation

The fact genetic inactivation of \(LT_\alpha\) conveys host resistance to prostate
cancer raised an interesting possibility that \(LT_\alpha\) may be targeted for the purpose
of immune prevention. Since aged \(LT_\alpha^{-}\) mice developed chronic inflammation,
one has to be concerned with potential autoimmune side effects of this treatment
(29, 30). In order to achieve this goal, we compared the inflammatory response
when mice were treated with 3 weekly administration of soluble murine \(LT_\beta\)Rlglg or
Human IgGFc, starting at 4, 6 or 11 weeks of age. The mice were sacrificed 4
weeks after completion of the treatments. As shown in Table 1 and Fig. 3, while
infiltrates in liver and lung was observed in mice received their first dose at 4
weeks, no inflammation or tissue injury were observed when the treated was
initiated at 6 or 11 weeks.

We have recently reported strong clonal deletion in transgenic mice
TRAMP/TGB that both express TCR specific for SV40 large T cells and SV40
large T antigen (35, 36). The clonal deletion was characterized by massive
reduction of CD8^+V\(\beta^8\)hi transgenic T cells (35). These features were
recapitulated in the double transgenic mice receiving IgG Fc control (Fig. 4a).
Interestingly, treatment with LTβRIg resulted in a 6-fold increase in DP and nearly 3-fold increase in the CD8 SP subset (Fig. 4b lower panel and Fig. 4c). Correspondingly, the number of transgenic CD8 T cells was more than doubled in the spleen. In mice lacking the large T antigen, no increase of transgenic T cells in the thymus was conferred by fusion protein (Fig. 4f-h). In contrast the fusion proteins actually reduced the number of transgenic T cell in the thymus. Therefore, the LTβRIg expand SV40 T antigen-specific T cells only if the antigen was present.

To determine whether LTβRIg prevented deletion of antigen-specific T cells, we compared % of apoptotic cells by staining with Annixin V. As shown in Fig. 5, LTβRIg significantly reduced % of apoptotic cells regardless of the subsets of the transgenic thymocytes. This treatment, however, has no effect on apoptosis of T cells in the spleen. Therefore, the increase of transgenic T cells in the TRAMP/TGB mice is likely due to rescue of T cells from clonal deletion in the thymus.

**Short-term treatment with LTβRIg reduces the progression of primary prostate cancer and prevented metastasis**

LTβRIg binds LTα with high affinity. To test whether LTβRIg treatment can significant affect the progression of prostate cancer, we treated the TRAMP mice with 3 weekly injections of either LTβIg or control IgG, starting at six weeks. At 30 weeks the volume of the prostate were measured the MRI. As shown in
Fig. 6a, on average, the LTβR Ig treatment at 6 weeks caused greater than 50% reduction in the prostate volume (P<0.01).

We carried out histological analysis to characterize the effect of LTβR Ig treatment on the development of metastasis. As shown in Fig. 6b, 4 of 7 control-Ig treated TRAMP mice developed metastasis in lung and/or liver, consistent with previous reports by others (33). Importantly, none of the LTβR Ig treated mice developed metastasis. Moreover, the lack of autoimmune disease is further supported by lack of inflammation in any of the organ studied (Fig. 6b and data not shown). Therefore, transient treatment of LTβR Ig reduced the site of primary lesion and completely prevented metastasis without provoking lymphocyte infiltration into organs.
Discussion

It is difficult to use cancer vaccine as preventive measures for those with genetic predisposition because of a multitude of mechanisms of immune tolerance, including clonal deletion to tissue-specific antigens (26, 35, 36) and clonal anergy (37) as well as unpredictability of tumor antigens (23-25). Here we devised a non-antigen-based strategy of immune prevention that in theory can be applicable to tumors from a variety of tissue origin. The foundation of the strategy is the critical role for LTα in clonal deletion of T cells specific for peripheral antigen (29, 30). Using TCR transgenic mice as the basic readout, we have demonstrated that short-term treatments with soluble LTβRIg rescued cancer-reactive T cells that would be otherwise deleted in the thymus. Corresponding to this, we found that TRAMP mice that received short term treatment of soluble LTβRIg at six weeks have significantly reduced sizes at 30 weeks. More importantly, this treatment completed prevented the development of metastasis. Since targeted mutation of LTα limits clonal deletion of SV40 T antigen-specific T cells and inhibits development of spontaneous prostate cancer, prevention by LTβRIg is likely due to its binding to LTα.

It has been demonstrated that transgenic mice expressing SV40 T antigens developed tumors concomitant with development of T-antigen-specific T cells (38). Therefore, merely priming antigen-specific T cells is insufficient to prevent tumor development. The quality of T cells, such as the antigens recognized and affinity for cancer antigens, also likely matters. Our data
presented in this studies indicated that blockade of LTα can efficiently prevent deletion of two lines of high affinity transgenic T cells specific for an antigen expressed in prostate specific fashion as a transgene.

A major advantage of the LTα-blockade based immune prevention is the potential applicability to a number of different cancer types regardless of tumor antigens involved. Although it remains to be tested whether this strategy is applicable to human, it is of interest to note the association between LTα polymorphism and risk of prostate cancer in man (39-41).

Since the prevention is to be applied to high-risk healthy patients, a primary concern is its potential autoimmune side effect. It has been reported that germline mutation of LTα cause multiple organ infiltration (29). Our extensive analysis of the LTβRIg-treated mice indicated no lymphocyte infiltration into organs if the treatment was initiated after 4 weeks. The side effect when treated at 4 weeks of age is probably due to more active thymopoiesis at younger age. Since treatment at six week have significant preventive effect, our data demonstrate that it is possible to identify appropriate window in which cancer immune prevention can be achieved without overt risk of autoimmune diseases. Taken together, this study has opened a new avenue to develop an immune intervention that prevents cancer development. This approach represents a major departure from the principle of cancer vaccine as it alleviates the need to identify tumor antigen. It is envisaged that subjects that carries high risk allele may be treated with reagents to block LTα or other critical pathway for tolerance
to periphery antigen in order to reduce their future cancer risk and improve clinical outcome if they do develop cancer.
Materials and Methods

Experimental animals  WT, TRAMP mice expressing the SV40 Tag controlled by rat probasin regulatory elements and Lt\(\alpha^{+/-}\) mice, all in the C57BL/6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were bred at the animal facilities of the Ohio State University (Columbus, OH) and the University of Michigan (Ann Arbor, MI). Transgenic TGB and TAG-I mice expressing TCR specific for different epitope of SV40 large T antigen presented by different MHC loci have been described (42) (32).

Generation of TRAMP mice expressing TGB TCR (TGB-TRAMP) was described (35). Lt\(\alpha^{+/-}\)TRAMP, Lt\(\alpha^{+/-}\)-TRAMP and Lt\(\alpha^{-/-}\)-TRAMP mice were obtained by breeding Lt\(\alpha^{+/-}\) mice with Lt\(\alpha^{+/-}\)-TRAMP mice. The TAG-1 mice were bred with Lt\(\alpha^{-/-}\) mice to obtain Lt\(\alpha^{+/-}\)-TAG-1 mice, which were crossed with the Lt\(\alpha^{+/-}\)-TRAMP mice to produce Lt\(\alpha^{+/-}\)-TAG-1TRAMP, Lt\(\alpha^{+/-}\)-TAG-1TRAMP and Lt\(\alpha^{-/-}\)-TAG-1TRAMP mice.

LT\(\beta\)RIgFc treatment  For cancer prevention, 6 weeks old TRAMP mice were treated with 3 weekly injections of 100µg LT\(\beta\)RIgFc or control IgGFc, intraperitoneally. Treated mice were examined at least weekly for palpable tumor at lower abdomen. The prostate volume was measured by MRI at 30 weeks. Mice were euthanized at 32 weeks, and internal organs were collected for histology analysis.

For rescue of clonal deletion, 6 weeks old TRAMP/TGB mice were treated with 3 weekly injection of 100µg LT\(\beta\)RIgFc or control IgGFc, intraperitoneally.
Two weeks after the last treatment, the mice were sacrificed and the total thymocytes and splenocytes were harvested and stained with fluorochrome-conjugated anti-CD4 (RM4.5), anti-CD8 (53-6.7), and anti-Vß8.1+8.2 (MR5-2) antibodies and analyzed by flow cytometer LS2 (Becton & Dickinson, Mountainview, CA).

To test potential autoimmune side effects, 4 weeks, 6 weeks and 11 weeks old TRAMP mice were treated with 100µg LTβRIgFc or control IgGFc every week, total 3 injections intraperitoneally. Two weeks after the last treatment, the mice were sacrificed and peripheral organs were collected. Tissue sections from peripheral organs were stained with hematoxylin and eosin (H&E).

**Histology** Mouse organs were fixed with 10% buffered formalin and were paraffin embedded. Tissue sections were stained with hematoxylin and eosin (H&E), and examined under a microscope. All pathological examinations were performed without knowing the treatment and the genotypes of the mice. At least three sections, 25 micron apart, were examined for each organ to ensure comprehensive evaluation.

**Magnetic resonance imaging (MRI) of prostate.** The progression of prostate cancer in the TRAMP model was measured by MRI as described (34).
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References


Table 1. Inflammation induced by LTβIg at 4 but not 6 or 11 weeks.

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Fig 1. LTα deficiency prevents clonal deletion of tumor-reactive T cells in the TRAMP mice. LTα+/+, LTα+/- and LTα-/- Tag-ITRAMP mice were sacrificed at 6 weeks for analyses. Thymocytes (a-c) and splenocytes (d, e) were harvested and analyzed by flow cytometry, using antibodies specific for Vβ7 (transgenic TCRβ), CD4 and CD8. a. Number of Vβ7+ cells in the thymus. Data shown are means and SEM of cell numbers (n=8). b, FACS plots depicting the distribution of CD4 and CD8 markers among the Vβ7+ thymocytes (b, left panels) or CD8 and Vβ7 among total thymocytes (b, right panels), and Vβ7+ splenocytes (d). The data in the left panel are from one representative mouse per group and similar data were obtained in 2 independent experiments, involving a total of 8 mice per group. c. e. Number of different subsets of transgenic Vβ7+ T cells in the thymi (c) and spleens (e). Data shown are means and SEM of cell numbers (n=8).

Fig. 2. LTα deficiency inhibits development of prostate cancer.
The tumor incidence of Lta+/+TRAMP, Lta+/-TRAMP and Lta-/- TRAMP mice were diagnosed by double blind histology examination by two individuals at 34 weeks; while the prostate volumes were measured by MRI at 30 weeks. a. Representative local prostate images of Lta+/+TRAMP, Lta+/-TRAMP and Lta-/- TRAMP mice. The prostate were identified with thick white outlines. b. The prostates sizes of Lta+/+TRAMP, Lta+/-TRAMP and Lta-/- TRAMP mice at 30
weeks old. c. Targeted mutation of LT$\alpha$ resulted in reduction of prostate cancer incidence and elimination of distal metastasis. The raw data for incidence are provided on top of bars, while the $P$ value shown in the panels are obtained by $X^2$ analyses for gene dose effects. The malignancy and metastasis were diagnosed by two independent and double blind evaluations of at least three slides per organ, including, heart, liver, lung, kidney, pancreas and intestine, 25 microns apart.

**Fig. 3. Identification of a time window to avoid lymphocyte infiltration associated with LT$\beta$Rlg treatment.** 4, 6 and 11 weeks old C57B6 mice received 3 weekly i.p. injections with 100 µg of either soluble murine LT$\beta$Rlg or Human IgGFc. The mice were sacrificed 4 weeks after the last injection. Peripheral organs were collected for H&E staining. a. Lymphocyte infiltration into liver was only observed when the treatment was initiated at 4 weeks, but not 6 or 11 weeks. b. Infiltration to lung was only observed if the treatment was initiated at 4 weeks of age.

**Fig. 4. LT$\beta$Rlg treatment rescued tumor reactive T cells from clonal deletion in the thymus.** TRAMP/TGB (a-e) or TGB (e-j) transgenic mice received 3 weekly injections (i.p.) of 100 µg of either soluble LT$\beta$Rlg or Human IgGFc, starting at 6 weeks of age. The mice were sacrificed 2 weeks after the last injection. Thymocytes (a-c, f-h) and splenocytes (d, e, i, j) were harvested and analyzed by flow cytometry using antibodies specific for CD4, CD8 and V$\beta$8.
a, f. Number of $V\beta^8^+$ thymocytes. b, d, g, i. Representative plots depicting distribution of CD4, CD8 and transgenic TCR$\beta$ among thymocytes. FACS plots are from gated $V\beta^8^+$ cells, except for the two right panels in Fig. 4b, which represented that of total thymocytes. Similar data were obtained from two independent experiments, each involving 4 mice per group. The numbers of different subsets of $V\beta^8^+$ transgenic thymocytes (c, h) and splenocytes (e, j) are presented in bar graphs as means and SEM, involving 8 mice per group.

**Fig. 5. LT$\beta$Rlg reduced apoptosis of transgenic T cells in the TRAMP/TGB transgenic mice.** Thymocytes and splenocytes of the TRAMP/TGB mice as described in Fig. 4 legends were stained with antibodies against $V\beta^8$, CD4 and CD8 in conjunction with Annexin V. a. LT$\beta$Rlg treatment on TRAMP/TGB mice reduced the percentage of apoptotic cells in the thymus, mainly at the DP stage. b. LT$\beta$Rlg had no impact on apoptosis of transgenic T cells in the spleen. Plots depict apoptotic cells among different subsets of thymocytes (a) spleen cells (b). The numbers in the panels are means and SEM of the % of apoptotic cells, summarized from two independent experiments, each with 4 mice per group (n=8).

**Fig. 6. LT$\beta$Rlg treatment reduces size of prostate cancer and prevented metastasis.** a. Prostate volumes as measured by MRI. Male TRAMP mice received 3 weekly i.p. injections with either 100 µg of soluble murine LT$\beta$Rlg or Human IgGFc at 6 weeks old. The prostate volume was measure at 30 weeks.
The upper panels show representative local images of Human IgGFc treated and LTβR Ig treated TRAMP mice. The prostate were identified with thick white outlines. The lower panels depict the sizes of individual prostates (n=7). b. Histological analysis of tumor metastasis. TRAMP mice that received 3 weekly treatment of control Ig or LTβR Ig starting at 6 weeks were sacrificed at 33 weeks after MRI analysis at week 30. H&E sections were examined double blind by a pathologist for metastatic lesions in all internal organs, including liver, lung, kidney, colon, heart and pancreas. Metastases (to lung and/or liver) were found in 4/7 control Ig treated and none of the LTβR Ig-treated mice. The differences in the rate of metastasis is statistically significant (P=0.012).
Fig. 1.
Fig. 2
Fig. 3a

a. Liver

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b. Lung

Fig. 3b
Fig. 5

**a**

- Vβ8+
  - DN
    - HlgGFc: 43.7 ± 3.6
    - LTβ Rlg: 26.2 ± 4.8
  - DP
    - HlgGFc: 55.3 ± 3.1
    - LTβ Rlg: 12.8 ± 3.6
  - CD4SP
    - HlgGFc: 67.3 ± 4.4
    - LTβ Rlg: 40.4 ± 4.2
  - CD8SP
    - HlgGFc: 44.6 ± 2.8
    - LTβ Rlg: 21.3 ± 3.7

**b**

- Vβ8+
  - CD4SP
    - HlgGFc: 17.0 ± 1.8
    - LTβ Rlg: 15 ± 0.7
  - CD8SP
    - HlgGFc: 8.7 ± 2.2
    - LTβ Rlg: 7.1 ± 2.4
Fig. 6.
Dendritic cells in the thymus contribute to T-regulatory cell induction

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Central tolerance is established through negative selection of self-reactive thymocytes and the induction of T-regulatory cells (T\(_\text{Rs}\)). The role of thymic dendritic cells (TDCs) in these processes has not been clearly determined. In this study, we demonstrate that in vitro, TDCs not only play a role in negative selection but in the induction of T\(_\text{Rs}\). TDCs include two conventional dendritic cell (DC) subsets, CD8\(_{\text{lo}}\)Sirp\(_\text{a}^+\) (CD8\(_{\text{lo}}\)Sirp\(_\text{a}^+\)) and CD8\(_{\text{hi}}\)Sirp\(_\text{a}^–\) (CD8\(_{\text{hi}}\)Sirp\(_\text{a}^–\)), which have different origins. We found that the CD8\(_{\text{hi}}\)Sirp\(_\text{a}^+\) DCs represent a conventional DC subset that originates from the blood and migrates into the thymus. Moreover, we show that the CD8\(_{\text{lo}}\)Sirp\(_\text{a}^+\) DCs demonstrate a superior capacity to induce T\(_\text{Rs}\) in vitro. Finally, using a thymic transplantation system, we demonstrate that the DCs in the periphery can migrate into the thymus, where they efficiently induce T\(_\text{R}\) generation and negative selection.

thymic selection | migratory dendritic cells | tolerance

Tolerance to self-antigens is established in the thymus. Developing thymocytes undergo stringent selection to eliminate self-reactivity (1). Developing T cells that recognize self-peptide with a sufficiently high affinity can encounter two fates: (i) deletion through negative selection or (ii) differentiation into T-regulatory cells (T\(_\text{Rs}\)). T\(_\text{Rs}\) express the transcription factor Foxp3 (2–4) and can suppress self-reactive T cells that have escaped negative selection (5, 6). During mouse ontogeny, T\(_\text{Rs}\) appear in the thymus 3 days after birth (7). Deficiency in T\(_\text{R}\) development or function results in multiorgan autoimmunity (6).

A role for thymic dendritic cells (TDCs) in negative selection (8–12) and for thymic epithelial cells (TECs) in negative selection and T\(_\text{R}\) induction has been demonstrated (9, 13–16). The role of TDCs in these processes has not been clearly determined (8–12) and for thymic epithelial cells (TECs) in negative selection (17, 18), and in light of a recent study demonstrating the potential of human TDCs to induce T\(_\text{Rs}\) in vitro (19), the possible role of TDCs in T\(_\text{R}\) induction in vivo needs careful dissection using mouse models.

In mouse thymus, three subsets of DCs have been identified. The plasmacytoid dendritic cell (pDC) and two conventional dendritic cell (cDC) subsets defined based on CD11c and Sirp\(_\text{a}\) expression: the CD8\(_{\text{hi}}\)Sirp\(_\text{a}^+\) cDCs (≈30% of cDCs, Sirp\(_\text{a}^+\) TDCs hereafter) and the CD8\(_{\text{lo}}\)Sirp\(_\text{a}^+\) cDCs (≈70% of cDCs, Sirp\(_\text{a}^–\) TDCs hereafter) (20, 21). Sirp\(_\text{a}^+\) TDCs develop from intrathymic lymphoid precursors (22, 23). The origin of Sirp\(_\text{a}^+\) TDCs is less clear, although one study demonstrated that the CD8\(_{\text{hi}}\)CD11b\(^+\) cDCs (equivalent to Sirp\(_\text{a}^+\) cDCs) migrate into the thymus from the periphery (24). The role of the individual TDC subsets in T-cell selection is yet to be determined.

In addition to the contribution of medullary thymic epithelial cells (mTECs) to T\(_\text{R}\) generation (16), in this study, we demonstrate that TDCs make a significant contribution to T\(_\text{R}\) induction as well as negative selection. This was established in vivo using two bone marrow (BM) chimeric mouse models in which the hematopoietic-derived compartment was impaired in antigen presentation (MHC class II [MHCII]\(^{\text{+/–}}\)) or T-cell activation (B7\(^{\text{+/–}}\)). Using an in vitro culture system, we established that the Sirp\(_\text{a}^+\) TDCs played the major role in T\(_\text{R}\) induction when compared with other DC subtypes. This functional capacity of the Sirp\(_\text{a}^+\) TDCs correlates with a unique set of properties, particularly their maturity, their chemokine production, and their migratory origin. These findings suggest that a subset of TDCs migrating from the periphery makes a specialized contribution to T\(_\text{R}\) induction in the thymus.

Results

TDCs Contribute to T\(_\text{R}\) Induction and Negative Selection In Vivo. To dissect the contribution of DCs from that of mTECs in the induction of T\(_\text{Rs}\), two different in vivo systems were used. In the first, irradiated C57BL/6 (B6) WT CD45.1 recipients were reconstituted with BM from MHCII\(^{-/–}\) or B6 WT (CD45.2) mice. In MHCII\(^{-/–}\) BM chimeras, the host epithelial cells can still present antigen via MHCII, whereas the BM-derived cells, including TDCs, cannot. In the second system, irradiated CD45.1 recipients were reconstituted with B7\(^{–/–}\) BM (lacking CD80 and CD86) or WT BM for controls. Because expression of MHCII and costimulatory molecules CD80 and CD86 is essential for the induction of thymic-derived T\(_\text{Rs}\) (5, 14, 15, 19, 25, 26), these systems enabled us to discern the contribution of DCs to T\(_\text{R}\) induction.

Because some DCs are radioresistant, it was important to establish whether TDCs in the chimeras were all of donor origin (27, 28). Staining the TDC-enriched light density cell fraction for donor-derived DCs 6 weeks after BM reconstitution demonstrated that >98% of DCs were of donor origin (MHCII\(^{–/–}\)). Using an in vitro culture system, we established that the Sirp\(_\text{a}^–\) TDCs played the major role in T\(_\text{R}\) induction when compared with other DC subtypes. This functional capacity of the Sirp\(_\text{a}^–\) TDCs correlates with a unique set of properties, particularly their maturity, their chemokine production, and their migratory origin. These findings suggest that a subset of TDCs migrating from the periphery makes a specialized contribution to T\(_\text{R}\) induction in the thymus.

To assess the effect on thymocyte development in mice lacking MHCII on DCs, the proportion and total numbers of the individual donor-derived thymocyte populations were determined (Fig. 1 C–E). Total thymic cellularity was comparable between the MHCII\(^{+/–}\) and WT BM chimeras [supporting information (SI) Table S1], and the numbers of CD4\(^+\)CD8\(^{–}\) double-negative, CD4\(^+\)CD8\(^{–}\) double-positive, and CD8\(^+\)CD4\(^{–}\) (CD8\(^{+}\)hereafter) T-cell popula-
tions were not significantly different between the two groups (Table S1). There was a 20% increase in the number of CD4+CD8− (CD4+ hereafter) thymocytes in the MHCIIE−/− BM chimeras, however, suggesting that there was incomplete negative selection (Fig. 1D). Syngeneic mixed leukocyte reaction assays confirmed that the CD4+ thymocytes contained auto-reactive T cells (data not shown). Concomitant with this increase was a statistically significant 30% decrease in the number of CD4+CD25Foxp3+ Tregs (P = 0.008) (Fig. 1E).

To test the function of the Treg in the WT and MHCIIE−/− chimeras, they were sorted and used in an in vitro Treg suppression assay. The Tregs from both groups were functional (Fig. 1F).

In the second BM chimeric system, B7−/− mice were used. Initially, it was established that B7−/− mice have a deficiency in the proportion of Tregs that equated to a 94% decrease (Fig. S1A). To establish if this was attributable to cells in the hemopoietic or epithelial cell compartment, four cohorts of chimeras were set up. CD45.1 WT or CD45.1 B7−/− mice were reconstituted with CD45.2 WT or B7−/− BM and analyzed for Treg development 8 weeks after reconstitution. Total thymic cellularity did not differ between the four cohorts (data not shown). There was a 50% decrease in the number of thymic Tregs in the B7−/− to WT chimeric mice, however (Fig. 1G; Fig. S1B).

Overall, these results suggest a nonredundant role for TDCs in the induction of thymic Treg and in the negative selection of self-reactive CD4+ thymocytes.

**DCs Induce Antigen-Specific Tregs and Negative Selection In Vivo.** Treg induction and negative selection of self-reactive CD4+ thymocytes require self-peptide presentation on MHCIIE via an antigen-presenting cell (14, 15). To address Treg induction by DCs in an antigen-specific system, Rag2−/− OTII T-cell receptor (TCR) transgenic (tg) mice (which lack OVA-specific Treg because of the absence of the OVA antigen) were crossed with CD11cOVA tg mice (membrane-bound OVA expressed under the CD11c promoter). In these Rag2−/− OTII/CD11cOVA (Rag2−/− O/OVA) double-tg mice, OVA is expressed on CD11c+ TDCs and can influence the development of CD4+ T cells that express the OVA-specific TCR (29). To follow development of newly formed thymocytes from the double-tg BM cells, irradiated WT CD45.1 recipients were reconstituted with the BM of CD45.2 Rag2−/− O/OVA mice or Rag2−/− OTII mice for controls. Thymocytes were analyzed by flow cytometry 6 weeks later. Total cellularity of the Rag2−/− O/OVA BM chimeric thymuses was reduced compared with controls (Fig. 2A). The presentation of OVA by DCs in Rag2−/− O/OVA BM chimeric mice led to the deletion of the majority of OTII+CD4+ cells, as seen by a >90% reduction in the total number of CD4+CD4+Vα2 OTII T cells compared with controls (Fig. 2B and C). Furthermore, there was a clear induction of OTII Tregs in the thymus of Rag2−/− O/OVA BM chimeras (mean 15 ± 2% of OTII+CD4+ cells) compared with the controls (0.1% of OTII+CD4+ thymocytes). This represented a greater than 150-fold increase in Treg numbers in the thymus of Rag2−/− O/OVA BM chimeras compared with controls (Fig. 2B and D).

Overall, these results demonstrate that DCs are capable of Treg induction and negative selection in an antigen-specific manner.
Sirpα⁺ TcDCs Are More Mature in Surface Phenotype Than the Sirpα⁻ TcDCs. Because TDCs were involved in T₉ generation and negative selection, we investigated the contribution of the TDC subtypes to these processes. We compared TDCs for expression of MHCI and costimulatory molecules, because these are important in T₉ induction and negative selection (19, 30–34). We then compared the TDCs with their splenic DC (SDC) equivalents. TDCs and SDCs were segregated into pDCs and cDCs, which could be further segregated as Sirpα⁺ CD80 and Sirpα⁻ CD8+ cDCs (21) (Fig. 3A). Strikingly, the Sirpα⁺ TcDCs expressed higher levels of MHCI and CD86 and slightly increased levels of the activation marker CD69 and the costimulatory molecules CD40 and CD80 compared with Sirpα⁻ TcDCs (Fig. 3B). This difference was not observed between the DC subsets in the spleen, where both cDC subsets expressed comparable levels of these markers (refs. 35, 36; Fig. 3B). Nor was there a difference in expression of these molecules in thymic versus splenic pDCs (Fig. 3C). Thus, in the steady state, Sirpα⁺ TcDCs are phenotypically more “mature” than other TDC subtypes.

Sirpα⁺ TcDCs Are More Efficient at Inducing Functional T₉s In Vitro. To compare the activity of each TcDC subset to induce T₉s, sorted TcDC subsets were cocultured with syngeneic CD4⁺ CD8⁻ CD25⁻ thymocytes (which contain T₉ precursors) for 5 days. To maintain T-cell survival, an optimal level of IL-7 was added (37). The number of T₉s that developed in these cultures was enumerated. The Sirpα⁺ TcDCs were the most efficient at inducing T₉s, as shown by the higher number of CD4⁺ CD25⁻ Foxp3⁺ cells in the cultures (Fig. 3D and E). In the cultures containing Sirpα⁻ TcDCs, there was also some level of T-cell activation, as evidenced by a population of CD4⁺ CD25⁺ Foxp3⁻ cells (Fig. 3D and data not shown). This T-cell activation was accompanied by T-cell proliferation and a total number of T cells within the cultures (Fig. S2A). Given that the proportion of T₉s induced in Sirpα⁺ TcDC cocultures (14 ± 5%) was also significantly higher compared with Sirpα⁻ TcDC cocultures (9 ± 1% (Fig. 3D)), it was clear that the increased number of T₉s could not be attributable solely to a higher absolute number of T cells generated. Furthermore, we determined that the T₉ induction observed was attributable to de novo generation and not to proliferation of preexisting CD4⁺ CD25⁻ Foxp3⁻ cells within the starting population of thymocytes by using Foxp3-GFP mice to gate out CD4⁺ CD25⁻ Foxp3⁺ cells (Fig. S2B).

T₉ generation in vitro was thymus specific. When TDCs were cultured with splenic CD4⁺ CD25⁻ naïve T cells rather than thymic CD4⁺ CD25⁻ T cells, no T₉ induction was observed (Fig. S2C), even in the presence of T-cell activation and proliferation (Fig. S2D). Conversely, when SDCs were cocultured with thymic CD4⁺ CD25⁻ T cells, few T₉s were generated (data not shown).

To test the function of in vitro–derived T₉s, T₉s were sorted as CD4⁺ CD25⁺ CD62L⁺ and used in a T₉ suppression assay. CD62L was included as a marker to exclude activated T cells. In vitro derived T₉s were able to suppress T-cell proliferation (Fig. 3F).

Sirpα⁺ TcDCs Produce Chemokines and Attract CD4⁺ Thymocytes. The chemokine-mediated migration of developing thymocytes through the thymus ensures their interaction with the appropriate thymic stromal cells. We examined chemokine production as a factor that may explain the effectiveness of the Sirpα⁺ TcDCs in inducing T₉s. To test whether the DC-expressed chemokines were chemotactic for T₉s, we examined chemokine engagement through RT-PCR, comparing the TDC and SDC subsets, macrophages, and thymic mTECs. The mTECs expressed significantly higher levels of CCL19, CCL21, and CCL25, higher than the DC subsets (Fig. S3B). In contrast, CCL17 and CCL22 were expressed at very high levels only by the Sirpα⁺ TcDCs (Fig. S3A). The expression of CCL22 by the Sirpα⁺ TcDCs was confirmed at the protein level by intracellular chemokine staining (Fig. S3B).

CCL17 and CCL22 both bind to CCR4. Using RT-PCR, we found that the CD4⁺ thymocytes expressed the highest levels of CCR4 (Fig. S3C), a finding consistent with other studies (38). To test whether the DC-expressed chemokines were chemotactic for CD4⁺ thymocytes, migration assays were performed. Sorted TDC and SDC subsets were cultured alone for 3 h. The supernatants were then used as a source of chemotactins for CD4⁺ thymocytes, seeded in transwells, and incubated for 2 h. The supernatants from the Sirpα⁺ TcDC cultures showed the greatest capacity to attract CD4⁺ thymocytes (Fig. S3D). Thus, the Sirpα⁺ TcDCs, through their chemokine production, have a special capacity to attract newly formed CD4⁺ T cells.

CD11c⁻ Sirpα⁺ CD11b⁻ cDCs Are Found in Blood and Migrate into the Thymus. A number of observations have led to the suggestion that the TDC subsets have different developmental origins, with a major proportion of the TcDCs being derived from an early intrathymic precursor (24, 39). To test the origin of each TcDC, the earliest intrathymic precursors (Lineage “Thy-1⁺c-kit⁺”) that have DC po-
tential were transferred intrathymically into sublethally irradiated CD45.1 recipient mice. DC generation was analyzed 2 weeks after transfer. The cDCs that developed from the intrathymic precursors were mainly CD8+ Sirpα− (Fig. 4A).

In contrast, the CD8−CD11b+ TDC subset has been shown to migrate in paraibiotic mice from the circulation into the thymus of the conjoined mouse (24). To determine whether the Sirpα+ cDCs correspond to this population, the CD11c+ DCs within mouse blood were characterized. Total peripheral blood mononuclear cells were enriched for DCs. The preparation was then stained for DC markers. Gating on CD11c+ cells revealed that more than 70% of the blood DCs were Sirpα+CD11b+ (Fig. 4B). Among the blood DCs, 25% expressed high levels of MHCII, indicating that immature and mature DCs were present in mouse blood.

To determine whether these blood DCs migrate to the thymus, white blood cells from CD45.1 mice were transferred i.v. into nonirradiated CD45.2 recipients and the phenotype of donor-derived cells in the recipient thymus was determined 3 days later by gating for CD45.1+/CD11c+CD45RA+ cDCs. Expression of Sirpα, CD11b, CD8, and MHCII was determined on this population. (C–E) Thymic lobes from OTII tg CD45.2+ mice crossed to CD45.1+ WT mice were grafted under the kidney capsule of CD45.2+CD11cOVA tg or WT recipients. (C) The phenotype of recipient-derived CD45.2+CD45.1+ DCs in the grafted thymic lobes from WT and CD11cOVA TG mice was determined. The recipient CD45.2+CD45.1+CD11c+CD45RA+ cDCs were gated for, and the expression of CD8 and Sirpα was determined. The level of expression of MHCII was determined on Sirpα+ and Sirpα− cDCs. (D) The total number of CD45.1+CD4+Vα2+Vβ5+ cells (OTII) was calculated in OTII lobes grafted into WT or CD11cOVA tg recipients. Data are the mean of three independent experiments (error bars, ±SD) (n = 11–21). * P < 0.05. (E) CD45.1+CD4+Vα2+Vβ5+ cells in the OTII lobes from WT and CD11cOVA tg recipients (as in D) were further analyzed for CD25 and Foxp3 expression. The total number of CD45.1+CD4+Vα2+Vβ5+CD25+Foxp3+ Tregs was calculated. Data are the mean of three independent experiments (error bars, ±SD) (n = 11–21). * P < 0.05.

**Impact of Migrating DCs on T-Cell Development.** To determine the impact of circulating DCs on thymic T-cell selection, day 1 neonatal thymic lobes from CD45.1/OTII tg mice were grafted under the kidney capsule of recipient CD45.2 WT or CD45.2 CD11cOVA tg mice. This system allows recipient DCs to migrate into the grafted thymic lobes via the blood. Therefore, the effects of peripherally derived CD45.2 CD11cOVA migrating DCs on OTII T-cell development in the grafted lobes could be assessed. The kinetics of DC migration were determined. At day 7, before the recipient BM progenitors had contributed to the TDC population, the DCs entering the thymic lobes were predominantly the Sirpα+ cDCs (80 ± 5%; data not shown). We therefore waited a further 3–5 days to see the effects of these incoming DCs on T-cell development. Thymic lobes were removed 10–12 days after transplantation, and the phenotype of the incoming CD45.2+ DCs and the resident CD45.1+ OTII T cells was studied.

At day 10, DCs in the grafted thymic lobes were analyzed for DC markers to assess the phenotype of the host-derived CD45.2+ migrating DCs. Of these CD11c+ cells, 54 ± 6% were mature MHCIIhi CD8−Sirpα+ cDCs, 4 ± 1% were mature CD8+SIRpα− cDCs, and the remaining were MHCIIloCD8−Sirpα− cDCs. The total number of CD45.1+CD4+Vα2+Vβ5+ cells (OTII) was calculated in OTII lobes grafted into WT or CD11cOVA tg recipients. Data are the mean of three independent experiments (error bars, ±SD) (n = 11–21). * P < 0.05.

Thymocyte populations were analyzed by flow cytometry. The number of CD45.1+OTII+CD4+Vα2+Vβ5− T cells was reduced in lobes grafted into CD11cOVA tg mice compared with controls (Fig. 4D), whereas the number of CD45.1+Vα2+Vβ5− CD4+ was similar in both groups (data not shown), suggesting that antigen-specific negative selection of OTII T cells was occurring. In addition, there is more than twofold increase in the number of OTII Foxp3+/Tregs was seen in the lobes grafted into CD11cOVA tg mice compared with controls (Fig. 4E). Together, these results indicate that DCs migrating into the thymus from the periphery can induce negative selection and antigen-specific Treg development.

**Discussion.** The present study demonstrates a role for mouse TDCs in Treg differentiation as well as negative selection. In the absence of a MHCII-expressing hematopoietic compartment, we found a 30% reduction in the total number of polyclonal T cells and an increase in the number of self-reactive CD4 T cells in the thymus. This demonstrates that in addition to mTECs (16, 40), BM-derived cells make a significant contribution to Treg generation and negative selection of CD4 T cells in a steady-state mouse. In addition, a 50% reduction in Treg numbers was observed when the hematopoietic compartment lacked expression of CD80 and CD86. Although...
these BM chimeras indicated that a BM-derived cell was important for T<sub>R</sub> induction, the in vitro coculture system indicated that only TDCs, and not B cells and macrophages, were efficient in inducing T<sub>Rs</sub>. Thus, taken together, it appears that TDCs are the major hematopoietic cells that contribute significantly to T<sub>R</sub> generation and negative selection of CD<sub>4</sub> T cells in vivo. Previous studies have discounted a nonredundant role for DCs in T<sub>R</sub> induction (41, 42).

The irradiation protocol used (850–900 rad), which may not be sufficient to completely ablate host-derived cells, coupled with the later time point for analysis (8–10 weeks), may have contributed to these results, however. Mice with reduced thymic cellularity and a profound increase in CD<sub>4</sub><sup>+</sup> thymocyte cell numbers were observed in previous reports (41). We also see similar results in MHCII<sup>−/−</sup> BM chimeras at later time points. These mice have reduced thymic cellularity and show immune cell infiltration into organs—an initial sign of autoimmunity (data not shown). At this stage, the massive accumulation of autoreactive CD<sub>4</sub><sup>+</sup> T cells in the thymus has masked the changes in T<sub>R</sub> numbers.

Apart from the issue of their quantitative contribution to the total T<sub>R</sub> population, our results now demonstrate that TDCs can induce Ag-specific T<sub>R</sub>.

Materials and Methods

**Mice.** All mice were bred under specific pathogen-free conditions. B<sup>7−/−</sup> mice were purchased from The Jackson Laboratory and maintained in the University Laboratory Animal Research Facility at the University of Michigan. All other mice were obtained from The Walter and Eliza Hall Institute animal breeding facility. C57BL/6 (B6) mice 6–8 weeks of age were used for isolation of DCs and thymocytes. B<sub>6</sub> CD4<sup>+</sup> mouse 10 weeks of age were used as BM recipients. The mouse strains used included OTI tg (CD<sub>4</sub><sup>+</sup> T cells expressing the TCR specific for MHCII-restricted Ova peptide) (48) on a B6, CD4<sup>+</sup>, or Rag2<sup>−/−</sup> background; IA/A<sup>−/−</sup> (MHCII<sup>−/−</sup>) (49); B7<sup>−/−</sup>; and CD11cOVA tg mice that express membrane-bound ovalbumin (amino acids 323–339) under control of the CD11c promoter (29, 51).

**BM Chimeras.** CD4<sup>+</sup> BM recipients were lethally irradiated with two doses of 5.5 Gy (3 h apart) and then received 5 × 10<sup>6</sup> CD4<sup>+</sup> donor BM cells i.v. from B<sub>6</sub> or MHCII<sup>−/−</sup> mice or from Rag2<sup>−/−</sup>OITI/CD11cOVA double-tg mice. For B7<sup>−/−</sup> chimeras, CD4<sup>+</sup> BM recipients were lethally irradiated with 8.0 Gy of total body irradiation. A total of 5 × 10<sup>6</sup> T-cell depleted B<sub>6</sub> WT or B7<sup>−/−</sup> donor BM cells were injected i.v. into the recipients the next day. Chimeras were analyzed by flow cytometry 6–8 weeks after reconstitution.

**Antibodies.** Details can be found in **SI Experimental Procedures**.

**Isolation of DCs.** Details can be found in **SI Experimental Procedures**.

**Isolation of T lymphocytes.** Details can be found in **SI Experimental Procedures**.

**Carboxyfluorescein Succinimidyil Ester Labeling.** Details can be found in **SI Experimental Procedures**.

**Isolation of Thymic B Cells, Macrophages, and mTECs.** Details can be found in **SI Experimental Procedures**.

**T<sub>R</sub> Suppression Assay.** Details can be found in **Supplementary Experimental Procedures**.

**Generation of T<sub>Rs</sub> In Vitro.** In vitro T<sub>R</sub> induction assays were performed in triplicate in a round-bottom 96-well plate with 1 × 10<sup>6</sup> sorted thymic splenic DC subsets from CD4<sup>+</sup>2 and 2 × 10<sup>3</sup> sorted CD<sub>4</sub><sup>+</sup> CD<sub>25</sub><sup>−</sup> thymocytes from CD4<sup>+</sup>1 mice, cultured together with an optimal concentration of IL-7 for 5 days. T<sub>R</sub>s were assessed by staining for CD45<sup>+</sup>1 (A201.1), CD4, CD25, and Foxp3. When GFP-Foxp3 mice were used as the CD4<sup>+</sup> thymocyte source, thymocytes (2 × 10<sup>4</sup>) on a B6, CD45.1, or Rag2<sup>−/−</sup> background; IA/A<sup>−/−</sup> (MHCII<sup>−/−</sup>) (49); B7<sup>−/−</sup> (50); and CD11cOVA tg mice that express membrane-bound ovalbumin (amino acids 323–339) under control of the CD11c promoter (29, 51).

**Quantitative PCR.** Quantitative PCR was performed for chemokine gene expression by DC subsets as previously described (56). Further details can be found in **Supplementary Experimental Procedures**.

**Cell Migration Assay.** Sorted TDC and SDC subsets (5 × 10<sup>5</sup> in 600 μl) were cultured in a 24-well plate for 3 h. The supernatant was removed and placed in the base of transwell chambers (5.0-μm pore size; COSTAR). Sorted CD4<sup>+</sup> CD<sub>25</sub><sup>−</sup> thymocytes (2 × 10<sup>6</sup>) were placed in the top of the chamber and allowed to migrate for 2 h at 37 °C. The number of cells that had migrated was enumerated using fixed numbers of beads as a calibration standard.

**In Vivo DC Migration Assay.** Details can be found in **Supplementary Experimental Procedures**.

**Staining Blood DCs.** Details can be found in **Supplementary Experimental Procedures**.

**Thymic Grafting.** Thymic lobes from 1-day-old donor mice were grafted under the kidney capsule of anesthetized 8-week-old recipient mice using a procedure described elsewhere (57). At specified times postgrafting, grafted thymic lobes were recovered and processed individually. Thymic lobes were digested in collagenase/DNase and analyzed by flow cytometry.

**Statistical Analysis.** Statistical significance was assessed by the two-tailed unpaired Student’s t test. Differences with P values less than 0.05 were considered significant.

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IN THIS ISSUE, PSYCHOLOGY
Correction for the “In This Issue” summary entitled “Universal displays of pride and shame,” which appeared in issue 33, August 19, 2008, of Proc Natl Acad Sci USA (105:11587–11588).

The authors note that the figure is copyrighted by Bob Willingham and is reprinted with permission. The online version has been corrected. The figure and its corrected legend appear below.

Blind athletes (Right) show pride in victory like sighted athletes (Left). [Reproduced with permission (Copyright 2004, Bob Willingham).]

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PERSPECTIVE

The authors note that a reference was inadvertently omitted from their article. On page 4607, right column, in Conclusion: How Do New Gene Clusters Form?, line 17, the reference callout “(109–111)” should instead read “(109–111, 113).” The added reference appears below.


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DEVELOPMENTAL BIOLOGY

The authors note that to a printer’s error, the affiliation information for some authors appeared incorrectly. The correct affiliation for V. Havlicek and U. Besenfelder is “Reproduction Centre-Wiesbaden, University of Veterinary Medicine, 1210 Vienna, Austria”; and the correct affiliation for H. Lehrach and J. Adjaye is “Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany.”

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APPLIED BIOLOGICAL SCIENCES

The authors note that the author name Christian Steinkulher should have appeared as Christian Steinkulher. The author line has been corrected online. The corrected author line appears below.

Claudia Colussi, Chiara Mozzetta, Aymone Gurtner, Barbara Illi, Jessica Rosati, Stefania Straino, Gianluca Ragone, Mario Pescatori, Germana Zaccagnini, Annalisia Antonini, Giulia Minetti, Fabio Martelli, Giulia Piaggio, Paola Gallinari, Christian Steinkulher, Emilio Clementi, Carmela Dell’Aversana, Luca Altucci, Antonello Mai, Maurizio C. Capogrossi, Pier Lorenzo Puri, and Carlo Gaetano

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IMMUNOLOGY

The authors note that due to a printer’s error, in the Abstract, beginning on line 6, “TDCs include two conventional dendritic cell (DC) subtypes, CD8loSirpahi/lo (CD8loSirpahi/lo) and CD8hiSirpalo/lo (CD8hiSirpalo/lo), which have different origins. We found that the CD8hiSirpalo/lo DCs represent a conventional DC subset that originates from the blood and migrates into the thymus” should instead read: “TDCs include two conventional dendritic cell (DC) subtypes, CD8loSirpahi/lo (CD8loSirpahi/lo) and CD8hiSirpalo/lo (CD8hiSirpalo/lo), which have different origins. We found that the CD8hiSirpalo/lo DCs represent a conventional DC subset that originates from the blood and migrates into the thymus.”

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