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14. ABSTRACT 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.						
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Table of Contents

Introduction.....4

Body.....5-7

Key Research Accomplishments.....8

Reportable Outcomes.....9

Conclusions.....10

References.....11

Appendices.....

(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 lymphotoxin (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-D^b*-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 $\text{TCR}_\beta^+ \text{NK1.1}^+$, as well as $\text{TCR}_\beta^+ \alpha\text{-Galcer/CD1d}^+$ (iV α 14 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 *LT α* 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 LT β 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.

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(7) Reportable Outcomes:

Manuscripts:

1. Penghui Zhou, Xincheng Zheng, Huiming Zhang, Yang Liu and Pan Zheng. B7 Blockade Re-Balances Regulatory T cells and Tumor-reactive T Cells for Prevention and Therapy of Prostate Cancer. *Clinical Cancer Research*. 2009. 15:960-970. (Appendix 1).
2. Penghui Zhou, Xianfeng Fang, Ping Yu, Mingzhao Zhu, Yang-Xin Fu, Lizhong Wang, Yang Liu and Pan Zheng. Targeting Lymphotoxin-mediated Negative Selection to Prevent Prostate Cancer in Mice with Genetic Predisposition. 2009. Submitted. (Appendix 2).
3. Proietto AI, van Dommelen S, Zhou P, Rizzitelli A, D'Amico A, Steptoe RJ, Naik SH, Lahoud MH, Liu Y, Zheng P, Shortman K, Wu L. Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc Natl Acad Sci U S A*. 2008. 105:19869-74. (Appendix 3).

(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 administered 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 eradicate 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 threat 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 α* gene efficiently rescued tumor-reactive T cells, drastically reduced cancer incidence and almost completely ablated metastasis. Remarkably, short-term treatments with LT β 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.

**(9) References:
None.**

B7 Blockade Alters the Balance between Regulatory T Cells and Tumor-reactive T Cells for Immunotherapy of Cancer

Penghui Zhou,¹ Xincheng Zheng,² Huiming Zhang,¹ Yang Liu,¹ and Pan Zheng¹

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/B7 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/B7 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/B7 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 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/B7 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/B7 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

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P. Zhou and X. Zheng contributed equally to this study.

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Translational Relevance

Despite the conceptual advances in cancer immunotherapy, clinical development has been slow. Immunotherapy has thus far failed to show clear cut effect once cancers are established in advance stage. In this article, we showed that temporary blockade of B7 1 and B7 2 reduced the number of regulatory T cells and conveyed considerable therapeutic effects in transgenic mouse model of prostate cancer mice with spontaneous prostate cancer. To our knowledge, this is the first time that the prostate cancer in the transgenic mouse model of prostate cancer mice can be effectively treated when the large tumors can be shown. Mechanistically, we showed that transient blockade of B7 1/B7 2 resets the balance of regulatory T cells 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 (Food and Drug Administration 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.

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 transplantable 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 experimental 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 regiment, 4 to 6 week old TRAMP male 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 regiment, 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 days. 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 IgG 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 days. 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 Vβ8.1+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 (5×10^5 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 (3×10^5 per well) from control immunoglobulin or anti B7 treated TRAMP \times TGB ($H2^{b*}$) F_1 mice were cultured with the given concentrations of SV40 TAg K560 568 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 [3 H]thymidine pulsed (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 560 568 SEFLLEKRI (7) and HSV gB peptide gB498 505 SSIEFARL (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 µg/mL antibodies specific for CD3 (2C11, hamster IgG). CD3⁺ foci were counted using $\times 20$ microscope visual fields.

In vivo cytotoxicity assay. Spleen cells from C57BL/6 mice were pulsed with 10 µg/mL of either SV40 TAg 560 568 SEFLLEKRI or a control peptide HSV gB498 505 SSIEFARL 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 CFSE^{low} (SV40 TAg peptide) and CFSE^{hi} (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 was 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 was acquired using a T_1 weighted spin echo sequence (TR/TE = 880/13, field of view = 30×30 mm using a 128×128 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: $V = 4/3[(D_1 + D_2) / 4]^3\pi$, 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 pre necropsy MRI volumes with postnecropsy 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 vivo* 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^{lo}) and control HSV gB peptide pulsed (CFSE^{hi}) spleen cells. The spleens were harvested 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^{lo} and CFSE^{hi} 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 2K^k (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 V β 8⁺ 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 the 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

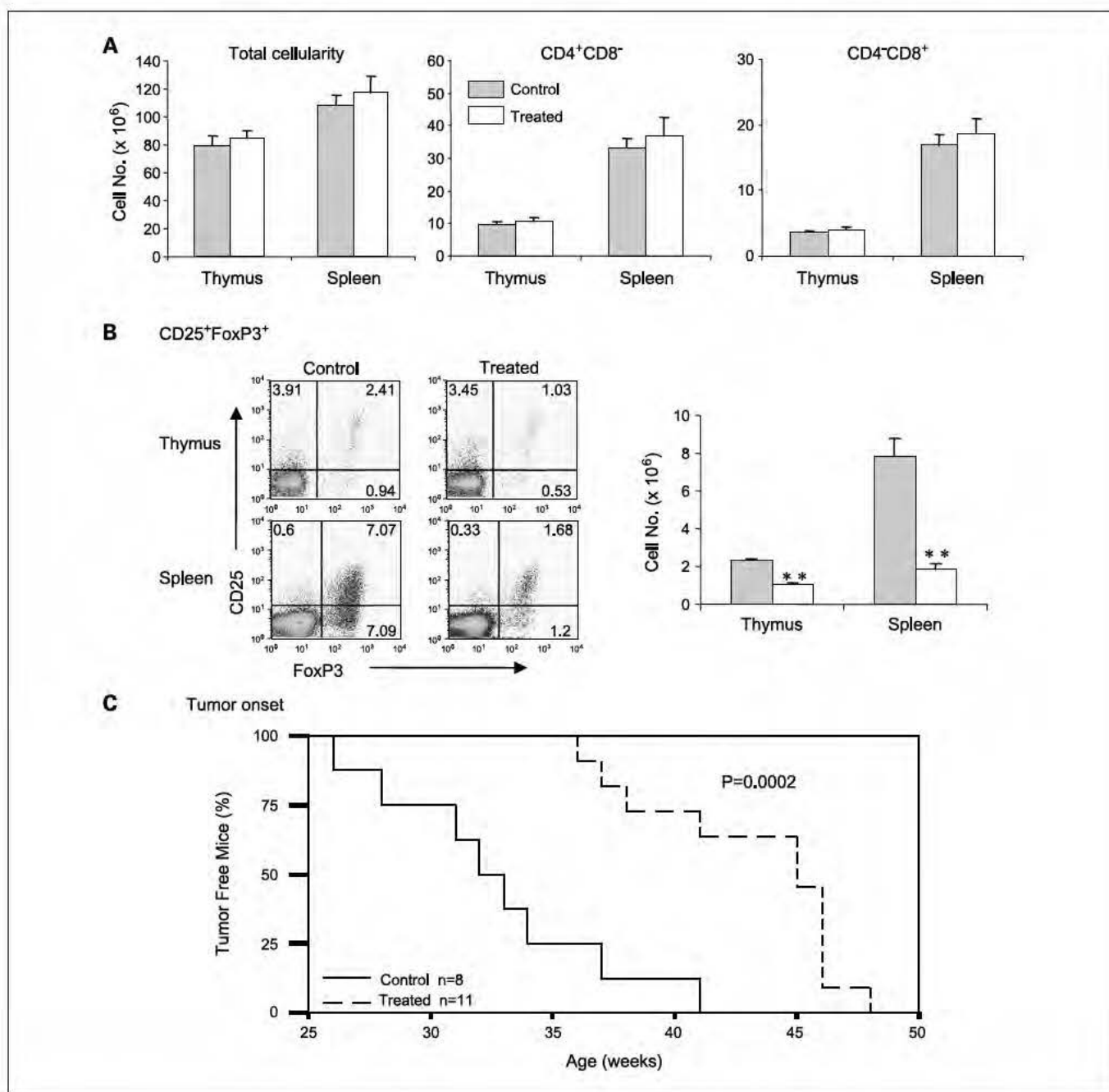


Fig. 1. Anti-B7-1/B7-2 antibody treatment reduced Treg in both the thymus and the periphery of normal mice and delayed the development of palpable tumors in TRAMP mice. **A** and **B**. C57BL/6 mice were injected intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μ g 3A12 and 100 μ g GL-1) or control IgG (1:1 mixture of 100 μ g hamster and 100 μ g rat IgG) at 6 wk old five times every other day. The mice were sacrificed 8 d after the last injection. Thymocytes and splenocytes were harvested and analyzed by flow cytometry. **A**, anti-B7-1/B7-2 antibody treatment did not alter the number of thymocytes, spleen cells, and CD4 and CD8 subsets. **B**, anti-B7-1/B7-2 antibody treatment reduced CD25⁺Foxp3⁺ cells in both thymus and spleen. Plots are from CD4⁺ cells among the lymphocyte gates. *, $P < 0.05$; **, $P < 0.01$, Student's t test. **C**. Kaplan-Meier analysis of tumor incidence. The experimental endpoint is 2 cm in tumor diameter as determined by palpation. Data have been repeated three times.

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

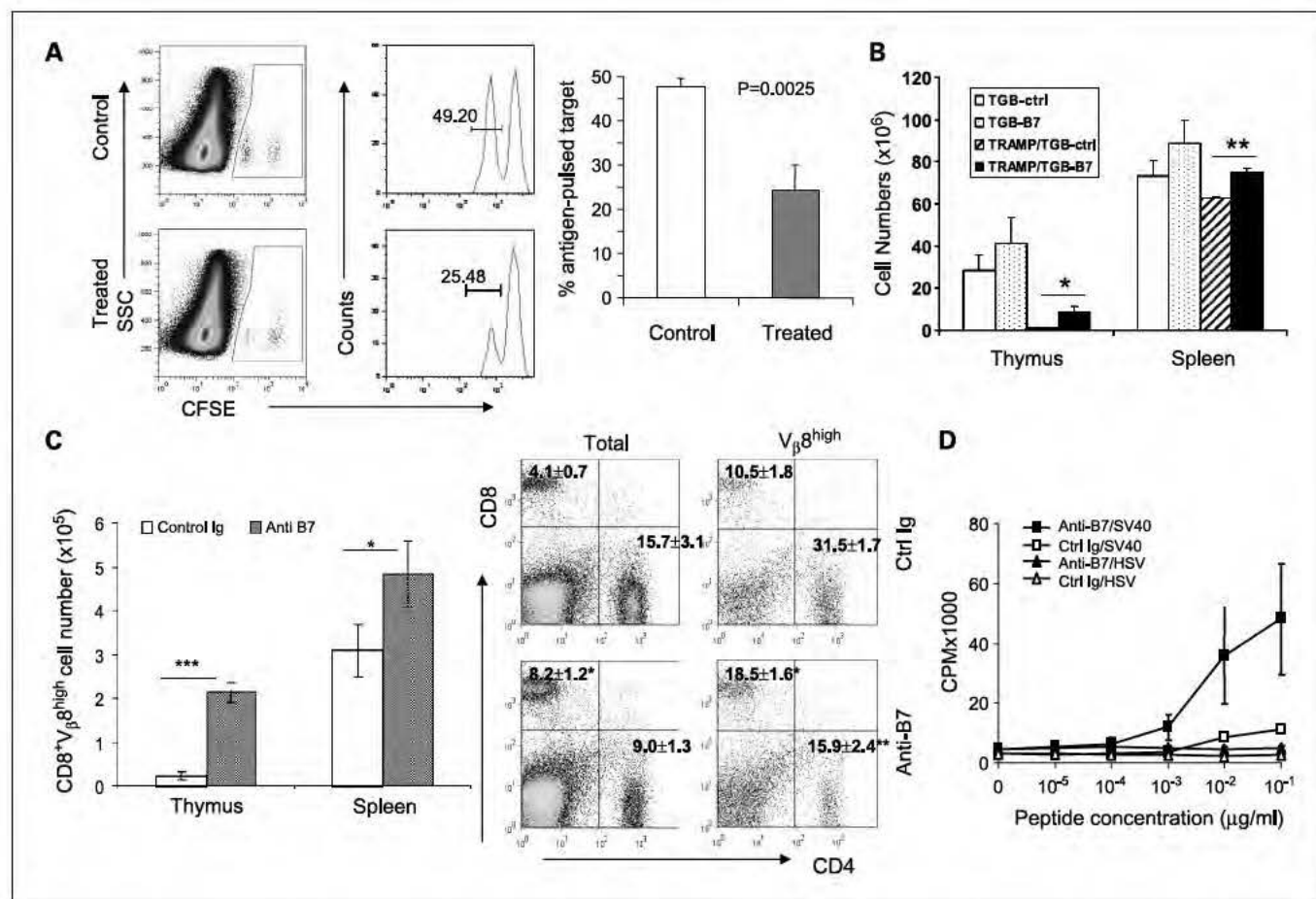


Fig. 2. Anti-B7-1/B7-2 antibody treatment enhanced TAG-specific CTL and partially rescued clonal deleted TAG-specific T cells in the TRAMP mice. **A**, 6-week-old male TRAMP mice were treated intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μ g 3A12 and 100 μ g GL-1) or control IgG (1:1 mixture of 100 μ g hamster and 100 μ g rat IgG) five times every other day. Two weeks after the first injection, mice received an intravenous injection of a 1:1 mixture of TAG peptide-pulsed (CFSE^{hi}) and control HSV peptide-pulsed (CFSE^{lo}) spleen cells (5×10^6 each). Twenty hours later, the spleens were harvested and analyzed by flow cytometry. *Left*, representative profiles; *right*, summary of two experiments involving a total of 6 mice per group. CFSE^{hi} and CFSE^{lo} cells are gated as indicated. The number shown in the gates are the percentage of gated cells. **B** to **D**, anti-B7 treatment rescued tumor-reactive T cells from clonal deletion. TRAMP/TGB double transgenic or TGB single transgenic mice that received sublethal irradiation (500 rad) were treated with either control immunoglobulin or anti-B7 mAbs five times every other days. The thymocytes and splenocytes were harvested on day 7 after final treatment and analyzed by flow cytometry. **B**, thymocyte and splenocyte cellularities in TGB single transgenic and TRAMP/TGB double transgenic mice treated with control immunoglobulin or anti-B7 antibodies. **C**, increase of CD8⁺V β 8^{high} T cells in thymus and spleen. *Left*, summary of CD8⁺V β 8^{high} cell number change in thymus and spleen from TRAMP/TGB double transgenic mice; *right*, representative profiles of CD4 and CD8 T cells in the spleens of control immunoglobulin or anti-B7-treated mice. The left flow cytometry shows those for total spleen cells, whereas the right flow cytometry shows those for gated V β 8^{high} cells. **D**, antigen reactivity of T cells rescued by anti-B7. The splenocytes from TRAMP/TGB double transgenic mice were stimulated with either SV40 TAG peptide or control HSV peptide for 72 h and pulsed with [³H] thymidine to determine the rate of T cell proliferation. **B** to **D**, mean \pm SE ($n = 3$). The conclusions have been confirmed with another independent experiment.

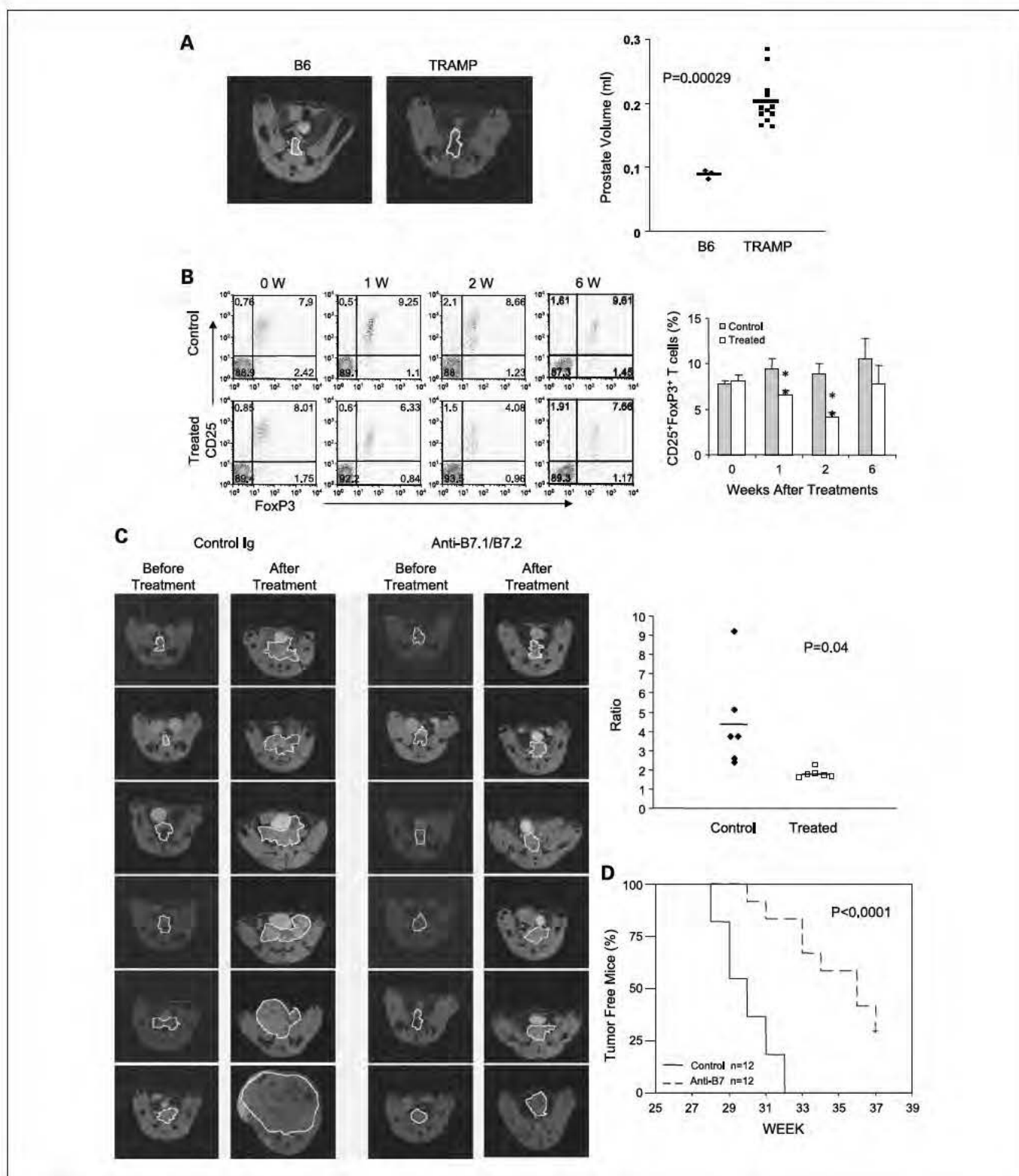


Fig. 3. Anti-B7-1/B7-2 mAb treatments of mice with established prostate cancer inhibited cancer progression. **A**, MRI measurement of prostate volumes of 25-week-old normal and TRAMP mice. *Left*, representative local images of male B6 and TRAMP mice. The prostate was identified with thick white outlines. *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 μ g 3A12 and 100 μ g GL-1) or control IgG (1:1 mixture of 100 μ g hamster and 100 μ g 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. **B**, 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.

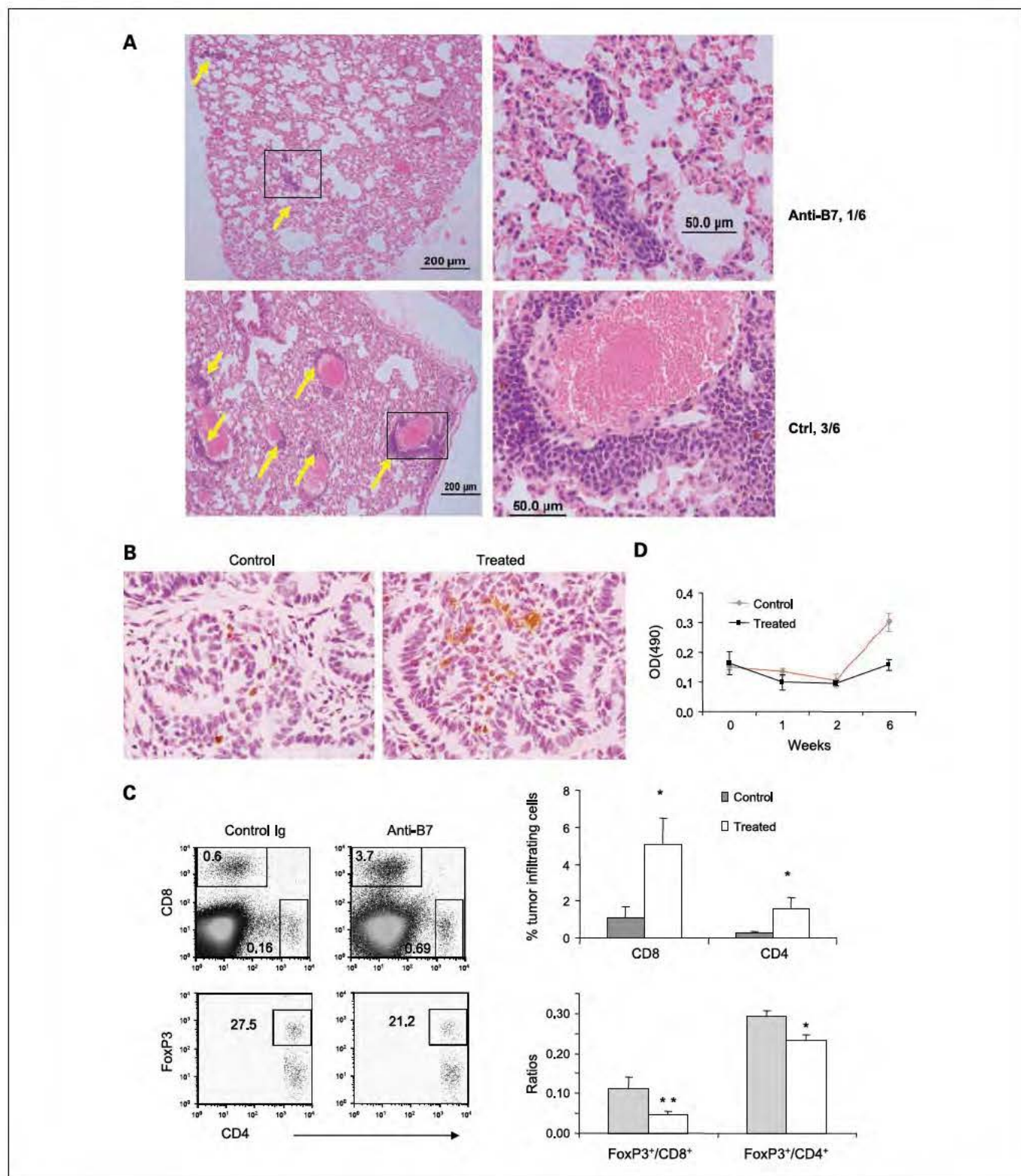


Fig. 4. Anti-B7 blockade in tumor-bearing mice reduces the number and size of metastatic lesions in the TRAMP mice and increases infiltration of T cells into tumors but does not cause autoimmunity. **A**, internal organs of mice from Fig. 3C were analyzed for metastatic lesions. Three sections of liver, lung, kidney, intestine, and heart, 30 μ m 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 \pm SE ($n = 6$). **D**, serum anti-double-stranded DNA antibodies. Data are A_{490} from an ELISA using 1:50 dilution of sera. Mean \pm 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 CD4 and CD8 T cells significantly decreased in anti B7 treated group (Fig. 4C, bottom right). Therefore, anti B7 treatment alters the ratio of Treg over effector T cells in the tumor, presumably in favor of local immune response.

A general concern for immunotherapy of cancer is autoimmune side effect. To determine whether autoantibodies were induced in tumor bearing TRAMP mice, the sera were collected at 1, 2, and 6 weeks after the start of anti B7 1/B7 2 antibody treatments. The anti double stranded DNA antibodies were detected by ELISA. As shown in Fig. 4D, although an increase in anti DNA antibodies was detected at 6 weeks after control immunoglobulin treatment, presumably due to tumor growth, such increase was not observed in the anti B7 treated mice. Histologic analysis showed no inflammation of internal organs in either group (data not shown). Therefore, anti B7 antibodies can induce significant protection against established tumor without eliciting autoimmune side effect.

Anti-B7 antibodies inhibit MC38 colon carcinoma cell growth. To confirm the general antitumor effect of anti B7 treatment, we tested it with MC38 colon carcinoma tumor model. Male C57BL/6 mice were injected 5×10^6 MC38 tumor cells subcutaneously. Ten days after injection, mice developed palpable tumors and were divided evenly into two groups based on the tumor sizes. MC38 tumor bearing mice were administered intraperitoneally with either anti B7 1/B7 2 mAbs (1:1 mixture of 100 μ g 3A12 and 100 μ g GL 1) or control IgG three times every other day. Peripheral blood samples were taken at 0 and 6 days, and spleens were collected at 14 days after completion of antibody treatments. As shown in Fig. 5A, at 6 and 14 days after anti B7 antibody treatment, the CD4⁺CD25⁺ Foxp3⁺ Treg cells were significantly reduced. Correspondingly, anti B7 treatment conferred a significant reduction in the growth rate of MC38 colon carcinoma ($P = 0.035$; Fig. 5B).

Transient depletion of Treg by anti-CD25 antibody delays established prostate cancer growth in TRAMP mice. To test whether transient depletion of Treg alone inhibits tumor growth, we treated 25 week old TRAMP mice with anti CD25 antibody to deplete CD4⁺CD25⁺ cells. Male 25 week old TRAMP mice were examined by MRI to measure prostate size and divided into two groups. The mice were treated intraperitoneally with either 1 mg anti CD25 mAbs (PC61) or 1 mg control rat immunoglobulin. Peripheral blood samples were taken at 0 and 3 days, and spleen were collected on day 35; 0 day is the day before injection. As shown in Fig. 6A, 99% of Foxp3⁺CD25⁺ cells were depleted in 3 days after anti CD25 treatment; however, the Foxp3⁺CD25⁺ 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

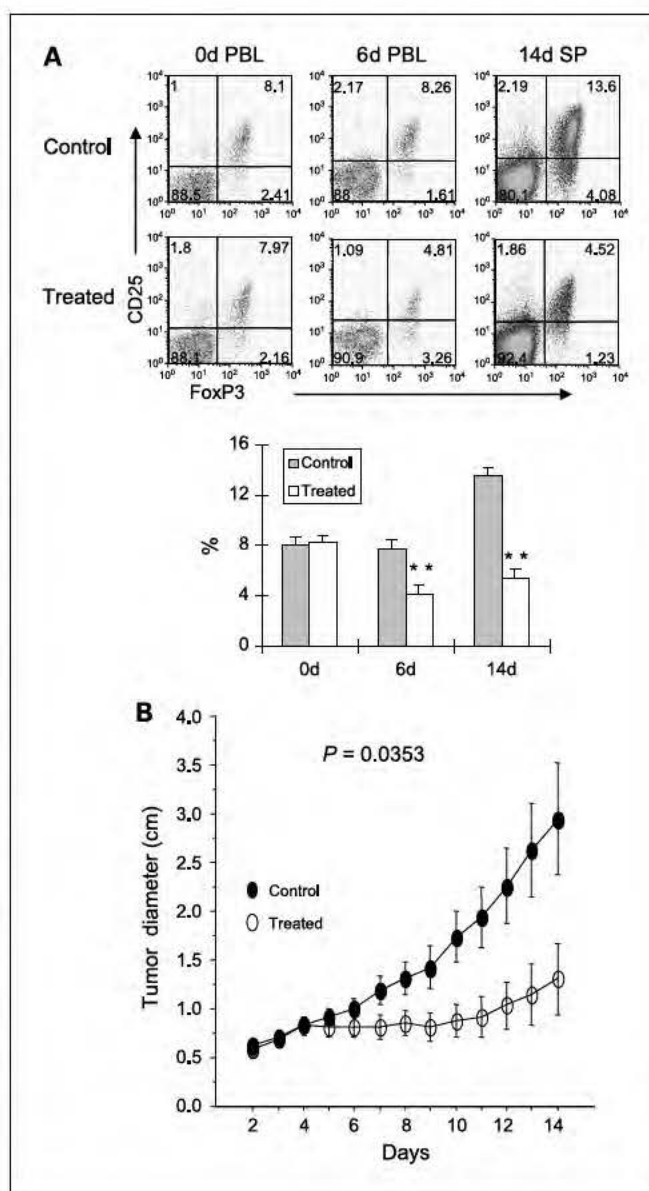


Fig. 5. Anti-B7-1/B7-2 mAb treatments of mice bearing MC38 colon carcinoma. Eight-week-old male C57BL/6 mice were injected subcutaneously with 5×10^5 MC38 tumor cells. Ten days after injection, mice were divided evenly into two groups based on the tumor sizes. The mice were administered intraperitoneally with either anti-B7-1/B7-2 mAbs (1:1 mixture of 100 μ g 3A12 and 100 μ g GL-1) or control IgG (1:1 mixture of 100 μ g hamster and 100 μ g rat IgG) three times every other day. Peripheral blood was taken at 0 and 6 d, and spleen was collected at 14 day; 0 day is the day before the administration of antibodies. Cells were stained for flow cytometry. Plots of gated CD4⁺ cells are presented. *A*, CD4⁺FoxP3⁺CD25⁺ cell number started to reduce following the first week of treatment. *Top*, representative profiles; *bottom*, summary data. *B*, anti-B7 treatment delayed growth of MC38 tumor (6 mice per group). Mean \pm SE of tumor diameters at different time points. Day 1 is defined as the day of first injection of antibody. The statistical significance is determined by Plois Fisher's test.

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

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 B7 1 and B7 2 has been explored for treatment of autoimmune diseases and transplant rejection (38). Recent studies that reveal a

critical role for B7 1/B7 2 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

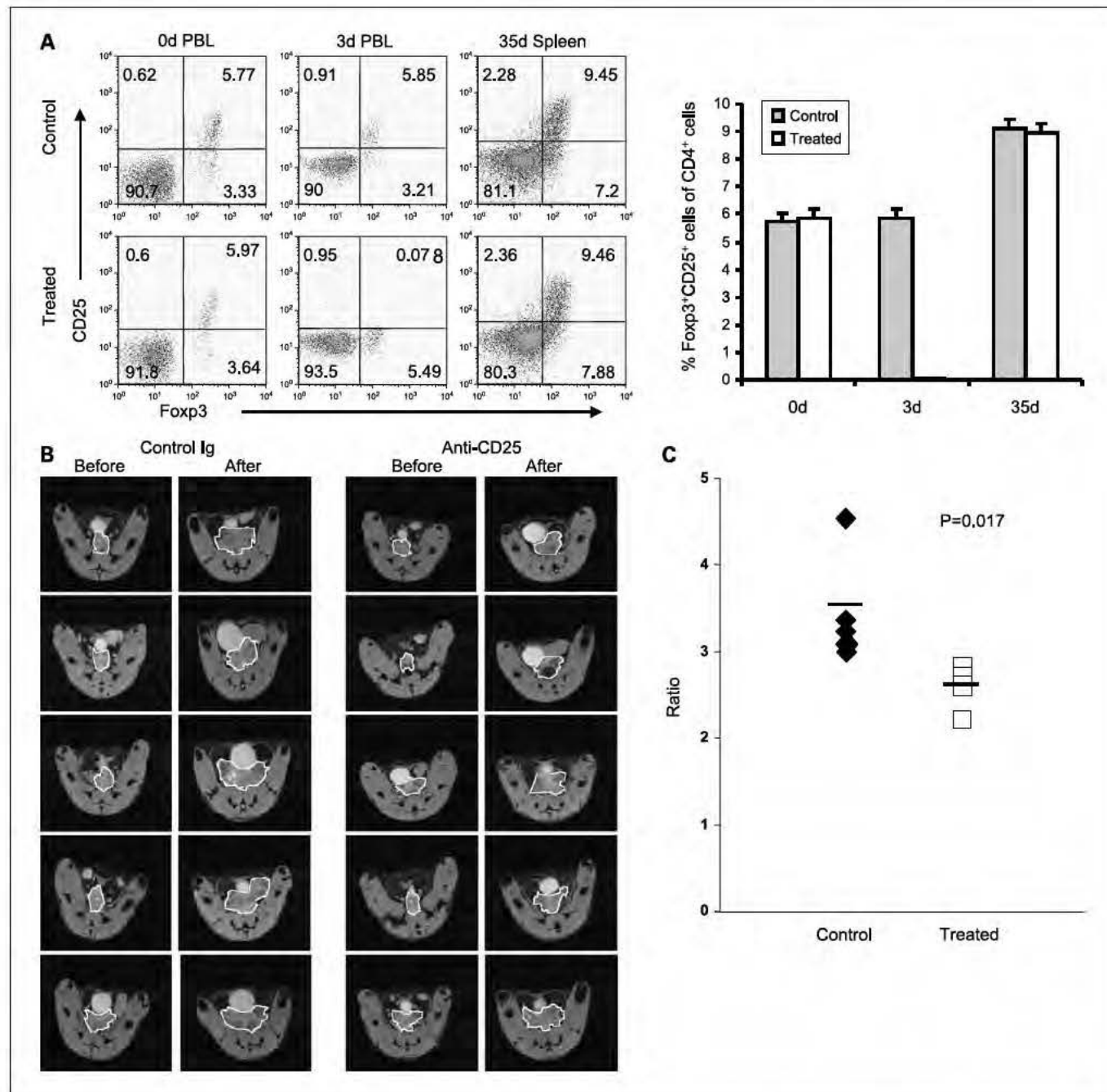


Fig. 6. Anti-CD25 treatments of mice with established prostate cancer inhibited cancer progression. **A**, anti-CD25 (clone PC61) treatment initiated at 25-week-old TRAMP mice transiently depleted Treg. Male TRAMP mice were administered intraperitoneally with one dose of anti-CD25 (1 mg/mouse) or control rat IgG (1 mg). 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 of gated CD4⁺ cells are shown. The conjugated anti-CD25 from a different clone 7D4 was used to avoid blocking by the depleting antibody. CD4⁺CD25⁺Fxp3⁺ cells were reduced at 6 d after anti-CD25 treatment but fully recovered at 35 d. **B**, MRI image of TRAMP mice at 25 and 30 wk (5 wk after the treatments with either control immunoglobulin or anti-CD25 antibody, 5 mice per group). **C**, summary data are ratio of prostate volumes at 30 wk versus 25 wk when the treatments started. The difference was compared by a Student's *t* test.

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 eradicate the tumors. Because the majority of tumors that developed in human have clonal origin, the malignant transformation is likely less frequent than 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 cells, 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 CTLA4Ig 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.

Acknowledgments

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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 threat 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 α* gene efficiently rescued tumor-reactive T cells, drastically reduced cancer incidence and almost completely ablated metastasis. Remarkably, short-term treatments with LT β Rlg 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 administered 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 threat 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 ($LT\alpha$) 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 $LT\alpha$ (31), we reveal here a valuable target for rescuing prostate cancer-reactive T cells and for cancer immune prevention. More importantly, transient blockade of $LT\alpha$ 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 *LT α* limits clonal deletion of SV40 T antigen-specific T cells

One of our groups has recently demonstrated a critical role for *LT α* 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 *LT α* 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 *LT α* 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 *LT α* 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 *LT α* -deficient mice (Fig. 1d, e). Remarkably, partial rescue was observed in the heterozygous mice (Fig. 1). Therefore, *LT α* play a critical role in clonal deletion of SV40-large T antigen-reactive T cells.

Targeted mutation of *LT α* inhibits development of spontaneous prostate cancer

To test the role for *LT α* 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 α* (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 α* 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 $LT\beta$ Rlg 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 $LT\beta$ Rlg 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 LT β Rlg 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 LT β Rlg expand SV40 T antigen-specific T cells only if the antigen was present.

To determine whether LT β Rlg prevented deletion of antigen-specific T cells, we compared % of apoptotic cells by staining with Annexin V. As shown in Fig. 5, LT β Rlg 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 LT β Rlg reduces the progression of primary prostate cancer and prevented metastasis

LT β Rlg binds LT α with high affinity. To test whether LT β Rlg treatment can significant affect the progression of prostate cancer, we treated the TRAMP mice with 3 weekly injections of either LT β Ig 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 LT β Rlg 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 LT β Rlg 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 LT β Rlg 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 LT β Rlg 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 $LT\alpha$ 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 $LT\beta RIg$ 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 $LT\beta RIg$ at six weeks have significantly reduced sizes at 30 weeks. More importantly, this treatment completely prevented the development of metastasis. Since targeted mutation of $LT\alpha$ limits clonal deletion of SV40 T antigen-specific T cells and inhibits development of spontaneous prostate cancer, prevention by $LT\beta RIg$ is likely due to its binding to $LT\alpha$.

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 $LT\alpha$ 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 $LT\alpha$ -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 $LT\alpha$ 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 $LT\alpha$ cause multiple organ infiltration (29). Our extensive analysis of the $LT\beta RIg$ -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 $LT\alpha$ 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-1 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 β RlgFc treatment For cancer prevention, 6 weeks old TRAMP mice were treated with 3 weekly injections of 100 μ g LT β RlgFc 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 100 μ g LT β RlgFc 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-V β 8.1+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 100 μ g LT β RlgFc 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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Table 1. Inflammation induced by LT β lg at 4 but not 6 or 11 weeks.

Organs	Liver		Lung		Kidney		Prostate	
treatment	hlg	LT β lg	hlg	LT β lg	hlg	LT β lg	hlg	LT β lg
4W	0/7	6/7	0/7	3/7	0/7	0/7	0/7	0/7
6W	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
11W	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Figure legends

Fig 1. LT α deficiency prevents clonal deletion of tumor-reactive T cells in the TRAMP mice.

LT α ^{+/+}, LT α ^{+/-} and LT α ^{-/-} 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 V β 7 (transgenic TCR β), CD4 and CD8. a. Number of V β 7⁺ 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 V β 7⁺ thymocytes (b, left panels) or CD8 and V β 7 among total thymocytes (b, right panels), and V β 7⁺ 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 V β 7⁺ T cells in the thymi (c) and spleens (e). Data shown are means and SEM of cell numbers (n=8).

Fig. 2. LT α deficiency inhibits development of prostate cancer.

The tumor incidence of *Lt α ^{+/+}TRAMP*, *Lt α ^{+/-}TRAMP* and *Lt α ^{-/-}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 *Lt α ^{+/+}TRAMP*, *Lt α ^{+/-}TRAMP* and *Lt α ^{-/-}TRAMP* mice. The prostate were identified with thick white outlines. b. The prostates sizes of *Lt α ^{+/+}TRAMP*, *Lt α ^{+/-}TRAMP* and *Lt α ^{-/-}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 $LT\beta RIg$ treatment. 4, 6 and 11 weeks old C57B6 mice received 3 weekly i.p. injections with 100 μg of either soluble murine $LT\beta RIg$ 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. $LT\beta RIg$ 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 $LT\beta RIg$ 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 β 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 β 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 β Rlg treatment on TRAMP/TGB mice reduced the percentage of apoptotic cells in the thymus, mainly at the DP stage. b. LT β 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 β 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 μ g of soluble murine LT β Rlg or Human IgGFc at 6 weeks old. The prostate volume was measured at 30 weeks.

The upper panels show representative local images of Human IgGFc treated and LT β Rlg 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 LT β Rlg 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 LT β Rlg-treated mice. The differences in the rate of metastasis is statistically significant (P=0.012).

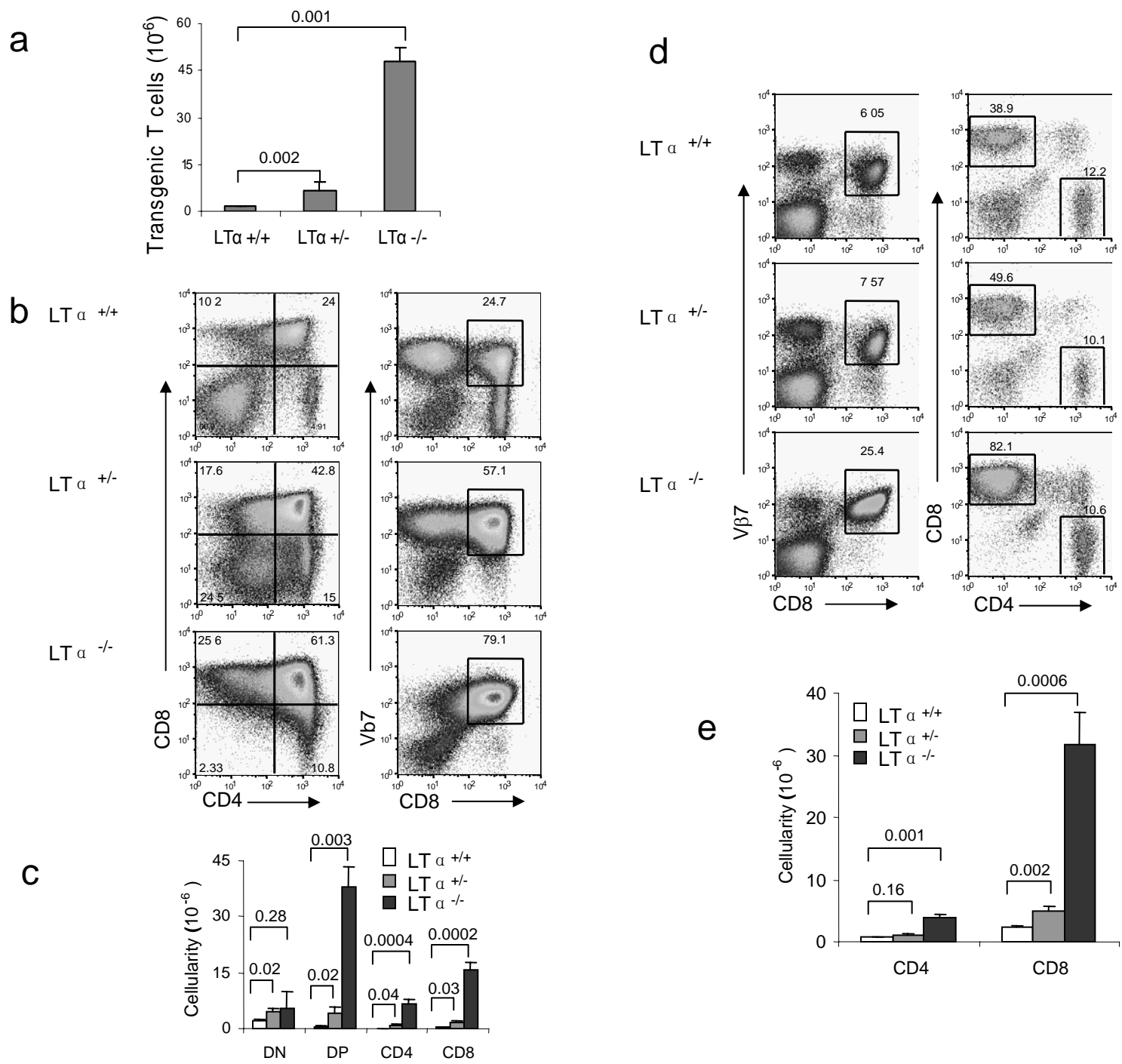


Fig. 1.

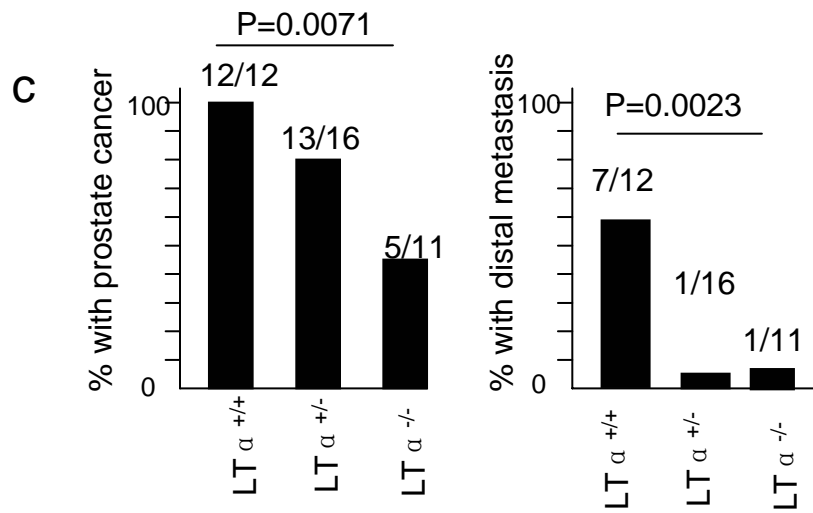
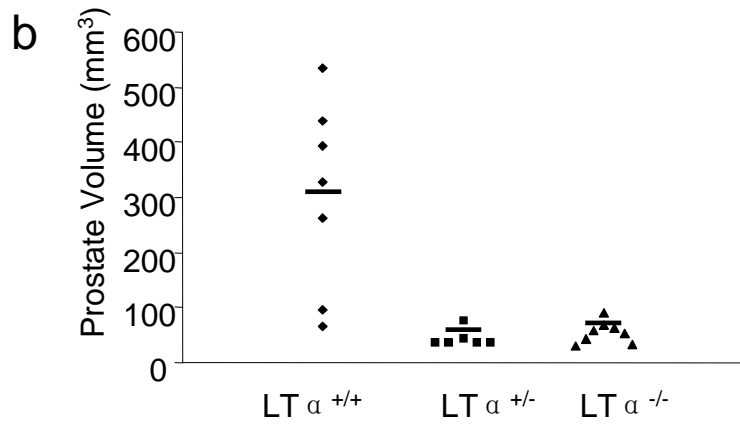
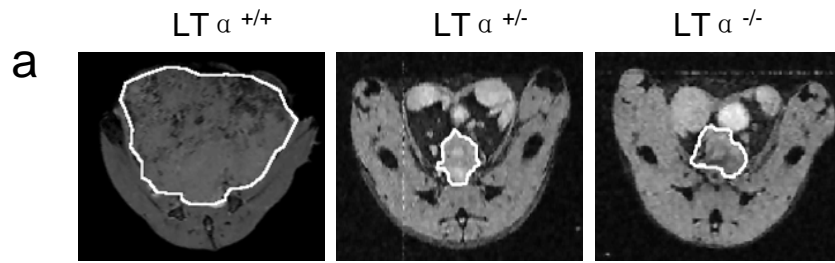


Fig. 2

a. Liver

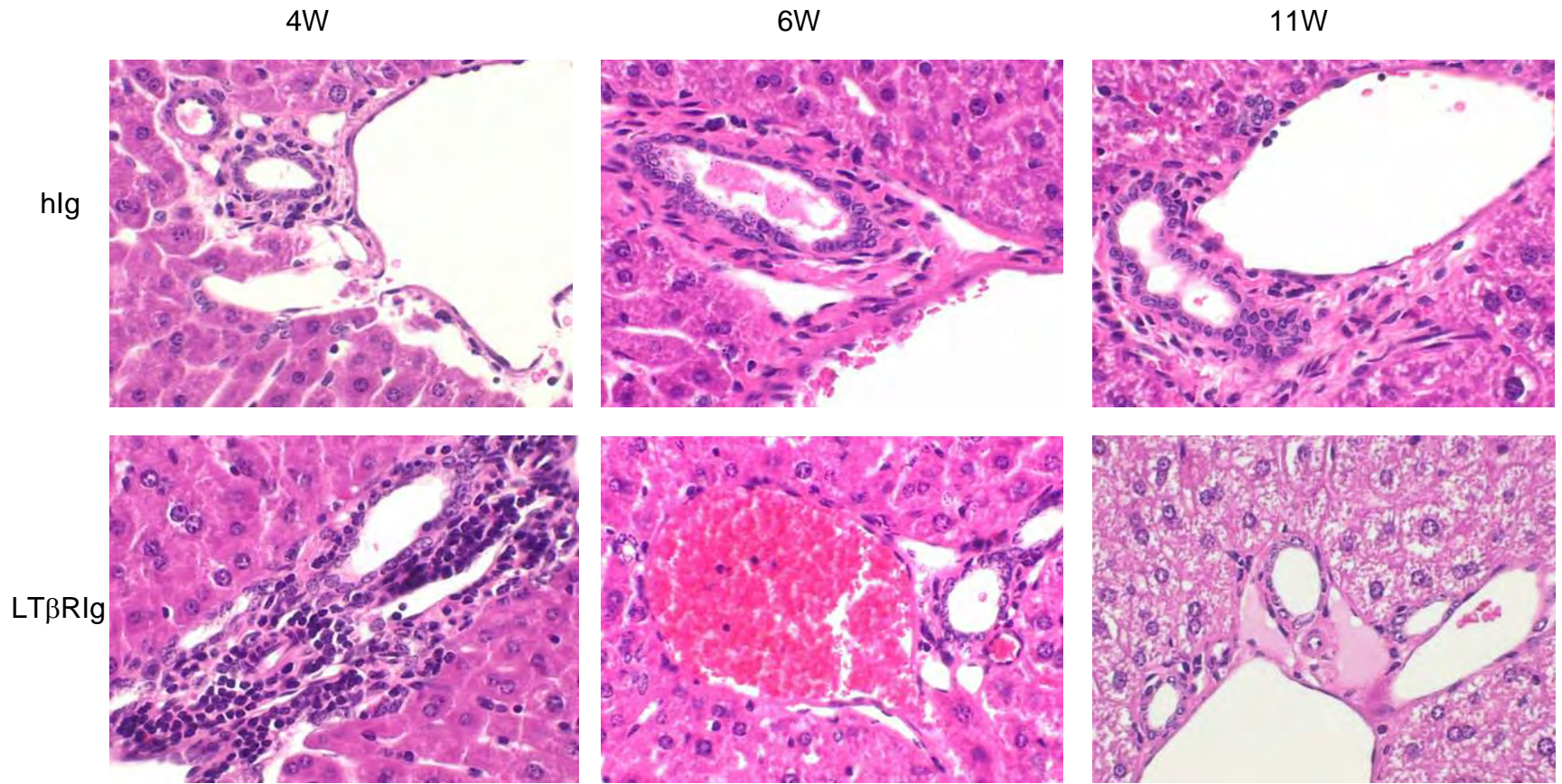


Fig. 3a

b. Lung

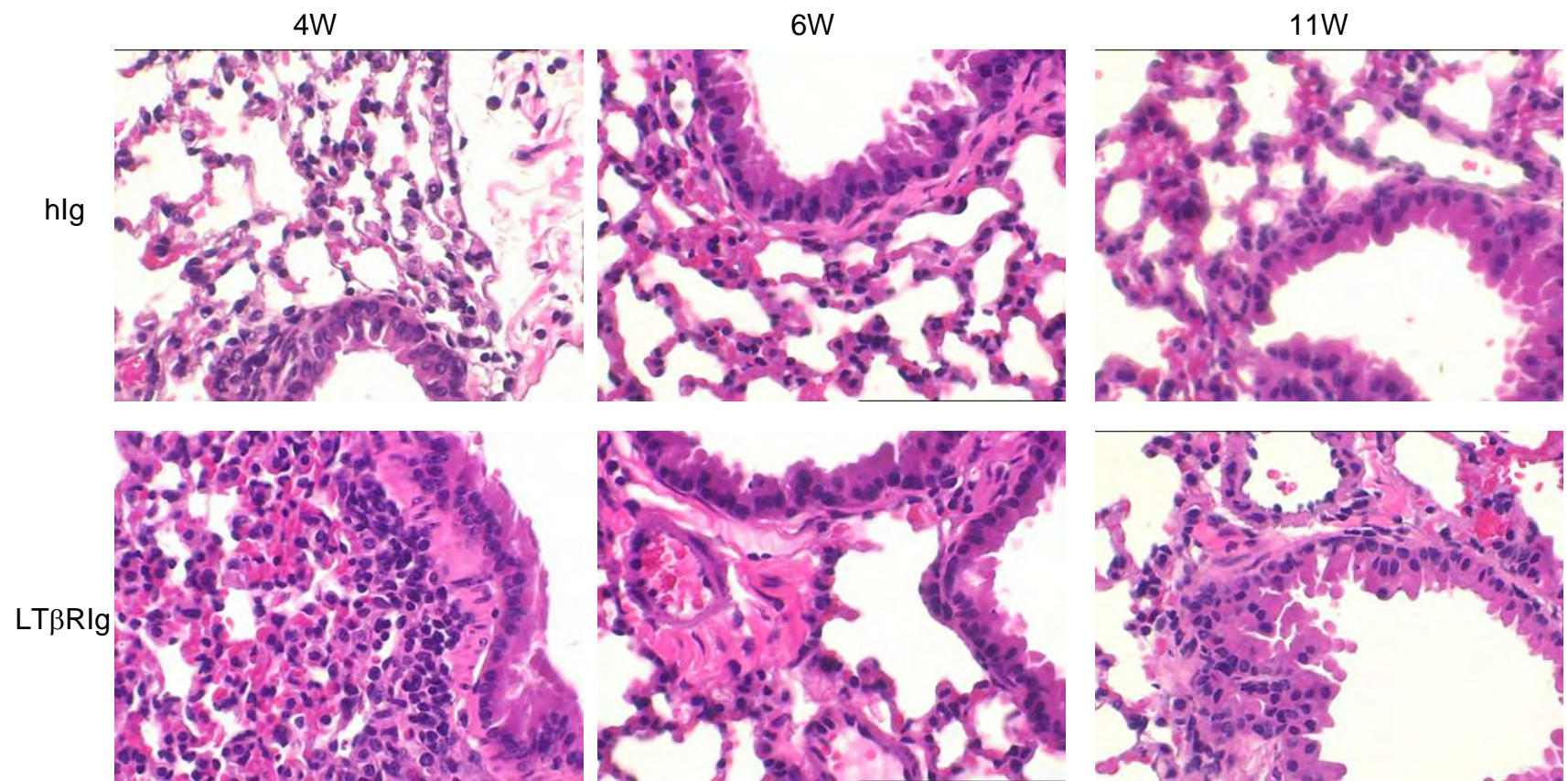


Fig. 3b

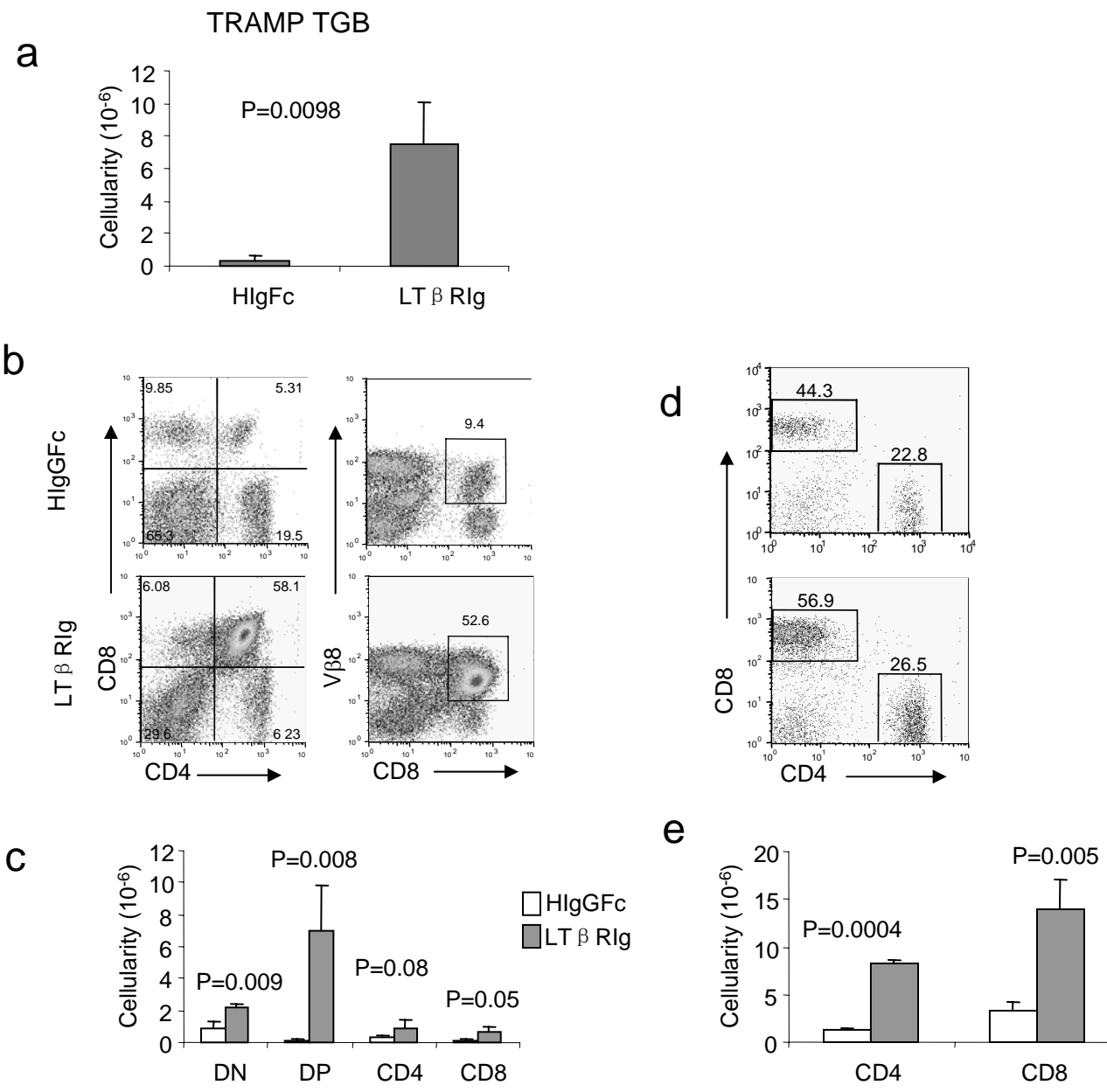


Fig. 4a-e

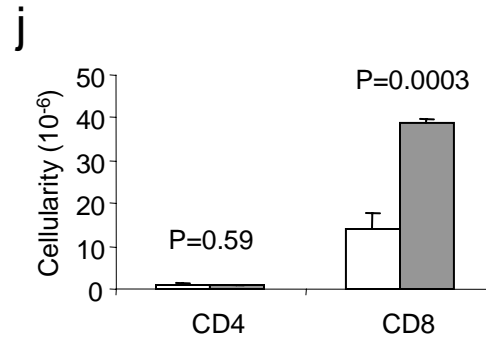
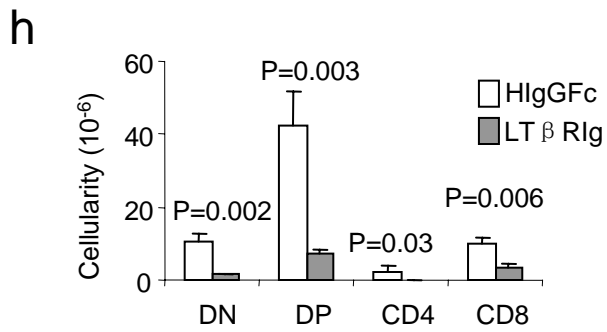
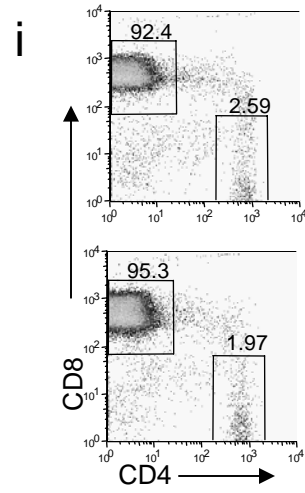
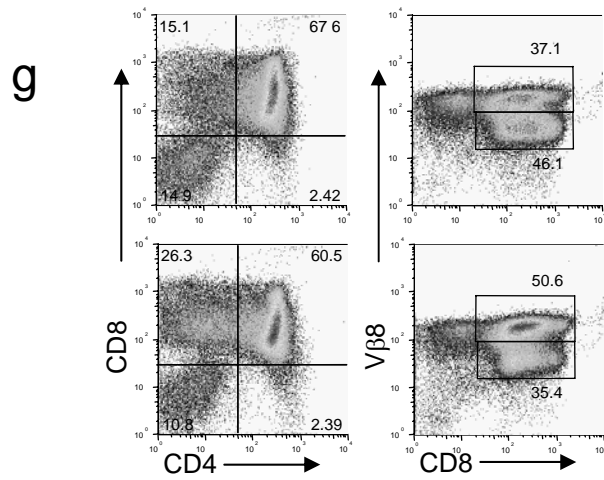
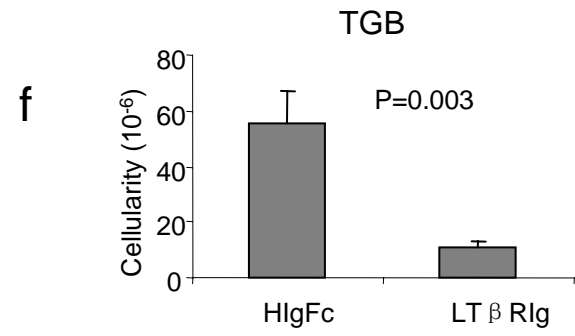


Fig. 4f-j

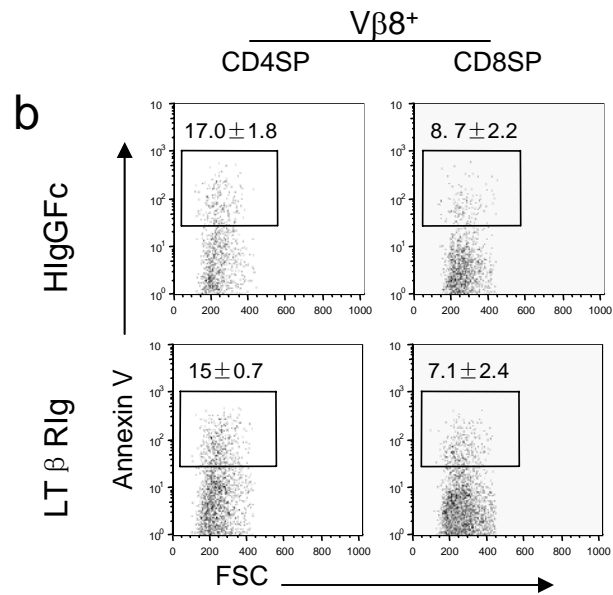
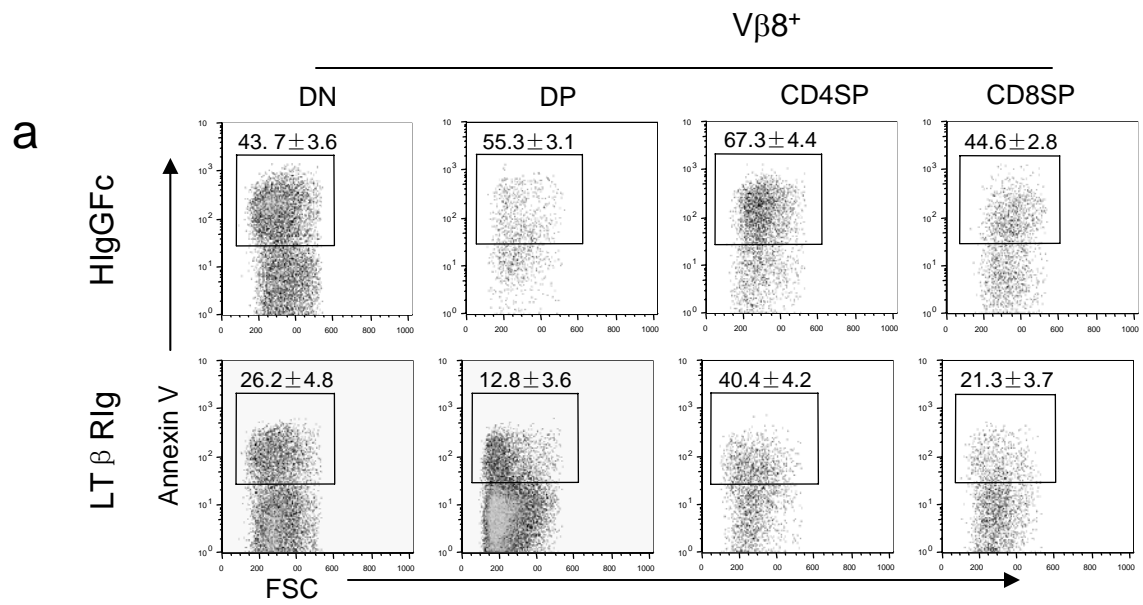


Fig. 5

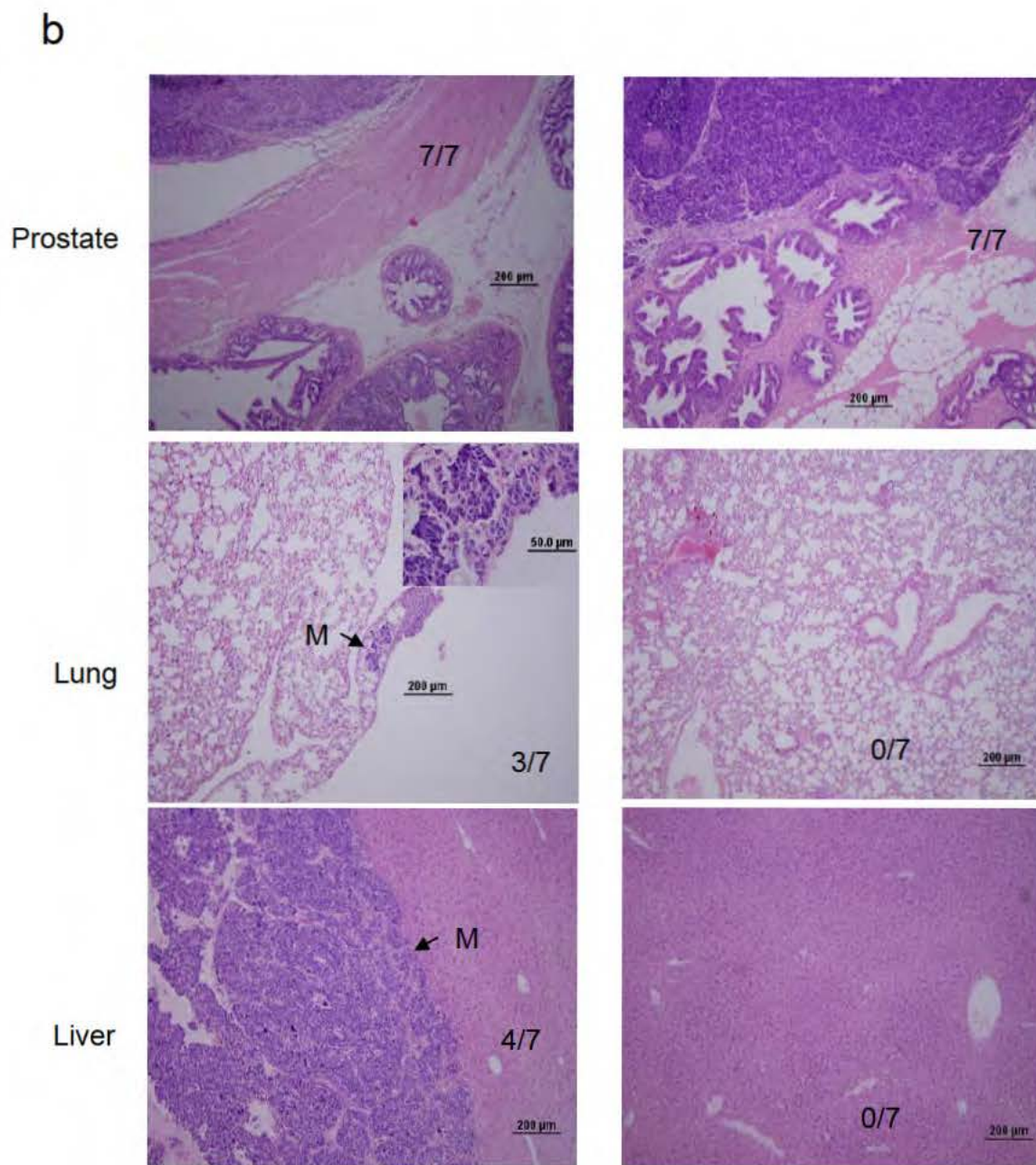
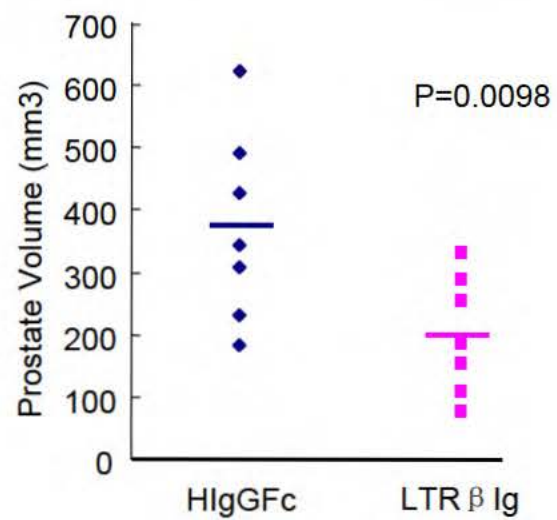
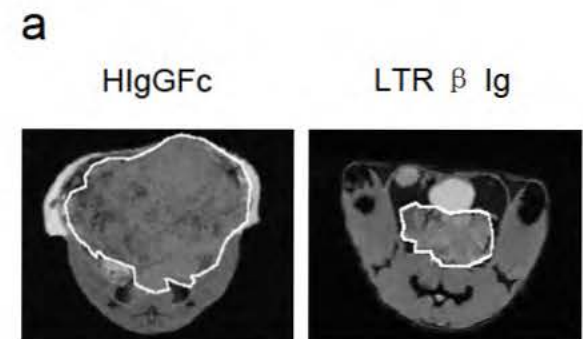


Fig. 6.

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_Rs). The role of thymic dendritic cells (TDCs) in these processes has not been clearly determined. In this study, we demonstrate that *in vivo*, TDCs not only play a role in negative selection but in the induction of T_Rs. TDCs include two conventional dendritic cell (DC) subtypes, CD8^{lo}Sirpα^{hi/+} (CD8^{lo}Sirpα⁺) and CD8^{hi}Sirpα^{lo/-} (CD8^{hi}Sirpα⁻), which have different origins. We found that the CD8^{hi}Sirpα⁺ DCs represent a conventional DC subset that originates from the blood and migrates into the thymus. Moreover, we show that the CD8^{lo}Sirpα⁺ DCs demonstrate a superior capacity to induce T_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_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_Rs). T_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_Rs appear in the thymus 3 days after birth (7). Deficiency in T_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_R induction has been demonstrated (9, 13–16). The role of dendritic cells (DCs) in T_R generation in the thymus is unclear, however. Given the importance of DCs in the generation of peripherally induced T_Rs (17, 18), and in light of a recent study demonstrating the potential of human TDCs to induce T_Rs *in vitro* (19), the possible role of TDCs in T_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 CD8α and Sirpα expression: the CD8^{lo}Sirpα^{hi/+} cDCs (≈30% of cDCs, Sirpα⁺ TcDCs hereafter) and the CD8^{hi}Sirpα^{lo/-} cDCs (≈70% of cDCs, Sirpα⁻ TcDCs hereafter) (20, 21). Sirpα⁻ TcDCs develop from intrathymic lymphoid precursors (22, 23). The origin of Sirpα⁺ TcDCs is less clear, although one study demonstrated that the CD8^{lo}CD11b⁺ cDCs (equivalent to Sirpα⁺ 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_R generation (16), in this study, we demonstrate that TDCs make a significant contribution to T_R induction as well as to negative selection. This was established *in vivo* using two bone marrow (BM) chimeric mouse models in which the hemopoietic-

derived compartment was impaired in antigen presentation (MHC class II [MHCII]^{-/-}) or T-cell activation (B7^{-/-}). Using an *in vitro* culture system, we established that the Sirpα⁺ TcDCs played the major role in T_R induction when compared with other DC subtypes. This functional capacity of the Sirpα⁺ TcDCs 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_R induction in the thymus.

Results

TDCs Contribute to T_R Induction and Negative Selection *In Vivo*. To dissect the contribution of DCs from that of mTECs in the induction of T_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_Rs (5, 14, 15, 19, 25, 26), these systems enabled us to discern the contribution of DCs to T_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⁻), indicating effective elimination of host DCs (Fig. 1A). The TDCs from the MHCII^{-/-} BM chimeras did not express MHCII (Fig. 1B). Furthermore, both cDC subsets were observed in similar proportions and number in WT and MHCII^{-/-} chimeras (data not shown).

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. 1C–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-

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The authors declare no conflict of interest.

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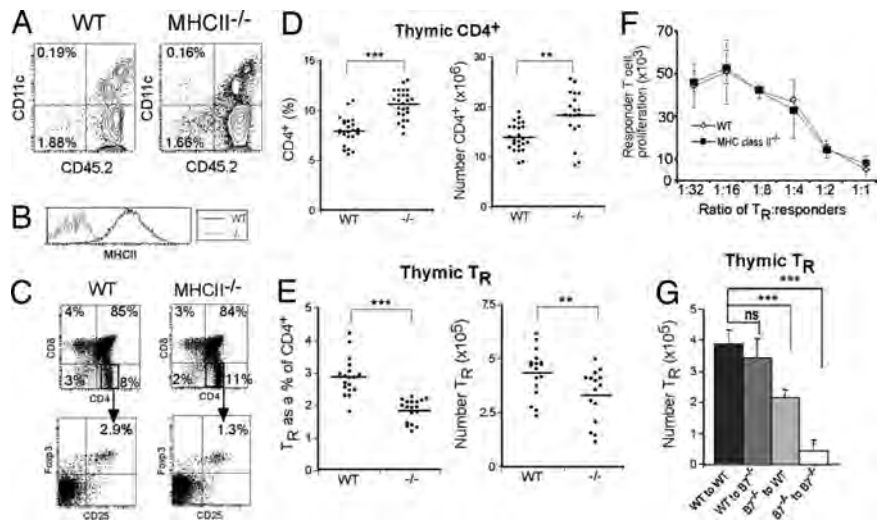
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Fig. 1. MHCII⁺ DCs contribute to negative selection and T_R induction. (A) The light density fraction of cells from the thymus of WT and MHCII^{-/-} BM chimeras was analyzed to determine the % of donor-derived DCs. More than 98% of CD11c⁺ cells were CD45.2⁺ donor-derived DCs in both BM chimera groups. (B) MHCII expression on CD11c⁺ TDCs in WT (black line) and MHCII^{-/-} (gray line) chimeras. (C) The % of double-negative, double-positive, CD4⁺, and CD8⁺ thymocytes (Upper) and CD4⁺CD25⁺Foxp3⁺ T_Rs (Lower). (D) The % and total number of CD4⁺ thymocytes in WT and MHCII^{-/-} BM chimeras. (E) The % and total number of T_Rs in WT and MHCII^{-/-} BM chimeras (n = 20–24 per group for D and E). (F) Suppressive activity of CD4⁺CD25⁺ thymocytes from MHCII^{-/-} or WT chimeras. Data are the mean (error bars, ±SD) of triplicate cultures from one of two experiments. (G) The number of T_Rs (CD4⁺Foxp3⁺) in the thymus of B6 to B6, B6 to B7^{-/-}, B7^{-/-} to B6, and B7^{-/-} to B7^{-/-} BM chimeras was analyzed by flow cytometry. Data are the mean of three independent experiments (error bars, ±SD) (n = 68 for G). *, P < 0.05; **, P < 0.001; ***, P < 0.0001.



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 MHCII^{-/-} 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⁺CD25⁺Foxp3⁺ T_Rs (P = 0.008) (Fig. 1E).

To test the function of the T_Rs in the WT and MHCII^{-/-} chimeras, they were sorted and used in an *in vitro* T_R suppression assay. The T_Rs 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 T_Rs 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 T_R 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 T_Rs 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 T_Rs and in the negative selection of self-reactive CD4⁺ thymocytes.

DCs Induce Antigen-Specific T_Rs and Negative Selection *In Vivo*. T_R induction and negative selection of self-reactive thymocytes

require self-peptide presentation on MHCII via an antigen-presenting cell (14, 15). To address T_R induction by DCs in an antigen-specific system, Rag2^{-/-} OTII T-cell receptor (TCR) transgenic (tg) mice (which lack OVA-specific T_Rs 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 CD45.2⁺CD4⁺Vα2⁺ OTII thymocytes compared with controls (Fig. 2B and C). Furthermore, there was a clear induction of OTII T_Rs 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 T_R 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 T_R induction and negative selection in an antigen-specific manner.

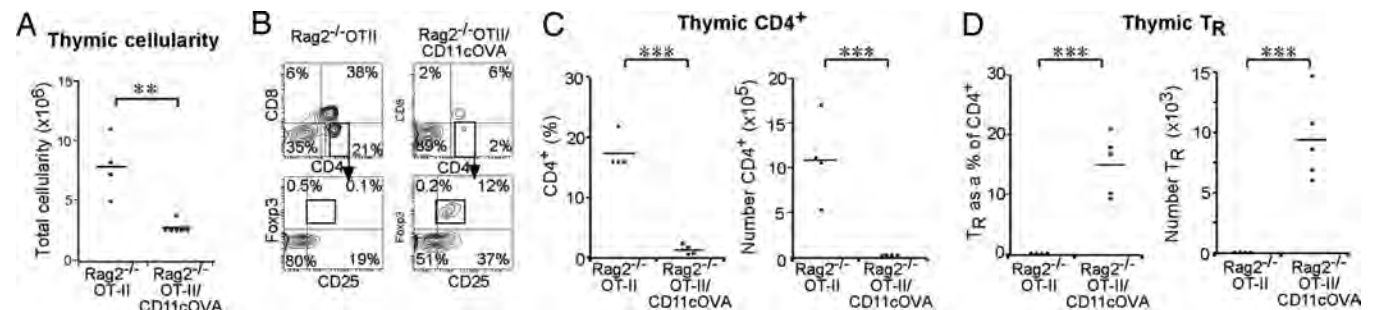


Fig. 2. OVA expressing DCs induce OTII T_Rs and deletion of OTII CD4⁺ T cells. Irradiated WT CD45.1 mice were reconstituted with double-tg Rag2^{-/-}OTII/CD11cOVA (Rag2^{-/-}O/OVA) BM or Rag2^{-/-}OTII BM as a control (n = 4–5 per group). (A) Total thymic cellularity of Rag2^{-/-}O/OVA and control BM chimeras. (B) CD45.2⁺Vα2⁺ thymocytes were gated, and the % of CD4⁺ and CD8⁺ thymocytes was determined. To assess T_R induction, CD4⁺ OTII⁺ thymocytes were gated for and expression of CD25 and Foxp3 was determined. The % and number of OTII⁺ CD4⁺ thymocytes (C) and the % and number of T_Rs (D) in Rag2^{-/-}O/OVA and control BM chimeras.

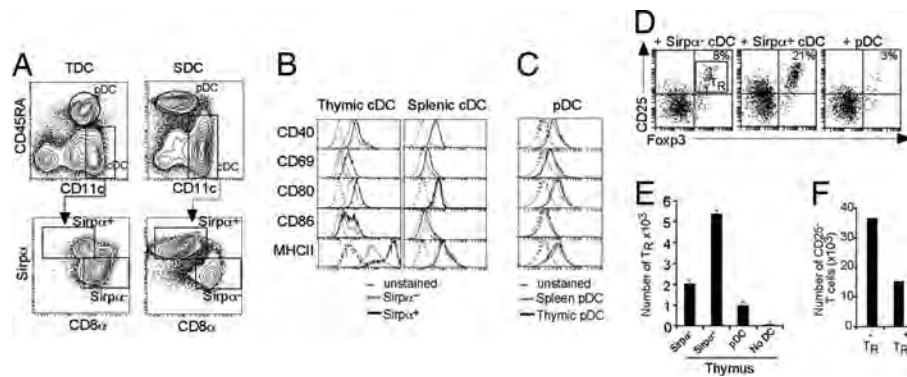


Fig. 3. $\text{Sirp}\alpha^+$ TcDCs are more mature and efficiently induce T_R *in vitro*. (A) Enriched TDCs and SDCs were segregated into pDCs ($\text{CD11c}^{\text{int}}\text{CD45RA}^+$) and cDCs (CD11c^+ CD45RA^-) and further segregated into $\text{CD8}^{\text{lo}}\text{Sirp}\alpha^+$ and $\text{CD8}^{\text{hi}}\text{Sirp}^-$ cDCs. Thymic and splenic $\text{Sirp}\alpha^+$ and $\text{Sirp}\alpha^-$ cDCs (B) and thymic and splenic pDCs (C) were analyzed for the expression of costimulatory molecules, CD69, and MHCII. (D) T_R ($\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$) induction by TDC subsets in the cocultures of TDCs and $\text{CD4}^+\text{CD25}^-$ thymocytes for 5 days. Data shown are representative of six experiments. (E) The number of T_R s induced was determined by flow cytometric analysis of the cultured cells of D using calibration beads ($n = 6$) (error bars, \pm SD). (F) Suppressive capacity of T_R s induced in cultures by $\text{Sirp}\alpha^+$ TcDCs. *In vitro*-derived T_R s were sorted as $\text{CD4}^+\text{CD25}^+\text{CD62L}^+$ and subject to a suppression assay as per Fig. 1F. The number of proliferating CFSE-labeled $\text{CD4}^+\text{CD25}^-$ T cells was determined 3 days later ($n = 2$).

$\text{Sirp}\alpha^+$ TcDCs Are More Mature in Surface Phenotype Than the $\text{Sirp}\alpha^-$ TcDCs. Because TDCs were involved in T_R generation and negative selection, we investigated the contribution of the TDC subtypes to these processes. We compared TDCs for expression of MHCII and costimulatory molecules, because these are important in T_R 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 $\text{Sirp}\alpha^+\text{CD8}^{\text{lo}}$ and $\text{Sirp}\alpha^-\text{CD8}^{\text{hi}}$ cDCs (21) (Fig. 3A). Strikingly, the $\text{Sirp}\alpha^+$ TcDCs expressed higher levels of MHCII and CD86 and slightly increased levels of the activation marker CD69 and the costimulatory molecules CD40 and CD80 compared with $\text{Sirp}\alpha^-$ 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, $\text{Sirp}\alpha^+$ TcDCs are phenotypically more “mature” than other TDC subtypes.

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

T_R generation *in vitro* was thymus specific. When TDCs were cultured with splenic $\text{CD4}^+\text{CD25}^-$ naive T cells rather than thymic $\text{CD4}^+\text{CD25}^-$ T cells, no T_R induction was observed (Fig. S2C), even in the presence of T-cell activation and proliferation (Fig.

S2D). Conversely, when SDCs were cocultured with thymic $\text{CD4}^+\text{CD25}^-$ T cells, few T_R s were generated (data not shown).

To test the function of *in vitro*-derived T_R s, T_R s were sorted as $\text{CD4}^+\text{CD25}^+\text{CD62L}^+$ cells and used in a T_R suppression assay. CD62L was included as a marker to exclude activated T cells. *In vitro* derived T_R s were able to suppress T-cell proliferation (Fig. 3F).

BM-derived cells that express MHCII within the thymus include B cells and macrophages. To exclude the possibility of T_R induction by those cells, the same coculture method was used. We demonstrated that the T_R induction capacities of both of these cell types were negligible (Fig. S2E).

$\text{Sirp}\alpha^+$ 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 $\text{Sirp}\alpha^+$ TcDCs in inducing T_R s. The expression of the genes encoding six chemokines known to be involved in thymocyte differentiation was examined by real-time (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. S3A). In contrast, *CCL17* and *CCL22* were expressed at very high levels only by the $\text{Sirp}\alpha^+$ TcDCs (Fig. S3A). The expression of *CCL22* by the $\text{Sirp}\alpha^+$ 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 $\text{Sirp}\alpha^+$ TcDC cultures showed the greatest capacity to attract CD4^+ thymocytes (Fig. S3D). Thus, the $\text{Sirp}\alpha^+$ TcDCs, through their chemokine production, have a special capacity to attract newly formed CD4^+ T cells.

$\text{CD11c}^+\text{Sirp}\alpha^+\text{CD11b}^+$ 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 $^- \text{Thy-1}^{\text{lo}}\text{c-kit}^+$) that have DC po-

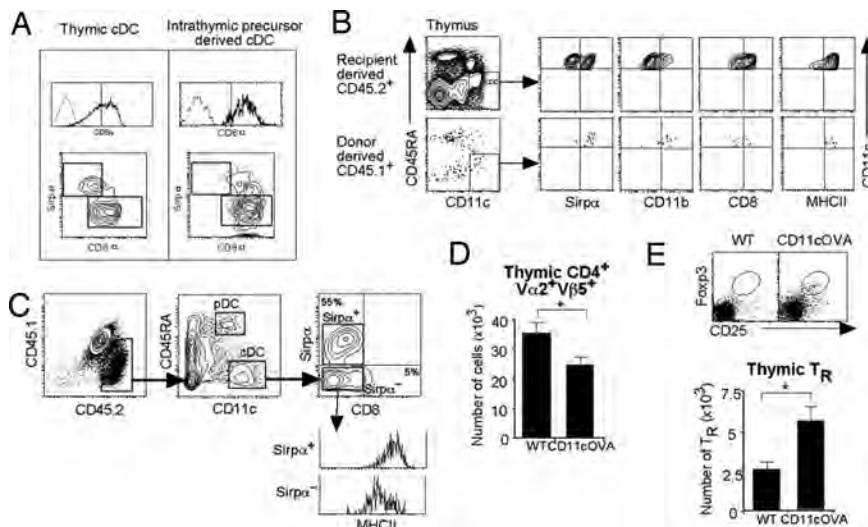


Fig. 4. $\text{Sirp}\alpha^+$ TcDCs originate from peripheral blood and can migrate into the thymus. (A) DC generation from purified Lineage⁻Thy-1^{lo}c-kit⁺ intrathymic precursors (CD45.2) was analyzed 2 weeks after precursor transfer. The intrathymic precursor-derived cDCs were mainly CD8⁺ $\text{Sirp}\alpha^-$ (Right). A representative contour plot of the normal TcDC subsets is shown (Left) for comparison. (B) White blood cells (20×10^6) from CD45.1 mice were transferred i.v. into nonirradiated CD45.2 recipients. The phenotype of donor-derived cells in the thymus of recipients was determined 3 days later by gating for CD45.1⁺CD11c⁺CD45RA^{lo} cDCs. Expression of $\text{Sirp}\alpha$, 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⁻ cDCs 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 $\text{Sirp}\alpha$ was determined. The level of expression of MHCII was determined on $\text{Sirp}\alpha^-$ and $\text{Sirp}\alpha^+$ cDCs. (D) The total number of CD45.1⁺CD4⁺V α 2⁺V β 5⁺ cells (OTII) was calculated in OTII lobes grafted into WT or CD11cOVA tg recipients. Data are the mean of three independent experiments (error bars, \pm SD) ($n = 11$ –21). *, $P < 0.05$. (E) CD45.1⁺CD4⁺V α 2⁺V β 5⁺ 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⁺V α 2⁺V β 5⁺CD25⁺Foxp3⁺ T_Rs was calculated. Data are the mean of three independent experiments (error bars, \pm SD) ($n = 11$ –21). *, $P < 0.05$.

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⁺ $\text{Sirp}\alpha^-$ (Fig. 4A).

In contrast, the CD8⁻CD11b⁺ TDC subset has been shown to migrate in parabiotic mice from the circulation into the thymus of the conjoined mouse (24). To determine whether the $\text{Sirp}\alpha^+$ TcDCs 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 $\text{Sirp}\alpha^+$ CD11b⁺ (Fig. S4). 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. Donor-derived cells made up 0.1% of total cells in the recipient thymus, and of these, 10% were CD11c⁺CD45RA⁻ cDCs. These cDCs were all $\text{Sirp}\alpha^+$ CD11b⁺CD8^{lo}MHCII^{hi} (Fig. 4B), correlating with DCs found circulating in the blood.

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 $\text{Sirp}\alpha^+$ cDCs ($80 \pm 5\%$; 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, $54 \pm 6\%$ were mature MHCII^{hi}CD8⁻ $\text{Sirp}\alpha^+$ cDCs, $4 \pm 1\%$ were mature CD8⁺ $\text{Sirp}\alpha^-$ cDCs, and the remaining were MHCII^{lo/int}CD8⁻ $\text{Sirp}\alpha^-$, the precursors of CD8⁺ $\text{Sirp}\alpha^-$ cDCs (Fig. 4C). The latter two populations represented newly formed cells derived from recipient BM progenitors that had seeded the thymic grafts.

Thymocyte populations were analyzed by flow cytometry. The number of CD45.1⁺OTII⁺CD4⁺V α 2⁺V β 5⁺ T cells was reduced in lobes grafted into CD11cOVA tg mice compared with controls (Fig. 4D), whereas the number of CD45.1⁺V α 2⁻V β 5⁻CD4⁺ was similar in both groups (data not shown), suggesting that antigen-specific negative selection of OTII⁺ T cells was occurring. In addition, a more than twofold increase in the number of OTII⁺Foxp3⁺ T_Rs 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 T_R development.

Discussion

The present study demonstrates a role for mouse TDCs in T_R differentiation as well as negative selection. In the absence of a MHCII-expressing hemopoietic compartment, we found a 30% reduction in the total number of polyclonal T_Rs 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 T_R generation and negative selection of CD4 T cells in a steady-state mouse. In addition, a 50% reduction in T_R numbers was observed when the hemopoietic compartment lacked expression of CD80 and CD86. Although

these BM chimeras indicated that a BM-derived cell was important for T_R induction, the *in vitro* coculture system indicated that only TDCs, and not B cells and macrophages, were efficient in inducing T_{RS} . Thus, taken together, it appears that TDCs are the major hemopoietic cells that contribute significantly to T_R generation and negative selection of $CD4^+$ T cells *in vivo*. Previous studies have discounted a nonredundant role for DCs in T_R 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 $CD4^+$ thymocyte cell numbers were observed in previous reports (41). We also see similar results in $MHCII^{-/-}$ 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 $CD4^+$ T cells in the thymus has masked the changes in T_R numbers.

Apart from the issue of their quantitative contribution to the total T_R population, our results now demonstrate that TDCs can induce Ag-specific T_R .

Three types of DCs are found in the mouse thymus (pDCs, $Sirp\alpha^+ CD8^+$ TcDCs, and $Sirp\alpha^+ CD8^-$ TcDCs), and these have counterparts in the human thymus (43). We show that the minor $Sirp\alpha^+$ TcDC subset is much more efficient than the other DCs at polyclonal T_R induction *in vitro*.

Why would the $Sirp\alpha^+$ TcDCs be more efficient in T_R generation? First, the $Sirp\alpha^+$ TcDCs are more mature, in terms of expression of MHCII and costimulatory molecule, than the other TDCs, consistent with the phenotype of migratory DCs. This may enable them to interact more efficiently with the $CD4^+$ thymocytes. Second, the $Sirp\alpha^+$ cDCs are more efficient at presentation of antigens on MHCII than the $CD8^+$ cDCs (44–46). Finally, we found that the $Sirp\alpha^+$ TcDCs express high levels of *CCL17* and *CCL22*, and this may facilitate an interaction with the $CD4^+$ thymocytes expressing high levels of *CCR4*. In a cell migration assay, $CD4^+$ thymocytes preferentially migrated toward supernatants from the $Sirp\alpha^+$ TcDCs. This provides a mechanism by which rare antigen-specific thymocytes can encounter their cognate antigen with a higher frequency.

Previous studies showed that migratory DC can induce negative selection of T cells specific for a peripherally expressed antigen (10). We now add to this picture by demonstrating that $Sirp\alpha^+$ cDCs or their immediate precursors present in blood migrate into the thymus and induce both negative selection and T_R development.

Would this process be detrimental to the host during a viral infection? Viral antigens in the periphery ferried to the thymus may induce T_{RS} , which could induce tolerance to the virus and potentially jeopardize a memory response. It is possible that thymic homing receptors or lymphoid egress receptors are down-regulated in DCs that have been activated by virus infection. Indeed, activated T cells down-regulate the egress receptor sphingosine 1-phosphate receptor-1, leading to retention of T cells in the lymphoid tissues (47). Whether this also occurs in activated DCs would be an interesting question to address in future studies.

In summary, we demonstrate that thymic DCs contribute to T_R induction *in vivo*. More significantly, we show that peripheral DCs can migrate into the thymus, where they induce the development of T_{RS} and the deletion of self-reactive $CD4^+$ thymocytes. Based on these observations, we propose a mechanism by which central tolerance to peripherally expressed antigens is induced by migrating DCs, a mechanism additional to the ectopic expression of peripheral antigens by mTECs.

Materials and Methods

Mice. All mice were bred under specific pathogen-free conditions. $B7^{-/-}$ 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. B6 $CD45.1$ mice 10 weeks of age were used as BM recipients. The mouse strains used included OTII tg ($CD4^+$ T cells expressing the TCR specific for MHCII-restricted Ova peptide) (48) on a B6, $CD45.1$, or $Rag2^{-/-}$ background; $IA/IA^{-/-}$ ($MHCII^{-/-}$) (49); $B7^{-/-}$ (50); and $CD11cOVA$ tg mice that express membrane-bound ovalbumin (amino acids 323–339) under control of the $CD11c$ promoter (29, 51).

BM Chimeras. $CD45.1$ recipient mice were lethally irradiated with two doses of 5.5 Gy (3 h apart) and then received 5×10^6 $CD45.2$ donor BM cells i.v. from B6 or $MHCII^{-/-}$ mice or from $Rag2^{-/-}$ OTII/ $CD11cOVA$ double-tg mice. For $B7^{-/-}$ chimeras, $CD45.1$ recipient mice were lethally irradiated with 8.0 Gy of total body irradiation. A total of 5×10^6 T-cell depleted B6 WT or $B7^{-/-}$ 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 Thymocytes. Details can be found in [SI Experimental Procedures](#).

Carboxyfluorescein Succinimidyl 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_R Suppression Assay. Details can be found in [Supplementary Experimental Procedures](#).

Generation of T_{RS} In Vitro. *In vitro* T_R induction assays were performed in triplicate in a round-bottom 96-well plate with 1×10^4 sorted thymic or splenic DC subsets from $CD45.2$ mice and 2×10^4 sorted $CD4^+ CD25^-$ thymocytes from $CD45.1$ mice, cultured together with an optimal concentration of IL-7 for 5 days. T_{RS} were assessed by staining for $CD45.1$ (A201.1), $CD4$, $CD25$, and $Foxp3$. When GFP- $Foxp3$ mice were used as the $CD4^+$ thymocyte source, thymocytes were sorted as $CD4^+ CD25^- Foxp3^-$ (excluding all $Foxp3^+$ cells). IL-7 used was from the culture supernatant of J558L cells transfected with the murine IL-7 cDNA in the BMG *neo* vector (55). To determine the optimal concentration of the IL-7 supernatant, the supernatant was titrated on IL-7-dependent pro-B cells (37).

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 (5×10^5 in 600 μ l) were cultured in a 24-well plate for 3 h. The supernatant was removed and placed in the base of transwell chambers (5.0- μ M pore size; COSTAR). Sorted $CD4^+ CD25^-$ thymocytes (2×10^5) 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

Correction for “Chemical Ecology Special Feature: The evolution of gene collectives: How natural selection drives chemical innovation,” by Michael A. Fischbach, Christopher T. Walsh, and Jon Clardy, which appeared in issue 12, March 25, 2008, of *Proc Natl Acad Sci USA* (105:4601–4608; first published January 23, 2008; 10.1073/pnas.0709132105).

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.

113. Balado M, Osorio CR, Lemos ML (2006) A gene cluster involved in the biosynthesis of vancomycin, a chromosome-encoded siderophore produced by *Vibrio anguillarum*. *Microbiology* 152:3517–3528.

www.pnas.org/cgi/doi/10.1073/pnas.0812594106

DEVELOPMENTAL BIOLOGY

Correction for “Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo,” by W. A. Kues, S. Sudheer, D. Herrmann, J. W. Carnwath, V. Havlicek, U. Besenfelder, H. Lehrach, J. Adjaye, and H. Niemann, which appeared in issue 50, December 16, 2008, of *Proc Natl Acad Sci USA* (105:19768–19773; first published December 8, 2008; 10.1073/pnas.0805616105).

The authors note that due 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-Wieselburg, 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

Correction for “HDAC2 blockade by nitric oxide and histone deacetylase inhibitors reveals a common target in Duchenne muscular dystrophy treatment,” by Claudia Colussi, Chiara Mozzetta, Aymone Gurtner, Barbara Illi, Jessica Rosati, Stefania Straino, Gianluca Ragone, Mario Pescatori, Germana Zaccagnini, Annalisa Antonini, Giulia Minetti, Fabio Martelli, Giulia Piaggio, Paola Gallinari, Christian Steinkulher, Emilio Clementi, Carmela Dell’Aversana, Lucia Altucci, Antonello Mai, Maurizio C. Capogrossi, Pier Lorenzo Puri, and Carlo Gaetano, which appeared in issue 49, December 9, 2008, of *Proc Natl Acad Sci USA* (105:19183–19187; first published December 1, 2008; 10.1073/pnas.0805514105).

The authors note that the author name **Christian Steinkulher** should have appeared as **Christian Steinkuhler**. 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, Annalisa Antonini, Giulia Minetti, Fabio Martelli, Giulia Piaggio, Paola Gallinari, Christian Steinkuhler, Emilio Clementi, Carmela Dell’Aversana, Lucia Altucci, Antonello Mai, Maurizio C. Capogrossi, Pier Lorenzo Puri, and Carlo Gaetano

www.pnas.org/cgi/doi/10.1073/pnas.0813311106

IMMUNOLOGY

Correction for “Dendritic cells in the thymus contribute to T-regulatory cell induction,” by Anna I. Proietto, Serani van Dommelen, Penghui Zhou, Alexandra Rizzitelli, Angela D’Amico, Raymond J. Steptoe, Shalin H. Naik, Mireille H. Lahoud, Yang Liu, Pan Zheng, Ken Shortman, and Li Wu, which appeared in issue 50, December 16, 2008, of *Proc Natl Acad Sci USA* (105:19869–19874; first published December 10, 2008; 10.1073/pnas.0810268105).

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, CD8^{lo}Sirpα^{hi/+} (CD8^{lo}Sirpα⁺) and CD8^{hi}Sirpα^{lo/-} (CD8^{lo}Sirpα⁻), which have different origins. We found that the CD8^{hi}Sirpα⁺ 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, CD8^{lo}Sirpα^{hi/+} (CD8^{lo}Sirpα⁺) and CD8^{hi}Sirpα^{lo/-} (CD8^{hi}Sirpα⁻), which have different origins. We found that the CD8^{lo}Sirpα⁺ DCs represent a conventional DC subset that originates from the blood and migrates into the thymus.”

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