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13. ABSTRACT (Maximum 200 Words)

This is the first annual report on the grant "Rescuing high avidity T cells for prostate cancer immunotherapy". The purpose of the grant proposal is to rescue high avidity tumor-antigen specific T cells that can respond effectively to prostate cancer cells and delay the development of prostate cancer in the TRAMP mouse model. The innovative idea is based on the hypothesis that blockade of the T cell costimulatory pathway in adults will inhibit the deletion of high avidity tumor antigen specific T cells. We have proposed three specific aims in the grant, (1). Identify the cells in thymus that express peripheral tumor antigen to induce clonal deletion of tumor antigen reactive T cells. (2). Examine whether anti-B7 antibody treatment in TRAMP mice can rescue the tumor-antigen specific T cells that are otherwise deleted. (3). Determine the thymic function in prostate cancer patients undergoing hormonal therapy. In the past funding period, we have completed the specific aim 1 and obtained promising preliminary data in specific aim 2. Through bone marrow radiation chimera mice experiments that we proposed in specific aim 1, we have shown that expression of the self antigen in thymic epithelial cells is both necessary and sufficient to induce clonal deletion. Surprisingly, while bone marrow-derived peripheral antigen expressing cells failed to induce clonal deletion, they did cause the activation-induced cell death of autoreactive cells in the secondary lymphoid organs. Thus, the BM-derived PAE have a distinct function in the maintenance of tolerance to tissue-specific antigens.

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(4) Introduction

Most tumor antigens have the same sequences as the endogenous genes. In addition to their over-expression in the tumors, essentially all of these antigens are expressed in some normal tissues, which is analogous to the so-called peripheral antigen. Most recent studies showed that many of the peripheral antigens are expressed in specialized cells in the thymus, and can induce central tolerance of their specific T cells. However, it is not clear whether mechanisms responsible for tolerizing peripheral antigen in the thymus are responsible for tolerizing potential cancer-specific T cells. We have recently taken a double-transgenic approach to study the mechanism of immune tolerance to a transgenic antigen, SV40-large T antigen (Tag), in the transgenic adenocarcinoma in mouse prostate (TRAMP) mice. We have showed that central tolerance plays an important role in immune tolerance to TRAMP mice. In addition, we have published strong evidence that blockage of T cell costimulatory pathway can break the central tolerance for a large array of autoreactive T cells. In this proposal, we have hypothesized that blockade of the T cell costimulatory pathway in adults would inhibit the deletion of high avidity tumor antigen specific T cells. The rescued high avidity tumor-antigen specific T cells can respond effectively to prostate cancer cells and delay the development of prostate cancer in the TRAMP model. We proposed to carry out the following specific aims. (1). Identify the cells in thymus that express peripheral tumor antigen to induce clonal deletion of tumor antigen reactive T cells. (2). Examine whether anti-B7 antibody treatment in TRAMP mice can rescue the tumor-antigen specific T cells that are otherwise deleted. (3). Determine the thymic function in prostate cancer patients undergoing hormonal therapy.

During the first funding period, we have completed the studies outlined in specific aim 1. We have generated the bone marrow (BM) radiation chimera mice to study the existence, contribution and mechanism of the hematopoietic peripheral antigen expressing (PAE) cells in tolerance to tissue-specific antigens. Our results revealed that BM-derived PAE exist in both central and secondary lymphoid organs and that the expression of peripheral antigens in the BM-derived cells does not correlate with *aire* expression. Using double transgenic mice expressing TCR specific for a model antigen expressed under the control of the prostate-specific promoter, we show that expression of the self antigen in PAE of non-hematopoietic origin is both necessary and sufficient to induce clonal deletion. Surprisingly, while BM-derived PAE failed to induce clonal deletion, they did cause the activation-induced cell death of autoreactive cells in the secondary lymphoid organs. Thus, the BM-derived PAE have a distinct function in the maintenance of tolerance to tissue-specific antigens. Meanwhile, we have also generated promising preliminary data in specific aim 2. We have shown that anti-B7 antibody treatment in adult TRAMP mice prolonged the mice survival for more than 10 weeks. We will continue to study the molecular mechanism that leads to the success of the antibody treatment as outlined in specific aim 2.

(5) Body of Annual Report

Task 1. *What are the cells in thymus that express tumor-associated antigen that induce clonal deletion of potentially tumor antigen reactive T cells? (Month 1-18).* (Completed).

a. To breed TRAMP mice with TCR transgenic mice TG-B to produce 4 different types of F1 mice: WT F1 and Tag^+/TCR^- F1 (to be used as recipient mice); Tag^-/TCR^+ F1 and Tag^+/TCR^+ F1 (to be used as bone marrow donor mice). 10-15 F1 mice for each group are needed. (Months 1-12). (Completed).

b. To perform the bone marrow irradiation chimera experiments. Each animal experiment cycle requires 12 weeks. Three independent experiments will be performed. Average 5 mice per group with 4 groups in each experiment. (Months 4-15). (Completed).

c. To perform in vitro experiments, such as immunofluorescent study, Immunohistochemical studies, and lymphocyte functional assays (Months 13-18). (Completed).

d. To perform the modified bone marrow irradiation chimera experiment involving surviving surgery (thymectomy and thymus engrafting). Each animal experiment cycle requires 16 weeks. Three independent experiments will be performed. Average 5 mice per group with 4 groups in each experiment (Months 7-18). (These were alternative experiments to compliment the Task 1-b and 1-c. Since the bone marrow chimera experiments gave us conclusive results, it is not necessary for us to perform Task 1-d).

We have completed the Task 1 and the results have been summarized in a manuscript that submitted to European Journal of Immunology. The manuscript "Expression of tissue-specific autoantigens in the hematopoietic cells leads to activation-induced cell death of autoreactive T cells in the secondary lymphoid organs" is attached as Appendix 1.

During the process, we had made some unexpected observations related to the function of costimulatory molecules in early stages of T cell development. The manuscript has submitted to the Journal of Immunology. We were asked to submit a revision with encouraging positive reviews. We are currently performing additional experiments that requested by the reviewers. The manuscript "B7-CD28 Interaction Promotes Proliferation and Survival but Suppresses Differentiation of $CD4^-CD8^-$ T Cells in the Thymus" is attached as Appendix 2.

Task 2. ***Will anti-B7 antibody treatment in adult animal rescue the tumor-antigen specific T cells that are otherwise deleted? (Month 1-36).*** **(In progress).**

a. To test the effect of anti-B7 antibody treatment on the clonal deletion of SV40 Tag specific transgenic T cells in F1 Tag^+/TCR^+ double transgenic mice. Each animal experiment cycle requires 16 weeks. Average 5 mice per group with 2 groups in each experiment. 4 different starting ages will be used. Three independent experiments for each age will be performed. (Months 1-18). (In progress).

b. Test the effect of anti-B7 antibody treatment on the TRAMP mice with polyclonal T cell repertoire. Each animal experiment cycle requires 16 weeks.

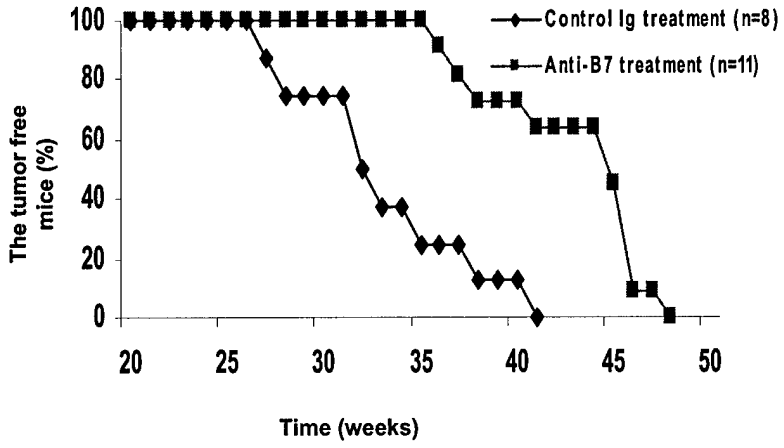
Average 5 mice per group with 2 groups in each experiment. 4 different starting ages will be used. Three independent experiments for each age will be performed. (Months 7-24). (In progress).

c. *We will observe the tumor development in TRAMP mice to test whether the transient block of T cell development by anti-B7 antibody treatment can delay tumor progression. (Months 7-36). (In progress).*

d. *We will determine the time window that optimizes the effect of anti-B7 antibody treatment in combination with radiotherapy and chemotherapy (Months 25-36).*

We have generated very promising preliminary data in Task 2. We have treated the TRAMP mice with either control Ig (100 μg Hamster IgG and 100 μg Rat IgG, in a volume of 200μl, injected intraperitoneally, treatment started at 12 weeks, twice a week, total 5 injections) or anti-B7 antibodies (anti-B7-1, 100 μg, anti-B7-2, 100 μg, in a volume of 200μl, injected intraperitoneally, treatment started at 12 weeks, twice a week, total 5 injections). We continued to monitor the mice with tumor incidence. As shown in Fig. 1, anti-B7 treatment drastically delayed the development of prostate cancer in TRAMP mice. In the next funding period, we will focus on Specific Aim 2 to study the molecular mechanism of anti-B7 treatment.

Fig. 1. The adult anti-B7 treatment delays the development of prostate cancer in TRAMP mice



Task 3. To examine the thymic function in human prostate cancer patients (Months 1-36). **(In Progress).**

a. *Obtain prostate cancer patients' consents and collect clinical information (Month 1-36).*

b. Coordinate with urologists and establish schedule to collect blood samples from prostate cancer patients undergo different therapy modules (Month 4-36).

c. Establish reliable experimental procedure to isolate different subsets of lymphocytes from peripheral blood mononuclear cell fractions. Identify reliable PCR condition to detect the levels of TRECs from T lymphocytes. To set up real-time PCR and QC-PCR protocol to get good reproducibility. (Month 1-6).

d. To perform QC-PCR to detect TRECs from lymphocytes from prostate cancer patients. (Month 7-36).

e. To perform statistical analysis on data (Month 25-36).

Due to the prolonged period of human subject protocol approval process, we just started to focus our effort on establishing the experimental procedure to detect TRECs from lymphocytes using real-time PCR.

(6) Key Research Accomplishments

- We have generated the bone marrow (BM) radiation chimera mice to study the existence, contribution and mechanism of the hematopoietic peripheral antigen expressing (PAE) cells in tolerance to tissue-specific antigens.
- We have shown that BM-derived PAE exist in both central and secondary lymphoid organs.
- We have examined the expression of peripheral antigens in the BM-derived cells and demonstrated that its expression does not correlate with *aire* expression.
- Using double transgenic mice expressing TCR specific for a model antigen expressed under the control of the prostate-specific promoter, we have shown that expression of the self antigen in PAE of non-hematopoietic origin is both necessary and sufficient to induce clonal deletion.
- We have demonstrated that while BM-derived PAE failed to induce clonal deletion, they caused the activation-induced cell death of autoreactive cells in the secondary lymphoid organs. Thus, the BM-derived PAE have a distinct function in the maintenance of tolerance to tissue-specific antigens.
- We have shown that anti-B7 antibody treatment in adult TRAMP mice prolonged the mice survival for more than 10 weeks.

(7) Reportable Outcomes:

1 Tianyu Yang, Beth McNally, Soldano Ferrone, Yang Liu and **Pan Zheng**. 2003. A single nucleotide deletion leads to rapid degradation of *TAP-1* mRNA in a melanoma cell line. *J. Biol. Chem.* 278:15291-6

2 Huiming Zhang, Jonathan Melamed, Ping Wei, Karen Cox, Wendy Frankel, Robert R. Bahnson, Nikki Robinson, Ron Pyka, Yang Liu and **Pan Zheng.** 2003. Concordant down-regulation in expression of proto-oncogene PML and the major histocompatibility antigen HLA class I in high grade prostate cancer. *Cancer Immunity* 2003; 3:2.

3 Yang Liu, Jian Xin Gao, Xue-Feng Bai, Xingluo Liu, Huiming Zhang and **Pan Zheng**. 2003. Costimulatory molecules in T cell development, activation and effector function: similar activity, opposite consequences. In "B7-CD28 Family Molecules in Immune Responses" Ed. L. Chen.. Landes Publishing House, Houston, TX.

4 Yang Liu, Jian-Xin Gao, Huiming Zhang, Xue-Feng Bai, Jing Wen, Xincheng Zheng Jinqing Liu, **Pan Zheng.** 2003 Why are mice with targeted mutation of costimulatory molecules prone to autoimmune attack? *Ann. NY. Acad. Sci.* 987:307-308.

5 **Pan Zheng,** Xincheng Zheng, Jian-Xin Gao, Huiming Zhang, Terrence Geiger, Yang Liu. 2003. Central tolerance in a prostate cancer model TRAMP mouse. *Ann. NY. Acad. Sci.* 987:322-323.

6 Xincheng Zheng, Lijie Yin, Yang Liu and **Pan Zheng.** 2004. Hematopoietic peripheral antigen expressing cells impose activation-induced cell death of autoreactive T cells in the secondary lymphoid organs. Submitted to *Euro. J. Immunol.* (Appendix 1).

7. Xincheng Zheng, Jian-Xin Gao, Xing Chang, Yin Wang, Yan Liu, Jin Wen, Jian Jiang, Yang Liu and **Pan Zheng.** 2004. B7-CD28 Interaction Promotes Proliferation and Survival but Suppresses Differentiation of $CD4^-CD8^-$ T Cells in the Thymus. *J. Immunol.* (under revision). (Appendix 2).

(8) Conclusions:

In summary, we have made important progress during the first funding period to achieve our goal. We have followed the Statement of Work closely. We have completed the Task 1 and made important and promising progress in Task 2.

Our progress is fundamentally important for future development of new ways for prostate cancer immunotherapy. Various ideas and methods have been developed to augment anti-tumor immunity. Most of the therapies aim at activation of T cells that are already present in peripheral T cell repertoire. However, our preliminary data provide definitive evidence that high avidity tumor antigen specific T cells are deleted through central tolerance. As such, the existing approaches aim at expanding what is likely to be low avidity tumor reactive T cells. This proposal will explore an innovative idea that high avidity tumor antigen specific T cells can be rescued from clonal deletion by blockade of T cell costimulatory pathway. We anticipate that the rescued T cells will be much more powerful in the combat against tumor. The relevance of our study is further increased by two important factors. First, utilization of spontaneous prostate cancer model will allow us to build a solid experimental foundation for a novel immunotherapy of prostate cancer. Second, the widely used hormonal therapy of prostate-cancer patients may create a new wave of T cell thymopoiesis among them. The random gene re-arrangement of TCR gene during the process may provide a new source of high-avidity prostate cancer-specific T cells to be rescued by our novel approach.

(9) References:

None.

Expression of tissue-specific autoantigens in the hematopoietic cells leads to activation-induced cell death of autoreactive T cells in the secondary lymphoid organs

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Summary

Many tissue-specific antigens are expressed in specialized cells, called peripheral antigen-expressing cells (PAE), in the thymus, and induce central tolerances. While the thymic medullary epithelial cells are the prototypic PAE that express peripheral antigens in an *aire*-dependent mechanism, some studies also describe bone marrow (BM) derived dendritic cells (DC) and macrophages as PAE in both the thymus and in the secondary lymphoid organs. However, their role in the tolerance to tissue-specific antigens is not elucidated. Here we use BM radiation chimera mice to study the existence, contribution and mechanism of the hematopoietic PAE in tolerance to tissue-specific antigens. Our results reveal that BM-derived PAE exist in both central and secondary lymphoid organs and that the expression of peripheral antigens in the BM-derived cells does not correlate with *aire* expression. Using double transgenic mice expressing TCR specific for a model antigen expressed under the control of the prostate-specific promoter, we show that expression of the self antigen in PAE of non-hematopoietic origin is both necessary and sufficient to induce clonal deletion. Surprisingly, while BM-derived PAE failed to induce clonal deletion, they did cause the activation-induced cell death of autoreactive cells in the secondary lymphoid organs. Thus, the BM-derived PAE have a distinct function in the maintenance of tolerance to tissue-specific antigens.

Introduction

The expression of peripheral antigen in the thymus by the PAE serves to project a "shadow of immunological self" in the thymus [1]. By constitutively expressing antigens assumed to be limited in the peripheral organs, PAE ensures that tolerance to tissue-specific antigens be imposed during T cell development and thus reassures the importance of central tolerance in self-nonself discrimination [2-10].

Recent studies established medullar thymic epithelial cells (mTECs) as the prototypic PAE cell phenotype in the thymus [6-9]. The expression of a diverse set of genes that encompassed cell surface proteins, enzymes, hormones, structural proteins, which are all restricted tissue distribution has been detected in mTECs [9, 11, 12]. The expression of these genes correlates to the risk of autoimmunity in experimental models of autoimmune diseases. More recently, it has been demonstrated that mutation of *aire*, a nuclear protein with preferential expression in the mTEC, abrogates the expression of a large array of the peripheral antigens in the thymus [1] and prevents deletion of T cells specific for antigens expressed under the control of tissue-specific promoters [13].

Meanwhile, it has also been reported that dendritic cells (DC) and macrophages are possible PAE candidates. In support of this contention are thymus cell fraction studies [3, 4], which show that thymic insulin-expressing cells segregate into a low-density fraction that is enriched with DC and macrophages. In addition to that, several reports demonstrated the co-localization of insulin and other pancreatic hormones with markers of the DC and macrophage-lineage in the murine and human thymus [5, 10] .

The function and mechanisms of these putative PAE in self-tolerance, however, have not been systematically analyzed.

In the analysis of the mechanism of immune tolerance in the transgenic mouse prostate cancer model (TRAMP), we have demonstrated that in addition to non-DC, DC constitutes a substantial portion of thymic PAE expressing the SV40 large T antigen under the control of a prostate-specific promoter [14] . While the PAE in TRAMP mice caused a complete deletion of the SV40 large T antigen-specific T cells, the subset of PAE responsible for the clonal deletion was not identified. Here we used irradiation chimera mice to demonstrate that radio-resistant PAE is necessary and sufficient to cause clonal deletion of the Tag-specific T cells. Surprisingly, bone-marrow-derived PAE cause activation-induced cell death of self-reactive T cells in the spleen. Our results established novel function and mechanisms of the bone-marrow derived PAE in the induction of tolerance of T cells to tissue-specific antigens in the secondary lymphoid organs.

Results

Radio-resistant PAE is necessary and sufficient to induce clonal deletion in the thymus

TG-B mice expressed, at high levels, a T cell receptor from a $CD8^+$ cytotoxic T cell clone that recognized SV40 Tag presented by MHC class I molecule $H\text{-}2K^k$ [15]. We recently demonstrated that the transgenic T cells are deleted in TRAMP/TG-B double transgenic ($H\text{-}2^{bxk}$) mice due to PAE in the thymus [14]. In this study, the chimera mice were produced by transferring bone marrows from $TG\text{-}B^+$ mice with or without SV40 large T (Tag) transgene to lethally irradiated $TG\text{-}B^-$ mice with or without SV40 Tag transgene (Table.1). Except recipients in group I, all the other groups of chimera mice synthesized the SV40 Tag in the non-hematopoietic cells of the recipients and/or in the bone marrow-derived donor cells. To confirm that both radio-resistant recipient cells and bone-marrow derived donor cells express the Tag in the thymus, we used a previously described method [14], based on RT-PCR plus probing with products by Southern blot, to determine expression of Tag in the thymi of the chimera mice. As shown in Fig. 1, Tag mRNA was detectable in the thymi of groups II, III and IV chimera mice, although the amounts detected in group III was significantly lower than those in group II and IV. This result is consistent with our immunohistochemical analysis of SV40 Tag protein expression in the thymus [14], which revealed that while both DC and non-DC expressing Tag, most of the Tag-expressing cells lacked CD11c. As expected, no Tag mRNA was detected in group I thymi.

To analyze the functions of two different lineages of PAE in the thymus, we analyzed the fate of Tag-reactive T cells by flow cytometry. In comparison to group I, the

total thymocytes recovered from reconstituted thymi were also clearly reduced in groups II and IV, but not in group III (Table 1). Moreover, thymi from groups I and III had essentially identical subset distribution, while those from groups II and IV were depleted of $CD8^+CD4^-$ T cells (data not shown). Among the T cells that express high levels of transgenic TCRβ, the reduction of the mature antigen-specific T cells was even more pronounced (Fig. 2). While groups I and III had comparable numbers of antigen-specific T cells, a more than 5-fold reduction in the number of mature CD8 T cells was observed among groups II and IV. Since the common feature of groups II and IV is their shared origin of radio-resistant PAE, our results demonstrated that expression of Tag in the recipient thymic stoma cells is sufficient to induce clonal deletion. In addition, since no clonal deletion was observed in group III chimera that have bone marrow-derived PAE, expression of Tag in the recipient radio-resistant PAE is also necessary for the clonal deletion of autoreactive T cells.

Bone marrow-derived PAE caused activation-induced cell death of self-reactive T cells in the secondary lymphoid organs

As shown in Fig. 3, in comparison with group I of chimera, groups II and IV had a substantially reduced number of transgenic T cells in the spleen. This reduction roughly correlated to that found in the thymi (Fig. 2). Surprisingly, although group III had an essentially identical number of mature transgenic T cells in the thymi as that of group I (Fig. 2), the number of transgenic T cells from the spleen was approximately 3-5 fold less than that in group I (Fig. 3). These results suggest that bone marrow-derived PAE can reduce the number of autoreactive T cells in the secondary lymphoid organ.

The reduction of the number of T cells resulted in reduced response of T cells to in vitro stimulation by the cognate peptide. As shown in Fig. 4a, spleen cells from groups II and IV did not proliferate to the Tag peptide, while group III mice mounted a significant, although much reduced proliferation. After in vitro stimulation, potent CTL can be elicited from group I, but not group IV spleen cells (Fig. 4b). In most experiments, recall CTL response was not detectable in group II spleens, while a much reduced (about 100-fold less as judged by E/T ratio) CTL activity could be elicited from group III. In some experiments, however, low, but significant CTL response was detected in groups II and III. Thus, optimal tolerance to self antigens requires both lineages of PAE.

It has been demonstrated that host APC can cross-present tissue specific antigens and thereby cause activation-induced cell death (AICD) of T cells [16]. However, it has not been tested whether hematopoietic cells could express tissue-specific antigens and induce AICD in the secondary lymphoid organ. To determine if this is the case, we compared spleen cells in groups I and III for activation markers and signs of programmed cell death. As shown in Fig. 5a, the transgenic T cells from group III were clearly being stimulated as substantial proportions expressed CD69, CD25 and CD24, which are activation markers for T cells. Interestingly, the difference in down-regulation of memory marker CD62L was much less pronounced, which is consistent with the fact that activation of T cells in group III did not lead to stronger CTL recall response (Fig. 4b).

Instead of inducing strong memory markers, we observed that group III T cells in the spleen had elevated expression of Fas which was critical for activation-induced cell

death (Fig. 5b). Tunnel assay revealed about a 5-fold increase in the proportion of cells undergoing programmed cell death (Fig. 5c). This result demonstrated that PAE of hematopoietic origin induced activation-induced cell death of self-reactive T cells.

Peripheral antigen expression by PAE in the secondary lymphoid organs does not correlate with *aire* expression

It has been demonstrated that *aire* is preferentially expressed in the mTEC and functioned as a transcriptional regulator to control the peripheral organ specific antigen expression in the thymus [1, 13]. Interestingly, a significant expression of *aire* can be detected in the spleen, although the level is about 10% of what is found in the thymus [1]. To determine whether *aire* expression correlates with the synthesis of autoantigens in the secondary lymphoid organs, we compared $CD11c^+$, $CD11c^-$ and total spleen cells for the expression of *aire* and a panel of autoantigens that are found in the PAE in the thymus. As shown in Fig. 6, enrichment of $CD11c^+$ cells increased the *aire* mRNA by about 10-fold, while elimination of the $CD11c^+$ cells reduced the *aire* mRNA by more than 10-fold. Thus, DC are the primary *aire*-expressing cells in the spleen. SV40 Tag expression is neither enriched nor depleted in the $CD11c^+$ population. Of the three "organ-specific" autoantigens that are found in mTEC, but not in DC and macrophages in the thymus [17], insulin and cytochrome P450 1a2 mRNA were found at significant and comparable levels in total spleen cells, as well as $CD11c^+$ and $CD11c^-$ spleen cells. In contrast, GAD67 mRNA is barely detectable in $CD11c^+$ cells, and depletion of the $CD11c^+$ did not reduce GAD67 mRNA. These results make two points. First, expression of tissue-specific antigens in the spleen is not limited to the SV40 Tag

transgene. Second, the expression of the "organ-specific" autoantigens does not correlate with *aire* expression.

Discussion

Peripheral antigen expressing cells (PAE) in the thymus constitutively express antigens assumed to be limited to the peripheral organs [4]. Although thymic medullar epithelial cells are considered the major PAE in the thymus [7, 9], several groups have reported *de novo* synthesis of peripheral antigens in hematopoietic cells in the thymus [5, 10, 18]. However, the relative contribution of the two types of PAE in the thymus for clonal deletion in the thymus has not been addressed. Our previous study established that the TRAMP mice express prostate specific antigen in both lineages of PAE [14]. In order to identify which subset of PAE induces clonal deletion, we made bone marrow chimera mice that express the peripheral antigen in only one lineage. Using deletion of SV40 large T antigen-specific transgenic T cells as a basic readout, we demonstrated that expression of the peripheral antigens in the thymic epithelial cells is sufficient to induce clonal deletion. Since no clonal deletion is observed in mice that have bone-marrow derived PAE only, expression of peripheral antigens in the thymic epithelial cells is also necessary for clonal deletion. Given the fact that thymic mTEC is the major source of PAE in the thymus for the majority of antigens analyzed [9], our conclusion may be generally applicable to clonal deletion of tissue-specific antigens. This is also compatible with recent genetic data that the mutation of *AIRE*, which prevented the expression of many tissue-specific antigens in the mTEC [1], inhibits clonal deletion of tissue-specific T cells in the thymus [13].

In light of previous reports that the expression of minor H, allogeneic MHC and VSAg in either thymic epithelial cells or bone-marrow derived cells is sufficient to induce clonal deletion [19-22], it is surprising that expression of peripheral antigens by bone

marrow-derived cells in the thymus does not cause clonal deletion. Since our analysis indicates that the level of expression is lower among the hematopoietic APC (this study), and since the number of bone-marrow derived PAE is substantially lower than the thymic epithelial cells [14], the requirement for de novo synthesis on thymic stromal cells may be simply due to its more abundant expression of peripheral antigen.

Another important observation documented in this study relates to expression and function of PAE in the secondary lymphoid organs. Although the existence of PAE in secondary lymphoid organs has been suggested [10], it is unclear if the antigen expression is *aire*-dependent. Our data demonstrated that the expression of the peripheral antigens in the APC does not correlate with the level of expression of *aire.* As such, the expression is unlikely controlled by *aire*. Our data further demonstrated that the bone marrow-derived PAE reduces the number of autoreactive T cells in periphery and causes activation-induced cell death of self-reactive T cells in the secondary lymphoid organs. The localization of cells undergoing programmed cell death is consistent with the notion that PAE in the secondary lymphoid organs caused activation-induced cell death, although an imprinting by thymic hematopoietic PAE during T cell development cannot be ruled out at this stage. These two lines of evidence establish a novel function and mechanism of the bone-marrow derived PAE in the induction of tolerance of T cells to tissue-specific antigens. It is likely that this mechanism complements the previously established mechanism by which the host APC cross-present tissue antigen to induce activation-induced cell death [23, 24].

Taken together, we have demonstrated that two populations of PAE played distinct roles in the induction of tolerance of self-reactive T cells. The non-

hematopoietic PAE, presumably the medullar epithelial cells, induced clonal deletion in the thymus. Perhaps because of lower levels of expression, the hematopoietic cells are neither necessary nor sufficient to induce clonal deletion in the thymus. However, the hematopoietic PAE induces AICD in the secondary lymphoid organs. Since clonal deletion induced in the thymus is rarely complete under physiological conditions, optimal tolerance may require concerted actions of both populations of PAE.

Materials and methods

Experimental animals TRAMP mice expressing the SV40 Tag controlled by rat probasin regulatory elements in C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). TG-B mice were kindly sent by Dr. Geiger from St. Jude's Children's Hospital [25]. TRAMP and TG-B mice were bred at the animal facilities of the Ohio State University (Columbus, OH). Mice were typed for SV40 Tag or TCR expression by isolation of mouse tail genomic DNA. The PCR-based screening assays were described previously [14].

Generation of irradiation bone marrow chimera Four groups of chimera mice with different donor and recipient combinations from different TRAMP x TG-B ($H\text{-}2^{bxk}$) F1 phenotypes are presented in Table 1. Briefly, the lethally irradiated (1000 Rad) mice were reconstituted with bone marrow from femurs and tibias of the donor mice after the T cells were depleted with anti-CD4 (Gk1.5) and anti-CD8 (TIB210) monoclonal antibody. A total of $1.0x10^7$ T cell-depleted bone marrow cells were injected i.v. through tail vein to the recipient mouse. All experiments were performed after 8 weeks of bone marrow reconstitution.

Antibodies The fluorescence conjugated antibodies anti-CD4 (RM4.5), anti-CD8 (53-6.7), anti-Vβ8.1+8.2 (MR5-2), anti-CD25 (PC61), anti-HSA (M1/69), anti-CD69 (H1.2F3), anti-CD62L, anti-CD28 (37.51), anti-Fas Ligand (MFL3) and APO-DIRECT Kit were purchased from BD Pharmingen (San Diego, CA).

Peptide synthesis Peptides, SV40 Tag 560-568 SEFLLEKRI [14] and HSV gB peptide gB498-505: SSIEFARL [26], were synthesized by Research Genetics, Inc. (Huntsville, AL). The peptides were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and diluted in PBS or culture medium before use.

Proliferation of T cells to antigenic peptides and CTL assays T cell proliferation assay and CTL assay have been previously described [14].

Fractionation of splenic $CD11C^+$ and $CD11C^-$ cells and real-time PCR The splenic $CD11C^+$ and $CD11C^-$ cells were fractionated according to a protocol previously described [27]. Briefly, the collagenase (Sigma) solution (1mg/ml in 10mM Hepes-NaOH, pH 7.4) was injected into the spleen before the spleen was spliced and incubated with additional collagenase solution for 60 min at 37°C. The single cell suspension was obtained by passing through a steel mesh. The red blood cells were lysed with 1x lysis buffer (0.15M NH_4Cl, 1.0mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.4). The viable cells were used as total spleen cell population. The $CD11C^+$ and $CD11C^-$ cells were magnetically separated by CD11C Microbeads and LS^+ positive selection column according to manufacturer's protocol (Miltenyi Biotec Inc., Auburn, CA). Total RNA was extracted from total spleen cells, $CD11C^+$ and $CD11C^-$ cells. Total RNA (1 μg/sample) was pretreated with RNase-free DNase I before cDNA synthesis by using Superscriptase II and oligo(dT) (Invitrogen, Carlsbad, CA). The real time PCR was carried out in ABI PRISM 7700 Cycler (Applied Biosystems, Foster City, CA) by using QuantiTect SYBR green PCR kit (Qiagen) according to manufacturers' protocols. The

oligonucleotide primers used in real time PCR were: SV40 Tag: F: 5'-GCTACACTGTTTGTTGCCCA -3'; R: 5'- CCCCCACATAATTCAAGCAA-3'; Aire: F: 5'-ACCATGGCAGCTTCTGTCCAG-3'; R: 5'- GCAGCAGGAGCATCTCCAGAG-3'[9]; Insulin I: F: 5'-TATAAAGCTGGTGGGCATCC-3'; R: 5'- GGGACCACAAAGATGCTGTT-3'; Insulin II: F: 5'- TTTGTCAAGCAGCACCTTTG-3'; R: 5'-GTCTGAAGGTCACCTGCTCC-3'; GAD67: F: 5'- ATCGTGCAAGCAAGGAAGCA -3'; R: 5'- GCAAGAGACCTCGGATAGAAGAGT-3'; Cytochrome P450 1a2: F: 5'-GCTGCCATATCTAGAGGCCTTCAT-3'; R: 5'- TGGTTGACCTGCCACTGGTTTA-3'; the ribosome L-19: F: 5'-CTGAAGGTCAAAGGGAATGTG-3'; R: 5'-GGACAGAGTCTTGTGATCTC-3'. The relative amount of the gene was normalized by using the CT values of the sample and the corresponding standard curve. The target gene expression level was quantified by the ratio between the target gene and the ribosome L-19 as reference gene.

Acknowledgement

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Table 1. Total thymocytes reconstituted from different groups of bone marrow chimera (Mean ± SD). Data shown are representative of 6 independent experiments. (*: P<0.001 compared with group I and III).**

Group	Donor (TRAMP/TG-B)	Recipient (TRAMP/TG-B)	Thymocytes ($x10^6$)
I	-/+	-/-	53.1± 6.3
II	-/+	+/-	31.6 ± 2.5***
III	+/+	-/-	49.2 ± 3.5
IV	+/+	+/-	25.7 ± 2.7***

Figure legend

Fig. 1. Expression of SV40 Tag mRNA in reconstituted thymus and spleen from recipients of different groups. RT-PCR results of L-19 amplification (25 cycles) were shown as an agarose gel image. The SV40 Tag PCR products (35 cycles) were separated by agarose gel electrophoresis and transferred to the Hybond N+ membrane. The membrane was hybridized with an HRP-labeled probe and signals were detected by ECL direct nucleic acid labeling and detection system (Amersham).

Fig. 2. Clonal deletion of Tag-specific T cells in the thymus. Thymi from reconstituted chimera mice were harvested 8 weeks after bone marrow transplantation. Thymocytes were stained with anti-CD4, anti-CD8 and anti-Vβ8.1+8.2 antibodies. The data presented are the subset distribution (%) of gated $V\beta8^{high}$ T cells. A representative profile from each group (left) and combined results (right) from 6 independent experiments (8-12 in each group) are shown. $P<0.001$ when comparing groups II, IV to groups I, III.

Fig. 3. Peripheral deletion of autoreactive T cells by bone marrow-derived PAE in the spleen. The spleens from reconstituted chimera mice were harvested 8 weeks after bone marrow transplantation. Splenocytes were stained with anti-CD4, anti-CD8 and anti-Vβ8.1+8.2 antibodies. The data presented are the subset distribution (%) of total splenocytes. A representative profile from each group (left) and the combined results (right) from 6 independent experiments (8-12 in each group) are shown. Note that in

some experiments, reconstitution of non-transgenic CD8 T cells expressing lower levels of Vβ8 can be observed, and this population has been excluded from the analysis. $P<0.001$ when comparing group II and IV to I or III, group III to I respectively. $P<0.01$ when comparing group IV to group II.

Fig. 4. The functions of mature SV40 Tag-specific T cells in the spleen. The spleens from reconstituted chimera mice were harvested 8 weeks after bone marrow transplantation. The data presented are proliferation assay (a) and the cytotoxicity assay (b) to SV40 Tag peptides 560-568 and the control peptide HSV-gB from 6 independent experiments (8-12 in each group). Representative experiments reflecting two patterns of CTL responses are presented in Fig. 4b.

Fig. 5. Bone marrow-derived PAE causes activation-induced cell death of self-reactive T cells in the secondary lymphoid organs. The spleens from reconstituted chimera mice were harvested 8 weeks after bone marrow transplantation. Splenocytes were stained with anti-CD4, anti-CD8, anti-Vβ8.1+8.2 and the antibodies against different activation markers (a) or Fas (b). The cells undergoing apoptosis were visualized by FITC conjugated dUTP (c). The numbers in the panels are the % of the population (a and c) or mean fluorescence intensity (b) of the gated $CD8^{+}V_{\beta}8^{high}$ T cells.

Fig. 6. The expression of *aire* does not correlate with that of "organ-specific autoantigens" in the spleen. a. Expression of *aire* and SV40 large T antigens in the spleen cells from irradiation chimera (group III, donor, TG-B/TRAMP, recipient,

B6xB10.BR). Data shown are a summary of three independent experiments. b. Expression of *aire,* insulin, GAD and cytochrome c P450 in the spleen from irradiation chimera. Data shown are a summary of 4 independent experiments involving either group III (donor, TG-B/TRAMP, recipient, B6xB10.BR) or group I (donor, TG-B/B6, recipient, B6xB10.BR) chimera mice. The spleens were harvested and fractionated into $CD11c^+$, $CD11c^-$ subpopulations. Real time PCR was carried out to detect the expression level of different genes. The relative amount of the gene was normalized by using the CT values of the sample and the corresponding standard curve. The target gene expression level was quantified by the ratio between the amount of target gene and the housekeeping gene ribosome L-19 within the same sample. Statistical analysis was done with Student T test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

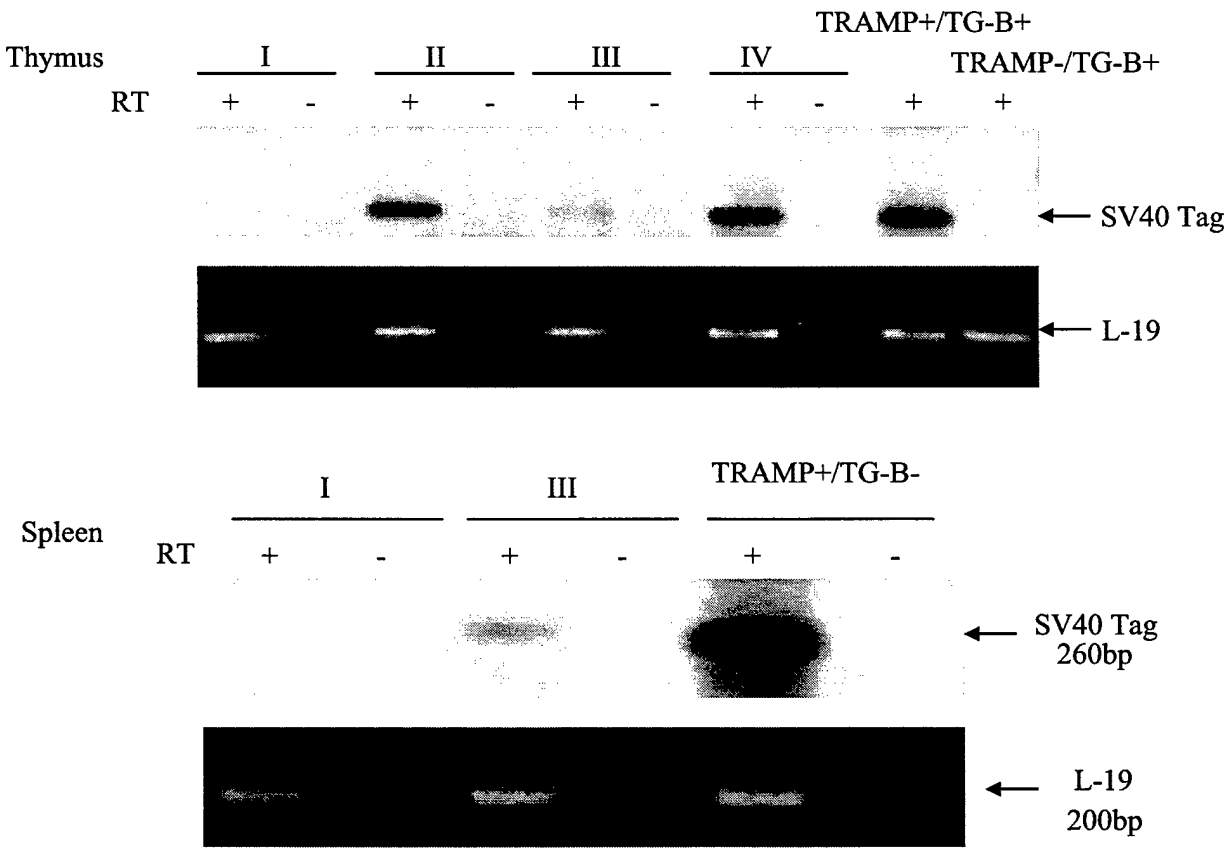

TRAMP+/TG-B+
Thymus
I
II
III
IV
TRAMP-/TG-B+
RT
+ - + - + - + - + +
SV40 Tag
L-19
TRAMP+/TG-B-
I
III
Spleen
RT
+ - + - + -
SV40 Tag
260bp
L-19
200bp

Fig.1

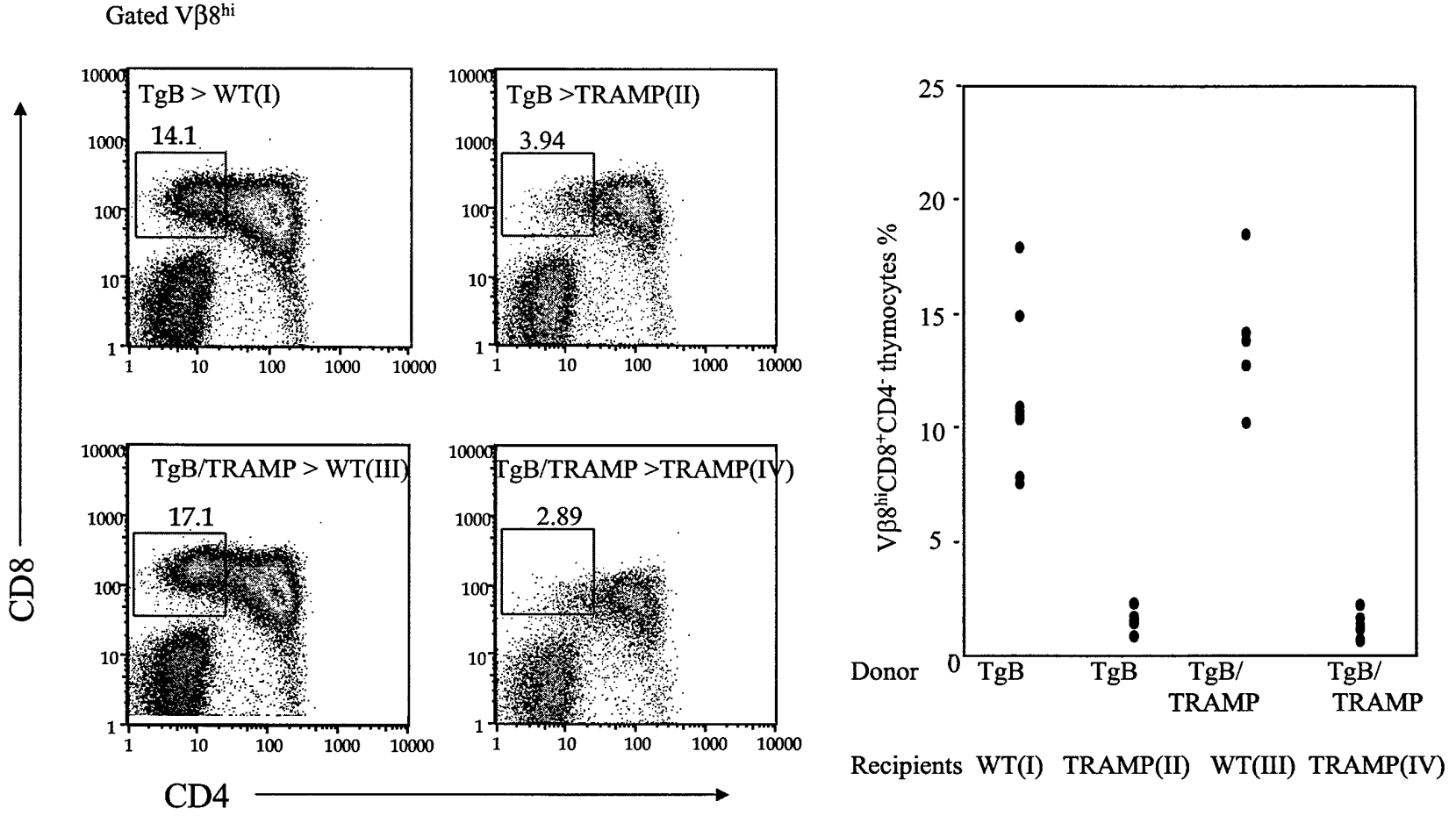
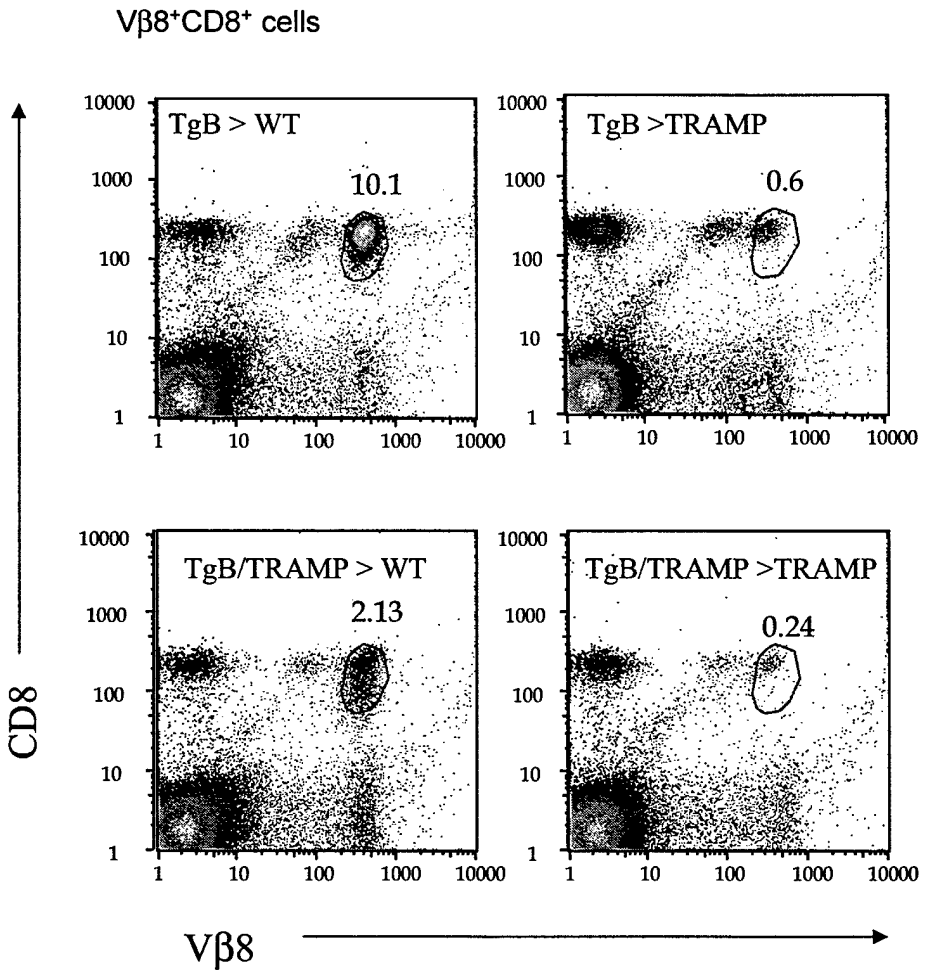

Gated Vβ8hi
TgB > WT(I)
14.1
TgB >TRAMP(II)
3.94
TgB/TRAMP > WT(III)
17.1
TgB/TRAMP >TRAMP(IV)
2.89
CD8
CD4
Vβ8hiCD8+CD4- thymocytes %
Donor
TgB
TgB
TgB/ TRAMP
TgB/ TRAMP
Recipients WT(I) TRAMP(II) WT(III) TRAMP(IV)

Fig. 2


Vβ8+CD8+ cells
TgB > WT
10.1
TgB >TRAMP
0.6
TgB/TRAMP > WT
2.13
TgB/TRAMP >TRAMP
0.24
CD8
Vβ8
10000
1000
100
10
1

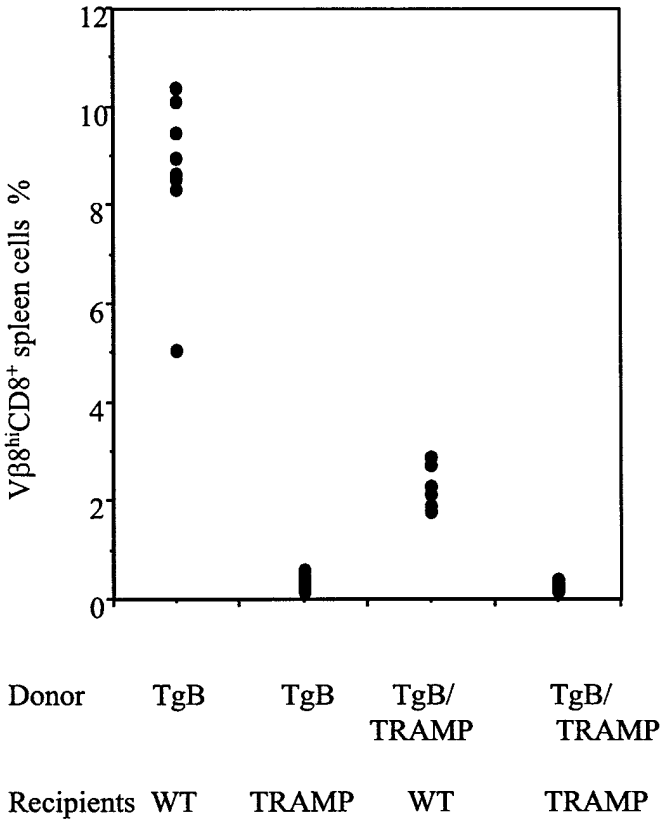

Vβ8hiCD8+ spleen cells %
12
10
8
6
4
2
0
Donor TgB TgB TgB/ TRAMP TgB/ TRAMP
Recipients WT TRAMP WT TRAMP

Fig. 3

a

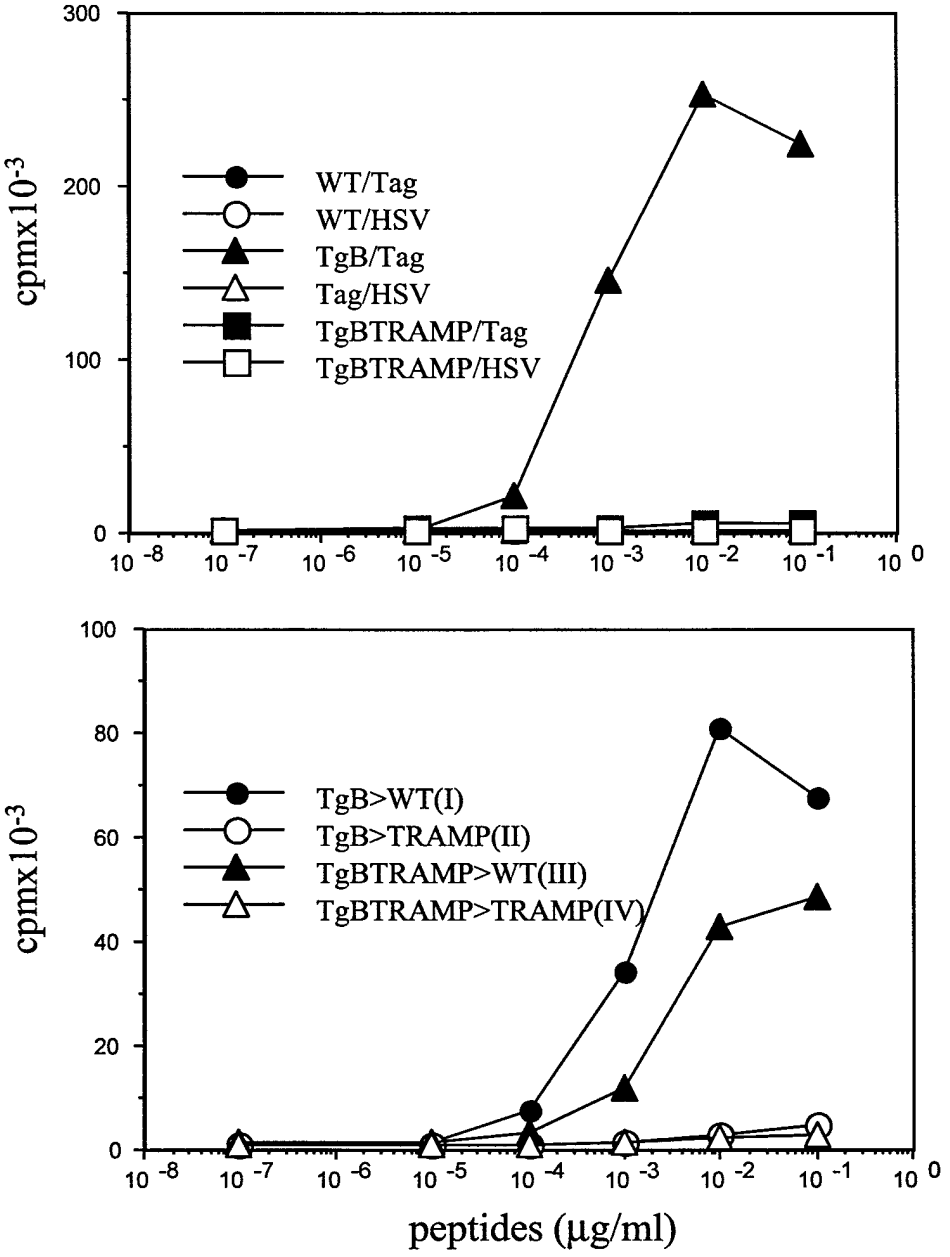

300
200
100
0
cpmx10-3
WT/Tag
WT/HSV
TgB/Tag
Tag/HSV
TgBTRAMP/Tag
TgBTRAMP/HSV
10 -8
10 -7
10 -6
10 -5
10 -4
10 -3
10 -2
10 -1
10 0
100
80
60
40
20
0
cpmx10-3
TgB>WT(I)
TgB>TRAMP(II)
TgBTRAMP>WT(III)
TgBTRAMP>TRAMP(IV)
peptides (µg/ml)

Fig. 4a

b

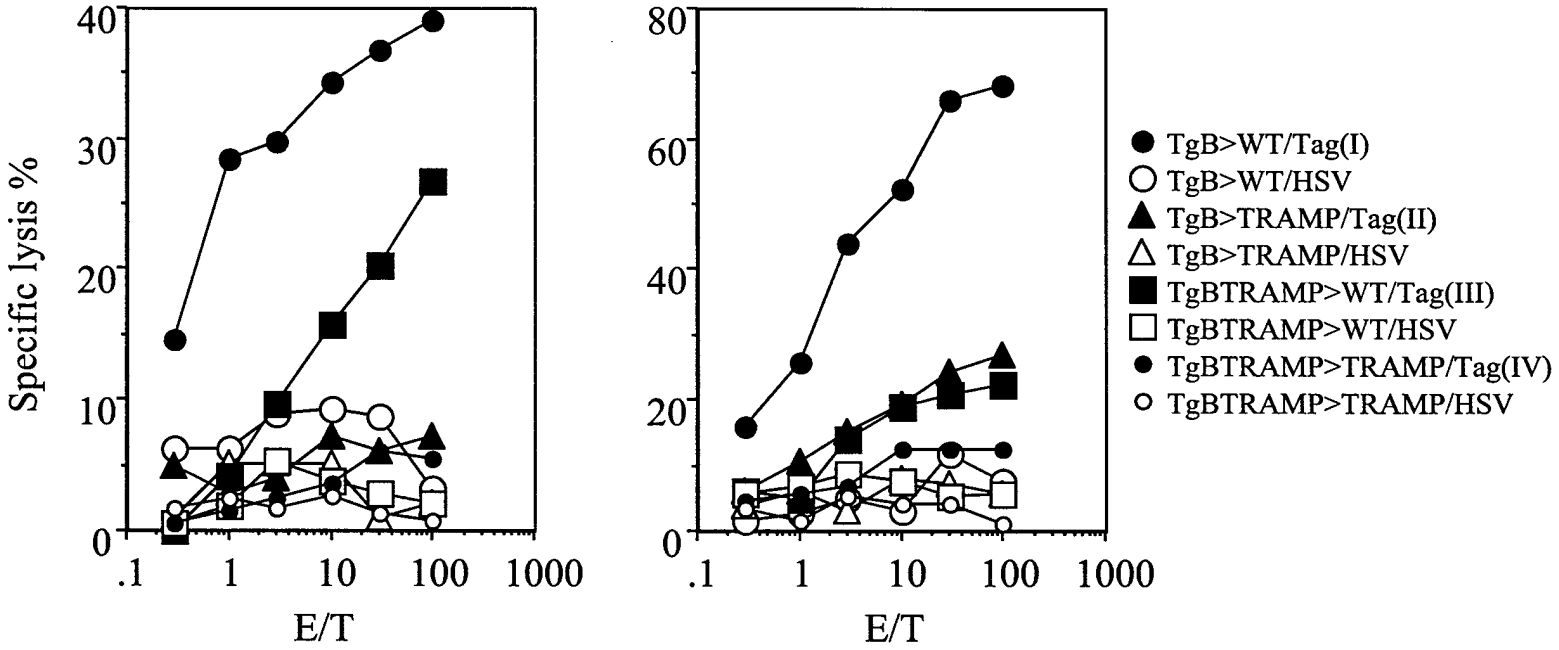

Specific lysis %
40
30
20
10
0
.1
1
10
100
1000
E/T
80
60
40
20
0
.1
1
10
100
1000
E/T
TgB>WT/Tag(I)
TgB>WT/HSV
TgB>TRAMP/Tag(II)
TgB>TRAMP/HSV
TgBTRAMP>WT/Tag(III)
TgBTRAMP>WT/HSV
TgBTRAMP>TRAMP/Tag(IV)
TgBTRAMP>TRAMP/HSV

Fig. 4b

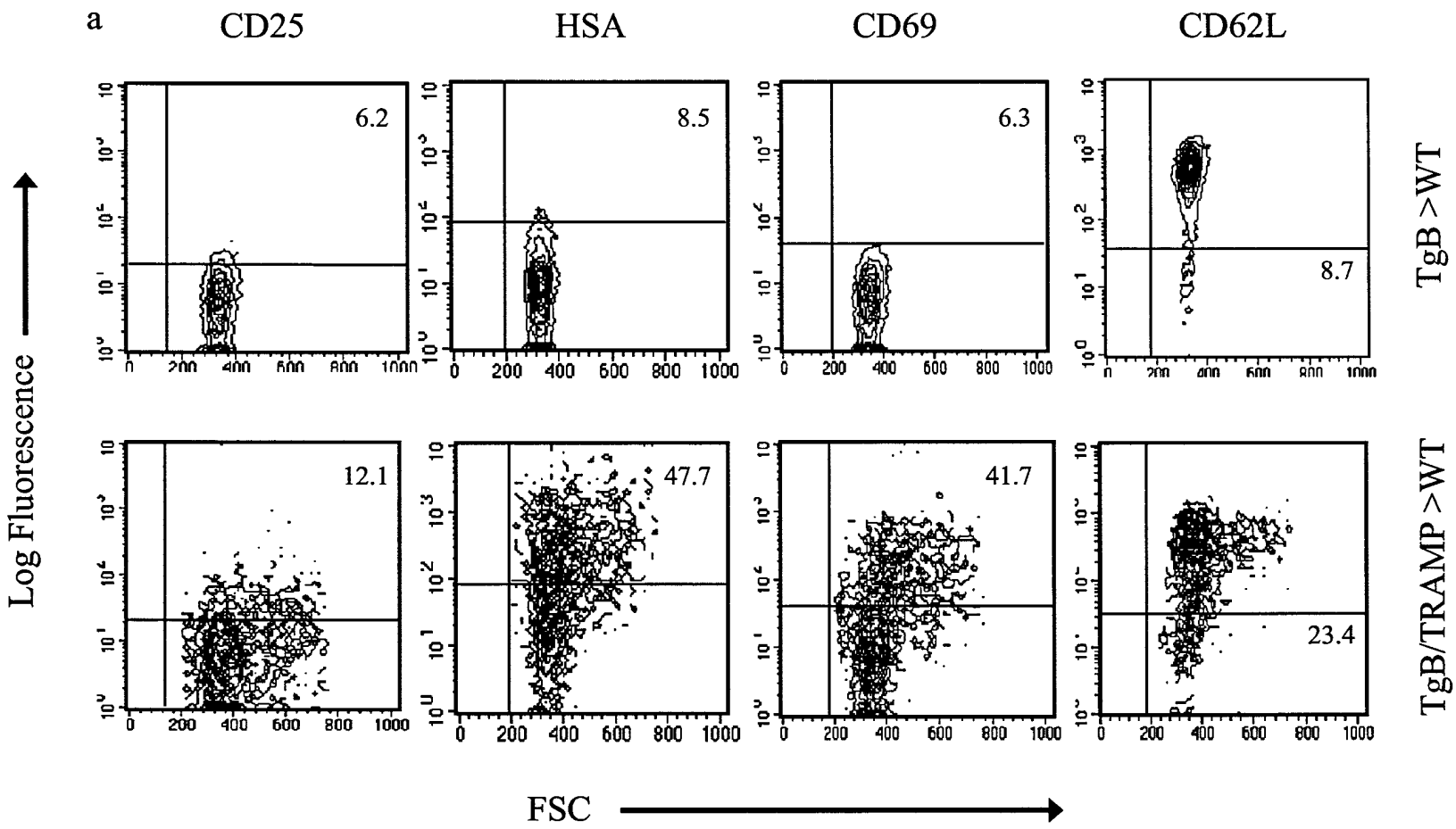

a
CD25
HSA
CD69
CD62L
6.2
8.5
6.3
8.7
12.1
47.7
41.7
23.4
TgB >WT
TgB/TRAMP >WT
Log Fluorescence
FSC

Fig. 5a

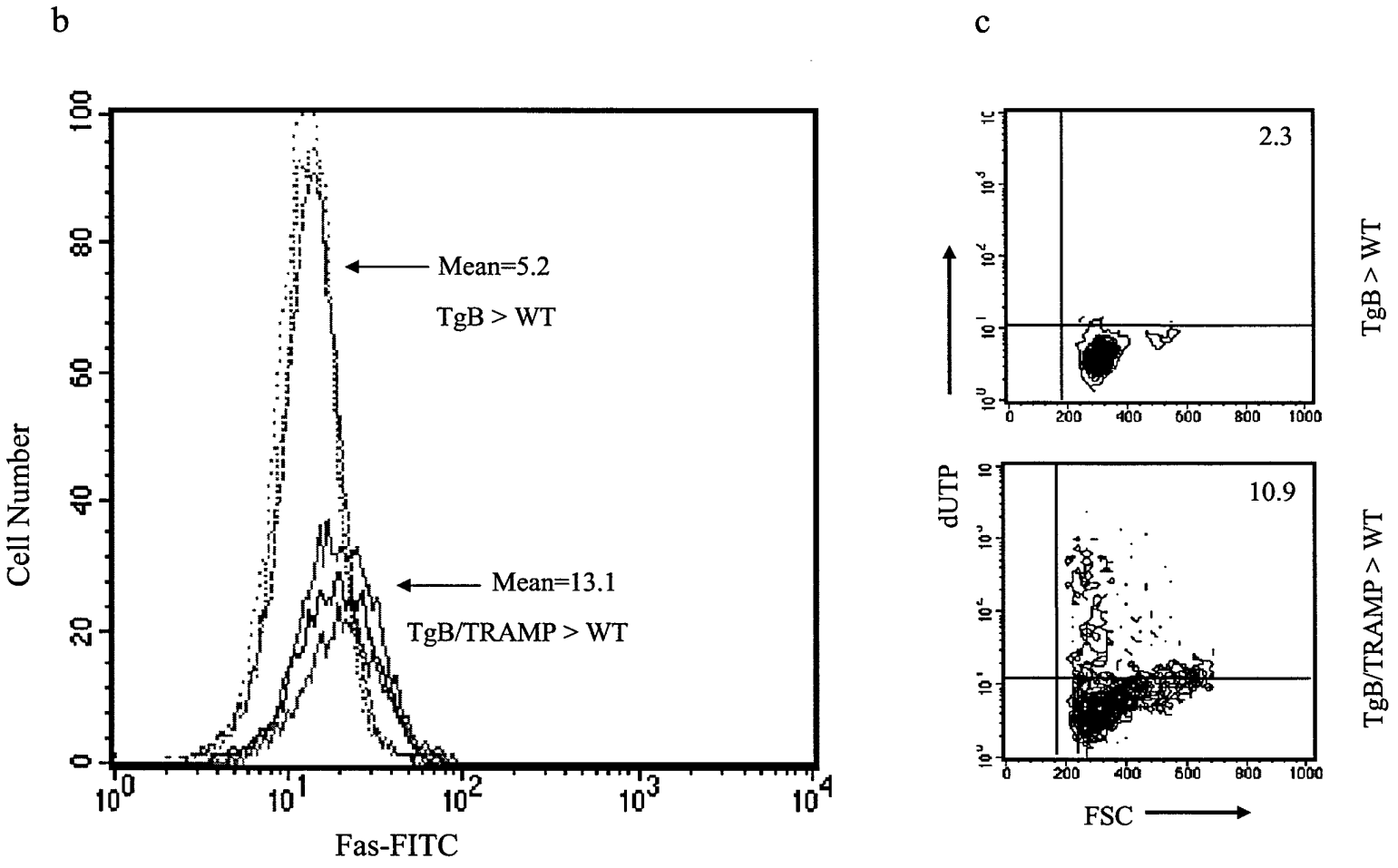

b
c
Cell Number
Fas-FITC
Mean=5.2
TgB > WT
Mean=13.1
TgB/TRAMP > WT
2.3
10.9
dUTP
FSC
TgB > WT
TgB/TRAMP > WT

Fig. 5b, c

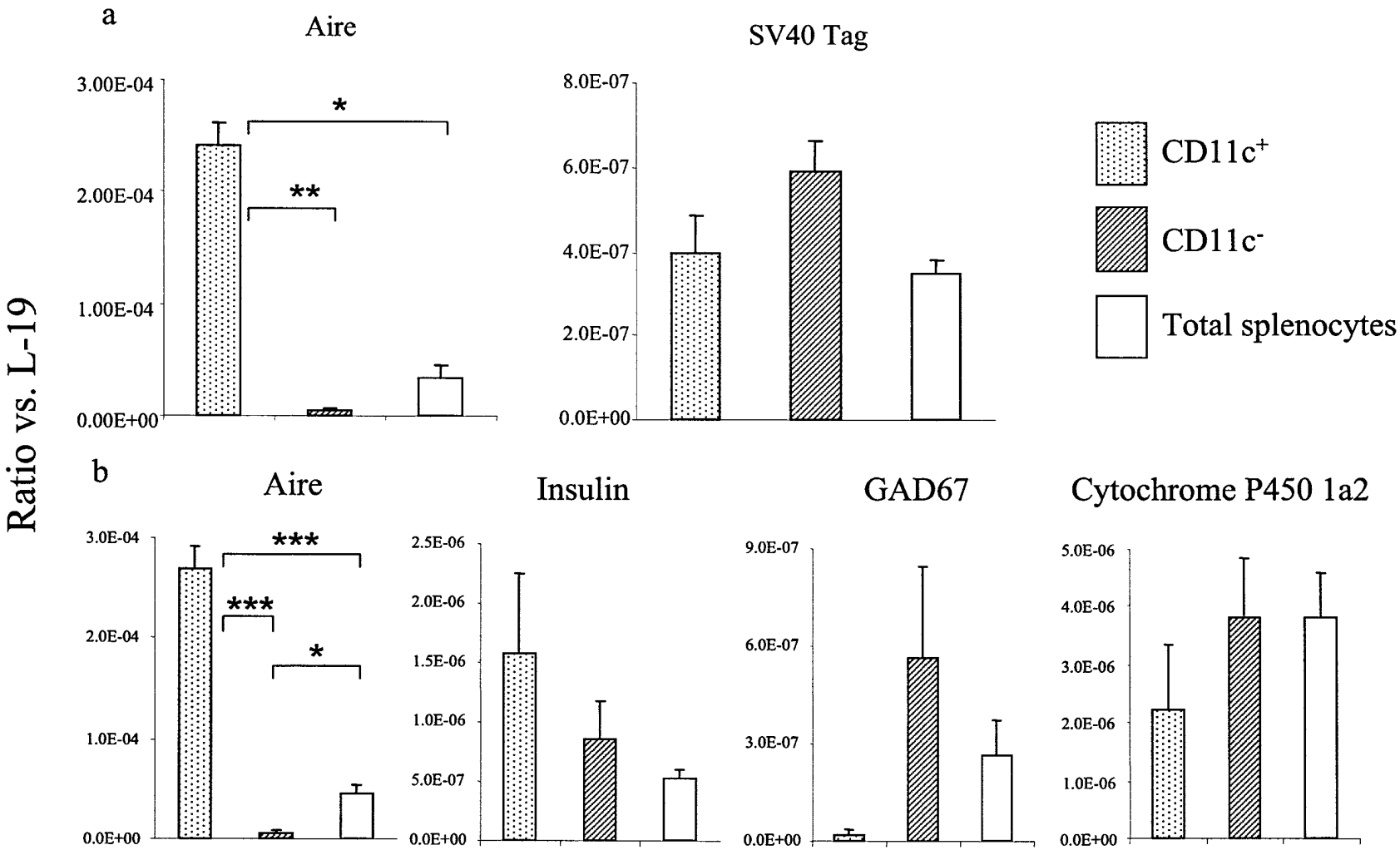

a
Aire
SV40 Tag
Ratio vs. L-19
3.00E-04
2.00E-04
1.00E-04
0.00E+00
8.0E-07
6.0E-07
4.0E-07
2.0E-07
0.0E+00
*
**
CD11c+
CD11c-
Total splenocytes
b
Aire
Insulin
GAD67
Cytochrome P450 1a2
3.0E-04
2.0E-04
1.0E-04
0.0E+00
***
***
*
2.5E-06
2.0E-06
1.5E-06
1.0E-06
5.0E-07
0.0E+00
9.0E-07
6.0E-07
3.0E-07
0.0E+00
5.0E-06
4.0E-06
3.0E-06
2.0E-06
1.0E-06
0.0E+00

Fig. 6

B7-CD28 Interaction Promotes Proliferation and Survival but Suppresses Differentiation of $CD4^-CD8^-$ T Cells in the Thymus

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Key words: T cell development, costimulatory molecules, T Cell Receptor β selection

Abstract

Costimulatory molecules play critical roles in the induction and effector function of T cells. More recent studies revealed that the costimulatory molecules enhance clonal deletion of autoreactive T cells as well as generation and homeostasis of the $CD25^{+}CD4^{+}$ regulatory T cells. However, it is unclear if the costimulatory molecules play any role in the proliferation and differentiation of early T cell progenitors. Here we report that targeted mutations of B7-1 and B7-2 substantially reduces the proliferation and survival of $CD4^{-}CD8^{-}$(DN) T cell progenitors. Perhaps as a result of reduced proliferation, the accumulation of RAG-2 protein in the DN thymocytes is increased in B7-deficient mice, which may explain the increased expression of TCR gene and accelerated transition of $CD25^{+}CD44^{+}$ (DN3) to $CD25^{-}CD44^{+}$ (DN4) stage. Qualitatively similar but quantitatively less striking effects were observed in mice with targeted mutation of CD28, but not CTLA4. Taken together, our results demonstrate that development of early T cell progenitor in the thymus is subject to modulation by the B7-CD28 costimulatory pathway.

Introduction

The T cells are educated in the thymus to gain immune competence. Mature T cells migrate into second lymphoid organ where they encounter antigens, expand and differentiate into effector cells. The activated T cells are dispatched to target tissues to mediate effector function. As the major costimulators in T cell activation, B7-1 and B7-2 was first demonstrated to play a major role in the activation and differentiation of T cells in the secondary lymphoid tissues (1-3). Subsequently, the notion of T cell costimulation has been extended to T cell effector function in target tissues, including tumors (4-7) and normal tissues during autoimmune destruction (8, 9). Accumulating data from several groups, including that of ours, have shown that B7/CD28 modulate clonal deletion by proper elimination of autoreactive T cells (10-14). Moreover, it has been shown that costimulation is critical for generation and homeostasis of the $CD25^{+}CD4^{+}$ regulatory T cells (Treg) (15-18). Since all of these stages involves interaction of antigen-specific receptors with MHC: peptide complex, it can be suggested that T cell costimulation is an important parameter whenever TCR are engaged by MHC:peptide complex. An interesting issue that has not been addressed in whether T cell costimulation participate the β-selection, in which the rearranged TCRβ paired with pTα to mediate proliferation and differentiation of early progenitor T cells.

The earliest T cell progenitor expresses neither TCR nor co-receptor CD4 or CD8, and is usually referred as double negative (DN) T cells for the lack of

CD4 and CD8. They are subdivided according to the expression of surface markers CD44 and CD25. DN1, which is $CD44^+CD25^-$, contains cells that are committed to lymphoid lineage but maintain the potential to develop into T cells, B cells, or natural killer (NK) cells (19). With the increased CD25 expression, DN1 becomes $CD44^+CD25^+$ and are called DN2 cells. Cells at this stage are committed to T-cell lineage and therefore are also called pro-T cells. Growth factors, such as interleukin 7 (IL-7) and stem cell factor (SCF, c-kit ligand), play important roles in this developmental step (20-24). DN3 or early pre-T population down-regulate CD44 and is characterized by $CD44^-CD25^+$. At this stage, the T cell receptor TCRβ chain locus is rearranged by recombination activation gene (*RAG*)-dependent mechanism. This leads to assemble of the pre-TCR complex consisting of CD3, pTα and TCRβ chains. Disruption of the complex causes a complete arrest at DN3, as shown in *Rag*- (25-27), TCRβ- (28) and pTα (29) deficient mice. As further maturation occurs, cells lose expression of CD25 to become $CD44^-CD25^-$ DN4. The DN4 progress to the $CD4^+CD8^+$ DP via an immature single positive (ISP) stage and then go through positive and negative selection to become $CD4^+$ or $CD8^+$ singe positive T cells.

While it is clear that the survival of DN4 requires rearrangement of TCRβ and expression of pTα, very little is known about other cell surface interactions during the early phase of T cell maturation, which is generally coupled with rapid T cell proliferation. In the process of studying the effect of B7 blockade on the development of antigen specific T cells in the thymus, we observed that anti-B7

antibodies have significant effect on the development of early T cell progenitors. To substantiate this observation, we systematically analyzed the maturation and proliferation of the T cell progenitors in mice with targeted mutation of B7-1 and B7-2, CD28, and CTLA-4. Our results demonstrate targeted mutations of B7-1 and B7-2 or CD28 diminish the proliferation and survival of DN4 T cells and accelerated DN3 to DN4 transition, most likely by increasing accumulation of the RAG-2 protein and enhancing TCR rearrangement.

Materials and Methods

Experimental animals WT, B7-1(-/-)B7-2(-/-) (), CD28(-/-) () and TRAMP () C57BL6/j mice and TRAMP mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CTLA-4(-/-) mice in B6 background has been described (). All animals were maintained in the University Laboratory Animal Research Facility at the Ohio State University under specific-pathogen-free conditions.

Adult and perinatal treatment with B7-1/2 or CTLA-4 monoclonal antibodies Adult treatment with anti-B7-1 (3A12, hamster IgG) and anti-B7-2 (GL-1, rat IgG2a) monoclonal antibodies were carried out at 8-9 weeks of age. The mice received 5 intraperitoneal injections (i.p.) of 100 μg/mAb/mouse over a 10 day period and were sacrificed 1 week after the last injection. Normal hamster IgG (Rockland Inc., Gilbertsville, PA, USA) and rat IgG (Sigma, St. Louis, MO, USA) were used as controls.

Antibodies and Flow cytometry Both cell surface markers and intracellular staining were analyzed by flow cytometry (Becton Dickinson, Mountain View, CA). The fluorescence conjugated antibodies anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD25 (PC61), anti-CD28 (37.57), anti-CTLA/4 (UC10-4F10-11), anti-TCR β chain (H57-597) were purchased from BD PharMingen (San Diego, CA, USA). The fixation and permeabilization solution kit (cytofix/cytoperm^{TM}, BD pharmingen, San Diego, CA, USA) was used for intracellular staining according to manufacture's protocol.

To measure proliferation of thymocytes in vivo, mice were injected intraperitoneally (i.p.) with BrDU (1 mg/mouse in 100 µl PBS). Four hours later, the mice were sacrificed and single thymocytes were prepared. BrDU incorporation was detected by flow cytometry with a BrDU Flow Kit, as described by manufacturer (BD PharMingen).

The apoptotic thymocytes were determined by their binding the Annexin V. After cell surface markers staining, the cells were resuspended in staining buffer with Annexin V (BD PharMingen) and were stained in room temperature for 15 minutes. The samples were analyzed by flow cytometry within one hour.

Western blot and real-time RT-PCR The total thymocytes were depleted twice with anti-CD4 (GK1.5), anti-CD8 (2.4.3), anti-TCRγδ (GL-3), anti-I-A^b (AF6-120.1) and magnetic Dynabeads (DYNAL, Oslo, Norway) according to manufacture's manual. The RAG-2 protein level was detected by Western blot. Briefly, Cells were lysed in a lysis buffer consisted of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM DTT,1 mM Na_3O_4, 2 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM PMSF. Total protein extracts (100 µg) were fractionated on a 12% SDS-PAGE and transferred to Hybond-P membrane (Amersham Biosciences, Buckinghamshire, England). The transferred membrane was blotted by a goat polyclonal antibody against RAG-2 (Santa Cruz Biotechnology, Inc. CA, USA). Mouse monoclonal β-actin antibody (Sigma) was used for internal blotting control.

Total RNA was extracted from DN thymocytes, treated with RNase-free DNaseI (Invitrogen, Carlsbad, CA). cDNA was synthesized with Superscriptase II and oligo(dT) (Invitrogen). The real time PCR was carried out in ABI PRISM 7700 Cycler (Applied biosystems, Foster city, CA) using a QuantiTect SYBR green PCR kit (Qiagen Inc, Valencia, CA) according to manufacturers' protocols and real time PCR conditions. The relative quantification of expression level in B7KO mice was compared with it from wild type B6 mice after normalization with internal control (ribosome L-19) respectively. As shown by following:

Comparative expression level= $2^{-(\Delta C_T B7KO - \Delta C_T B6)}$

$\Delta C_T = C_T$(target gene)- C_T(L-19), which represents the difference between the two CT values of two PCRs for the same initial template amount. The oligonuceotide primers were: the RAG-1 forward primer: 5'-TGCAGACATTCTAGCACTCTGG–3'; reverse primer: 5'–ACATCTGCCTTCACGTCGAT–3'; the RAG-2 forward primer: 5'–CACATCCACAAGCAGGAAGTACAC–3'; reverse primer: 5'–TCCCTCGACTATACACCACGTCAA-3'; the pre-TCRα forward primer: 5'-AGCTTCTGGCTGCAACTGGGTCAT-3'; reverse primer: 5'-TACCTGCCGCTGTGTCCCCCCGAG; TCR Vα2 forward primer: 5'-CAATAAAAGGGAGAAAAAGC-3'; reverse primer: 5'-AAGTCGGTGAACAGGCAGAG-3'; TCR Vα4 forward primer: 5'-AGCAGCAGAGGKTTTGAAGC-3'; reverse primer: 5'-GGCACATTGATTTGGGAGTC-3' (30); CD8 β chain forward primer: 5'-CTGCTTTGAACTGCTGCAAG-3'; reverse primer: 5'-

GGAAGAGTACATGGTGGCGT-3'; The ribosome L-19 forward primer: 5'-CTGAAGGTCAAAGGGAATGTG-3'; reverse primer: 5'-GGACAGAGTCTTGTGATCTC-3' (31) is used as internal control.

Antimitotic drug treatment Eight week-old mice received 3 injections of either Demecolcine (Sigma) at 200 μg/mouse/injection or PBS at 4-hour intervals. This is followed by two daily treatments with same amount drug. The BrdU was injected in conjunction with the last treatment. The mice were sacrificed 4 hours later to harvest thymocytes for analyses.

Statistical Analysis Data were statistically analyzed with two-tailed student T-test.

Results

1. Impact of defective B7-1, B7-2 and their receptors on the DN subsets

In the process of studying the effect of anti-B7-1/2 antibodies for T cell development in vivo, we observed that anti-B7-1/2, but not control Ig, caused a substantial reduction of DN3 and a major increase of DN4 in thymus of TRAMP mice (31) (Fig. 1). A statistically significant reduction was also observed in the DN2 subset. These effects were also observed when adult C57BL/6 mice and perinatal BALB/c mice received the same treatments (data not shown). These data suggest that B7-1 and B7-2 play a critical role in the maturation of progenitor T cells. To substantiate this observation, we compare mice deficient for B7-1 and B7-2 with their WT controls for DN subsets. As shown in Fig. 2, B7-deficient mice had an increased DN4 and decreased DN2 and DN. As expected, the effect of target mutation is more severe than what was found in the mice that received anti-B7 antibodies.

B7-1 and B7-2 interact with two known receptors, CD28 and CTLA-4 on T cells. A putative third receptor has been suggested (32, 33), although its identity remains elusive. As a first step to determine the receptors that may be involved in the alteration of DN3 and DN4, we first analyzed cell surface expression of CD28 and intracellular accumulation of the CTLA-4 among DN3 and DN4 subsets. As shown in Fig. 3. In comparison to isotype control, CD28 is expressed on about 13% of DN3 and 70-80% of DN4. CTLA-4, on the other hand, is expressed on about 80% of DN3 cells, and 40% of DN4. To determine

the role for CD28 and CTLA-4 in development of DN, we compared WT, B7KO, CD28KO and CTLA-4KO for the DN subsets. As shown in Fig. 2, targeted mutation of CD28 had significant effects on the distribution of DN. Qualitatively, reductions in DN2 and DN3 and an increase in DN4 parallele to what was found in B7-KO. However, targeted mutation of CD28 is less effective than those of the B7-1 and B7-2. To determine if CTLA4 function explains the differences between CD28KO and B7KO, we compared thymi havested from 15-day old CTLA-4(+/-) and CTLA-4(-/-) mice for the DN maturation and proliferation. At this point, the overall subset distribution of SP, DN and DP subsets is grossly normal () and no autoimmune disease is observed. Fig. 4 shows the profiles of DN thymocytes in two week-old CTLA-4(+/-) and CTLA-4(-/-) mice. No overt change in the relative amounts of DN3 and DN4 was observed. Thus, CTLA-4 alone is not responsible for the costimulation by B7-1 and B7-2 in the early stage of thymocyte development.

2. Role for B7-1/2 and CD28 in proliferation and programmed cell death of DN

An important feature of the DN thymocytes is their high rate of proliferation (34, 35). To test whether B7-deficiency affects its proliferation, we pulsed WT, CD28 and B7-deficient mice with BrdU and measured the DNA synthesis of the ex vivo DN by flow cytometry 4 hours later. As shown in Fig. 5, in agreement with previous publications (36, 37), two major waves of proliferation was observed in DN2 (about 20%) and DN4 (about 40%) in the WT mice, and

somewhat less pronounced, but still substantial proliferation was found in DN3 (about 10%). Interestingly, less than 8% of the DN4 from B7-deficient mice incorporated BrdU. As expected, this reduction corresponds to a reduction of the proportion of DN4 with large forward scatters. Thus, targeted mutation of B7 suppress both enlargement and DNA synthesis of the DN4 cells. While less pronounced than what was found in the B7-deficient mice, DN4 from CD28-deficient mice also showed about 2-fold reduction in the BrdU incorporation.

An interesting issue is whether the CD28 and B7 deficiencies affect the survival of DN thymocytes. Here we investigated the number of cells underlying programmed cells death in the WT, B7-1/2- and CD28-deficient mice, by staining with the FITC-tagged Annexin V. As shown in Fig. 6, while high number of DN1 and DN4 are recognized by Annexin V, the difference was found only in DN4, as B7-1/2 and CD28-deficient DN4 had more Annexin V^+ cells than the WT counterpart. These results revealed that knocking out B7 and CD28 had similar effect in causing death of DN4.

3. Targeted mutation of B7 and CD28 induce TCR expression among DN4

An important feature of the small DN4 T cells is the expression of cell surface pre-TCR complex. To test the effect of costimulation on the up-regulation of TCRβ, we compared DN1-4 from WT, B7-1/2-, CD28-deficient mice for the expression of TCR. Although a significant proportion of $CD44^{hi}CD25^-$ (DN1) cells express TCRβ on the cell surface, these cells belongs to the NKT lineages, as has been reported by others (38). In addition, no $TCR\beta^{hi}$

thymocytes were found with the DN2 populations. A small number of DN3, and about 20% of DN4 thymocytes express TCRβ at significant levels. Interestingly, in the B7KO mice, the overwhelming majority (nearly 80%) of DN4 express TCRβ, while more than 60% of DN4 in the CD28-deficient mice are $TCR\beta^{hi}$ (Fig. 7). Thus, costimulation by B7-1/2 and CD28 genes inhibit the expression of the TCRβ on DN4.

4. Elimination of cycling thymocytes recapitulate the basic features of DN subsets in mice with targeted mutations of B7-1/2 or CD28

Given the overall role of B7-CD28 interaction in promoting T cell proliferation and survival (), it is of great interest to determine whether the altered DN subset distribution and enhanced expression TCR is a consequence of reduced TCR proliferation and survival. To test this hypothesis, we treated mice with demecolcine which kills cells undergoing mitosis. As shown in Fig. 8a, three consecutive treatments with demecolcine removed the overwhelming majority of cells within the DN thymocytes. When the CD25 and CD44 expression were analyzed, it is clear that deletion of mitotic thymocytes caused a drastic increase of DN4 and reduction in DN3, which recapitulate the phenotype of B7 blockade. At the same time, the expression of TCR is significantly increased among DN3 and DN4 in mice that received anti-mitotic treatments. Thus, anti-mitotic treatment recapitulates the effect of B7 blockade.

5. Accumulation of RAG-2 and increased expression of rearranged TCRα in mice with targeted mutation of B7-1/2

It is well established expression of both RAG-1 and RAG-2 is critical for transition from DN3 to DN4, as thymocyte development in mice lacking RAG-1 or RAG-2 in blocked at DN3 (). Since both TCR over-expression and increased DN4 can be explained by increased RAG activity, we analyzed expression and accumulation of RAG-1/2 mRNA by RT-PCR and RAG-2 protein by Western blot. As shown in Fig. 9a and b, purified DN cells from WT mice expressed significantly less RAG-2 protein than wild-type mice. This increase in B7KO DN cells is most likely due to post-transcriptional mechanisms, as the RAG-1/2 mRNA was not increased. Corresponding to increased RAG-2 protein, real time RT-PCR revealed a 2-3 folds increase in Vα2 and Vα4 expression among the DN from B7-deficient mice in comparison to WT mice.

6. Subset distribution of thymocytes among age-matched WT, CD28(-/-) and B7(-/-) mice

Since the DN4 cells are the immediate precursor for the DP cells, it is possible that the abnormal DN development may be associated with altered thymocyte subsets. As shown in Table 1, mutations in B7 and CD28 genes resulted in an 10-20% increase in $CD4^-CD8^-$ thymocyte and a small but statistically highly significant decrease in $CD4^+CD8^+$ T cells. The % of single positive CD4 and CD8 T cells was substantially increased.

Discussion

The T cell progenitors undergo rapid proliferation and phenotypic transitions before they emerge as the $CD4^+CD8^+$ T cell precursors for positive and negative selection (). However, the cell-surface interactions that guide the first phase of T cell development are still poorly understood. The potential involvement of costimulatory molecules in this phase has not been investigated. Here we provide several lines of evidence that demonstrate a critical contribution of costimulatory molecule B7-1/2, and their receptor CD28.

The most clear-cut demonstration of the impact of costimulation on DN development is the alteration of the relative amounts of $CD44^-CD25^+$ (DN3) and $CD44^-CD25^-$ (DN4) cells in mice with inactivated B7-1/2 and CD28. In the WT mice, there are 2-3 times more DN3 than DN4 cells, while B7-deficient mice have three-fold more DN4 than DN3 cells. Theoretically, there are at least four potential mechanisms that can account for the reduction of DN3 in B7-1/2 and CD28-deficient mice, namely, the proliferation and survival of DN3, a decreased transition from DN2 to DN3, an increased transition from DN3 to DN4, and reduced rate of transition between DN to DP. Our data effectively ruled out the first possibility as the proliferation and survival of DN3 was not inhibited by the targeted mutations. A CD28-mediated decrease in DN2 to DN3 transition is not probable, as the DN2 lacks cell surface CD28 expression, while very small proportion of DN3 expresses CD28.

More rapid transition from DN3 to DN4 is likely to contribute to both decrease in DN3 and increase in DN4, as these changes cannot be accounted for by death or proliferation in DN3 and DN4. This interpretation is also supported by increased accumulation of RAG-2, which mediate a critical checkpoint between DN3 and DN4. Paradoxically, despite the overall trend of decreased proliferation and survival, the numbers of total DN and especially DN4 is significantly increased. This can be explained only if one assume that progression from DN4 to DP is kinetically slower in mice with mutations of B7-1, B7-2 and CD28. This interpretation is consistent with the fact that DP subset is decreased in the mutant mice. The decrease is statistically highly significant although not large numerically. However, the decrease is surprising if one consider the decrease in the context previous works, which showed that costimulation blockade decrease clonal deletion. As such the decrease in DP resulted from delayed DN to DP progression may be overshadowed by the increase of DP caused by defective clonal deletion. The link between increased $CD4^+CD8^-$ and $CD4^+CD8^-$ cells can be attributed directly to abnormal clonal deletion, although altered differentiated DN may have an indirect effect.

Our analysis of proliferation and programmed cell-death of the T cell progenitors in the thymus reveals two interesting points.

First, in parallele to what was described for activation and effector function of mature T cells, the costimulatory molecule B7-1 and B7-2 promote proliferation and survival of T cell progenitors. Thus, in comparison to WT thymocytes, increased proportion of DN4 in B7- and CD28-deficient mice were undergoing

programmed cell death. Conversely, substantially reduced % of DN4 cells in B7- or CD28-deficient mice incorporated BrdU. Interestingly, in mice with mutations of either B7 or CD28, the proportion of dividing and apoptotic cells appear to have increased among DN2, and DN3 subsets. However, since these two subsets are diminished in the mutant mice, the changes in proportions does not translated into increased numbers of cells undergoing division or apoptosis. Differences in apoptosis and proliferation were also noted among DN1 subset that are equally presented in WT and mutant mice. However, since our study does not differentiate true DN1 and those that express TCR and CD44 (mostly NKT cells), the significance of the difference in DN1 is unclear at this point.

Second, the function of B7-CD28 interaction in promoting division of DN thymocyte may explain the more rapid DN3 to DN4 transition in mice with mutations of B7 or CD28. This is due to the fact that this transition requires RAG activity restricted to the G0/G1 phase of the cell cycle (39, 40). Indeed, RAG-2 protein is accumulated at G0/G1 and its expression level decreases rapidly at the G1-S transition of the cell cycle by cytoplasmic sequestration and ubiquitin-dependant degradation (41). Our analysis of RAG-2 protein and TCR gene expression clearly demonstrated that DN from B7-deficient mice have increased accumulation of RAG-2 protein and increased expression of rearranged TCR genes. The link between cell division and DN3-DN4 transition is supported by our data that anti-mitotic treatment results in changes of DN thymocytes that are not unlike those observed in B7- and CD28-deficient mice, including the ratio of DN3/DN4 and cell surface TCR.

An interesting issue is the identity of B7 receptors that may be involved in early stage of T cell development. Although mutations of B7 and CD28 have qualitatively similar phenotype, mutation of B7 has significantly more severe effect than that of CD28. A natural question is whether this is attributable to CTLA4, the other known B7 receptor. Although CTLA4 protein is expressed early among DN2 cells, targeted mutation of CTLA4 does not affect DN subset composition. Moreover, in vivo treatment with anti-CTLA4 antibody also had no effect (data not shown). It is therefore unlikely that CTLA4 is involved in the differentiation of DN. It is possible that other unidentified B7 receptor () may participate this process. Regardless of what the additional receptor may be, our results extended the functional spectrum of T cells that are modulated by costimulatory pathway. It would be of interest to analyze whether this abnormal DN development contributes to increased predisposition to autoimmune diseases in mice with targeted mutations of B7 (), CD28 () and perinatal blockade of B7-1 and B7-2 ().

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Table 1. Impact of targeted mutations of B7-1/2 and CD28 on the subsets of T cells in the thymus (N=5)

Mice	Thymocyte.	Subsets (5)			
	No. ($x10^{-6}$)	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^-CD8^+$	$CD4^+CD8^-$
WT	88.0±24.28	3.02+0.16	83.62+0.92	3.37+0.35	9.99+0.5
B7(-/-)	69.80+7.69	3.64+0.35	77.32+1.26	4.43+0.39	14.62+0.90
CD28(-/-)	59.00+16.76	3.32+0.17	75.53+1.7	4.60+0.63	16.55+1.40
PB7KO/WT	0.15	0.007	0.00002	0.002	0.0001
PCD28KO/WT	0.06	0.02	0.00001	0.005	0.00001

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Figure legend

Fig. 1. The effect of anti B7-1 and B7-2 mAbs on the DN subsets composition. The mice were treated with either anti B7-1 and B7-2 mAbs or control Ig at 8-9 weeks old. Thymocytes were harvested 1 week after last treatment and stained with anti-CD4, anti-CD8, anti-CD44 and anti-CD25 florescence conjugated antibodies. Total viable cells (upper panels) or $CD4^-CD8^-$ DN populations (lower panels) are gated. P value indicates the statistic difference between two groups on individual subset.

Fig. 2. The distribution of DN subsets in the thymi of WT, B7KO and CD28KO mice. B7KO, CD28KO and C57BL/6 mice were sacrificed at 8 weeks old and thymocytes were harvested and stained anti-CD4, anti-CD8, anti-CD44 and anti-CD25 florescence conjugated antibodies. Total viable cells (upper panels) or $CD4^-CD8^-$ DN populations (lower panels) are gated. Contour graphs depict thymocytes subsets of one mouse in each group. Means and S.D. are given in the panels. The experiments have been done twice, using a total of 9 mice per group.

Fig. 3. The expression of CD28 and CTLA-4 on different DN subsets. Wild type C57BL/6 mice were sacrificed at 8 weeks old and thymocytes were harvested. Cells are stained with anti-CD4/CD8, anti-CD44 and anti-CD25, anti-CD28 and intracellularlly stained with anti-CTLA-4 florescence conjugated antibodies. The

number indicates the percentage of CD28 and CTLA-4 or isotype control Ig positive thymocytes from individual subsets. Data shown are contour graphs of one representative from 3 mice per group.

Fig. 4. CTLA-4 alone is not responsible for the effect of B7-1/2 in DN thymocyte development. CTLA-4(+/-) and CTLA-4(-/-) thymi from two week-old mice were harvested and stained anti-CD4, anti-CD8, anti-CD44 and anti-CD25 florescence conjugated antibodies. Data shown are representative of three independent experiments.

Fig. 5. Target mutation of B7 and CD28 increase the proliferation among DN3 but decrease the proliferation among DN4. 8-9 week-old B7KO, CD28KO and C57BL/6 mice were injected with BrdU intravenously and then harvest thymus 4 hours after BrdU injection and stained with anti-CD4/CD8, anti-CD44 and anti-CD25 and intracellularlly stained with anti-BrdU florescence conjugated antibodies. The numbers in the panels indicate means and SD of the percentage of thymocytes incorporated with BrdU from individual subsets. The P values of student t tests are also provided. Data shown are representative of 2 independent experiments involving a total of 9 mice in each group.

Fig. 6. Target mutation of B7 and CD28 promote programmed cell death among DN4. B7KO, CD28KO and C57BL/6 mice were sacrificed at 8-9 weeks old and thymocytes were harvested and stained anti-CD4/CD8, anti-CD44 and anti-CD25

antibodies and Annexin V. Data shown are representative of 3 mice in each group.

Fig. 7. Target mutation of B7 and CD28 increase TCR expression among DN4. Thymocytes from 8-9 week-old B7KO, CD28KO and C57BL/6 mice were stained with anti-CD4/CD8, anti-CD44 and anti-CD25, anti-TCR β chain (lower panels) or isotype control Ig (upper panels) antibodies. Data shown are representative of 3 mice in each group.

Fig. 8. Anti-mitotic treatment recapitulates the effect of defective T cell costimulation on DN development. a. The efficiency of anti-mitotic treatment cells as revealed by reduction in BrdU incorporation. Data shown are means and SD (n=4) of the % $BrdU^+$ T cells within the DN subsets. b. Anti-mitotic treatment increases DN4 while reducing DN2 and DN3. c. Anti-mitotic treatment increases TCR expression on DN3 and DN4 thymocytes. Data shown in b and c are representative contour graphs from one mouse in groups of 4 mice.

Fig. 9. Increased accumulation of RAG-2 protein and rearranged TCRα in DN population of B7-1/2 deficient mice. Total thymocytes from 8-9 week-old B7KO and C57BL/6 mice were collected and DN thymocytes were purified by removing cells expressing CD4, CD8, TCRγδ and $I\text{-}A^b$. In each experiment, 3 thymi per goup were combined to isolate DN cells for protein and total RNA extraction. The experiments were repeated twice. (b). The RAG-2 protein was detected with

western blot. (b). The expressions of *RAG-1*, *RAG-2, pre TCRα*, TCR *Vα2* and *Vα4*, and *CD8 β* genes were detected with real time RT-PCR. ** P<0.01, ***P<0.001.

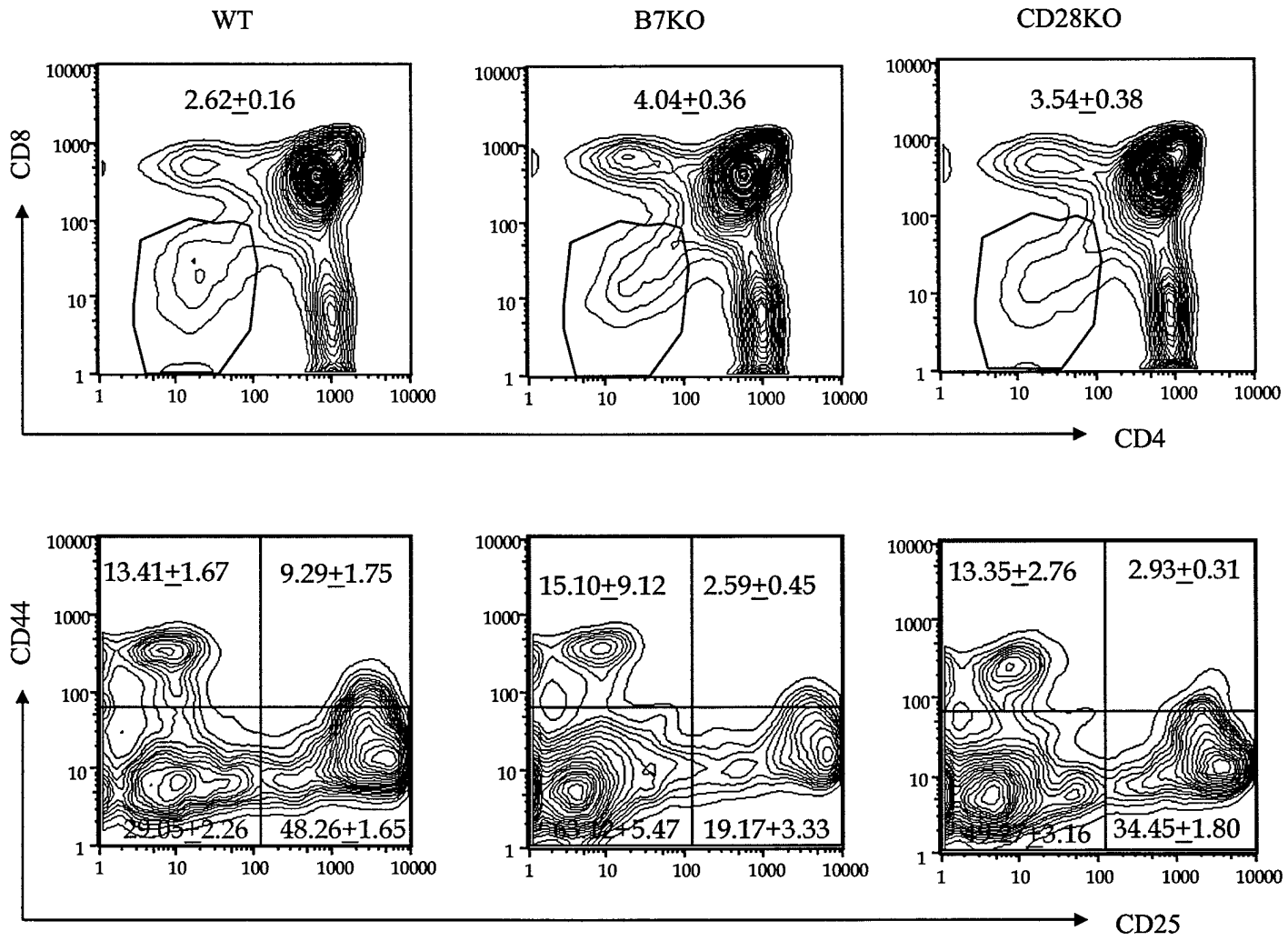

WT
B7KO
CD28KO
CD8
CD4
2.62±0.16
4.04±0.36
3.54±0.38
CD44
CD25
13.41±1.67
9.29±1.75
29.05±2.26
48.26±1.65
15.10±9.12
2.59±0.45
19.17±3.33
13.35±2.76
2.93±0.31
34.45±1.80
1
10
100
1000
10000

Fig. 1

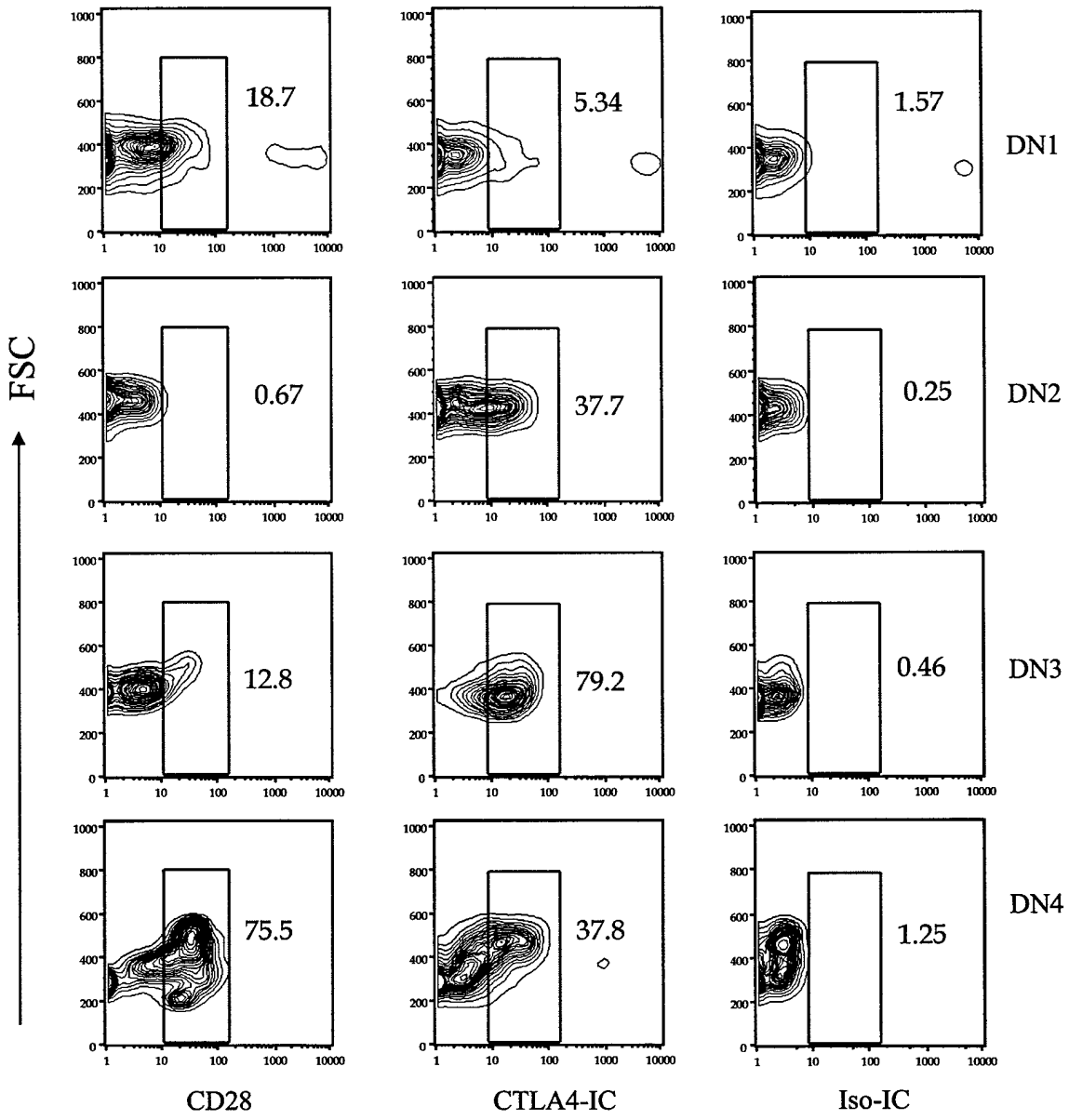
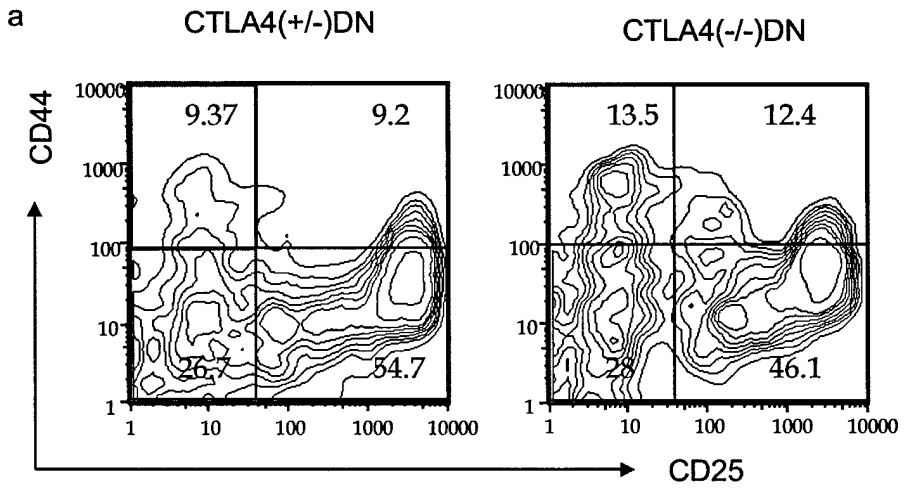

FSC
18.7
5.34
1.57
DN1
0.67
37.7
0.25
DN2
12.8
79.2
0.46
DN3
75.5
37.8
1.25
DN4
CD28
CTLA4-IC
Iso-IC

Fig. 2


a
CTLA4(+/-)DN
CTLA4(-/-)DN
CD44
CD25
9.37
9.2
26.7
54.7
13.5
12.4
28
46.1
10000
1000
100
10
1
1
10
100
1000
10000

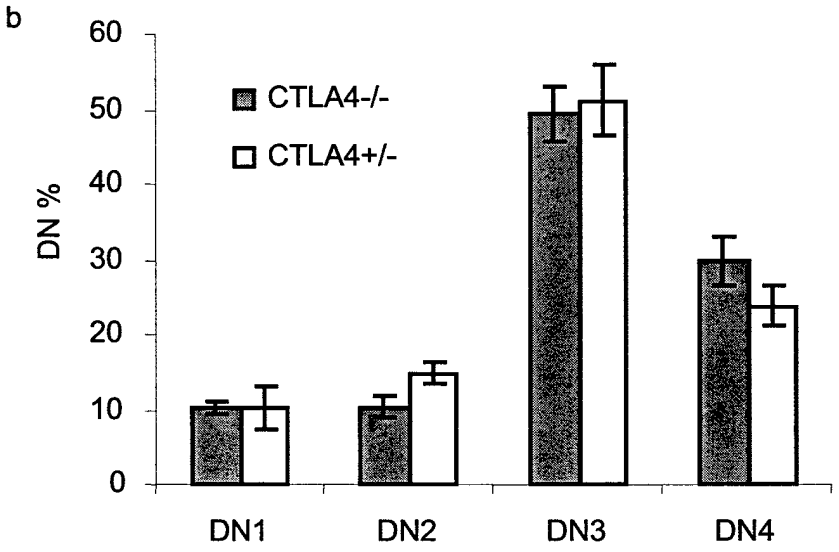
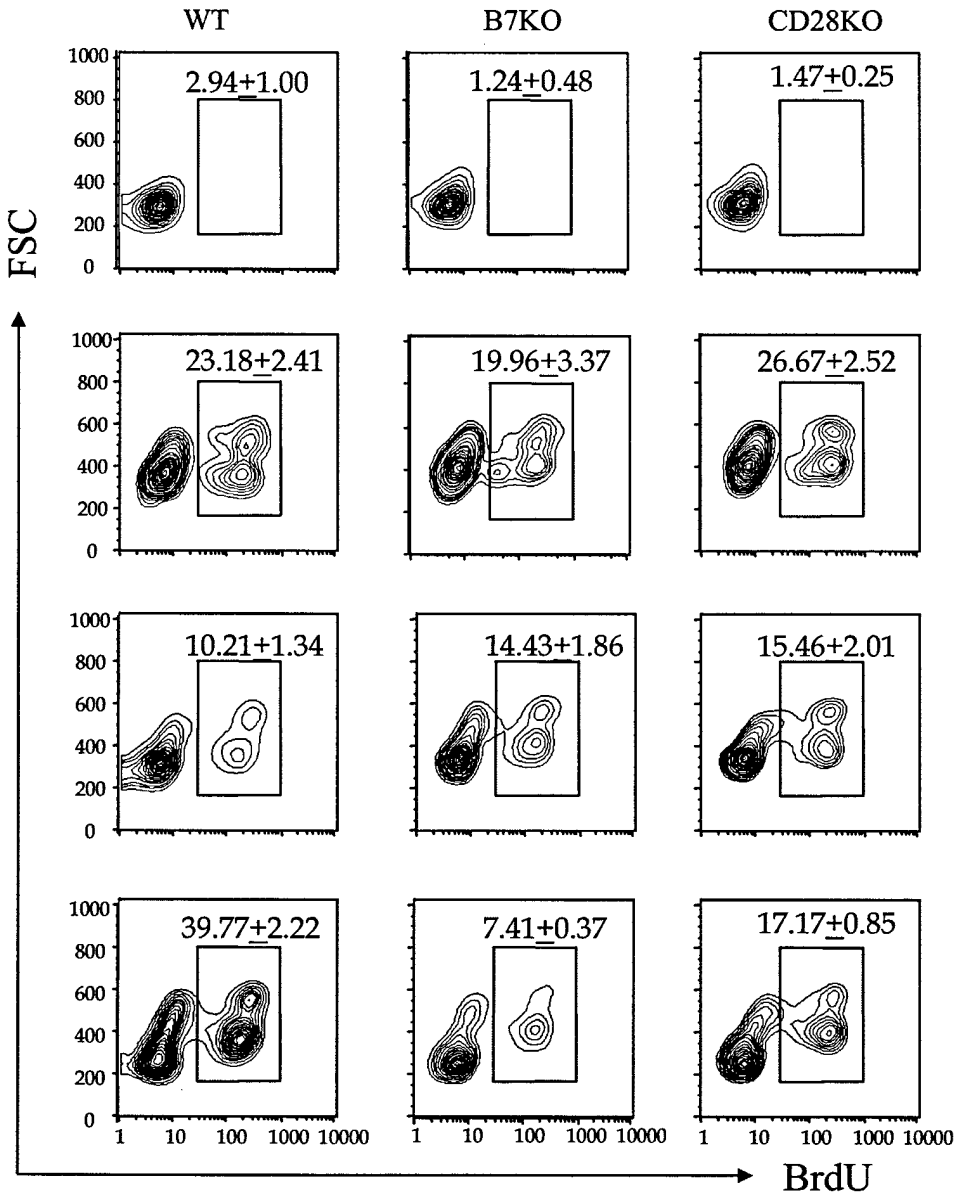

b
DN %
CTLA4-/-
CTLA4+/-
60
50
40
30
20
10
0
DN1
DN2
DN3
DN4

Fig. 3



DN1

WT vs B7KO P=0.022
WT vs CD28K) P=0.028
B7KO vs CD28KO P=0.425

DN2

WT vs B7KO P=0.17
WT vs CD28K) P=0.09
B7KO vs CD28KO P=0.01

DN3

WT vs B7KO P=0.01
WT vs CD28K) P=0.004
B7KO vs CD28KO P=0.48

DN4

WT vs B7KO $P<10^{-5}$
WT vs CD28K) $P<10^{-5}$
B7KO vs CD28KO $P<10^{-5}$

Fig. 4

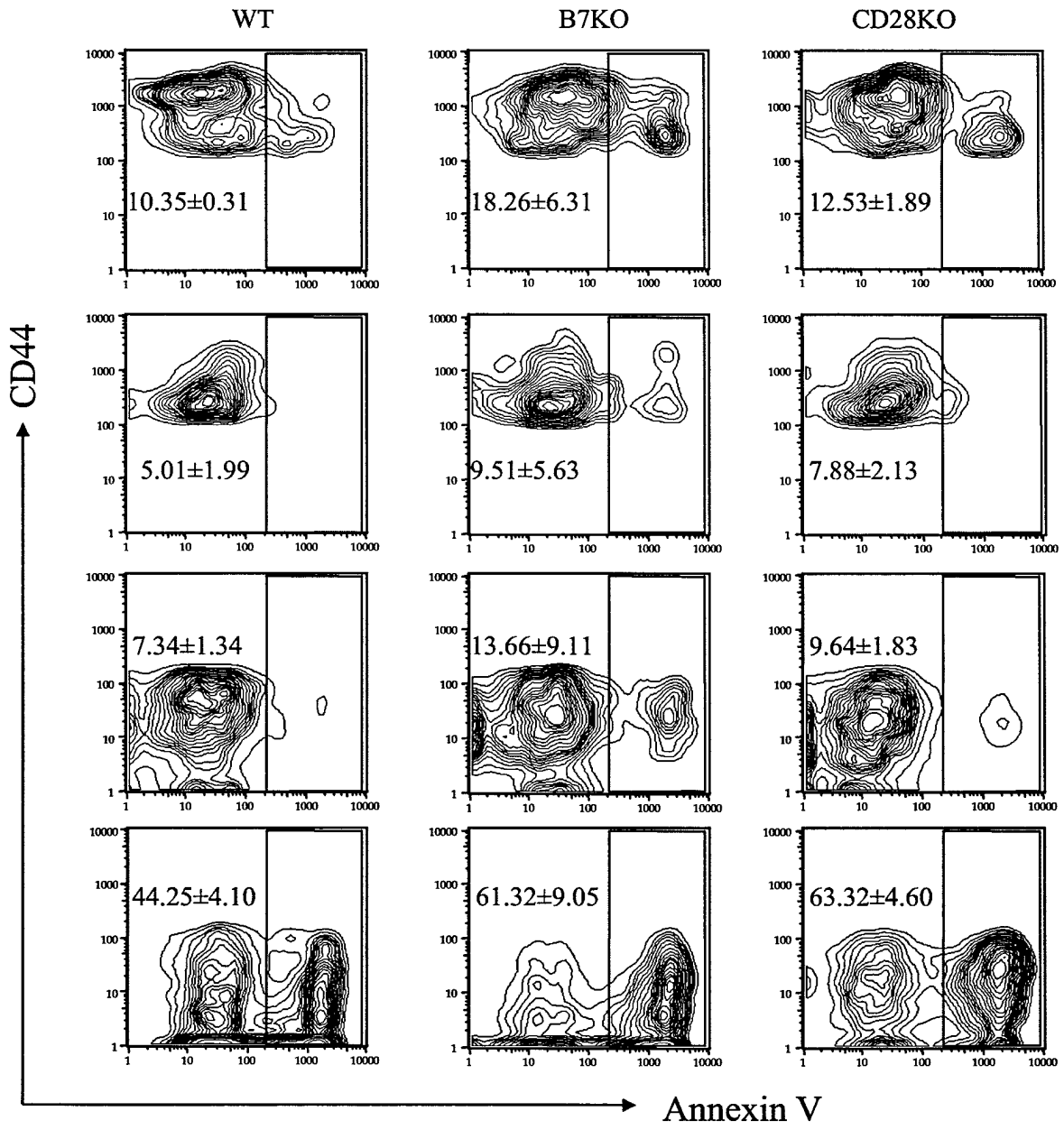

WT
B7KO
CD28KO
DN1
DN2
DN3
DN4
10.35±0.31
18.26±6.31
12.53±1.89
5.01±1.99
9.51±5.63
7.88±2.13
7.34±1.34
13.66±9.11
9.64±1.83
44.25±4.10
61.32±9.05
63.32±4.60
CD44
Annexin V
WT vs B7KO P=0.04
WT vs CD28K) P=0.005
B7KO vs CD28KO P=0.75

Fig. 5

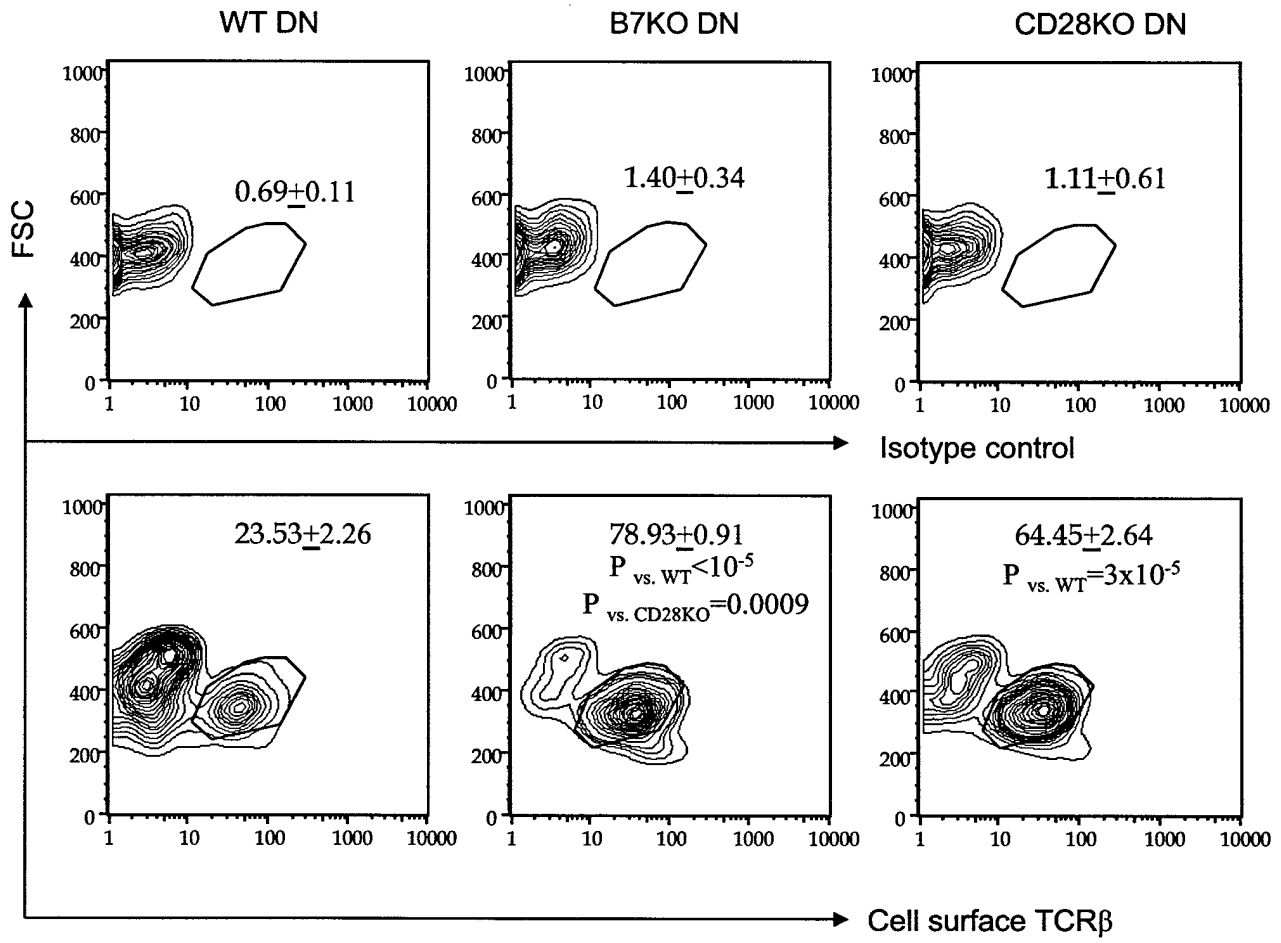

WT DN
B7KO DN
CD28KO DN
FSC
0.69±0.11
1.40±0.34
1.11±0.61
Isotype control
23.53±2.26
78.93±0.91
P vs. WT<10-5
P vs. CD28KO=0.0009
64.45±2.64
P vs. WT=3x10-5
Cell surface TCRβ

Fig. 6

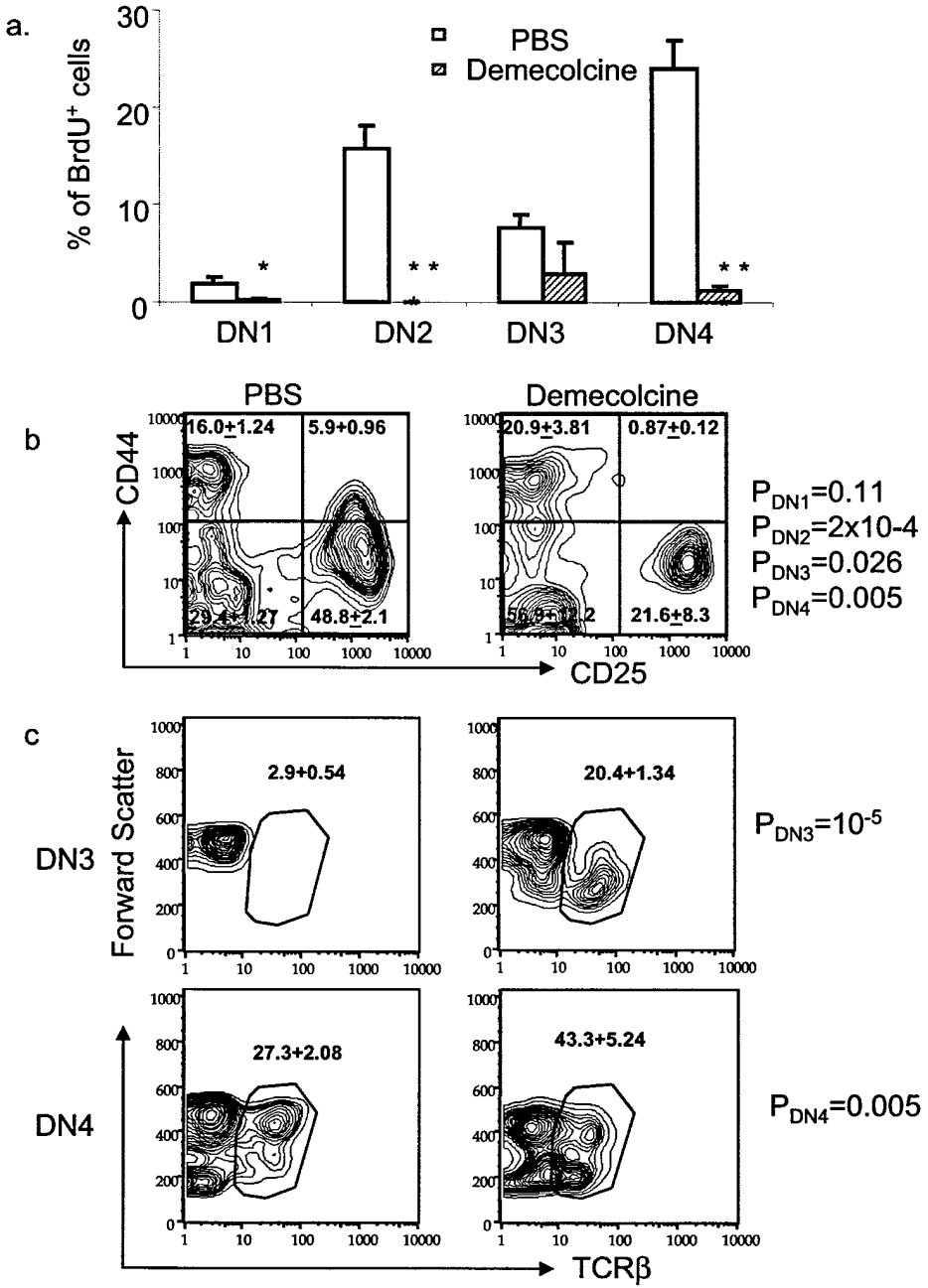

a.
% of BrdU+ cells
30
20
10
0
PBS
Demecolcine
*
* *
* *
DN1
DN2
DN3
DN4
b
PBS
Demecolcine
CD44
16.0±1.24
5.9+0.96
20.9±3.81
0.87±0.12
48.8±2.1
21.6±8.3
CD25
P_DN1=0.11
P_DN2=2x10-4
P_DN3=0.026
P_DN4=0.005
c
DN3
Forward Scatter
2.9+0.54
20.4+1.34
P_DN3=10-5
DN4
27.3+2.08
43.3+5.24
P_DN4=0.005
TCRβ

Fig. 7

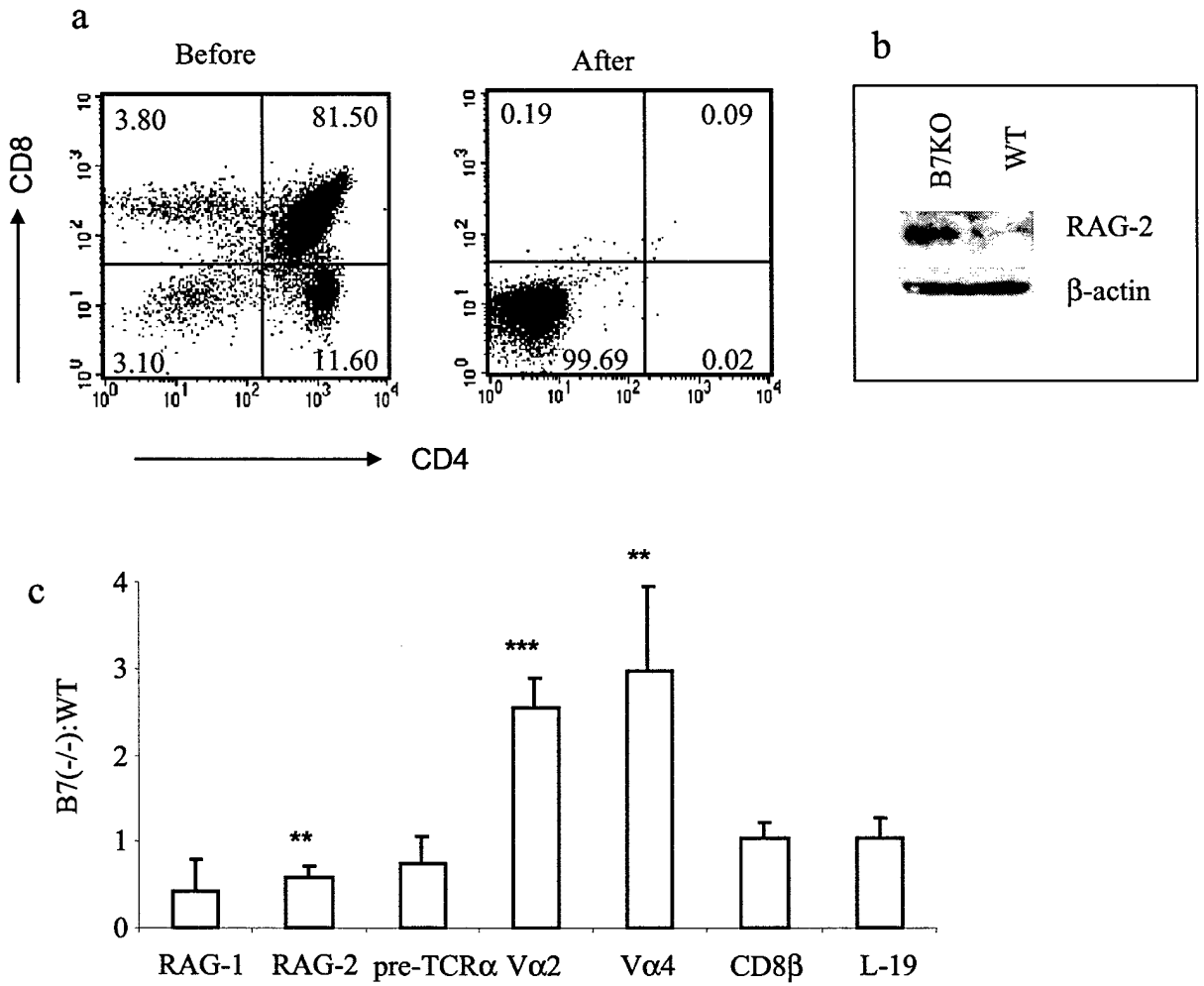

a
Before
After
CD8
CD4
3.80
81.50
3.10
11.60
0.19
0.09
99.69
0.02
b
B7KO
WT
RAG-2
β-actin
c
B7(-/-):WT
RAG-1
RAG-2
pre-TCRα
Vα2
Vα4
CD8β
L-19
**
***
**

Fig. 8