

## Article

# Deficiency of Mouse CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Regulatory T Cells in Xenogeneic Pig Thymus-Grafted Nude Mice Suffering from Autoimmune Diseases

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Xenogeneic thymus transplantation can efficiently induce specific immune tolerance to donor antigens in athymic recipients. However, many nude mice suffer from autoimmune diseases (AID) for over 10 weeks after xenogeneic thymus transplantation. CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T (Treg) cells were recently determined to play a pivotal role in keeping immune tolerance in humans and mice. Thus, we investigated this subpopulation of Treg cells in the periphery of pig thymus-grafted nude mice suffering from AID. Our results showed that the expression of Foxp3, CTLA-4 and GITR on mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells and the ratio of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells to CD4<sup>+</sup> T cells were significantly decreased in the periphery of pig thymus-grafted nude mice suffering from AID, compared with healthy pig or mouse thymus-grafted nude mice. Furthermore, mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells in pig thymus-grafted nude mice suffering from AID showed more severe deficiency in immunosuppressive function compared with the counterpart in xenogeneic pig or syngeneic thymus-grafted nude mice without AID. Thus, the decreased frequency, altered phenotype and functional deficiency of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in pig thymus-grafted nude mice may contribute to the development of AID in this model. *Cellular & Molecular Immunology*. 2008;5(5):325-332.

**Key Words:** regulatory T cell, Foxp3, autoimmune disease, pig, thymus transplantation

## Introduction

Xenotransplantation provides a promising strategy to solve the shortage of donor human organs for transplantation in clinics. Pigs are considered the best candidates for xenotransplantation donors because of their similarity in physiological and anatomical aspects with humans (1-3). Xenogeneic thymus transplantation is an effective strategy to achieve specific immune tolerance to donor antigens (4-6). The reconstitution of mouse functional CD4<sup>+</sup> T cells could be achieved in thymectomized, T cell-depleted mice grafted

with xenogeneic pig thymus tissue and these cells were tolerant to both donors and recipients mainly by intragraft clonal deletion (5), which was evidenced by the observation that porcine MHC mediated positive selection of mouse thymocytes, and both pig and host MHCs were involved in negative selection of mouse thymocytes in pig thymic grafts in this model (7, 8). However, most of pig thymus-grafted nude mice and 10% of grafted thymectomized mice showed severe development of autoimmune diseases (AID) evidenced by pathological and immunohistochemical examinations of livers, lungs and kidneys of mice with wasting syndrome (9). Similarly, other murine models received xenogeneic thymus grafts displayed multi-organ autoimmune syndrome, characterized by the occurrence of wasting, thyroiditis, gastritis, lacrimal gland infiltration and the production of auto-antibodies (10-12). It was demonstrated that mouse CD4<sup>+</sup> T cells played a key role in the development of AID because marked mouse CD4<sup>+</sup> T cell infiltration in organs without detectable pig cells were observed (9).

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**Abbreviations:** AID, autoimmune diseases; cpm, counts per minute; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; FCM, flow cytometry; FITC, fluorescein isothiocyanate; Foxp3, forkhead box p3; GITR, glucocorticoid-induced tumor necrosis factor receptor family related receptor; LN, lymph node; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PI, propidium iodide.

CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells, characterized by the expression of Foxp3, CTLA-4 and GITR, represent 5-10% of peripheral CD4<sup>+</sup> T cells in healthy adult mice and humans (13-16) and play a key role in maintaining self tolerance and immune balance *in vivo* by suppressing the effector functions of CD4<sup>+</sup>CD25<sup>-</sup> T cells (17-20). Accumulating data demonstrated that deficiency of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells was closely correlated with the development of AID, such as diabetes (21), thyroiditis (22), bowel disease (23) and systemic lupus erythematosus (24). Co-transfer of naïve syngeneic splenocytes significantly prevented the occurrence of AID in secondary recipients caused by adoptive transfer of syngeneic splenocytes from pig thymus-grafted athymic mice (9, 12), indicating a defective generation of regulatory lymphocytes in pig thymus-grafted athymic mice. However, our recent studies showed that normal level of mouse CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells presented in the periphery of pig thymus-grafted nude mice when mice did not suffered from AID. These mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells displayed immunosuppressive function on the effector CD4<sup>+</sup>CD25<sup>-</sup> T cells, though it was somewhat less efficient as mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells maturing in syngeneic mouse thymi (25). The relationship between CD4<sup>+</sup>CD25<sup>+</sup> Treg cells and AID in this model thus needs to be clarified. In the present study, the frequency and function of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were studied in pig thymus-grafted nude mice suffering from AID.

## Materials and Methods

### Animals

BALB/c (H-2<sup>d</sup>, female) and nude mice were purchased from Beijing Laboratory Animal Research Center (Beijing, China). All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

### Thymus transplantation

Fetal mouse thymic tissues were isolated from pregnant BALB/c mice (on D17-19). Fetal pig thymic tissues were isolated from pregnant sows (on D70) purchased from China University of Agriculture Sciences (Beijing, China). Thymic tissues were kept in cold RPMI 1640 medium (Invitrogen, Beijing) (~1 mm<sup>3</sup>) and then were implanted under the recipient double kidney capsules of nude mice under anesthesia with intraperitoneal injection of ketamine (0.08 mg/kg; Bayer, Shawnee, Kansas).

### Monoclonal antibodies (mAbs) and reagents

The following mAbs were purchased from BD Biosciences Pharmingen (San Diego, CA) or eBioscience (San Diego, CA): fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 mAb (RM4-5; rat IgG2a), phycoerythrin-CY5 (PE-CY5) labeled anti-mouse CD4 mAb (H129.19; rat IgG2a), FITC-labeled anti-mouse CD8a mAb (53-6.7; rat IgG2a), PE-

labeled anti-mouse CD8a mAb (53-6.7; rat IgG2a), FITC-labeled anti-mouse CD25 mAb (7D4; rat IgM), PE-labeled anti-mouse CD25 mAb (PC61.5; rat IgG1), PE-labeled anti-mouse GITR mAb (DTA-1; rat IgG2b) and PE-labeled anti-mouse CD152 mAb (BNI3; mIgG2a). In addition, PE-labeled anti-mouse Foxp3 mAb (FJK-16s; rat IgG2a) and its staining kit were obtained from eBioscience (San Diego, CA). Rat anti-mouse FcR mAb (2.4G2; IgG2b) was produced by 2.4G2 hybridoma (ATCC, Rockville, Maryland) in our laboratory.

The culture medium used in the present study was RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES and 50 µM 2-ME (Sigma, St. Louis, MO). Mitomycin C (C<sub>15</sub>H<sub>18</sub>N<sub>4</sub>O<sub>5</sub>) was obtained from Kyowa Hakko Co, Ltd. (Tokyo, Japan). <sup>3</sup>H-thymidine was purchased from China Institute of Atomic Energy (Beijing, China).

### Cell preparation

Mouse spleen and lymph nodes (LNs, including cervical, inguinal and axillary LNs) were harvested. Single cell suspensions were prepared by grinding the tissues with the plunger of a 5-ml disposable syringe and were then suspended in cold PBS (containing 0.1% BSA). Splenocytes were treated with a hemolysis buffer (17 mM Tris-HCl and 140 mM NH<sub>4</sub>Cl, pH 7.2) to remove RBCs.

### Purification of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells

CD4<sup>+</sup>CD25<sup>-</sup> T cells from spleens of nude mice grafted with syngeneic thymus and enriched CD4<sup>+</sup>CD25<sup>+</sup> T cell population from spleens of nude mice grafted with syngeneic or xenogeneic thymi were purified by using MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity for CD4<sup>+</sup>CD25<sup>-</sup> T cells was more than 98% and the purity for CD4<sup>+</sup>CD25<sup>+</sup> T cells was more than 90% as determined by flow cytometry (FCM) in each experiment. Purified cells were suspended in complete RPMI 1640 medium.

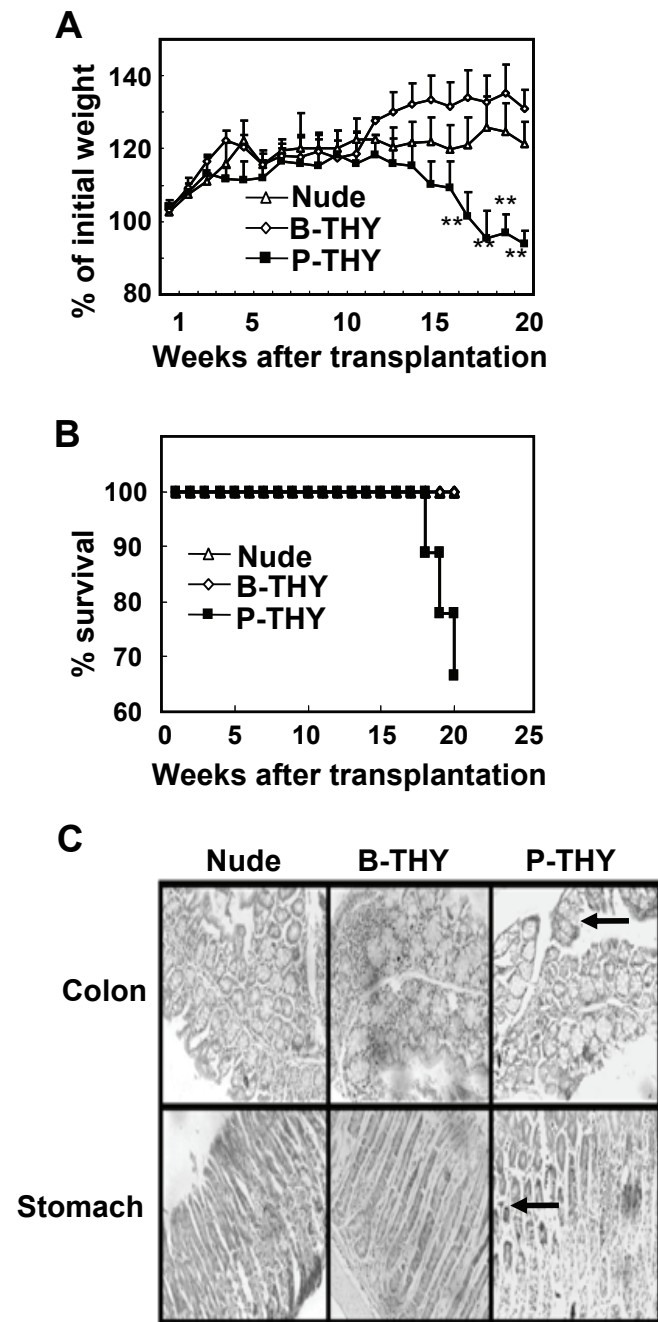
### Immunofluorescence staining and FCM

Cells were incubated with 2.4G2 to block FcRs and then incubated with an optimal concentration of mAbs for 30 min at 4°C in the dark. Cells were washed three times, resuspended by FCM buffer (PBS with 0.1% BSA and 0.1% NaN<sub>3</sub>) and assayed using FACSCalibur (Becton Dickinson, CA). In some experiments, non-viable cells were excluded using the vital nucleic acid stain propidium iodide (PI). The data were analyzed with CellQuest software.

For intracellular staining, cells were incubated with PE-Cy5-labeled anti-CD4 and FITC-labeled anti-CD25 mAbs. After washed, these cells were then stained with PE-labeled anti-mouse CD152 mAb or PE-labeled anti-mouse Foxp3 mAb, according to the instruction offered by the manufactory (eBiosciences, San Diego, CA).

### The proliferation of T cells to Con A

CD4<sup>+</sup>CD25<sup>-</sup> cells (1 × 10<sup>5</sup> cells/well) were cultured in U-bottom,



**Figure 1. The weight, survival and pathological changes of nude mice grafted with xenogeneic pig thymus.** (A) The weight change of nude mice after thymus transplantation. (B) The survival of nude mice after thymus transplantation. (C) The histological changes of colon and stomach in nude mice after thymus transplantation. More than 5 mice in each group were done.

96-well plates with syngeneic accessory cells ( $1 \times 10^5$  splenocytes/well, pretreated with 30  $\mu\text{g/ml}$  mitomycin C at 37°C for 30 min), 2  $\mu\text{g/ml}$  Con A and the indicated numbers of syngeneic  $\text{CD4}^+\text{CD25}^+$  T cells isolated from different mice for 72 h at 37°C in 5%  $\text{CO}_2$  (26).  $^3\text{H}$ -thymidine (0.5  $\mu\text{Ci}$ , 185 GBq/mmol; Atomic Energy Research Establishment, China)

was added into each well for the last 18 h. Cells were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland). Samples were assayed in a Liquid Scintillation Analyzer (Beckman Instruments, America). Values are presented as counts per minute (cpm) of triplicate wells.

#### Histology

Stomachs and colons were harvested from sacrificed experimental animals and fixed in 6% neutral formalin. Samples were embedded in paraffin, sectioned (5  $\mu\text{m}$ ), and stained with H&E for routine pathology.

#### Statistical analysis

All data are presented as mean  $\pm$  SD. Student's unpaired *t* test was used to compare groups. A *p* value of less than 0.05 was considered to be statistically significant.

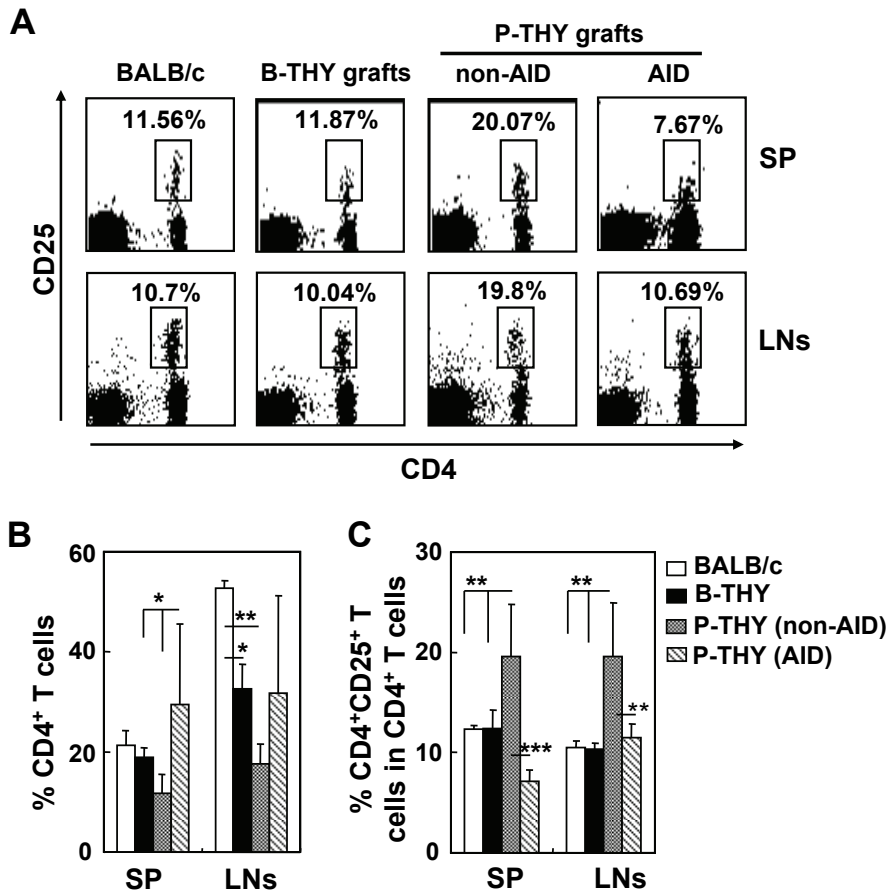
### Results

#### Occurrence of AID in nude mice after xenogeneic pig thymus transplantation

The occurrence of AID was determined by the appearance of symptoms such as wasting, body growth retardation, survival decrease and was confirmed by the pathological changes. AID were not observed in nude mice grafted with syngeneic BALB/c mouse thymus (B-THY). In contrast, some nude mice grafted with pig thymus (P-THY) showed clinical symptoms of AID, such as body weight loss by 12 weeks after pig thymus transplantation (Figure 1A). Some of these nude mice began to die by 18 weeks post-transplantation of pig thymus while nude mice grafted with syngeneic thymus survived well (Figure 1B). The symptoms of AID were also characterized by tissue destruction of colon and stomach (Figure 1C). Thus, these results confirmed that nude mice with xenogeneic pig thymus grafts would develop AID as previously reported (9).

#### The level of mouse $\text{CD4}^+\text{CD25}^+$ T cells in the periphery of nude mice grafted with either xenogeneic pig or syngeneic mouse thymus

It is now widely recognized that  $\text{CD4}^+\text{CD25}^+$  Treg cells play a critical role in keeping peripheral immune tolerance in mice and humans. Therefore, we investigated the ratio of  $\text{CD4}^+\text{CD25}^+$  T cells in  $\text{CD4}^+$  T cells in BALB/c nude mice grafted with B-THY or P-THY. As shown in Figure 2, mouse  $\text{CD4}^+$  T cells recovered efficiently in the periphery of nude mice after grafted with either syngeneic mouse or xenogeneic pig thymus compared with euthymic mice as reported previously (9). However, the percentages of mouse  $\text{CD4}^+\text{CD25}^+$  T cells in pig thymus-grafted nude mice suffering from AID were significantly lower than those in mice without AID or nude mice grafted with mouse thymus ( $p < 0.001$ , Figure 2C), though healthy nude mice with pig thymus grafts showed even higher levels of  $\text{CD4}^+\text{CD25}^+$  T cells in the periphery compared with nude mice with syngeneic mouse thymus grafts or euthymic control mice ( $p < 0.001$ , Figure 2C).



**Figure 2.** The levels of CD4<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> T cells in spleens and LNs of nude mice after thymus transplantation. (A) Cells from spleens or LNs of mice grafted with syngeneic or xenogeneic thymic tissue were stained with FITC-anti-mCD4 and PE-anti-mCD25 mAbs. The percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells to CD4<sup>+</sup> T cells were analyzed. (B) The percentages of CD4<sup>+</sup> T cells in spleens and LNs. (C) The ratio of CD4<sup>+</sup>CD25<sup>+</sup> T cells to CD4<sup>+</sup> T cells were further analyzed by Student's *t* test and shown. Results were shown as mean  $\pm$  SD (*n* = 3-5 in each group). \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001 compared with the indicated group.

*The decreased frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in pig thymus-grafted nude mice suffering from AID*

Foxp3 plays a key role in the development and function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells and is considered as the authoritative marker for this subpopulation of T cells (16). We thus investigated the expression of Foxp3 in mouse CD4<sup>+</sup> T cells derived in pig thymus grafts in this model. The percentage of Foxp3<sup>+</sup> cells in CD4<sup>+</sup>CD25<sup>+</sup> T cells and MFI of Foxp3 molecules in CD4<sup>+</sup>CD25<sup>+</sup> T cells in healthy pig or mouse thymus-grafted nude mice were similar to the counterparts in euthymic control mice (Figures 3A-3C, *p* > 0.05). However, in pig thymus-grafted nude mice suffering from AID, the percentage of Foxp3<sup>+</sup> cells in CD4<sup>+</sup>CD25<sup>+</sup> T cells and MFI of Foxp3 molecules in CD4<sup>+</sup>CD25<sup>+</sup> T cells were significantly decreased compared with other groups (Figures 3A-3C). Consistently, the ratio of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells to CD4<sup>+</sup> T cells was significantly lower in pig thymus-grafted nude mice suffering from AID than those in syngeneic thymus-grafted nude mice or euthymic control BALB/c mice (Figure 3D, *p* < 0.001).

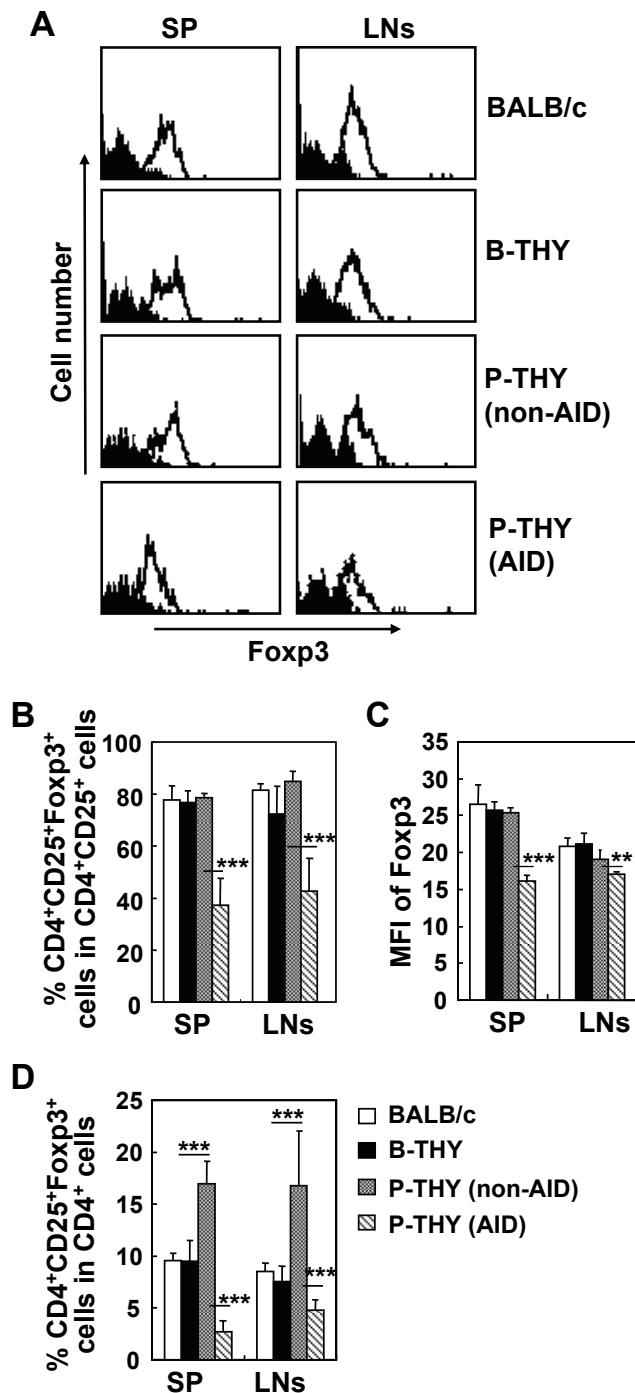
In addition to Foxp3, CTLA-4 and GITR, the co-stimulators expressed on CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, are tightly involved in modulating the immunosuppressive function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (27, 28). More mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells in pig thymus-grafted nude mice without AID showed dominant expression of CTLA-4 and GITR compared to

those in syngeneic thymus-grafted nude mice and euthymic control BALB/c mice (Figure 4, *p* < 0.001). However, the expressions of CTLA-4 and GITR on CD4<sup>+</sup>CD25<sup>+</sup> T cells in pig thymus-grafted nude mice suffering from AID were significantly lower than those in pig thymus-grafted nude mice without AID (Figure 4, *p* < 0.001).

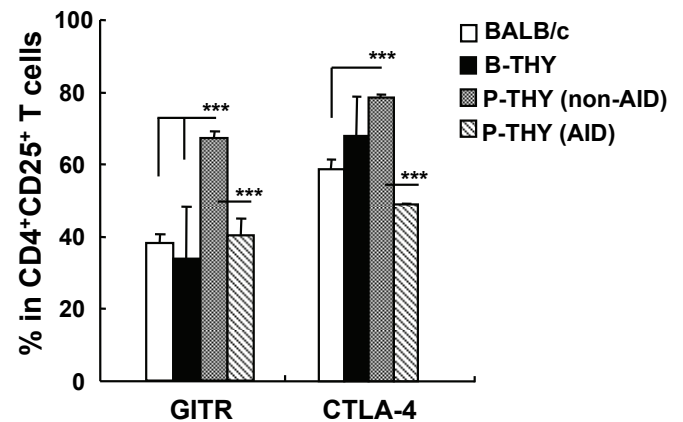
*The significantly reduced immunosuppressive function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in pig thymus-grafted nude mice suffering from AID*

To evaluate the function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in the periphery of pig thymus-grafted nude mice with or without AID, we enriched mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells from splenocytes of euthymic control BALB/c mice or nude mice grafted with syngeneic or xenogeneic thymi using FACS. Enriched mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from nude mice grafted with syngeneic thymus displayed a similar and dose-dependent suppressive function on the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells stimulated by Con A compared with those derived from euthymus control BALB/c mice (Figure 5). However, mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from pig thymus-grafted nude mice showed significantly lower suppressive function on the proliferation of mouse CD4<sup>+</sup>CD25<sup>-</sup> T cells to Con A than those from euthymus control BALB/c mice or syngeneic thymus-grafted nude mice. Importantly, the suppressive function of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells separated from pig





**Figure 3.** The levels of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in spleens and LNs of mice grafted with syngeneic or xenogeneic thymus. (A) Cells from spleens and LNs of mice grafted with syngeneic or xenogeneic thymus were stained with PE-CY5-anti-mCD4, FITC-anti-mCD25 and PE-anti-mFoxp3 mAbs. The expression of Foxp3 in CD4<sup>+</sup>CD25<sup>+</sup> T cells was analyzed by flow cytometry. (B) The percentage of Foxp3<sup>+</sup> cells in CD4<sup>+</sup>CD25<sup>+</sup> T cells from spleens and LNs. (C) The median fluorescence intensity (MFI) of Foxp3 in CD4<sup>+</sup>CD25<sup>+</sup> T cells. (D) The ratio of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells to CD4<sup>+</sup> T cells in spleens and LNs. Results were shown as mean  $\pm$  SD (n = 3-5 in each group). Data were representative of three independent experiments with identical results. \*\*p < 0.01, and \*\*\*p < 0.001 compared with the indicated group.



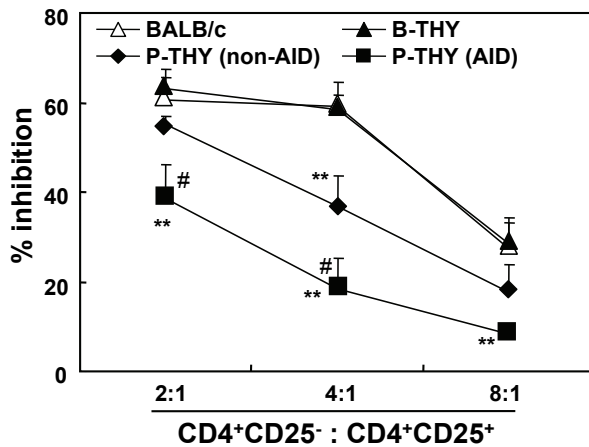
**Figure 4.** The expression of GITR and CTLA-4 on CD4<sup>+</sup>CD25<sup>+</sup> T cells in the spleens of mice after thymus transplantation. Cells from spleens of mice grafted with syngeneic and xenogeneic thymus were stained with PE-CY5-anti-mCD4, FITC-anti-mCD25, and mouse GITR or CTLA-4, respectively. The average percentages of GITR<sup>+</sup> and CTLA-4<sup>+</sup> cells in CD4<sup>+</sup>CD25<sup>+</sup> T cells were shown. Results were shown as mean  $\pm$  SD (n = 3-5 in each group). \*\*\*p < 0.001 compared with the indicated groups.

thymus-grafted nude mice with AID was severely reduced compared with those in pig thymus-grafted nude mice without AID (Figure 5).

## Discussion

It is demonstrated that mouse CD4<sup>+</sup> T cells develop normally in xenogeneic pig thymic grafts in which pig MHCs mediate positive selection and both pig and mouse MHCs participate in negative selection in pig thymus-grafted thymectomized mice (8, 29). T cells maturing in xenogeneic pig thymus grafts were tolerant to donor antigens mainly by thymic clonal deletion, whereas CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were involved in as well (5). However, it is reported that AID usually occurred in xenogeneic thymus recipients including nude mice or rats (9-12, 30). The autoimmune symptoms were also observed in the present study as wasting, growth retardation, decreased survival and destruction of colon and stomach in nude mice by 12 weeks after fetal pig thymus transplantation, while syngeneic thymus-grafted nude mice did not show detectable pathological changes.

AID in the secondary recipients caused by adoptive transfer of syngeneic splenocytes from xenogeneic thymus-grafted animals with AID could be prevented by the co-transfer of syngeneic splenocytes from euthymic animals indicating that a deficiency in regulatory immune cells may exist in xenogeneic thymus-grafted athymic animals (9, 10, 12). In the present study, we detected the frequency and function of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in peripheral lymphoid tissues of pig thymus-grafted nude mice with or without AID. Significant high levels of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells were detected in pig thymus-grafted nude mice without AID as reported before (25). However, markedly decreased



**Figure 5. Reduced immunosuppressive ability of mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells from pig thymus-grafted nude mice with AID.** Cell proliferation assay was determined by <sup>3</sup>H-thymidine incorporation. CD4<sup>+</sup>CD25<sup>+</sup> T cells were stimulated with 2 µg/ml Con A in the presence of the indicated numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells from spleens of mice grafted with syngeneic or xenogeneic thymus. Cultures were incubated for 4 days and pulsed with <sup>3</sup>H-thymidine for the last 18 h. Data were representative of two independent experiments with identical results. \*\**p* < 0.01 compared with BALB/c or syngeneic BALB/c thymus-grafted BALB/c nude mice. #*p* < 0.05 compared with pig thymus-grafted BALB/c nude mice without AID.

frequency of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in pig thymus-grafted nude mice with AID was observed. The reasons for the decreased frequency of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in this model with AID are not clear. It may be caused by the poor survival of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells maturing in xenogeneic thymic grafts in the periphery, which should be investigated in the future.

Foxp3, a conservative transcription factor, is a convincing marker for CD4<sup>+</sup>CD25<sup>+</sup> Treg cells and plays a critical role in the development and function of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (14, 16). CTLA-4 and GITR are expressed predominantly on CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (31, 32), although they are also expressed constitutively at low levels on conventional CD4<sup>+</sup>CD25<sup>+</sup> T cells (31, 33), and are closely related with the negative regulation of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (20, 27, 34-36). Impressively, mouse CD4<sup>+</sup>CD25<sup>+</sup> T cells in pig thymus-grafted nude mice with AID expressed significantly less Foxp3, CTLA-4 and GITR molecules. Consistently with the phenotype changes, CD4<sup>+</sup>CD25<sup>+</sup> Treg cells separated from pig thymus-grafted nude mice with AID showed remarkably decreased immunosuppressive function compared with CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from either euthymic mice or pig thymus-grafted nude mice without AID. Thus, both decreased frequency and functional deficiency of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in pig thymus-grafted nude mice may be related to the occurrence of AID in this model. The reasons for the changes of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in these mice with AID need to be identified.

It is known that thymus, especially thymic epithelium

expresses broad auto-antigens by which auto-reactive T cells are clonally deleted and followed by tolerance. Recently, it was reported that medullary thymic epithelial cells (mTECs) might determine the development of CD4<sup>+</sup>CD25<sup>+</sup> Treg cell lineage because targeting of a model antigen to Aire<sup>+</sup> mTECs led to the generation of specific CD4<sup>+</sup>CD25<sup>+</sup> Treg cells independently of antigen transfer to dendritic cells (37). Thus, it is speculated that mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells should be selected by xenogeneic pig mTECs in our present model. Whether mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells selected by pig mTECs will function well to prevent autoimmunity in the periphery of pig thymus-grafted nude mice or not needs to be studied.

It is noted that, in some cases, pig thymus-grafted nude mice without AID or BALB/c thymus-grafted nude mice exhibited a reduction of CD4<sup>+</sup> T cells in spleens or LNs as compared to those in pig thymus-grafted nude mice or BALB/c mice. The reasons for the occurrence are not clear at this moment. It may be related to grafted thymic tissue size, T cell recovery kinetics, and T cell expansion in different individual recipients with or without AID. However, it was demonstrated that mouse CD8<sup>+</sup> T cells always had poor recovery in xenogeneic pig thymus-grafted nude mice. The poor recovery of mouse CD8<sup>+</sup> T cells in the periphery of pig thymus-grafted athymic mice may be due to the poor migration ability of mouse CD8 single positive thymocytes from xenogeneic pig thymic grafts to the mouse periphery or/and the poor peripheral survival of mouse CD8<sup>+</sup> T cells positively selected by pig MHCs in pig thymic grafts in these models (38).

In summary, significantly low levels of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells with phenotype alteration including the expression of Foxp3, CTLA-4 and GITR were detected in pig thymus-grafted nude mice with AID. Enriched mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in pig thymus-grafted nude mice with AID displayed markedly impaired immunosuppressive ability. The decreased levels, altered phenotype and functional deficiency of mouse CD4<sup>+</sup>CD25<sup>+</sup> Treg cells may contribute to the development of AID in pig thymus-grafted nude mice.

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