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Development of mouse $CD4^+CD25^+Foxp3^+$ regulatory T cells in xenogeneic pig thymic grafts

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ABSTRACT

Xenogeneic thymus transplantation is an effective strategy to induce tolerance to donors mainly by clonal depletion of reactive T cells. Recent studies have shown that functional mouse $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) could efficiently populate in the periphery of athymic mice after grafting with neonatal pig thymus. However, it is still unknown whether xenogeneic thymus grafts could directly support the development of mouse $CD4^+CD25^+Foxp3^+$ Treg cells as an autogeneic counterpart. Our results show that the percentages of mouse $CD4^+CD8^-CD25^+$ thymocytes are similar among auto- and xenogeneic thymic grafts in thymic mouse recipients. Mouse $CD4^+CD8^-CD25^+$ thymocytes maturing in xenogeneic thymic grafts exhibit similar expressions of Foxp3, TCR, CTLA-4 and GITR as those in autogeneic thymic grafts. Moreover, mouse $CD4^+CD8^-CD25^+$ thymocytes maturing in xenogeneic thymic grafts showed a significant immunosuppressive function on the proliferation of $CD4^+CD25^-$ T cells stimulated with Con A or allogeneic antigens, although they showed weaker effects than those maturing in autogeneic thymic grafts. Therefore, the present data provides direct evidence for the ability of xenogeneic porcine thymus grafts to support the development of mouse naturally occurring $CD4^+CD25^+Foxp3^+$ Treg cells.

1. Introduction

Regulatory T cells (Treg), especially $CD4^+CD25^+$ Treg cells, play a key role in maintaining peripheral tolerance by active control of effector T cells, in addition to central tolerance induced by clonal deletion of T cells with high avidity for thymically expressed antigens. Depletion of functional $CD4^+CD25^+$ Treg cells leads to prominent autoimmune diseases such as diabetes, thyroiditis, bowel disease, and systemic lupus erythematosus [1–3]. This subpopulation of $CD4^+CD25^+$ Treg cells, which are mainly developed in the thymus, represents 5–10% of peripheral $CD4^+$ T cells in healthy adult mice and humans [4–8]. $CD4^+CD25^+$ Treg cells are characterized by several makers including Foxp3, CTLA-4, GITR and so on [9]. They are usually anergic but could potentially suppress the functions of $CD4^+CD25^-$ T or other immune cells [7,10]. Accumulating experimental and clinical evidence has demonstrated that $CD4^+CD25^+$ Treg cells are closely involved in transplant tolerance in humans and some mouse models [11–15].

Pigs are considered the best candidates for xenotransplantation because of their physiological and anatomical similarities to humans, and their efficient breeding and genetic manipulation [16–19]. Fetal or neonatal pig thymus (FP- or NP-THY) transplantation could reconstitute functional mouse $CD4^+$ T cells in thymectomized, T cell-depleted mice. These cells are specifically tolerant to donor pig antigens [20–22]. Recent studies demonstrated that a normal level of mouse $CD4^+CD25^+$ Treg cells was restored in the periphery of nude mice by grafting neonatal pig thymi [23]. These Treg cells shared similar characteristic expression of Foxp3, CTLA-4, GITR and CD62L with the counterparts of normal BALB/c mice. Also, they showed a similar T-cell receptor (TCR) V beta repertoire with normal BALB/c mice. More importantly, they exerted significant immunosuppression on the proliferation and interleukin-2 production of effector $CD4^+CD25^-$ T cells induced by mitogen, alloantigens and peptide antigens in vitro [23]. The enhanced immunosuppression of $CD4^+CD25^+$ Treg cells isolated from NP-THY-graft nude mice on the response of effector T cells to donor antigens was determined in vitro [23], suggesting that xenogeneic thymus transplantation could induce tolerance to donor antigens not only by clonal depletion but also possibly by the contribution of $CD4^+CD25^+$ Treg cells [21]. However, it is still unclear whether peripheral mouse $CD4^+CD25^+$ Treg cells develop in xenogeneic porcine thymic grafts in a similar manner as in host thymus or whether they develop in the periphery of this mouse model.

Abbreviations: 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; LNs, lymph nodes; mAbs, monoclonal antibodies; MLR, mix lymphocyte reaction; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; PI, propidium iodide; TCR, T-cell receptor.

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Therefore, we investigated the presence, characteristics and suppressive function of $CD4^+CD25^+Foxp3^+$ T cells in pig thymic grafts. Our results show that functional mouse $CD4^+CD25^+$ Treg cells could develop in xenogeneic porcine thymic grafts as efficiently as autogeneic thymic grafts in nude mouse recipients. The present study offers direct evidence that xenogeneic pig thymus supports the development of mouse $CD4^+CD25^+$ Treg cells.

2. Materials and methods

2.1. Animals and pig tissues

BALB/c mice (H-2^d, 8-week-old, female) and nude mice were purchased from the Beijing Laboratory Animal Research Center (Beijing, China). All mice were maintained in a specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. Pregnant sows (70 days) or 2-day-old pigs were purchased from the China University of Agriculture Sciences (Beijing, China). All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

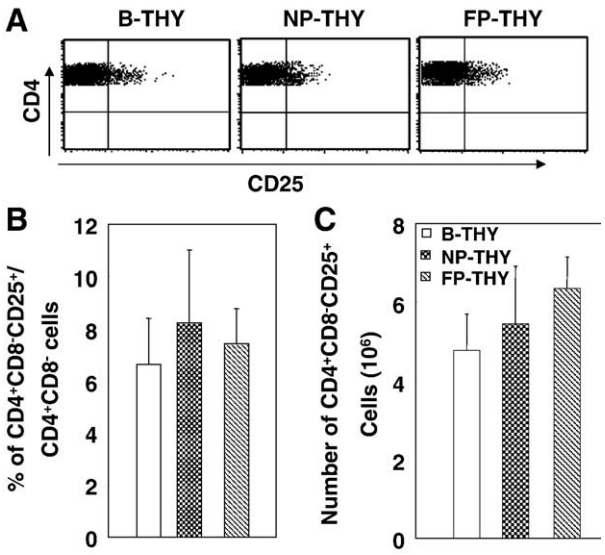

Fig. 2. The level of mouse $CD4^+CD8^-CD25^+$ thymocytes in auto- and xenogeneic thymic grafts. Cells from auto- and xenogeneic thymic grafts were stained with PE-CY5-anti-mCD4 mAb, FITC-anti-mCD8 mAb and PE-anti-mCD25 mAb and analyzed by FCM. (A) One representative of thymocytes of auto- and xenogeneic thymic grafts stained with three antibodies mentioned above and with gate on $CD4^+CD8^-$ cells. (B) The average ratio of $CD4^+CD8^-CD25^+$ to $CD4^+CD8^-$ thymocytes in auto- and xenogeneic thymic grafts. (C) The cell numbers of $CD4^+CD8^-CD25^+$ thymocytes in auto- and xenogeneic thymic grafts. Results were shown as mean±SD, and 5 mice in each group were done. No significant difference was detected among groups ($P>0.05$).

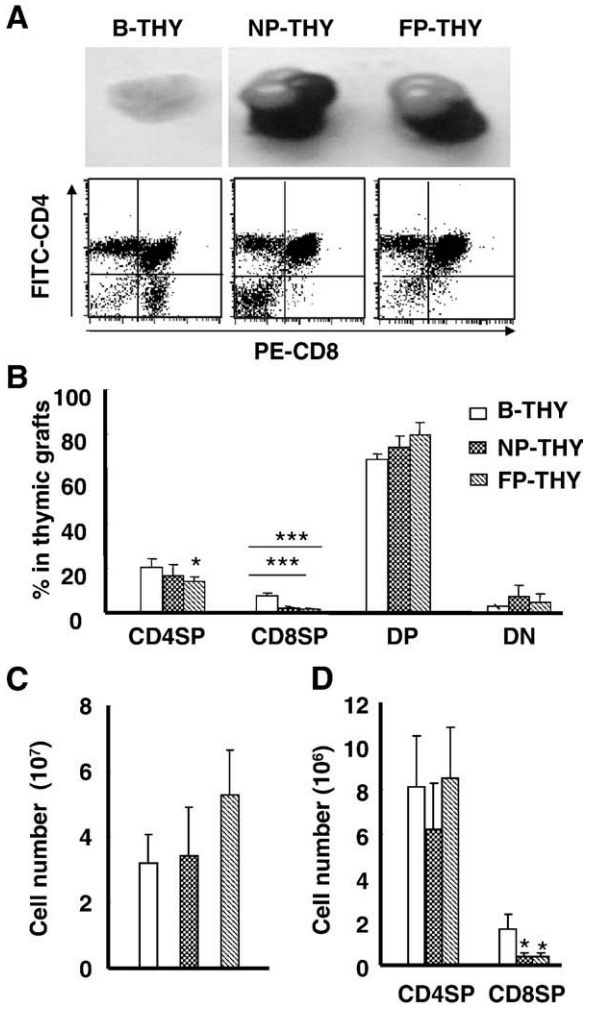

Fig. 1. Mouse thymocyte subpopulations in auto- and xenogeneic thymic grafts. By thymic transplantation for 10 weeks, thymocytes were stained with FITC-anti mCD4 mAb and PE-anti mCD8 mAb and analyzed by FCM. (A) One representative of thymocytes separated from auto- (, BALB/c thymic grafts, B-THY) and xenogeneic (neonatal or fetal pig thymic grafts, NP-THY or FP-THY) thymic grafts with anti-mCD4 and anti-mCD8 mAbs assayed by FCM ($n=3–5$). (B) The percentages of CD4SP, DP, DN and CD8 SP cells in different thymic grafts. (C) The total thymocyte number of thymic grafts. (D) The cell numbers of CD4 SP and CD8 SP thymocytes. Results were shown as mean±SD, and 5 mice in each group were done. *, $P<0.05$; ***, $P<0.001$ compared among the indicated groups.

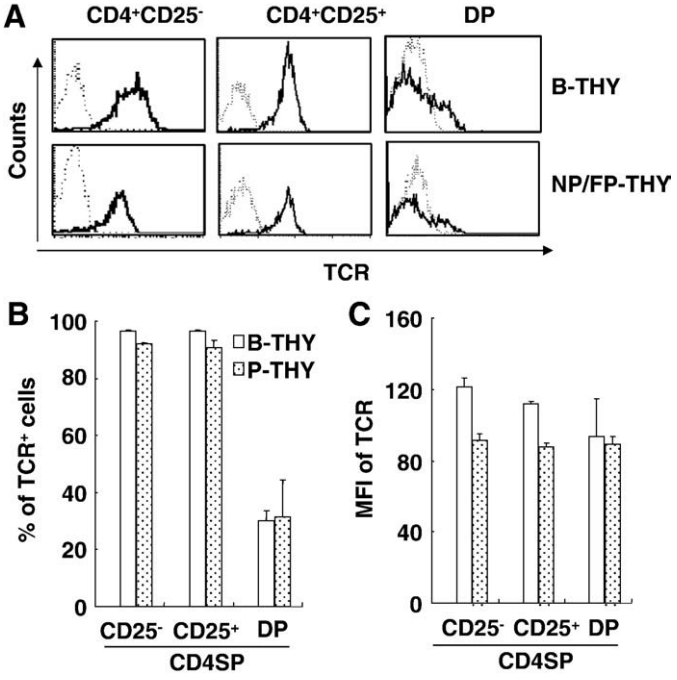

Fig. 3. Expression of mouse TCR on $CD4^+CD25^+$ thymocytes. CD4 SP cells were purified from thymocytes of auto- and xenogeneic thymic grafts and stained with PE-CY5-anti-mCD4 mAb, FITC-anti-mCD25 mAb and PE-anti-mTCR mAb. (A) One representative of TCR staining in $CD4^+CD25^-$, $CD4^+CD25^+$ and DP thymocytes. The broken and solid lines represent isotype control and specific mAb, respectively. (B) The percentages of TCR^+ cells in $CD4^+CD25^-$, $CD4^+CD25^+$ and DP thymocytes of each group. (C) The mean of TCR on $CD4^+CD25^-$, $CD4^+CD25^+$ and DP thymocytes of each group. Five mice in each group were performed. No significant difference was detected among groups ($P>0.05$).

2.2. Thymus transplantation

Mouse thymic tissues were isolated from 1-day-old BALB/c mice. Pig thymic tissues were isolated from fetal or 2-day-old neonatal pigs. Thymic tissues (~1 mm^3) were kept in cold RPMI 1640 medium (Invitrogen, Beijing) and were then implanted under the kidney capsules of BALB/c nude mice under anesthesia with intraperitoneal injection of ketamine (0.08 mg/g; Bayer, Shawnee, Kansas).

2.3. Monoclonal antibodies (mAbs) and reagents

The following mAbs were purchased from either 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 CD4mAb (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 CD62L mAb (SK11; rIgG2a), PE-labeled anti-mouse glucocorticoid-induced tumor-necrosis-factor-receptor (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 ($C_{15}H_{18}N_4O_5$) was obtained from Kyowa Hakko Co, Ltd. (Tokyo, Japan). [^{3}H] thymidine was purchased from China Institute of Atomic Energy (Beijing, China).

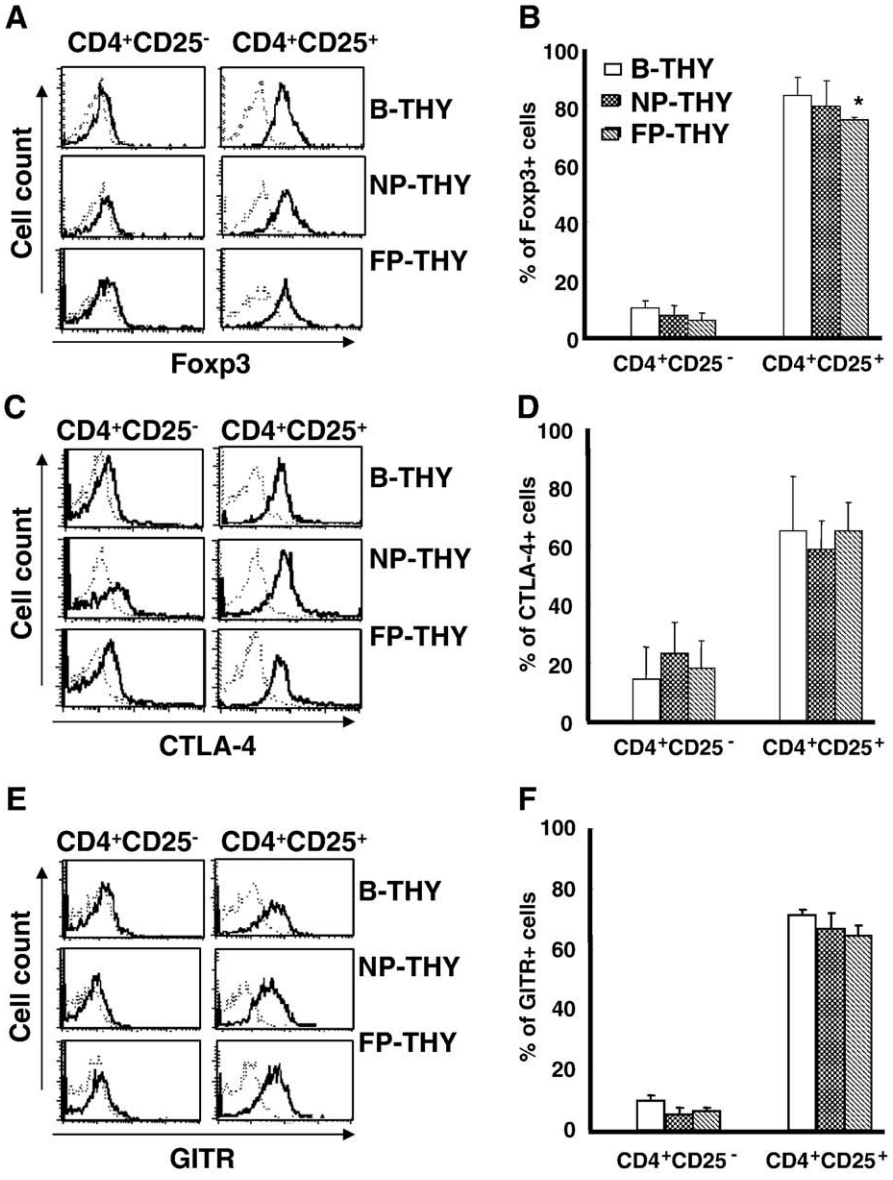

Fig. 4. Expression of mouse Foxp3, CTLA-4 and GITR on $CD4^+CD25^+$ thymocytes. CD4 SP cells were purified from thymocytes of auto- and xenogeneic thymic grafts, and were stained with PE-CY5-anti-mCD4 mAb, FITC-anti-mCD25 mAb and PE-anti-mFoxp3 mAb, PE-anti-mCTLA-4 mAb or PE-anti-mGITR mAb respectively. For detecting the expression of Foxp3 and CTLA-4, intracellular staining was performed. One representative and percentages of Foxp3 (A and B), CTLA-4 (C and D) or GITR (E and F) staining in $CD4^+CD25^-$ or $CD4^+CD25^+$ thymocytes in each group. The broken and solid lines represent isotype control and specific mAb, respectively. Data was presented as mean ± SD ($n=5$). *$P<0.05$ compared with the control mice.

2.4. Immunofluorescence staining and flow cytometry (FCM)

Control thymus or thymic grafts were harvested and single cell suspensions were prepared by grinding the tissues with the plunger of a 5-ml disposable syringe and were then suspended in cold FCM buffer (PBS with 0.1% BSA and 0.1% NaN_3). Thymocytes 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 and assayed using a FASCalibur flow cytometry (Becton Dickinson, CA). In some experiments, non-viable cells were excluded using the vital nucleic acid stain propidium iodide (PI). The data was analyzed with the CellQuest software.

For the intracellular staining, cells were incubated with PE-Cy5-labeled anti-CD4 and FITC-labeled anti-CD25 mAbs [24]. After washing, 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).

2.5. Purification of mouse $CD4^+CD25^+$ and $CD4^+CD25^-$ thymocytes

Firstly, thymic $CD8^+$ cells were deleted by anti-mCD8 mAb and complement. $CD4^+CD25^-$ T cells from $CD8^+$ depleted thymocytes of normal BALB/c mice and $CD4^+CD25^+$ T cells from $CD8^+$ cell-depleted thymocytes of thymus grafts were isolated using MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions, respectively. The purity for $CD4^+CD8^-CD25^-$ thymocytes was more than 95% and the purity for $CD4^+CD8^-CD25^+$ thymocytes was more than 90% as determined by FCM in each experiment. Purified cells were suspended in complete RPMI 1640 medium.

2.6. The proliferation of T cells to Con A

BALB/c $CD4^+CD8^-CD25^-$ thymocytes (1×10^5 cells/well) were cultured in U-bottom, 96-well plates with syngeneic accessory cells (1×10^5 splenocytes/well, pretreated with 30 μg/ml mitomycin C at 37 °C for 30 min), 2 μg/ml Con A and the indicated numbers of syngeneic $CD4^+CD8^-CD25^+$ thymocytes isolated from either auto- or xenogeneic thymus grafts for 72 h at 37 °C, 5% CO_2. 0.5 μCi [^{3}H] thymidine (185 GBq/mmol; Atomic Energy Research Establishment, China) was added to each well for the last 18 h [25]. Cells were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland). Samples were assayed in a Liquid Scintillation Analyzer (Beckmon Instruments, America). Values are presented as counts per minute (cpm) of triplicate wells.

2.7. Mixed lymphocyte reaction (MLR)

BALB/c $CD4^+CD8^-CD25^+$ thymoctyes were isolated from either control or TT mice as described above. BALB/c $CD4^+CD8^-CD25^-$ thymocytes were used as responder T cells. C57BL/6 splenocytes, which were pretreated with mitomycin C at the concentration of 30 μg/ml at 37 °C for 30 min, were used as allogeneic stimulator cells. Generally, 1×10^5 responder cells and 1×10^5 stimulator cells per well in RPMI1640 medium supplemented with 10% FCS were added in 96-well round-bottomed plates [26]. The indicated numbers of $CD4^+CD8^-CD25^+$ thymocytes were subsequently added to each well. Cells were cultured at 37 °C and 5% CO_2 for 4 days. 0.5 μCi [^{3}H] thymidine was added for the last 18 h. Cells were harvested with an automatic cell harvester (Tomtec, Toku, Finland). The radioactivity of each sample was assayed in a Liquid Scintillation Analyzer (Beckmon Instruments, America). Values are expressed as counts per minute (cpm) of triplicate wells.

2.8. Statistical analysis

All data is presented as the mean ± 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.

3. Results

3.1. The presence of mouse $CD4^+CD8^-CD25^+$ thymocytes in xenogeneic pig thymic grafts

The thymus is a vital central lymphoid organ where the majority of T cell subpopulations are generated through positive and negative selection. Naturally occurring $CD4^+CD25^+$ Treg cells expressing high affinity TCRs for self-peptide–MHC complexes could develop in the thymus by avoiding clonal deletion [27,28]. We initially observed the presence of mouse $CD4^+CD25^+$ Treg cells in pig thymic grafts by a FCM. BALB/c nude mice received autogeneic BALB/c thymus (B-THY), which were used as controls.

By 10 weeks after thymus transplantation, thymocytes from auto- and xenogeneic thymic grafts were stained by PE-anti-mCD8 mAb and FITC-anti-mCD4 mAb. As reported before [29], CD4 single positive (SP) cells showed similar population between these thymic grafts, whereas a significantly decreased percentage of single positive CD8 SP cells were observed in xenogeneic thymic grafts compared with autogeneic counterparts (Fig. 1A and B). Accordingly, the total cell number of thymocytes and CD4 SP cells among these groups shows no significant difference (Fig. 1C), but the cell number of CD8 SP cells shows a significant decrease in xenogeneic thymic grafts (Fig. 1D).

To determine the presence of $CD4^+CD25^+$ Treg cells in thymic grafts, we analyzed the thymic grafts by three-color staining with CY5-anti-mCD4, FITC-anti-mCD8 and PE-anti-mCD25 mAbs. The ratio of $CD4^+CD8^-CD25^+$ to $CD4^+CD8^-$ thymocytes and the cell number of $CD4^+CD8^-CD25^+$ thymocytes were similar among these thymic grafts (Fig. 2, $P>0.05$).

3.2. Phenotype of mouse $CD4^+CD8^-CD25^+$ thymocytes in auto- and xenogeneic thymic grafts

To characterize the phenotype of mouse $CD4^+CD8^-CD25^+$ thymocytes in xenogeneic thymic grafts, we compared the expression of $CD4^+CD25^+$ Treg cell-specific makers, which are usually used to describe the phenotype of naturally occurring $CD4^+CD25^+$ Treg cells including TCR, Foxp3, CTLA-4 and GITR, on $CD4^+CD25^+$ and $CD4^+CD25^-$ thymocytes in auto- and xenogeneic thymic grafts by a three-color FCM.

The conventional and mature $CD4^+$ T cells and $CD4^+CD25^+$ Treg cells will become TCR^{high} in the thymus of mice before their transfer to periphery. Identical expression of TCR on either $CD4^+CD25^-$ or $CD4^+CD25^+$ cells was observed among auto- and xenogeneic thymic grafts (Fig. 3). Foxp3 was one of the key transcription factors in the development and function of

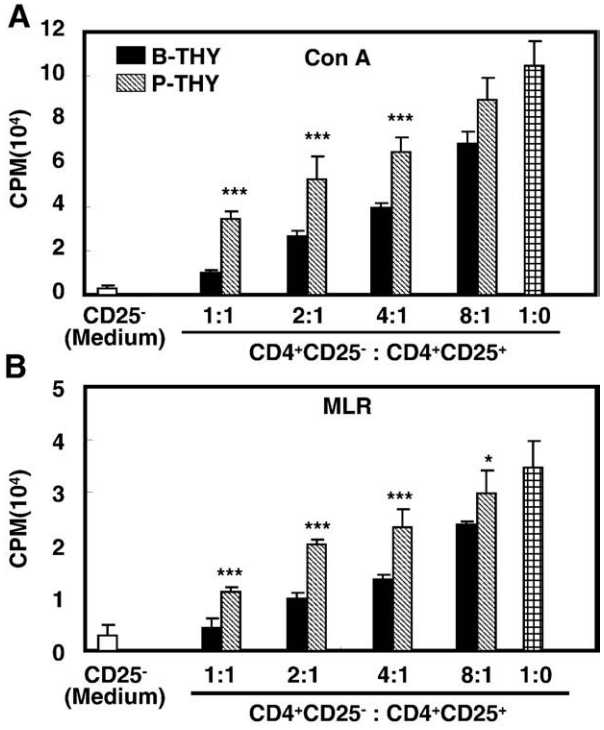

Fig. 5. Mouse $CD4^+CD8^-CD25^+$ T cells from auto- and xenogeneic thymic grafts showed significant immunosuppressive function on effector T cells. The proliferative assay was determined by [^{3}H]-TdR incorporation as described in Materials and methods. Thymic $CD4^+CD8^-CD25^-$ T cells were stimulated with Con A (A) or MMC-treated allogeneic splenocytes (B) at the ratio of 1:1 in the presence of the indicated numbers of $CD4^+CD8^-CD25^+$ thymocytes separated from either auto- or xenogeneic thymic grafts. Cultures were incubated for 4 days and pulsed with [^{3}H] thymidine for the last 18 h. The data is presented as mean ± SD of triplicate cultures. *$P<0.05$, ***$P<0.001$ compared among the indicated groups.

$CD4^+CD25^+$ Treg cells [30]. Foxp3 was highly expressed among $CD4^+CD8^-CD25^+$ thymocytes in contrast to $CD4^+CD8^-CD25^-$ thymocytes (Fig. 4A and B). More importantly, Foxp3 was highly expressed in $CD4^+CD8^-CD25^+$ thymocytes in xenogeneic thymic grafts as similarly as autogeneic counterparts (Fig. 4A and B). In addition, the expression of CTLA-4 and GITR on $CD4^+CD8^-CD25^-$ and $CD4^+CD8^-CD25^+$ thymocytes in auto- and xenogeneic thymic grafts was detected. $CD4^+CD8^-CD25^-$ thymocytes express significantly less CTLA-4 and GITR compared to $CD4^+CD8^-CD25^+$ thymocytes. $CD4^+CD25^+$ thymocytes in auto- or xenogeneic thymic grafts showed similar expression of CTLA-4 and GITR (Fig. 4C, D, E, and F).

3.3. Immunosuppressive function of mouse $CD4^+CD8^-CD25^+$ thymocytes maturing in xenogeneic pig thymic grafts

After determining that mouse $CD4^+CD8^-CD25^+$ thymocytes developing in xenogeneic thymic grafts have similar phenotypic features of the counterpart in autogeneic thymic grafts, we studied the immunosuppressive ability of these $CD4^+CD8^-CD25^+$ thymocytes in an in vitro classic suppressive assay. $CD4^+CD8^-CD25^+$ thymocytes were purified from thymocytes of different thymic grafts. As shown in Fig. 5, $CD4^+CD8^-CD25^+$ thymocytes developing in different thymic grafts displayed significant inhibition on the proliferation of syngeneic mouse $CD4^+CD8^-CD25^-$ T cells to Con A (Fig. 5A) or allogeneic stimulators (Fig. 5B) in a dose-dependent manner, although mouse $CD4^+CD8^-CD25^+$ thymocytes maturing in xenogeneic pig thymic grafts showed reduced immunosuppressive function.

4. Discussion

Specific tolerance across a widely disparate species barrier can be achieved by grafting of fetal or neonatal pig thymic and liver tissue to T cell and NK cell-depleted, thymectomized mice [19,21]. It has been shown that mouse $CD4^+CD25^+$ Treg cells could be reconstituted in the periphery of nude mice grafted with pig thymus [23]. In the present study, our results offer direct evidence supporting that mouse $CD4^+CD25^+$ Treg cells can develop normally in xenogeneic thymic grafts in this model.

The majority of peripheral $CD4^+CD25^+$ Treg cells are naturally matured in the thymus. Foxp3 is a conservative transcription factor, which programs the development and suppressive function of $CD4^+CD25^+$ Treg cells [31–33]. We observed that Foxp3 was highly expressed in mouse $CD4^+CD25^+$ thymocytes in xenogeneic thymic grafts in a similar level as those in autogeneic mouse thymic grafts. Furthermore, the expressions of CTLA-4 and CITR in mouse $CD4^+CD25^+$ thymocytes maturing in xenogeneic pig thymic grafts were identical to those in autogeneic thymic grafts or the reported results [34]. These data collectively suggest that xenogeneic pig thymic grafts have the ability to support the development of mouse $CD4^+CD25^+Foxp3^+$ Treg cells.

In the porcine thymus-grafted athymic mouse model, it has been demonstrated that pig MHCs mediate the positive selection of mouse thymocytes and both mouse and pig MHCs are involved in the negative selection of mouse thymocytes in xenogeneic thymic grafts in mice [35–37]. $CD4^+CD25^+$ Treg cell selection has been shown to require expression of self-antigen on radioresistant elements including cortical thymic epithelium (cTEC) and medullary thymic epithelium (mTEC) in the thymus [38]. It is speculated that expression of peptides in cTEC versus mTEC may be important in directing thymic $CD4^+CD25^+$ Treg cell selection [39]. In xenogeneic pig thymic grafts, mouse $CD4^+$ T cell positive selection was mediated by donor pig MHCs. Whether donor pig MHCs mediate the positive selection of mouse $CD4^+CD25^+$ Treg cells still needs to be determined.

The efficient immune regulatory function of thymic $CD4^+CD8^-CD25^+$ cells has been demonstrated by significantly suppressing the proliferation of autologous $CD4^+CD25^-$ thymocytes to allogeneic stimulation by a contact-dependent mechanism in vitro [40–42]. In our study, mouse $CD4^+CD25^+$ thymocytes maturing in xenogeneic pig thymic grafts significantly inhibited the response of $CD4^+CD25^-$ T cells to Con A or allogeneic antigens in a dose-dependent fashion. However, mouse $CD4^+CD25^+$ Treg cells obtained from xenogeneic pig thymic grafts showed a somewhat decreased immunosuppressive ability on effector T cells compared to autogeneic thymic counterparts. The reasons for the decreased immunosuppressive function of mouse $CD4^+CD25^+$ thymocytes are unclear so far, as it may be related to the xenogeneic donor MHCs-mediated positive selection in thymic grafts in these models.It was demonstrated that mouse $CD8^+$ T cells always had poor recovery in xenogeneic pig thymus-grafted nude mice. The poor recovery of mouse $CD8^+$ 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 and/or the poor peripheral survival of mouse $CD8^+$ T cells positively selected by pig MHCs in pig thymic grafts in these models [43].

In summary, efficient development of naturally occurring mouse $CD4^+CD25^+$ Treg cells in xenogeneic pig thymic grafts was observed. The mouse $CD4^+CD25^+$ Treg cells maturing in xenogeneic pig thymic grafts shared identical phenotypical characteristics with those maturing in autogeneic thymic grafts. More importantly, mouse $CD4^+CD25^+$ Treg cells maturing in xenogeneic pig thymic grafts show significant immunosuppressive ability on the responses of syngeneic effector T cells. Nevertheless, our present data provides direct evidence supporting the development of mouse functional $CD4^+CD25^+$ Treg cells in xenogeneic thymic grafts.

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