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Presence of Functional Mouse Regulatory CD4$^+$CD25$^+$T Cells in Xenogeneic Neonatal Porcine Thymus-Grafted Athymic Mice

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Xenotransplantation with porcine thymus is emerging as a possible means to reconstitute host cellular immunity and to induce immune tolerance in rodents and large animals. However, the presence of regulatory T cells (Treg cells) in this model needs to be determined. We herein demonstrated that efficient repopulation of mouse CD4$^+$CD25$^+$Treg cells was achieved in Balb/c nude mice by grafting neonatal porcine thymic tissue (NP THY). Mouse CD4$^+$CD25$^+$T cells expressed normal levels of Foxp3 in NP THY-grafted nude mice. Furthermore, these CD4$^+$CD25$^+$Treg cells showed significant inhibitory effects on the cell proliferation or interleukin-2 products of syngeneic T cells to alloantigens, Con A or a peptide antigen, although the potent immunosuppressive function might be lower than CD4$^+$CD25$^+$Treg cells in Balb/c mice. CD4$^+$CD25$^+$T cells in NP THY-grafted nude mice showed significantly stronger inhibition on the response to donor porcine antigens of CD4$^+$CD25$^-$T cells than CD4$^+$CD25$^+$Treg cells in Balb/c mice. Both CD4$^+$CD25$^+$Treg cells in NP THY-grafted nude and Balb/c mice prevented the development of autoimmune disease mediated by syngeneic CD4$^+$CD25$^-$T cells in a similar efficient way in the secondary recipients. These findings provide evidence for the potential involvement of CD4$^+$CD25$^+$Treg cells in keeping self-tolerance and transplant tolerance in this xeno-thymus transplantation model.

Key words: Regulatory T cells, thymus, xenotransplantation

Abbreviations: ATX, thymectomized; CTLA4, cytotoxic T-lymphocyte-associated protein 4; FCM, flow cytometry; FITC, fluorescein isothiocyanate; FP THY, fetal porcine thymus; GITR, glucocorticoid-induced tumor necrosis factor receptor; MFI, median fluorescence intensity; NP THY, neonatal porcine thymus; PE, phycoerythrin; PI, propidium iodide; Treg, regulatory T cells

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Introduction

The immune system protects a host from pathogens, distinguishes self from nonself structures and prevents nonessential and self-destructive immune responses through mechanisms of central and peripheral tolerance in vertebrates. In addition to some passive ways to maintain tolerance, such as clone deletion in the thymus and a state of unresponsiveness in the periphery through anergy and ignorance, CD4$^+$CD25$^+$ regulatory T cells (Treg) have been shown to play a crucial role in the maintenance of immune homeostasis and self-tolerance by counteracting the development and biological functions of potentially autoreactive T cells (1,2). The involvement of Treg cells in transplant immune tolerance has also been strongly evidenced (3–7). CD4$^+$CD25$^+$Treg cells represent approximately 10% of peripheral CD4$^+$T cells in mice and humans. In addition to the expression of CD25 molecule, mouse Treg cells are also characterized by the constitutive expressions of glucocorticoid-induce tumor necrosis factor receptor (GITR), cytotoxic T-lymphocyte—associated protein 4(CTLA4), CD45RB, CD122, CCR8, toll-like receptor-8, Foxp3 and CD103 molecules (3,8). However, it should be noted that many of these molecules are usually expressed by activated/memory nonTreg, too. Foxp3 is a much specific marker for Treg cells in mice.

Xenotransplantation has attracted a marked attention and interest in the past decades because it may be one of the possible approaches to solve the severe organ shortage of allogeneic human donors that greatly limits today's advancement in clinical transplantation. Pigs, especially miniature swine, because of their size and physiologic similarities to humans, have been widely recognized as the most likely xenograft donors to humans (9). However, the immune response to xenogeneic organs tends to be stronger than that toward allografts and the unacceptable high levels and long-term treatment of immunosuppression would likely be necessary to avoid xenograft rejection (10). Therefore, it is much practicable to induce specific immune tolerance to donor antigens by 'reeducation' of recipients' immune system, which would make

successful discordant xenogeneic porcine organ transplantation to humans possible without long lasting nonspecific immunosuppression.

Recent studies demonstrated that phenotypically and functionally mature mouse $CD4^+$T cells recovered in T and natural killer (NK) cell-depleted, thymectomized (ATX) immunocompetent or athymic mice by grafting xenogeneic fetal porcine thymus tissue (FP THY) or neonatal porcine thymus (NP THY) (11–13). Importantly, recipient mouse T cells maturing in porcine thymic grafts are specifically tolerant to pig antigens as determined *in vitro* and *in vivo* (11–14). Clone deletion mechanism has been clearly shown to play an important role in T cell immune tolerance to donor and host antigens in this model (12). Although no direct evidence, the preliminary results indicated that Treg cells might also participate in immune tolerance induction in this model (12). On the other hand, even with clear evidence showing that self-reactive T cells are efficiently deleted and efficient positive selection exists in FP THY grafts in this model (11,13,15–18), a high frequency of FP THY-grafted nude mice (16) or NP THY-grafted nude mice (unpublished data) suffered from autoimmune diseases. Thus, the presence of mouse Treg cells and, if presence, their functions in porcine thymus-grafted athymic mice require to be determined. In the present study, we have shown that (1) normal levels of mouse $CD4^+CD25^+$Treg cells were restored by grafting NP-THY in the periphery of Balb/c nude mice; (2) these Treg cells displayed normal phenotypes including the expression of Foxp3, CD62, CD45, GITR and CD25, as well as T-cell receptor (TCR) Vβ repertoire; (3) these $CD4^+CD25^+$Treg cells showed significant immunosuppression on the proliferation and interleukin (IL-2) products of effector $CD4^+CD25^-$T cells induced by mitogen (Con A), alloantigens and peptide antigens *in vitro*, although the function might be somewhat lower than Treg cells in Balb/c mice; (4) these Treg cells showed enhanced immunosuppression on response to donor antigens but not to the third party antigens of $CD4^+CD25^-$T cells compared with $CD4^+CD25^-$Treg cells of Balb/c mice and (5) these Treg cells had the ability to prevent from syngeneic $CD4^+CD25^-$T cell-mediated autoimmunity in syngeneic secondary recipients as efficiently as $CD4^+CD25^+$Treg cells of Balb/c mice did. Our present study offered the basic principle for the efficient peripheral reconstitution of host functional $CD4^+CD25^+$Treg cells by grafting xenogeneic thymic tissues.

Materials and Methods

Animals

C57BL/6 (H-2^b), Balb/c (H-2^d) and Balb/c nude mice were purchased from Institute of Genetics and Development, Chinese Academy of Sciences (Beijing, China). Balb/c DO11.10 mice (19), which are transgenic for the TCR specific for the immunodominant epitope of OVA peptide 323–339, were offered by animal facility (Shanghai, China). All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding and water. Neonatal pigs were purchased from 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.

Monoclonal antibodies (mAbs) and chemical reagents

The following mAbs were purchased from BD Biosciences PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 mAb (RM4-5), FITC-labeled rat anti-mouse CD8 mAb (53–6.7), FITC-labeled rat anti-mouse CD25 mAb (7D4), FITC-conjugated rat anti-mouse GITR mAb (DTA-1), FITC-labeled hamster anti-mouse TCR β-chain mAb (H57–597), phycoerythrin (PE)-labeled rat anti-mouse CD4 mAb, PE-labeled rat anti-mouse CD8α mAb (53–6.7) and Cy5-labeled anti-mouse CD25 mAb.

In addition, FITC-labeled anti-mouse Foxp3 mAb (FJK-16s) and its staining kit were obtained from eBioscience (San Diego, CA). Rat anti-mouse FcR mAb (2.4G2) was produced by 2.4G2 hybridoma (ATCC, Rockville, Maryland) in our laboratory. OVA were obtained from Sigma-Aldrich. Mitomycin C ($C_{15}H_{18}N_4O_5$) was obtained from Kyowa Hakko Co, Ltd. (Tokyo, Japan).

Immunofluorescence staining and flow cytometry (FCM)

Splenocytes (5×10^5) were prepared and washed once with FCM buffer (PBS, pH 7.2, containing 0.1% NaN_3 and 0.5% BSA) as described before (20). For two-color staining, cells were stained with PE-labeled anti-mouse CD4 mAb versus FITC-labeled anti-mouse TCR, CD25 mAb or the nonspecific staining control mAb, respectively. For three-color staining, cells were stained with PE-labeled anti-mouse CD4 mAb and Cy5-labeled anti-mouse CD25 mAb versus FITC-labeled anti-mouse CTLA-4, GITR mAb or the nonspecific staining control mAb. Nonspecific FcR binding was blocked by anti-mouse FcR mAb 2.4G2. At least 10 000 cells were assayed using a FASCalibur FCM (Becton Dickinson, CA), and data were analyzed with CellQuest software. Nonviable cells were excluded using the vital nucleic acid stain propidium iodide (PI). The percentage of cells stained with a particular reagent or reagents was determined by subtracting the percentage of cells stained nonspecifically with the negative control mAb from staining in the same dot-plot region with the anti-mouse mAbs. Certain molecule expression levels were determined as the median fluorescence intensity (MFI) of the cells positively stained with the specific mAb.

To determine the intracellular expression of Treg cell-specific transcription factor Foxp3 in $CD4^+CD25^+$cells, mouse splenocytes were first surface-stained with PE-labeled anti-mouse CD4 and Cy5-labeled anti-mouse CD25 mAbs as usual. These cells were subsequently stained with FITC-labeled anti-mouse Foxp3 mAb or nonspecific isotype control mAb, according to the manufacturer's protocol (eBioscience, San Diego, CA).

Transplantation procedures

Less than 1 day old neonatal pigs were deeply euthanized by anesthetic overdose (inhaled methoxyflurane). The thymic tissues were recovered and then placed in cold RPMI 1640 medium (Invitrogen, Beijing). Thymic xenotransplantation in Balb/c nude mice was carried out under anesthesia with intraperitoneal injection of ketamine (0.08 mg/g; Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (0.012 mg/g; Bayer, Shawnee, Kansas). One piece of neonatal porcine thymic tissue (2 mm^3) was grafted in the mediastinum (thoracic cavity of thymus *in situ*) and another piece under the kidney capsule of BALB/c nude mice.

Cells purification

$CD4^+CD25^+$Treg cells populations were isolated from mouse splenocytes using a $CD4^+CD25^+$Treg Cells Isolation Kit with MidiMACS™ Separator according to the manufacturer's protocols (Miltenyi, Bergisch Gladbach, Germany). Briefly, splenocytes were incubated with a Biotin-antibody cocktail against: CD8α (Ly2), CD11b (Mac-1), CD45R (B220), CD49B (DX5) and Ter-119, for 20 min at 4°C, and then with microbead-conjugated anti-biotin mAb (Bio318E7.2) and PE-labeled anti-CD25 mAb. The cell suspension was

loaded on a LD column, which is placed in magnetic field of a Magnetic Activator Cell Sorting (MACS) Separator. The remaining fraction in the column is the enriched $CD4^+$ cells. For the isolation of $CD4^+CD25^+$ cells, the $CD25^+$ PE-labeled cells in the enriched $CD4^+$ cells fraction were magnetically labeled with anti-PE microbeads. The magnetically labeled $CD4^+CD25^+$ cells were enriched from the $CD4^+$cells fraction by MACS sorting. Positive sorted $CD4^+CD25^+$ populations were always >95% as confirmed by FCM. The $CD4^+CD25^-$T cell fraction of Balb/c mice were usually used as responder cells.

Mixed lymphocyte reaction assay

Murine splenic $CD4^+CD25^+$Treg cells were isolated from either NP THY-grafted Balb/c nude mice or euthymic Balb/c mice as described above. Balb/c $CD4^+CD25^-$T cells were used as responder T cells. C57BL/6 or porcine splenocytes, which were pretreated with mitomycin C at the concentration of 30 μg/mL at 37°C for 30 min, were used as allogeneic or xenogeneic stimulator cells. In general, 5×10^4 responder cells (Balb/c $CD4^+CD25^-$T cells) and 5×10^4 stimulator cells (C57BL/6 or porcine splenocytes) per well in RPMI1640 medium supplemented with 10% FCS were added in 96-well round-bottomed plates. $CD4^+CD25^+$Treg cells were subsequently added to each well accordingly. Cells were cocultured at 37°C and 5% CO_2 for 96 h. 0.5 μCi of [^{3}H]Thymidine (185 GBq/mmol; Atomic Energy Research Establishment, China) was added for the last 18 h. Cells were recovered with an automatic cell recover (Tomtec, Toku, Finland). The radioactivity of each sample was assayed in a Liquid Scintillation Analyzer (Beckman Instruments, Fullerton, CA). Values are expressed as counts per minute of triplicate wells.

The cell proliferation to a peptide antigen of T cells

Splenic $CD4^+CD25^-$T cells sorted from TCR-transgenic DO11.10 mice using a MACS Sorting Isolation Kit were used as responder T cells. Wild-type Balb/c splenocytes were treated with mitomycin C and used as APCs. DO11.10 $CD4^+CD25^-$T cells (5×10^4) were cultured with 5×10^4 antigen presenting cells (APCs) and 200 μg/mL OVA peptide 323–339 in 96-well plates in the presence of different numbers of $CD4^+CD25^+$T cells. Cells were cultured in complete RPMI1640 medium at 37°C and 5% CO_2 for 96 h. [^{3}H]Thymidine (0.5 μCi) was added for the final 18 h. Cells were recovered and [^{3}H]thymidine incorporation was measured as described above.

IL-2 production assay

$CD4^+CD25^-$T cells were stimulated with mitogen Con A or allogeneic antigens in the presence or absence of syngeneic $CD4^+CD25^+$Treg cells for 72 h. The supernatant were recovered and the levels of IL-2 were detected by an ELISA kit (BD Biosciences).

Adoptive transfer mouse model

Balb/c nude mice received an i.v. injection of 10^6 syngeneic Balb/c $CD4^+CD25^-$T cells with or without Balb/c $CD4^+CD25^+$Treg cells. The body weights were followed weekly.

Statistical analysis

All data are presented as the mean ± SD. Student's unpaired *t*-test for comparison of means was used to compare groups. A p value less than 0.05 was considered to be statistically significant.

Results

Peripheral recovery of mouse $CD4^+$ T cells in Balb/c nude mice after grafting with NP THY

It has been demonstrated that FP THY grafts enable efficient reconstitution of mouse $CD4^+$T cells in T and NK cell-depleted ATX B6 or nude mice (13,21). Significantly enhanced mouse $CD4^+$T cell reconstitution by grafting NP THY in alternative locations was observed in T and NK cell-depleted ATX B6 mice (22). To confirm the efficiency of mouse T cell recovery in immunodeficient Balb/c nude mice grafted with multiple pieces of neonatal pig thymic tissues, the levels of mouse T cells in the periphery were evaluated by FCM. As is shown in Figure 1, markedly higher percentages of mouse $CD4^+$T cells and, to a lower intensity, mouse $CD8^+$T cells were detected in the peripheral blood of NP THY-grafted nude mice by FCM at 6 weeks postNP THY-grafting, compared with control Balb/c nude mice that received none or neonatal porcine liver (NP LIV) alone. Consistently, the total cell numbers of mouse $CD4^+$T cells, but not $CD8^+$T cells in spleens and lymph nodes (LNs) of Balb/c nude mice grafted with NP THY were significantly higher than control Balb/c nude mice with or without NP LIV (Figure 1B and C). These data indicate that efficient peripheral repopulation of mouse $CD4^+$T cells in athymic mice could be achieved by grafting with multiple pieces of xenogeneic NP THY tissues. Thus, this model allowed us to investigate the peripheral reconstitution of mouse $CD4^+CD25^+$T cells by grafting xenogeneic thymus.

The presence of mouse $CD4^+CD25^+$ T cells in the periphery of NP THY-grafted Balb/c nude mice

Accumulated evidences have shown that $CD4^+CD25^+$Treg cells are a subpopulation of T cells with powerful immune regulating functions (1). CD25 has been widely used as a marker for Treg cells. In the present study, mouse $CD4^+CD25^+$Treg cells in the periphery immune system including peripheral blood, spleens and LNs were detected by two-color FCM after efficient recovery of mouse T cells in NP THY-grafted Balb/c nude mice (it usually took more than 8 weeks after NP THY grafting) was observed. As is shown in Figure 2, normal levels of mouse $CD4^+CD25^+$T cells in blood, spleens and LNs of NP THY-grafted Balb/c nude mice were detected as compared with wild-type Balb/c mice ($p > 0.05$, respectively).

The phenotype of mouse $CD4^+CD25^+$ T cells in NP THY-grafted Balb/c nude mice

As it is well known, CD25 is also expressed on activated T cells. To exclude the possibility that $CD4^+CD25^+$T cells in NP THY-grafted mice are a population of activated T cells, we studied the activation marker, CD69 and activated/memory T cell markers including CD44, CD45RB and CD62L on $CD4^+$ or $CD4^+CD25^+$cells in these mice by two- or three-color FCM, respectively. There were no significant differences for the expressions of these markers on $CD4^+$ or $CD4^+CD25^+$cells between NP THY-grafted Balb/c nude and wild-type Balb/c mice, respectively (data not show). We therefore concluded that $CD4^+CD25^+$T cells in NP THY-grafted Balb/c nude mice were not the activated T cells.

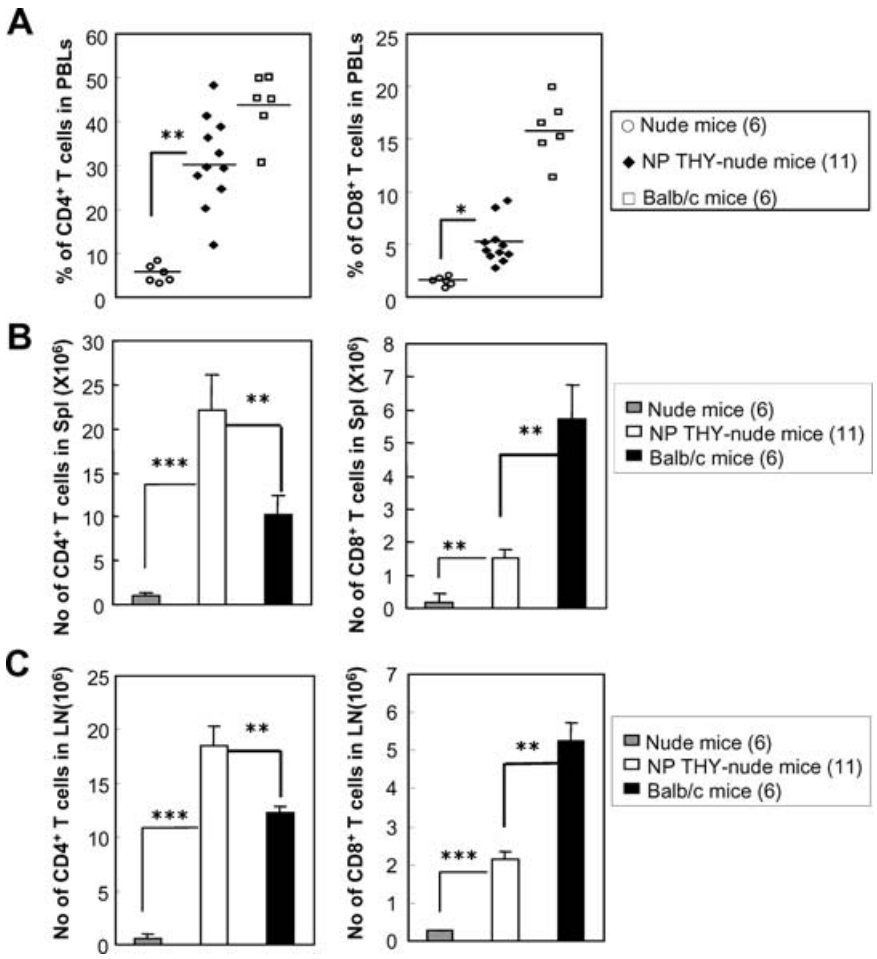


Figure 1: Peripheral repopulation of mouse CD4$^+$ T cells in Balb/c mice grafted with NP-THY. (A) Mean percentages of mouse CD4$^+$ or CD8$^+$ T cells in peripheral blood lymphocytes (PBLs) are shown. PBLs were collected at 8 weeks postpig thymus tissue grafting, and were stained with either anti-CD4 or anti-CD8 mAb plus anti-TCRβ mAb. (B) The total cell numbers of mouse CD4$^+$T and CD8$^+$T cells in spleens. (C) The total cell numbers of mouse CD4$^+$T and CD8$^+$T cells in LNs. NP THY-grafted nude mice and the control mice were sacrificed by 15 weeks postgrafting, the T cells in spleens and LNs were assayed. Some control Balb/c nude mice grafted with or without NP LIV were referred to as Balb/c nude mice. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared with Balb/c nude mice. The number of mice in each group is shown in brackets.

It has been recently reported that Foxp3 is a unique transcription factor in Treg cells (23–26). The expression of Foxp3 in mouse CD4$^+$CD25$^+$cells of NP THY-grafted Balb/c nude mice was determined by three-color intracellular staining FCM. As is shown in Figure 3, Foxp3 was predominately expressed in CD4$^+$CD25$^+$ cells, as indicated by that almost all CD4$^+$CD25$^+$ cells (89 ± 3%) expressed it, whereas almost no cells in CD4$^+$CD25$^-$cell subsets (1.06 ± 1.27%) expressed Foxp3 in wild-type Balb/c mice. This is consistent well with others' reports. Importantly, the majority of CD4$^+$CD25$^+$ cells in NP THY-grafted Balb/c nude mice expressed Foxp3 molecules with no significant difference with those in wild-type Balb/c mice. Furthermore, CD4$^+$CD25$^+$cells in both NP THY-grafted Balb/c nude mice and wild-type Balb/c mice expressed identical levels of Foxp3 as determined by the MFI of Foxp3 staining (Figure 3C).

The immunosuppressive function of mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice

We next examined the function of mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice using a classic suppressor cell assay in which the ability of enriched CD4$^+$CD25$^+$Treg cells by magnetic microbeads to suppress the alloantigen-induced proliferative responses of syngeneic CD4$^+$CD25$^-$T cells was tested *in vitro*. As is shown in Figure 4A, mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice or in normal Balb/c mice exhibited the capacity of suppressing the proliferation of syngeneic CD4$^+$CD25$^-$T cells induced by allogeneic stimulator cells in a dose-dependent manner. However, when the ratios of CD4$^+$CD25$^+$Treg cells: responder CD4$^+$CD25$^-$T cells were 1:1 or 1/2:1, the cell proliferation of CD4$^+$CD25$^-$T cells in the presence of CD4$^+$CD25$^+$Treg cells from NP THY-grafted Balb/c nude mice was significantly higher than those with CD4$^+$CD25$^+$Treg cells from euthymic Balb/c mice. These data indicate that the immunosuppressive ability of CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice was significantly reduced. In addition, the all CD4$^+$CD25$^+$T cells either from NP THY-grafted Balb/c nude mice or from euthymic Balb/c mice did not show significant cell proliferation to allo-antigens in this system (data not shown).

The immunosuppressive effects of CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice on a peptide

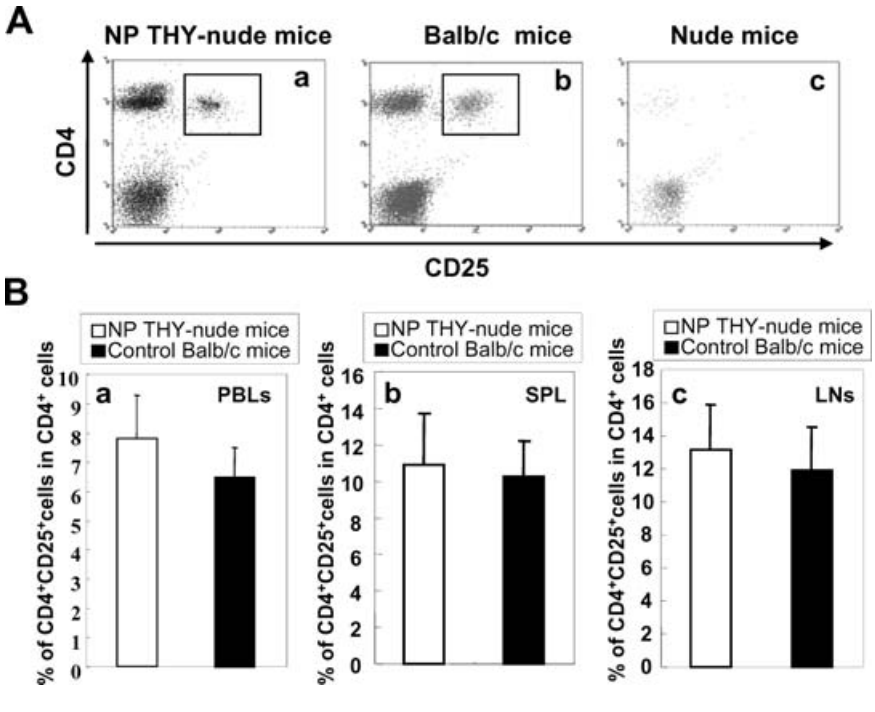
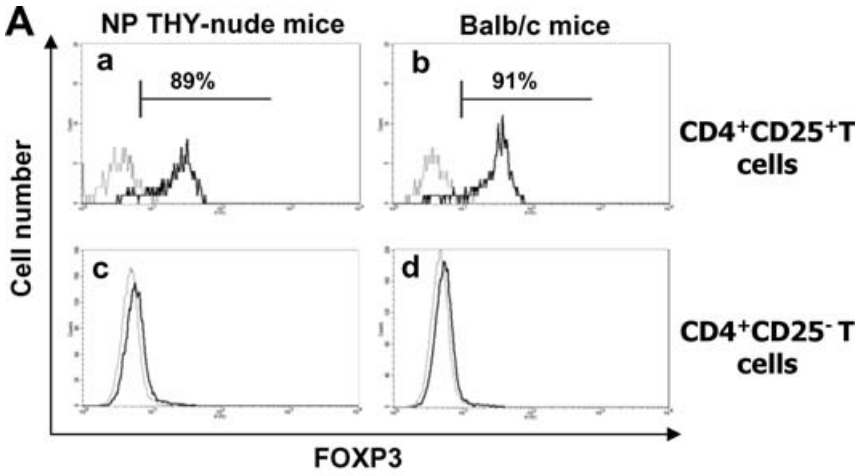


Figure 2: The presence of mouse CD4$^+$CD25$^+$Treg cells in the periphery of NP THY-grafted nude mice. (A) One representative for the mouse CD4 versus CD25 staining of PBLs in NP THY-grafted nude mice, wild-type Balb/c mice and Balb/c nude mice was shown. (B) The mean percentages of mouse CD4$^+$CD25$^+$cells in peripheral blood (a), spleens (b) and LNs (c), respectively. Mean values (± SEM) of four independent experiments are shown. There was no significant difference between NP THY-grafted Balb/c nude mice and control Balb/c mice.



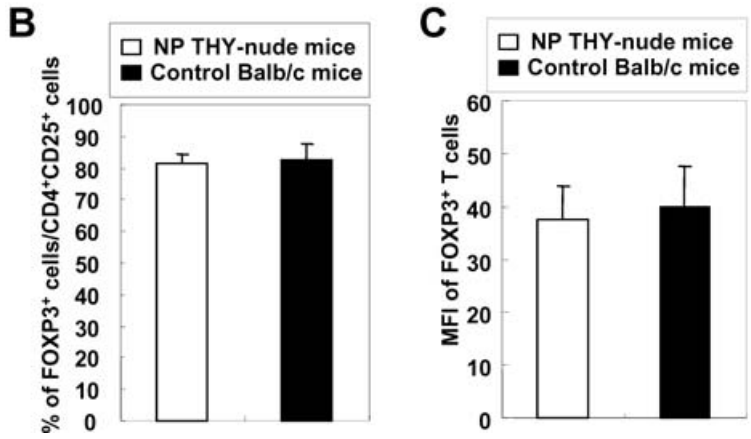


Figure 3: The expression of Foxp3 in mouse CD4$^+$CD25$^+$T cells in NP THY-grafted nude mice. Three-color intracellular staining FCM was employed to detect the expression of Foxp3 in different subsets of splenic T cells. (A) One representative was shown for the Foxp3 staining in mouse CD4$^+$CD25$^+$and CD4$^+$CD25$^-$cell fractions. The histogram represents FJK-16s staining (solid peak) and isotype control (dot peak), respectively, in mouse CD4$^+$CD25$^+$ (top panel) or CD4$^+$CD25$^-$cells (bottom panel). (B) The mean percentages of Foxp3$^+$cells in mouse CD4$^+$CD25$^+$cells. (C) The MFI of Foxp3 expression in mouse CD4$^+$CD25$^+$cells. Mean values (± SD) of four independent experiments are shown. No statistical difference was achieved among identical groups.

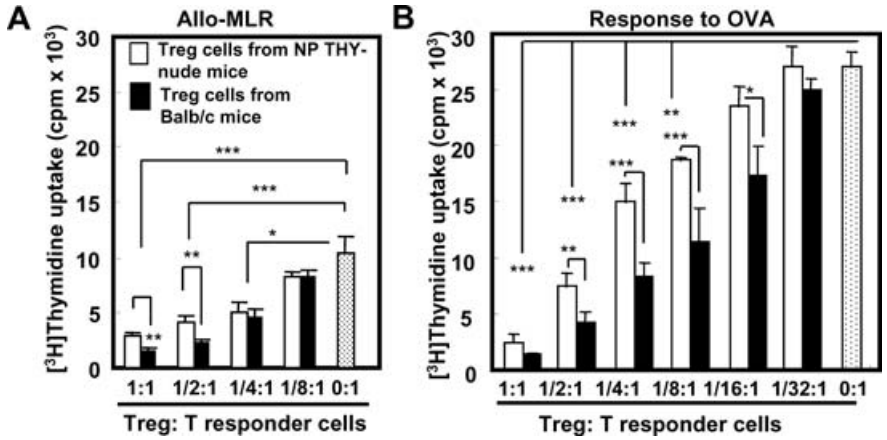


Figure 4: The inhibitory effects of mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted nude mice on the proliferative responses of mouse T cells to allo-antigens or a peptide antigen. The cell proliferation was determined by 3H-TdR incorporation as described in Materials and Methods. (A) The inhibitory effects of mouse CD4$^+$CD25$^+$Treg cells on the responses of mouse CD4$^+$CD25$^-$T cells to allo-antigens. (B) The inhibitory effects of mouse CD4$^+$CD25$^+$Treg cells on the responses of mouse CD4$^+$CD25$^-$T cells to a peptide antigen (OVA 323–339). The grey bars represent responder cells cultured with allo-stimulator cells or APCs plus OVA 323–339 alone, respectively. Results are expressed as the mean value of triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between the indicated groups. One representative of three independent experiments with similar results is shown.

antigen-specific response of effector T cells was also determined. TCR-transgenic DO11.10 mouse CD4$^+$CD25$^-$T cells, which recognize OVA peptide323–339, were used as responder cells, mitomycin C treated-Balb/c splenocytes as APCs. These cells were cocultured with OVA peptide323–339 at a final concentration of 200 μg/mL in the presence or absence of CD4$^+$CD25$^+$Treg cells from either NP THY-grafted Balb/c nude mice or euthymic Balb/c mice at various ratios between Treg cells and responder T cells. Cell proliferations were measured by incorporation of [^{3}H]thymidine. As is shown in Figure 4B, CD4$^+$CD25$^+$Treg cells could significantly inhibit OVA-induced T cell proliferation at the detected ratios, no matter CD4$^+$CD25$^+$Treg cells were from NP THY-grafted Balb/c nude mice or from euthymic Balb/c mice. Consistently with results in allo-mixed lymphocyte reaction assays, the immunosuppressive function of CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice was significant lower than that of normal Balb/c CD4$^+$CD25$^+$Treg cells ($p < 0.01$ or $p < 0.001$).

Furthermore, the immunosuppressive effect of CD4$^+$CD25$^+$Treg cells from NP THY-grafted Balb/c nude mice on the Con A or alloantigen-induced IL-2 production of CD4$^+$CD25$^-$T cells was detected. As is shown in Figure 5, CD4$^+$CD25$^+$Treg cells from both NP THY-grafted Balb/c nude mice and euthymic Balb/c mice showed significant inhibition on the IL-2 production of CD4$^+$CD25$^-$T cells, but the effects of CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice were weaker than those in euthymic mice. These data collectively indicated that mouse CD4$^+$CD25$^+$Treg cells maturing in xenogeneic NP THY were functional *in vitro*.

The presence of donor antigen-specific mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice

In an attempt to observe the presence of donor antigen-specific CD4$^+$CD25$^+$Treg cells in this mouse model, the effect of mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice on the response to donor or the third party porcine antigens of Balb/c CD4$^+$CD25$^-$T cells was detected *in vitro*. As is shown in Figure 6, CD4$^+$CD25$^+$Treg cells from both NP THY-grafted Balb/c nude mice and euthymic Balb/c mice significantly inhibited the response to donor and the third party porcine antigens of mouse CD4$^+$CD25$^-$T cells. However, the inhibiting effect of CD4$^+$CD25$^+$Treg cells from NP THY-grafted Balb/c nude mice on the response to donor porcine antigens of CD4$^+$CD25$^-$T cells was significantly stronger than its effect on the response to the third party porcine antigens of CD4$^+$CD25$^-$T cells. This preliminary data indicated that donor antigen-specific CD4$^+$CD25$^+$Treg cells might exist in NP THY-grafted Balb/c nude mice.

The effect of mouse CD4$^+$CD25$^+$Treg cells from NP THY-grafted Balb/c nude mice to prevent the occurrence of syngeneic CD4$^+$CD25$^-$T cell-mediated autoimmunity in vivo

To investigate the ability of mouse CD4$^+$CD25$^+$Treg cells maturing in NP THY grafts to inhibit the potential autoimmunity *in vivo*, we adoptively transferred Balb/c CD4$^+$CD25$^-$T cells with or without CD4$^+$CD25$^+$Treg cells of NP THY-grafted Balb/c nude mice to syngeneic Balb/c nude mice. As is shown in Figure 7, significant body weight loss was caused by CD4$^+$CD25$^-$T cells, whereas coinjection of Balb/c CD4$^+$CD25$^+$Treg cells

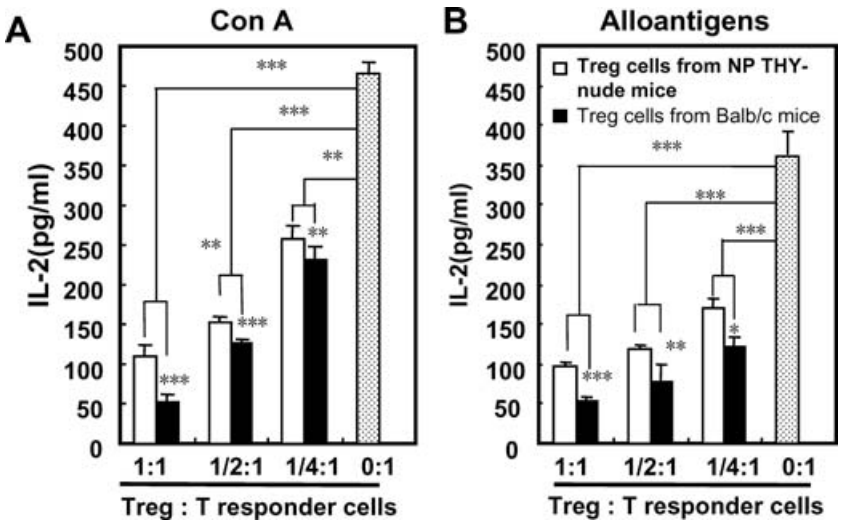


Figure 5: The inhibition of mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted nude mice on the IL-2 production of mouse T cells induced by Con A or allo-antigens. The IL-2 production was determined by ELISA as described in Materials and Methods. (A) The inhibitory effects of mouse CD4$^+$CD25$^+$Treg cells on the IL-2 products of mouse CD4$^+$CD25$^-$T cells induced by Con A. (B) The inhibitory effects of mouse CD4$^+$CD25$^+$Treg cells on the IL-2 products of mouse CD4$^+$CD25$^-$T cells induced by allo-antigens. The grey bars represent responder cells cultured with Con A or allo-stimulator cells without Treg cells. Results are expressed as the mean value of triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between the indicated groups. One representative of three independent experiments with similar results is shown.

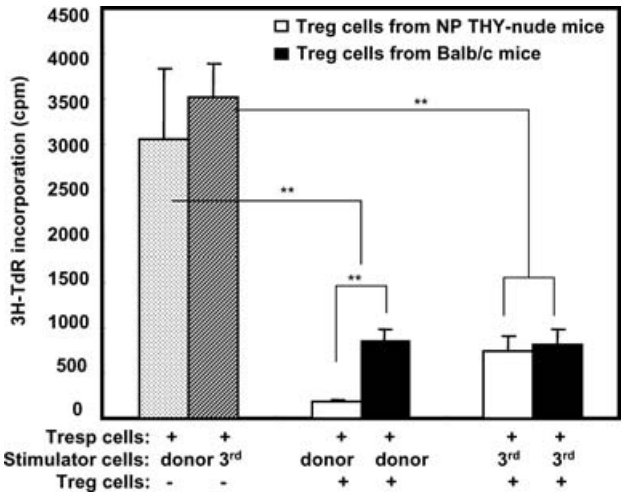


Figure 6: The presence of donor antigen-specific mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice. The cell proliferation to donor or the third party antigens of mouse CD4$^+$CD25$^-$T cells was determined by 3H-TdR incorporation as described in Materials and Methods. The gray bars represent responder cells alone cultured with donor or the third party porcine stimulator cells, respectively. The ratio between mouse CD4$^+$CD25$^+$Treg cells and mouse CD4$^+$CD25$^-$T cells was 1:1. Results are expressed as the mean value of triplicates. One representative of two independent experiments with identical results is shown. ** $p < 0.01$ between the indicated groups.

remarkably reversed it. Importantly, cotransfer of mouse CD4$^+$CD25$^+$Treg cells from NP THY-grafted Balb/c nude mice also significantly prevented the body weight loss caused by CD4$^+$CD25$^-$T cells (Figure 7A). This data indicate that mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted nude mice had the ability to prevent the autoimmune response *in vivo*.

Discussion

Physiological levels of mouse CD4$^+$CD25$^+$Treg cells were observed in NP THY-grafted Balb/c nude mice. These cells expressed normal levels of CD25, GITR, CTLA-4, CD44, CD62L, CD45RB and CD69 molecules as CD4$^+$CD25$^+$Treg cells in wild-type euthymic Balb/c mice, suggesting that mouse CD4$^+$CD25$^+$Treg cells displayed normal Treg cell phenotype in NP THY-grafted athymic mice. Studies in mice and humans have shown that Foxp3, a transcription factor, was expressed specifically in CD4$^+$CD25$^+$Treg cells, but not in CD4$^+$CD25$^-$ or CD8$^+$T cells, and was responsible for the immunosuppression activity of the CD4$^+$CD25$^+$Treg cells (27). Foxp3 plays a critical role in the generation, development and function of Treg cells (23,25,28). As detected by intracellular staining FCM, mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted nude mice expressed high levels of Foxp3 with no detectable difference to those in control Balb/c mice. Recent studies have provided evidence that GITR is preferentially expressed on murine CD4$^+$CD25$^+$Treg cells, when compared with to resting CD4$^+$CD25$^-$cells (29–31). Importantly, anti-GITR antibody was able to

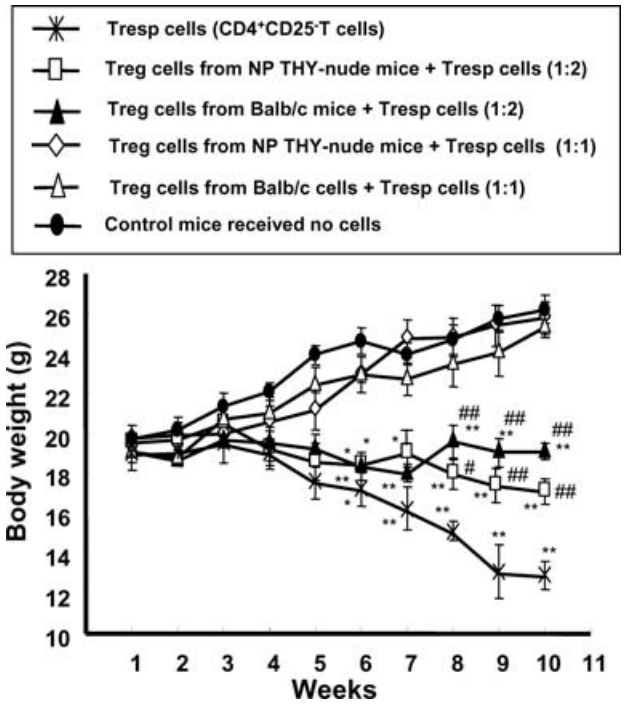


Figure 7: The effect of mouse CD4$^+$CD25$^+$Treg cells from NP THY-grafted nude mice on the occurrence of autoimmune disease induced by mouse CD4$^+$CD25$^-$T cells in an adoptive transfer mouse model. The secondary syngeneic Balb/c nude recipients received mouse CD4$^+$CD25$^-$T cells with or without mouse CD4$^+$CD25$^+$Treg cells either from Balb/c mice or from NP THY-grafted Balb/c nude mice as described in Materials and Methods. The body weights were followed weekly. Results are presented as Mean + SD. Five mice in each group were studied. * $p < 0.05$, ** $p < 0.01$ compared with mice received no cells. # $p < 0.05$, ## $p < 0.01$ compared with mice received mouse CD4$^+$CD25$^-$T cells alone.

abrogate CD4$^+$CD25$^+$Treg cell-mediated suppression and to induce spontaneous organ-specific autoimmune diseases (29). In addition, mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice expressed normally high levels of GITR and were polyclonal as indicated by the expression of a diversity of Vβ families as detected by FCM (data not shown). These CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice could significantly inhibit the cell proliferation and IL-2 production of syngeneic effector T cells stimulated by allo-antigens or a peptide antigen *in vitro*, albeit the suppression is lower than CD4$^+$CD25$^+$Treg cells from euthymic mice. Thus, phenotypically normal mouse CD4$^+$CD25$^+$Treg cells with a potent immunosuppressive function were developed in xenogeneic NP THY-grafted athymic mice.

CD4$^+$CD25$^+$Treg cells play an important role in maintenance of immune homeostasis and self-tolerance by counteracting with potentially autoreactive cells that have escaped negative selection in the thymus (32). Lots of evidence indicates that depletion of this population of cells results in multiorgan autoimmune diseases in a variety of strains of mice and in humans (33,34). Our present study showed that mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted nude mice were functional as determined by their efficient ability to block the alloantigen-induced cell proliferation and IL-2 production of CD4$^+$CD25$^-$T cells. Furthermore, the adoptive transfer study showed that mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted nude mice had the ability to prevent the occurrence of autoimmune disease mediated by CD4$^+$CD25$^-$T cells *in vivo*. Therefore, mouse CD4$^+$CD25$^+$Treg cells in NP THY-grafted nude mice were functional and had the potential ability to inhibit the autoimmunity. However, with the functional CD4$^+$CD25$^+$Treg cells present here and efficient intrathymic clonal deletion (16), porcine thymus-grafted nude mice suffered from autoimmune-like disease (data not shown) (16). Thus, the mechanisms for the sickness of these mice require further investigation.

On the other hand, it has been demonstrated that CD4$^+$CD25Treg cells play an important role in transplant tolerance in several animal models (4,6). It has been demonstrated that intrathymic clonal deletion played an important role in immune tolerance to xenogeneic donor porcine antigens in porcine thymus-grafted athymic mouse model. In the present study, we observed the presence of donor antigen-specific CD4$^+$CD25$^+$Treg cells in NP THY-grafted Balb/c nude mice. This data offered evidence for the possible involvement of CD4$^+$CD25$^+$Treg cells in xenogeneic immune tolerance.

Efficient mouse thymopoiesis and peripheral mouse CD4$^+$T cell repopulation occur when xenogeneic FP THY tissue is grafted to T and NK cell-depleted ATX mice (13). In this model, the reconstituted mouse T cells are functional and specifically tolerant to pig donor antigens (12,13). The previous studies showed that using NP THY grafts in T and NK cell-depleted ATX B6 mice allowed peripheral repopulation of mouse CD4$^+$T cells, but with markedly lower efficiency than that is observed with fetal pig thymus grafting (14,35). This difference may be due to the limited growth potential of neonatal pig thymic grafts (14). Recently, significantly enhanced mouse CD4$^+$T cell reconstitution by grafting NP THY in alternative locations was observed in T and NK cell-depleted ATX B6 mice (22). In the present study, we simultaneously grafted multipieces of NP THY into several locations of immunodeficient Balb/c nude mice, high levels of mouse CD4$^+$T cells in the periphery was observed, suggesting that grafting several pieces of NP THY tissues in different grafting locations could remarkably improve the efficiency of peripheral mouse CD4$^+$T cell repopulation. The level of mouse CD8$^+$T cells recovered in this model is still poor as observed in FP THY-grafted athymic mice (36). Consistently with the previous studies showing the enlargement of FP THY grafts and shrinking NP THY grafts in mouse recipients (11, 37), poor NP THY grafts were observed on

15 weeks after grafting (data not shown). Anyway, the amount of NP THY tissue grafted might be an important factor for the efficient mouse thymopoiesis and the reconstitution of peripheral cellular immunity.

In summary, functional polyclonal mouse $CD4^+CD25^+$Treg cells were observed in NP THY-grafted Balb/c nude mice as determined by their phenotypes including CD25, Foxp3 and GITR, as well as their potent immunosuppressive function. These findings provide evidence for the potential involvement of $CD4^+CD25^+$Treg cells in keeping host immune tolerance to self- or donor-antigens in xenogeneic thymus transplant models.

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