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Impact of activated invariant natural killer T cells on the expansion of regulatory T cell precursors in murine thymocytes *in vitro*

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ABSTRACT

Tolerance induction is a goal of clinical transplantation to prevent graft rejection without the lifelong use of immunosuppressive drugs. In a series of mouse studies, we previously reported that the establishment of mixed chimerism by treatment with a ligand for invariant natural killer T (iNKT) cells with CD40 signal blockade makes it possible to prevent allograft rejection without immunosuppressants, and this approach fails in thymectomized recipient mice. In this study, we showed that iNKT cells in murine thymocyte cultures are indispensable for the expansion of $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells as well as $CD4^+CD25^+Foxp3^-$ cells, which contained precursor Tregs (preTregs). After the culture of BALB/c mouse-derived thymocytes in the presence of α-galactosylceramide (α-GalCer), a representative ligand for iNKT cells, the ratio of $CD4^+CD25^+Foxp3^-$ preTregs to total $CD4^+CD8^-$ T cells was much higher than that of $CD4^+CD25^+Foxp3^+$ Treg cells, regardless of anti-CD40 L mAb treatment. The proliferation of $CD4^+CD25^+Foxp3^-$ cells, but not Treg cells, was significantly augmented, and the stability of Treg cells was not affected by α-GalCer. The expansion of thymocyte-derived Tregs was not inhibited by cytokine neutralization. However, *in vitro* thymus-derived $CD4^+CD25^+Foxp3^-$ cells expressed Foxp3 after IL-2 stimulation in a dose-dependent manner. These results collectively suggest that *in vitro* thymus-derived Treg cell expansion by α-GalCer treatment was caused by the proliferation of $CD4^+CD25^+Foxp3^-$ preTregs but not existing Treg cells.

1. Introduction

Despite significantly improved outcomes of organ transplantation by current immunosuppressive protocols, chronic rejection, which is the main negative prognostic factor, has not been satisfactorily resolved. The most important risk factor for developing de novo specific antibodies against donor antigens is nonadherence to immunosuppressant treatment [1]. Continuous immunosuppressive therapies also have several issues, such as the risks of infection and malignant neoplasms and high cost. Accordingly, the establishment of immunological tolerance, without requiring immunosuppressants, is an important goal. We previously developed a novel approach for the induction of immunological tolerance in which hematopoietic chimerism is established by the activation of invariant natural killer T (iNKT) cells using a liposomal formulation of alpha-galactosylceramide (Lipo-α-GalCer) with CD40 signal blockade in bone marrow-transplanted sublethally irradiated mice [2]. Lipo-α-GalCer upregulates the immunemodulatory functions of iNKT cells *via* the preferential presentation of

Abbreviations: α-GalCer, alpha-galactosylceramide; anti-CD40L mAb, anti-CD40 ligand monoclonal antibody; APCs, antigen presenting cells; DC, dendritic cell; IFN, interferon; IL, Interleukin; iNKT, invariant natural killer T cell; iTregs, induced Treg cells; Lipo-α-GalCer, liposomal α-galactosylceramide; mAb, monoclonal antibody; nTregs, natural occurring regulatory T cells; PTEN, phosphatase and tensin homolog deleted on chromosome 10; preTregs, regulatory T cell precursors; TCR, T-cell receptor; TGF-β, Transforming growth factor β; Treg, Regulatory T

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$CD21^{high}CD23^{low}$ B cells, resulting in peripheral $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cell expansion [3]. Lipo-α-GalCer combined with an anti-CD40 L neutralizing monoclonal antibody (mAb) effectively facilitates the engraftment of allogeneic hematopoietic cells *via* expanded Treg cell-mediated immunological regulation; the depletion of Treg cells by anti-CD25 monoclonal antibodies before treatment results in the failure to establish mixed chimerism [2]. Moreover, there are significantly fewer Treg cells in the spleen 7 days after bone marrow transplantation in thymectomized mice treated with our regimen than in euthymic mice [4]. Therefore, the presence of the thymus is indispensable for sufficient Treg cell expansion in our tolerance induction protocol.

Treg cells play an essential role in the preservation of self-tolerance and the prevention of autoimmunity [5,6]. There are at least two important functional populations of Treg cells, *i.e.*, natural occurring Treg cells (nTregs), which develop in the thymus, and induced Treg cells (iTregs), which differentiate from peripheral $CD4^+CD25^-$ T cells [7]. Several factors, especially Transforming growth factor β (TGF-β) and Interleukin (IL)-2, regulate the development of iTregs. Previous reports have demonstrated that peripheral $CD4^+CD25^-$ cells can be induced to express Foxp3 in the presence of TGF-β or IL-2 [8,9]. During T cell development in the thymus, T cell receptor (TCR) engagement and IL-2 are essential for the differentiation from $CD4^+$ thymocytes into $Foxp3^+$ Treg cells. After positive selection in the thymic cortex, auto-reactive CD4 single-positive thymocytes are removed from the thymic medulla in a process called negative selection [10]. However, some of these cells with high affinity to TCR differentiate into nTregs [11,12]. This is the initial step in the two-step process of thymic Treg cell development. The interaction between TCR and MHC II with co-stimulatory signals differentiates CD4 single-positive cells into $CD4^+CD25^+Foxp3^-$ Treg precursors (preTregs), which require cytokine signals to develop into Treg cells. In the second step, preTregs express Foxp3 and mature in the presence of IL-2 [13,14]. In this study, we evaluated the effect of Lipo-α-GalCer on thymocytes to elucidate the mechanism underlying Treg cell expansion after Lipo-α-GalCer stimulation using an *in vitro* culture system for murine thymocytes.

2. Materials and methods

2.1. Animals

Wild-type (WT) BALB/c ($H\text{-}2^d$) mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Vα14 NKT-deficient mice (BALB/c $Ja18^{-/-}$), as described in our previous study [2], were backcrossed for more than nine generations. Thymocytes from Foxp3-GFP DO11.10 BALB/c mice were kindly provided by Prof. Katsuaki Sato, University of Miyazaki. All mice were maintained in pathogen-free animal facilities at our institution. All animal experiments were performed according to protocols approved by an internal committee on the use and care of laboratory animals (AE17-104 and AE18-154).

2.2. Reagents

Lipo-α-GalCer was provided by REGiMMUNE Corp. (Tokyo, Japan) and the formulation was diluted in Roswell Park Memorial Institute Media 1640 (RPMI 1640; Life Technologies, Carlsbad, CA, USA) before use for stimulation experiments. Anti-CD40 L mAb (MR-1), anti-IL-2 mAb (S4B6-1), anti-IL-4 mAb (11B11), and anti-IL-10 mAb (JES5-2 A5) were purchased from Bio X Cell (West Lebanon, NH, USA) and diluted in Dulbecco's phosphate-buffered saline (D-PBS; Life Technologies) to the appropriate concentrations. Recombinant mouse IL-2 (Carrier-free) was purchased from Tonbo Bioscience (San Diego, CA, USA).

2.3. Flow cytometric analysis

Samples collected from each well were first washed with 2 ml of D-PBS and centrifuged at 630 × *g* for 5 min, followed by staining with Ghost Dye Red 780 (Tonbo Bioscience). Next, they were incubated with purified anti-mouse CD16/32 (BD Pharmingen, San Jose, CA, USA) to block nonspecific staining, followed by incubation with a monoclonal antibody mixture as follows: PE-Cy7-conjugated anti-CD4 (RM4-5), PerCp-Cy5.5-conjugated anti-CD8a (2.43), and FITC or APC-conjugated anti-CD25 (PC61.5) mAbs for the Treg analysis, PE-conjugated anti-CD1d (1B1) and PE-Cy7-conjugated anti-CD19 (1D3) mAbs for the B cell analysis. For intracellular staining, APC-conjugated anti-Foxp3 mAb (3G3), PE-conjugated-Ki67 mAb (B56), PE-conjugated anti-Bcl-2 mAb (3F11), PE-conjugated anti-PTEN mAb (REA270), and the Intracellular Fixation & Permeabilization Buffer Set (eBioscience, San Diego, CA, USA) were used as instructed. All antibodies were purchased from BD Pharmingen, eBioscience, Miltenyi Biotec (Bergisch Gladbach, Germany), or Tonbo Bioscience. Samples were acquired using the FACS Canto™ II (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.4. Isolation of thymocytes and cell cultures

Eight- to twelve-week-old BALB/c mice were sacrificed with CO_2 and subjected to thymectomy in sterile conditions. The isolated thymus was placed in Hanks' balanced salt solution (HBSS; Life Technologies) and immediately homogenized. The cells were filtered through a Falcon 70-μm cell strainer (Corning, NY, USA) to remove tissue debris and cells were hemolyzed with 1 ml of ACK Lysing Buffer (Lonza, Basel, Switzerland). After they were washed, thymocytes were resuspended in RPMI 1640 (Life Technologies) containing 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 100 × MEMNEAA, 1 × sodium pyruvate (100 mM, Life Technologies), 1 × penicillin–streptomycin (1 mM), and 0.5 × antibiotic/mycotic (all from Life Technologies). They were plated in 96-well plates (2×10^5 cells/100 μl/well) with or without Lipo-α-GalCer (100 ng/ml). To verify the effects of cytokines, anti-IL-2, 4, and 10 mAbs were administered for cytokine blockade.

2.5. Cell sorting and conversion of pre-tregs into Treg cells

Thymocytes from Foxp3-GFP DO11.10 BALB/c mice were cultured with Lipo-α-GalCer. These cells were collected from each well after 3 days, followed by enriched in CD4-positive cells using the Dynabeads Untouched Mouse CD4 Cells Kit (Life Technologies) before sorting. Subsequently, $CD4^+CD25^+GFP^-$ cells were sorted from CD4 single-positive cells labeled with anti-CD4, CD8a, and CD25 mAbs using MoFlo Astrios^{EQ} (Beckman Coulter, Los Angeles, CA, USA). The purity was greater than 95%. $CD4^+CD25^-Foxp3^-$ cells, used as a negative control, were also sorted. A total of 5×10^3 cells were stimulated with recombinant mouse IL-2 (10 ng/ml) for 24 h and GFP (Foxp3) expression was analyzed by flow cytometry.

2.6. Analysis of cytokine production

The levels of various cytokines (IL-2, IL-4, IL-10, and interferon (IFN)-γ) in the supernatants collected 72 h after culture were measured using the Cytometric Bead Array (BD Bioscience) and analyzed using FCAP Array Software.

2.7. Separated culture of wild-type and iNKT-Deficient thymocytes

Vα14 iNKT-deficient thymocytes from BALB/c $Ja18^{-/-}$ mice were co-cultured with Lipo-α-GalCer-stimulated or unstimulated thymocytes from WT BALB/c mice using trans-well system plates (24-well permeable support pore size 0.4 μm, Corning). A total of 2×10^5 WT thymocytes in 1000 μl of medium were plated in the lower well with or without 100 ng/ml Lipo-α-GalCer. Subsequently, the same number of Vα14 iNKT-deficient thymocytes in 100 μl of medium was loaded in the upper chamber. After 3 days of culture, the proportion of

$CD4^+CD25^+Foxp3^+$ cells among thymocytes in the upper chamber was analyzed by flow cytometry. Similar experiments were performed using splenocytes from $Ja18^{-/-}$ and WT BALB/c mice.

2.8. Deletion of B cell-depletion

B cells were depleted from hemolyzed freshly isolated thymocytes using CD19 MicroBeads, mouse (Miltenyi Biotec). The purity was greater than 95%. These cells were cultured with or without Lipo-α-GalCer following the same methods used in other experiments.

2.9. Statistical analysis

Student's *t*-tests or Mann–Whitney *U* tests were used for comparisons between two groups. For experiments with 3 or more groups, one-way ANOVA was performed to evaluate differences among groups, and Tukey's multiple comparison test was applied as a post-hoc test. All P-values were two sided, and $P < 0.05$ was considered statistically significant. All statistical analyses were performed using Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. $CD4^+CD25^+Foxp3^+$ cells and $CD4^+CD25^+Foxp3^-$ cells increased in cultured thymocytes with α-GalCer stimulation

To clarify the role of the thymus in Treg cell expansion by our original regimen using α-GalCer and CD40 blockade to induce transplant tolerance, we first examined whether Treg cells expand after the *in vitro* culture of thymocytes in the presence of α-GalCer and/or an anti-CD40 L neutralizing mAb. As shown in Fig. 1A, α-GalCer-CD1d tetramer$^+$TCRβ$^+$iNKT cells accounted for 0.81 ± 0.12% of thymocytes in WT BALB/c mice. The proportion of $CD4^+CD25^+Foxp3^+$ cells in CD4 single-positive cells did not differ between unstimulated and α-GalCer-simulated thymocytes on day 1. However, on day 3, the proportion and absolute number of $CD4^+CD25^+Foxp3^+$ cells were significantly higher in α-GalCer-stimulated thymocytes than in unstimulated thymocytes (Fig. 1B-D). Of note, in addition to $CD4^+CD25^+Foxp3^+$ cells, $CD4^+CD25^+Foxp3^-$ cells were also significantly expanded in α-GalCer-stimulated thymocytes on day 3 (Fig. 1B-D). Next, we evaluated the combined effects of α-GalCer and an anti-CD40 L neutralizing mAb *in vitro*. The proportions of $CD4^+CD25^+Foxp3^+$ cells and $CD4^+CD25^+Foxp3^-$ cells were comparable to those with α-GalCer alone. These results suggested that α-GalCer without CD40 signal blockade could promote the expansion of both $CD4^+CD25^+Foxp3^+$ cells and $CD4^+CD25^+Foxp3^-$ cells.

3.2. $CD4^+CD25^+Foxp3^-$ cells, but not $CD4^+CD25^+Foxp3^+$ cells, proliferated in α-GalCer-Stimulated thymocytes

The $CD4^+CD25^+Foxp3^+$ cells showed the phenotype of regulatory Treg cells; accordingly, we evaluated whether the expansion of $CD4^+CD25^+Foxp3^+$ cells is due to proliferation by the division of existing Treg cells, stabilization of existing Treg cells, or development from thymocytes. First, we analyzed the proliferation of $CD4^+CD25^+Foxp3^+$ cells and $CD4^+CD25^+Foxp3^-$ cells. Ki67 expression in $CD4^+CD25^+Foxp3^+$ cells did not increase on day 1 or day 3, but that in $CD4^+CD25^+Foxp3^-$ cells was significantly higher in α-GalCer-stimulated thymocytes than in unstimulated thymocytes on day 3 (Fig. 2A, B). We next analyzed the stability of $CD4^+CD25^+Foxp3^+$ cells. Flow cytometry showed that Bcl-2, an inhibitor of apoptosis, in $CD4^+CD25^+Foxp3^+$ cells 3 days after culture was slightly higher for α-GalCer thymocytes than for unstimulated thymocytes (Fig. 2C, D). However, PTEN, which is the main negative regulator of phosphoinositide 3-kinase and a stabilizer of $CD4^+CD25^+Foxp3^+$ cells, was not changed in α-GalCer-stimulated $CD4^+CD25^+Foxp3^+$ cells (Fig. 2C). These results suggested that the expansion of $CD4^+CD25^+Foxp3^+$ cells was not caused by the proliferation or stabilization of existing Treg cells in the thymus.

3.3. α-GalCer-Induced $CD4^+CD25^+Foxp3^-$ cells in thymocytes contained preTregs

Another potential explanation for the expansion of $CD4^+CD25^+Foxp3^+$ cells is that Tregs developed from $CD4^+CD25^+Foxp3^-$ preTregs, as suggested by the remarkable increase in these cells in thymocyte culture (*cf.* Fig. 1B, C). To evaluate the direct development of $CD4^+CD25^+Foxp3^+$ cells from $CD4^+CD25^+Foxp3^-$ cells *in vitro* in response to α-GalCer, we used an isolated population of $CD4^+CD25^+Foxp3^-$ cells in thymocytes derived from Foxp3-GFP reporter BALB/c mice. After 3 days of culture, $CD4^+CD25^+Foxp3^-$ cells were sorted by flow cytometry and stimulated with recombinant mouse IL-2 (rmIL-2) for 24 h. Sorting gates were defined as shown in Fig. 3A. The expression of Foxp3 in the sorted $CD4^+CD25^+Foxp3^-$ cells was augmented in an rmIL-2 dose-dependent manner, suggesting that $CD4^+CD25^+Foxp3^-$ cells could function as preTregs in the development of $CD4^+CD25^+Foxp3^+$ Treg cells (Fig. 3B).

3.4. Cell-to-cell contact was essential for the expansion of $CD4^+CD25^+Foxp3^+$ cells in α-GalCer-stimulated cultured thymocytes

We hypothesized $CD4^+CD25^+Foxp3^+$ cells in the thymocyte culture containing α-GalCer developed from $CD4^+CD25^+Foxp3^-$ cells that expanded *via* cytokines from α-GalCer-activated iNKT cells. As IL-2 is an essential cytokine for the homeostasis of nTregs and iTregs, we assessed the IL-2 concentration in the thymocyte culture. As shown in Fig. 4A, IL-2 was undetectable in the culture supernatants of thymocytes, whereas it was detected at high levels in the culture supernatants of splenocytes. To exclude the impact of small quantities of IL-2 (below the detection limit), we tested whether the blocking of cytokines affected the expansion of $CD4^+CD25^+Foxp3^+$ cells after α-GalCer stimulation. Thymocytes derived from WT mice were cultured in the presence of α-GalCer with an anti-IL-2, IL-4, or IL-10 neutralizing mAb. The proportion of $CD4^+CD25^+Foxp3^+$ cells was analyzed after 3 days. No anti-cytokine neutralizing mAb inhibited the expansion of $CD4^+CD25^+Foxp3^+$ cells derived from thymocytes in the presence of α-GalCer (Fig. 4B). Vα14 iNKT-deficient thymocytes from BALB/c $Jα18^{-/-}$ mice were co-cultured with α-GalCer-stimulated or unstimulated thymocytes from WT BALB/c mice. In this experiment, splenocytes from the same mice were also cultured as controls (Fig. 4C). After 3 days of co-culture with α-GalCer-stimulated WT splenocytes, $CD4^+CD25^+Foxp3^+$ cells in Vα14 iNKT-deficient splenocytes, which were cultured in the above well, increased, though these cells were not expanded when co-cultured with unstimulated splenocytes (Fig. 4D, left panel). However, neither $CD4^+CD25^+Foxp3^+$ cells nor $CD4^+CD25^+Foxp3^-$ cells in Vα14 iNKT-deficient thymocytes increased by co-culture with α-GalCer-stimulated WT thymocytes (Fig. 4D, right panel).

Taken together, these results indicated that the cell-to-cell interactions between iNKT cells and thymocytes might be indispensable for the development of Treg cells from preTregs *in vitro*.

4. Discussion

Our previous study of iNKT cell activation and CD40 blockade clearly indicated that the thymus in recipient mice is indispensable for the establishment of mixed chimerism, which could make it possible to prevent the rejection of skin grafts [4]. As shown Fig. 1, we observed a substantial quantity of iNKT cells in thymocytes derived from naïve mice and the remarkable proliferation of $CD4^+CD25^+Foxp3^-$ phenotype cells, rather than $CD4^+CD25^+Foxp3^+$ Treg cells, in the culture of mouse thymocytes upon the stimulation of iNKT cells by their specific

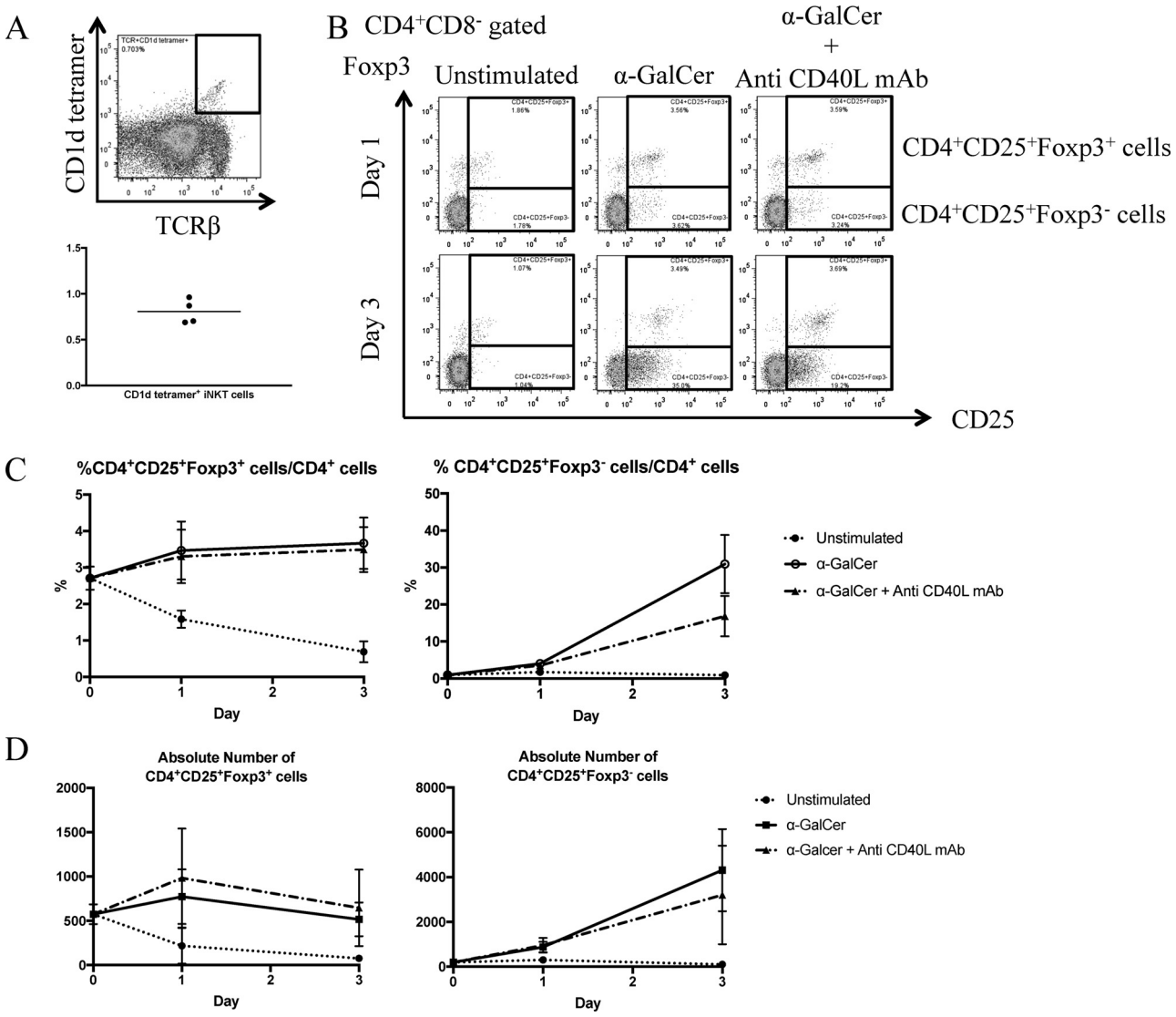

Fig. 1. Effect of α-GalCer on the proliferation of thymocytes *in vitro*.
Approximately 2×10^5 thymocytes isolated from naïve wild-type BALB/c mice (N = 4) were prepared. (A) Cells were stained with the fluorescently labeled α-GalCer-loaded CD1d-tetramer and anti-TCRβ mAb. The double-positive population of iNKT cells was analyzed by flow cytometry. (B) Cells were cultured in the absence (Unstimulated) or presence of α-GalCer (100 ng/ml) alone or together with anti-CD40 L mAb (10 ng/ml). The cells at day 1 or 3 after the culture were recovered and stained with fluorescence-anti-CD4 and anti-CD25 mAbs. Representative flow cytometry results are shown in the upper panel (day 1) and lower panel (day 3). (C) Proportions of $CD4^+CD25^+Foxp3^+$ cells and $CD4^+CD25^+Foxp3^-$ cells, and (D) Absolute number of $CD4^+CD25^+Foxp3^+$ cells and $CD4^+CD25^+Foxp3^-$ cells are plotted. α-GalCer, alpha-galactosylceramide; Anti CD40 L mAb, anti CD40 ligand monoclonal antibody; TCR, T cell receptor.

ligand, α-GalCer, regardless of anti-CD40 L mAb treatment. These results suggest that α-GalCer-activated iNKT cells preferentially exhibit the expansion of preTregs, rather than existing Treg cells in the thymus. Indeed, the ratio of Ki76-positive Treg cells in the thymus was much lower than that of preTregs (Fig. 2B). The expression of PTEN, which is an upstream inhibitor of lipid kinase PI3K-Akt signaling, is essential for the function and stabilization of Treg cells [15–17]. However, the enhancement of $CD4^+CD25^+Foxp3^+$ Treg cell stability by the up-regulation of PTEN was not observed (Fig. 2C). These results strongly suggest that *in vitro* expanded $CD4^+CD25^+Foxp3^+$ Treg cells after thymocyte culture in the presence of α-GalCer developed from $CD4^+CD25^+Foxp3^-$ cells. However, the magnitude of $CD4^+CD25^+Foxp3^+$ Treg cell expansion was quite low compared to that of $CD4^+CD25^+Foxp3^-$ cell expansion in the thymocytes cultured with α-GalCer (Fig. 1C). This might be explained by a low level of IL-2 production, which is indispensable for Treg development from preTregs, since $CD4^+CD25^+Foxp3^+$ Treg cells were expanded in the culture of isolated $CD4^+CD25^+Foxp3^-$ preTregs in an IL-2 dose-dependent manner (Fig. 3B). Indeed, the IL-2 concentration in the supernatant of the thymocyte culture with α-GalCer was close to the detection limit (Fig. 4A). The results of a Trans-well experiment support the importance of cell-to-cell interactions between activated iNKT cells and thymocytes for $CD4^+CD25^+Foxp3^+$ Treg cell development in our culture system (Fig. 4D). Taken together, we speculate that a low level of IL-2 produced by activated iNKT cells close to the $CD4^+CD25^+Foxp3^-$ preTreg population could promote $CD4^+CD25^+Foxp3^+$ Treg cell development. Since we did not perform staining of these cells with a mAb specific to T cells, the presence of CD4-positive cells other than T cells in the thymus cannot be completely excluded. However, we conclude that almost all of these populations were composed of T cells because $CD4^+CD25^+Foxp3^-$ cells derived from Foxp3-GFP reporter mice expressed Foxp3 in the presence of IL-2.

Stimulation by IL-2 converts preTregs into mature $CD4^+CD25^+Foxp3^+$ Treg cells [13,14]. It was not clear why iNKT cells failed to produce IL-2 in the thymocyte culture, even though a substantial population of iNKT cells was detected in thymocytes derived

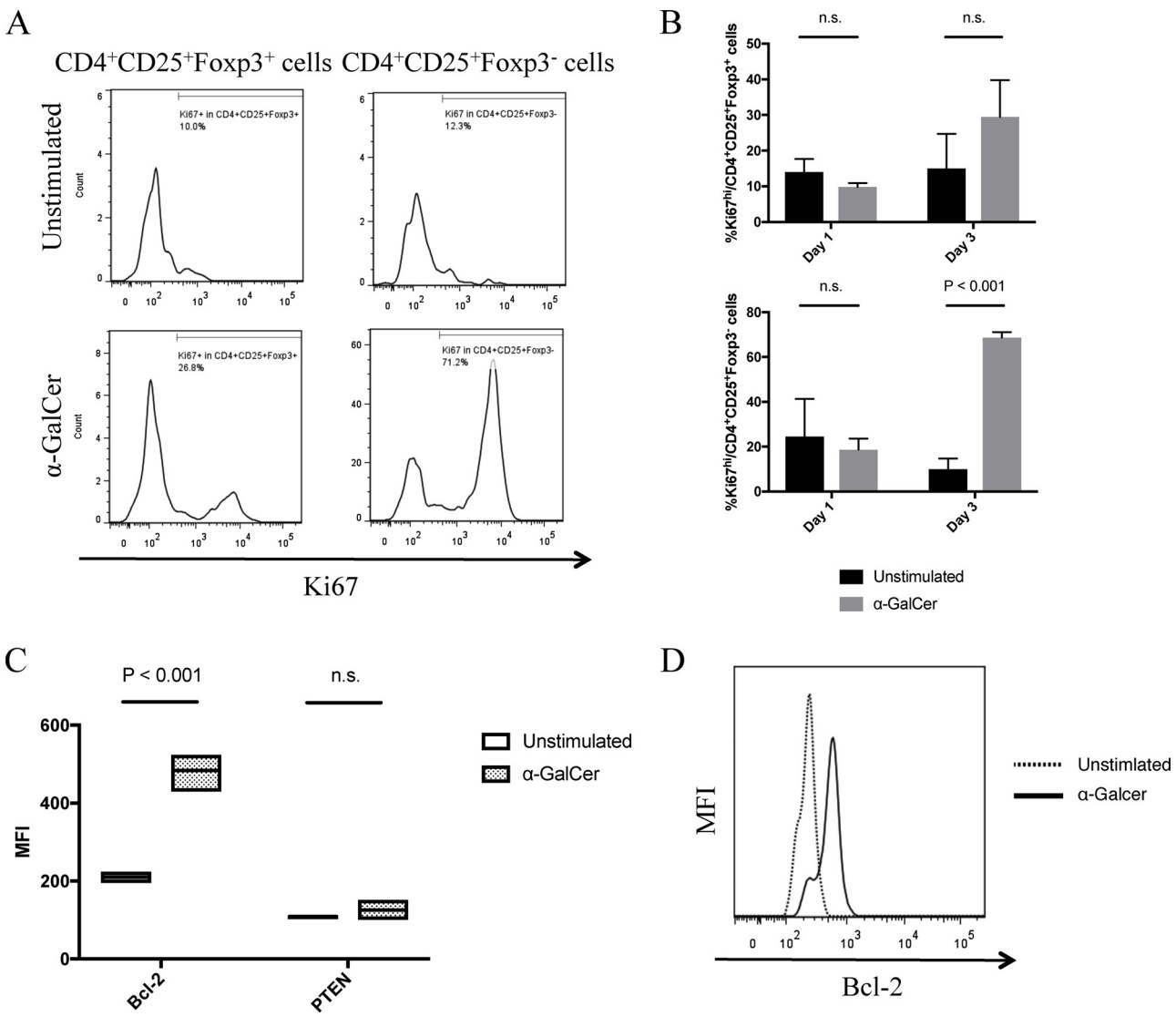

Fig. 2. Proliferation and stabilization of CD4$^+$ thymocytes after *in vitro* culture with α-GalCer.
Thymocytes derived from normal BALB/c mice (N = 4) were prepared. Anti-Ki67, Bcl-2, or PTEN mAb were incubated with whole cells on day 1 or day 3 after the *in vitro* culture of thymocytes in the absence (Unstimulated) or the presence of α-GalCer. (A) Representative histograms of Ki67 expression in CD4$^+$CD25$^+$Foxp3$^+$ cells and CD4$^+$CD25$^+$Foxp3$^-$ cells after 3 days are shown. (B) Proportions of Ki67-expressing CD4$^+$CD25$^+$Foxp3$^+$ cells or CD4$^+$CD25$^+$Foxp3$^-$ cells on day 1 and day 3 are plotted. (C) Expression of Bcl-2 or PTEN on cells after 3 days of culture was analyzed by flow cytometry using PE-conjugated anti-Bcl-2 mAb or PE-conjugated anti-PTEN mAb, respectively. MFI levels of Bcl-2 and PTEN in CD4$^+$CD25$^+$Foxp3$^+$ cells on day 3 are shown. (D) Representative histograms of Bcl-2 expression in CD4$^+$CD25$^+$Foxp3$^+$ cells after 3 days are shown. α-GalCer, alpha-galactosylceramide; MFI, mean fluorescent intensity; n.s., not significant; PTEN, Phosphatase and Tensin Homolog Deleted from Chromosome 10.

from naïve mice (Fig. 1A). In our regimen to establish mixed chimerism in mice, Lipo-α-GalCer was utilized owing to its higher efficacy compared with that of aqueous α-GalCer [2]. We have previously shown that Lipo-α-GalCer is incorporated into B cells, rather than dendritic cells (DCs) or macrophages [3]. We wondered if there are fewer CD1d-positive B cells in the thymus than in spleen. Using flow cytometry, we found that B cells accounted for less than 1% of the total thymocytes, and the CD1d^{high} population represented 6.2% of B cells, indicating that the number of α-GalCer-presenting B cells in the thymus is quite level (Supplemental Fig. 1A&B). As other potential α-GalCer-presenting cells, the involvement of DCs cannot be excluded because iNKT cells activated by α-GalCer-pulsed DCs produce multivalent cytokines, including IL-2 or particularly high levels of IFN-γ. However, we did not expect DCs expressing CD1d to play a role in the development of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells *in vitro* because the level of IFN-γ in the thymocyte culture supernatants was below the detection limit for flow cytometry (data not shown).

It has been reported that a limited niche supports Treg cell development. Previous reports have shown that thymic Treg cell development occurs despite a low frequency of preTregs [11,18]. Weist et al. have shown that the frequency of preTregs is not associated with Treg cell development, while existing Treg cells in the thymus limit new Treg cell development by competition for IL-2 [19]. Another question is why the preTregs population in the thymus could preferentially proliferate *in vitro* by iNKT activation. As this proliferation was required for cell-to-cell interactions between iNKT cells and thymocytes, we speculate that a low level of cytokines and/or cell surface molecules could stimulate proliferation.

The role of the anti-CD40 L mAb in the culture of thymocytes was not elucidated in this study. We evaluated the effect of blocking CD40-CD40 L signaling on α-GalCer dependent expansion of Treg cells and preTregs *in vitro*. However, there were no statistically significant differences in the effects between α-GalCer alone and α-GalCer combined with anti CD40 L mAb (Fig. 1C). This result suggested that there is no

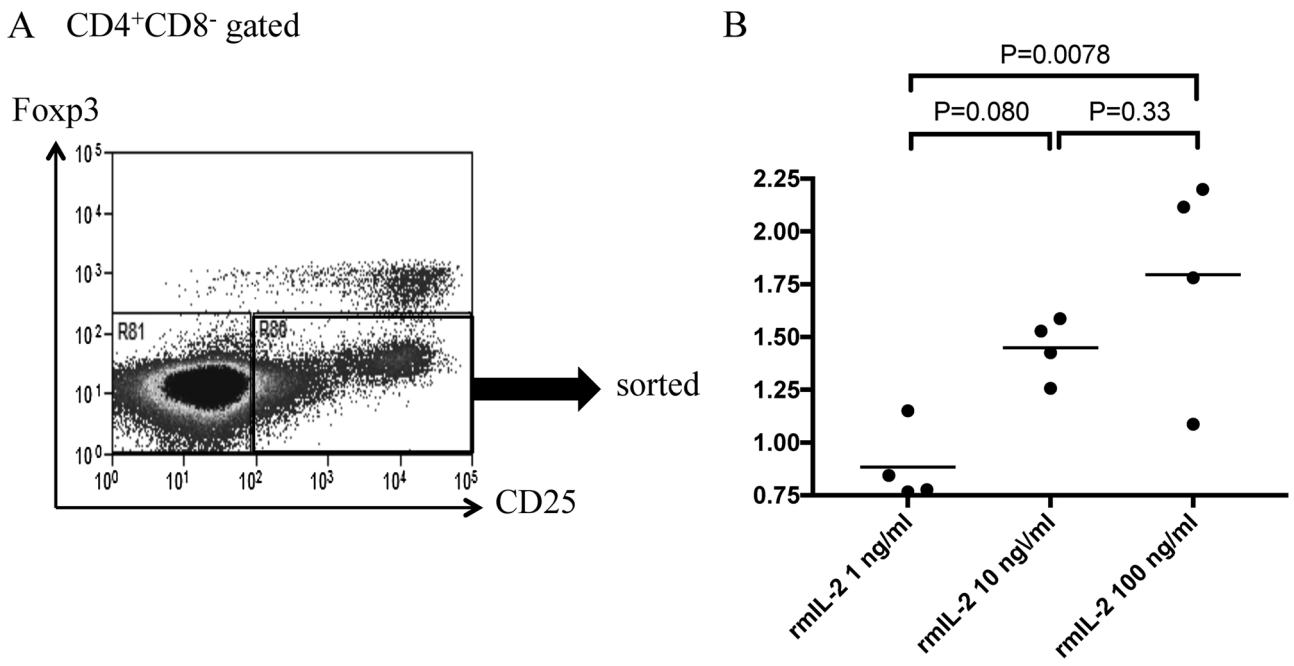

Fig. 3. Upregulation of Foxp3 in isolated thymic $CD4^+CD8^-CD25^+Foxp3^-$ cells after culture with IL-2.
(A) $CD4^+CD8^-CD25^+Foxp3^-$ cells were isolated from thymocytes derived from Foxp3-GFP DO11.10 mice (N = 4). Bold square shows the gate for sorting cells. (B) The sorted cells were cultured in the presence of rmIL-2 (1, 10, or 100 ng/ml) for 24 h. The ratio of Foxp3 expression in $CD4^+CD8^-CD25^+Foxp3^-$ cells to control ($CD4^+$ $CD8^-CD25^-$ cells) is shown. α-GalCer, alpha-galactosylceramide; IL, interleukin; rmIL-2, recombinant mouse IL-2.

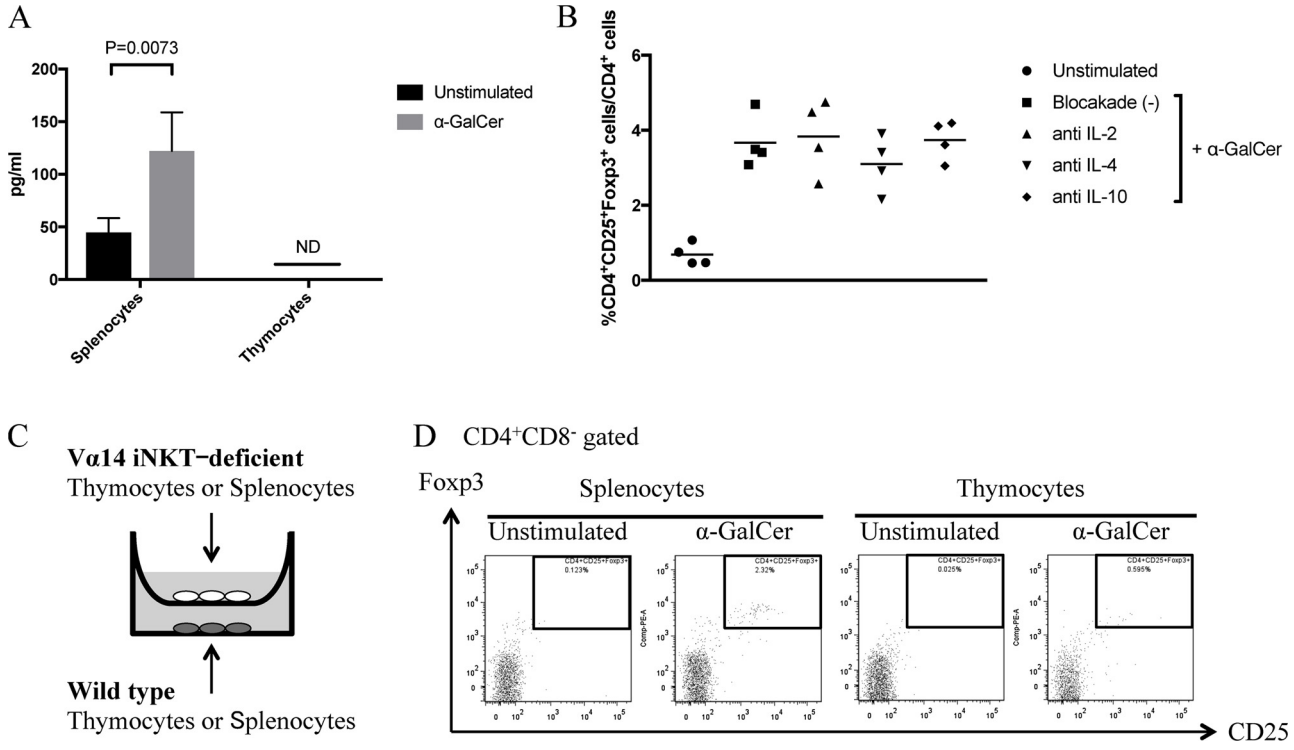

Fig. 4. Essential role of interactions between iNKT cells and thymocytes for Treg expansion.
(A) Splenocytes (N = 4) or thymocytes (N = 2) from wild-type BALB/c mice were cultured with α-GalCer. After 3 days of culture, the IL-2 concentrations in the supernatants were measured by a cytometric bead array. (B) The proportion of $CD4^+CD25^+Foxp3^+$ cells was analyzed by flow cytometry 3 days after the culture of thymocytes in the presence of α-GalCer with anti-IL-2 mAb (N = 4), anti-IL-4 mAb (N = 4), or anti-IL-10 mAb (N = 4), or without neutralizing mAb (N = 4). (C) Vα14 iNKT-deficient thymocytes from BALB/c $J\alpha18^{-/-}$ mice (upper well) were co-cultured with α-GalCer-stimulated or unstimulated thymocytes from wild-type BALB/c mice (bottom well). Splenocytes from the same mice were also cultured as a positive control. (D) Dot plots show the proportion of $CD4^+CD25^+Foxp3^+$ cells in Vα14 iNKT-deficient splenocytes (left panel) or thymocytes (right panel) cultured for 3 days in the upper wells. α-GalCer, alpha-galactosylceramide; iNKT cells, invariant natural killer T cells; mAb, monoclonal antibody. ND, not detected.

additional effect of blocking CD40-CD40 L signaling in the thymic culture. *In vivo*, on the other hand, CD40-deficient mice show a reduction in the number of Treg cells in the thymus and the CD40/CD40 L signal is important in thymic Treg cell homeostasis [20–22]. It is conceivable that other cells expressing CD40 migrated into the thymus from external areas and were involved in the development of Treg cells. In our system, to induce mixed chimerism, donor-derived DCs were detected in the thymus of recipient mice [4]. We speculate that systemic treatment with an anti-CD40 L mAb may not prevent Treg cell development from CD40-expressing DCs in the thymus but may mainly be involved in the induction of peripheral tolerance by the blockade of CD40/CD40 L signaling.

The contribution of DCs in the thymus and medullary thymic epithelial cells to Treg cell differentiation has been well established [23–26]. Recent reports have demonstrated that B cells or $CD19^+CD5^+CD1d^{hi}IL\text{-}10^+$ regulatory B cells in the thymus promote thymic Treg cell expansion [27–29]. We focused on thymic B cells because, as mentioned above, they preferentially present Lipo-α-GalCer. However, in our experiments, the depletion of $CD19^+$ B cells, including $CD1d^+$ B cells, from thymocytes before culture did not affect the outcome of Lipo-α-GalCer stimulation, *i.e.*, the expansion of Treg cells and $CD4^+CD25^+Foxp3^-$ preTregs (Supplemental Fig. 1C). This result indicated that other APCs in the thymus associated with Lipo-α-GalCer induced the development of Treg cells. We did not examine the contribution of these cells because it was difficult to completely and specifically remove them. A similar issue applies to B cell depletion. In this study, $CD19^+$ B cells were almost completely depleted, but we confirmed that a small quantity of $CD19^-B220^+$ B cells remained. These cells might have influenced the outcome of the study, suggesting that the identification of key APCs associated with Treg cell development after Lipo-α-GalCer stimulation is difficult.

In this study, we did not measure TGF-β, which is a fundamental cytokine for differentiation of peripheral Treg cells from $CD4^+CD25^-Foxp3^-$ naïve T cells [8]. The role of TGF-β during thymic Treg cell differentiation is controversial. It was previously reported that T cell responsiveness to TGF-β is not required for the development of thymic-derived Treg cells [30,31]. Moreover, we reasoned that TGF-β1 in culture supernatant probably could not be detected because TGF-β is noncovalently associated with latency-associated peptide to form the latent complex, which is attached to Treg cell surface molecule called GARP [32,33]. Therefore, we did not evaluate TGF-β in our experiment.

Our results clarified the mechanisms underlying thymic Treg cell expansion by α-GalCer and demonstrated an efficient *in vitro* Treg expansion method using α-GalCer and thymocytes. The immune suppressive capacity of Treg cells can ameliorate various T cell-mediated diseases, including rejection after transplantation and autoimmune diseases, in clinical settings [34,35]. In transplant tolerance, Treg cells are expanded in transplanted grafts and secondary lymphoid tissues after transplantation, suggesting that these cells have key roles in the establishment of tolerance [36,37]. The adoptive transfer of *ex vivo*-expanded Treg cells facilitates the establishment of mixed chimerism, thereby promoting engraftment [38,39]. The adoptive transfer of Treg cells has clinical benefits, but methods for the efficient generation of Treg cells *in vitro* have not been established [37,40]. Dijike et al. developed a novel Treg expansion method using the human thymus, which was discarded during pediatric cardiac surgery. Expanded thymic Treg cells have high purity and high suppressive function compared with those of expanded Treg cells from the blood, suggesting that the discarded thymus in humans is a potentially novel approach for therapeutic Treg cell expansion [41]. Our results, indicating that Treg cells and preTregs can be augmented by stimulating thymic iNKT cells with Lipo-α-GalCer *in vitro*, may also provide an effective *ex vivo* Treg cell expansion method.

In conclusion, we examined the effect of α-GalCer stimulation on thymocytes using an *in vitro* culture system. The stimulation of iNKT cells with α-GalCer led to the development of new Treg cells in thymocytes *via* the expansion of $CD4^+CD25^+Foxp3^-$ preTregs and not by the proliferation of resident Treg cells.

Declaration of interest

The authors of this manuscript have conflicts of interest to disclose as described by *Immunology Letters*. Y. Ishii is the executive chairman of REGiMMUNE Corp. The other authors have no other conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2018.11.013.

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