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Interleukin-2 drives immature double negative thymocytes into γδ T cells expressing Foxp3 on a stromal cell line *in vitro*

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ABSTRACT

γδ T cells are exported from the thymus as innate-like lymphocytes that can immediately respond to antigens. In the thymus, γδ T cells diverge into functionally distinct lineages. It is not known whether γδ T cells differentiate into regulatory cells that express Foxp3, which is an essential transcription factor for $CD4^+$ regulatory T cells. In this study, we analyzed $CD25^+$ immature thymocytes that give rise to both αβ and γδ thymocytes. These precursor cells have the potential to differentiate into $Foxp3^+$ γδ T cells on a stromal cell line, TSt4-Dll1. Development of $Foxp3^+$ γδ thymocytes in this culture was dependent on IL-2. IL-2 stimulation induced Id3, Egr1, and Egr3 expression in $CD25^+$ immature thymocytes, suggesting that it could activate signaling molecules that are downstream of TCR signaling. The induction of Foxp3 in precursor γδ T cells suggested that IL-2 could activate the Foxp3 gene early in thymocyte development.

1. Introduction

In early thymocyte development, the differential expression of CD25, CD44, and the early hematopoietic cell marker, c-Kit, defines four $CD4^-CD8^-$ double negative (DN) subsets: $CD44^+CD25^-$c-Kit^+ (DN1), $CD44^+CD25^+$c-Kit^+ (DN2), $CD44^-CD25^+$c-Kit^- (DN3), and $CD44^-CD25^-$c-Kit^- (DN4). The rearrangement of TCR β variable gene segments occurs in T lineage-committed DN3 cells. The first checkpoint for the rearranged αβ precursors is known as β-selection and it requires signaling through a pre-TCR consisting of a rearranged TCR β chain and the pre-Tα. Pre-TCR-mediated activation of early thymocytes leads to the expression of the early growth response gene (Egr) family members, Egr1, Egr2, and Egr3 [1]. Moreover, the development of γδ T cells also takes place at the DN3 checkpoint with signaling through complete TCR γδ complexes [2]. Unlike αβ T cells, γδ lineage differentiation does not depend on Notch signals [3]. The divergence of αβ versus γδ lineage can be controlled by the strength of pre-TCR and γδ TCR signals. Id-family molecules inhibit the E-box binding proteins, E2A and HEB, thus relieving the developmental block at the DN3 stage. Id3 is preferentially up-regulated in γδ lineage cells [4]. Although pre-TCR signals also up-regulate Id3 expression, the extent of Id3 up-regulation by pre-TCR versus γδTCR may play a role in lineage fate at the DN3 stage.

$Foxp3^+$ regulatory T cells (Treg) play an indispensable role in immunological tolerance. These cells are mainly generated in the thymus from positively selected $CD4^+$ thymocytes [5,6]. It is generally believed that a strong interaction with self-antigens is required for selection [7]. Co-stimulation with CD28 [8,9] and cytokine signaling are also involved in Treg development in the thymus [10]. Thymocytes that lack the common γ chain of cytokine receptors or the IL-2 receptor β (IL-2Rβ) chain failed to differentiate into Tregs for the establishment of tolerance [11]. Similarly, mice deficient for both IL-2 and IL-15 exhibited a significant reduction in Treg numbers [12]. In the body, autoreactive T cells are activated in the absence of Tregs, and this recognition of antigens is necessary for Treg-mediated suppression.

γδ T cells have diverse effector functions. They can be cytotoxic against infected cells and transformed cells and produce various cytokines that modulate protective immunity. Following recognition of stress-induced ligands, γδ T cells produce cytokines rapidly. Despite of these innate-like features, there are similarities in the effector functions of αβ T cells and γδ T cells. The functional specialization is controlled by distinct transcription factors, however, it is not known whether γδ T cells with regulatory functions require Foxp3 expression.

In this study, we analyzed the differentiation of DN thymocytes *in vitro* on a stromal cell line expressing Dll1, which supports the

Abbreviations: DN, double negative; DP, double positive; Egr, early growth response gene; Id3, inhibitor of DNA binding 3.

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differentiation of DN thymocytes into $CD4^+CD8^+$ double positive (DP). Foxp3 expression was induced unexpectedly in DN3 thymocytes cultured on the stromal cells in the presence of IL-2. These $Foxp3^+$ thymocytes maintained high expression of CD25 but did not express CD4, and they preferentially expressed TCRγδ. $Foxp3^+$-$TCR\gamma\delta^+$ cells were detected in the thymus at low frequency. These results suggested that the Foxp3 gene locus was opened by signaling events in early thymocyte development before divergence into αβ and γδ T cell lineages.

2. Materials and methods

2.1. Mice

Foxp3-IRES-EGFP knock-in mice (C57BL/6J) were kindly provided by B. Malissen (INSERUM, France). $TCR\beta^{-/-}$ and $TCR\delta^{-/-}$ mice were a generous gift from S. Fagarasan (RIKEN, Yokohama, Japan), which were backcrossed to C57BL/6J mice. All the mice were maintained under specific pathogen-free conditions in the animal facility at Tokyo University of Science and experimental studies were approved by the Animal Care and Use Committee of the university.

2.2. Monoclonal antibodies and reagents

Antibodies against CD4 (GK1.5), CD8 (53-6.7), CD3 (145-2C11), TCRγδ (UC7-13D5), c-Kit (2B8), CD25 (PC61.5), TCRβ (H57-597), Foxp3 (FJK-16s), conjugated to FITC, PE, APC or biotin and CD16/32 (93) for Fc blocking were purchased from eBioscience (San Diego, CA). Recombinant murine TGF-β1, IL-2, and IL-15 (R&D Systems, Minneapolis, MN) and IL-7 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used to stimulate cells. To neutralize TGF-β, monoclonal mouse IgG (Clone 1D11) (R&D systems, Minneapolis, MN) was used at 10 μg/ml [13]. Purified anti-TCRγδ (UC7-13D5, functional grade) and anti-CD28 (37.51, functional grade) were purchased from eBioscience (San Diego, CA).

2.3. Flow cytometry and cell sorting

Thymocyte cell suspensions were prepared from 6 to 10-week-old Foxp3-IRES-EGFP knock-in mice. DN thymocytes were first enriched by depleting $CD4^+$ and/or $CD8^+$ cells by using anti-CD4-labeled and anti-CD8-labeled I-MAG beads (BD Biosciences, San Jose, CA). Cells were then stained with FITC-labeled anti-CD4, anti-CD8, anti-CD3, and anti-TCRγδ, PE-labeled anti-CD25 and APC-labeled anti-c-kit. DN3 ($c\text{-}kit^-CD25^+$) cells in FITC-negative and GFP-negative cells were sorted using a FACSAriaII (BD Biosciences). Cells were analyzed using a FACSCalibur and CellQuest software (BD Biosciences).

2.4. Cell co-culture system

Cells were cultured in RPMI medium supplemented with 5% FCS, 100 mM L-glutamine, 10 mM 2-ME, and antibiotics. TSt-4/Dll1 stromal cells were kindly provided by H. Kawamoto (Kyoto University). Sorted DN3 thymocytes (1×10^6) were cultured on TSt-4/Dll1 with or without cytokines for 5 days.

2.5. Cell culture

Thymocytes were stimulated with plate-coated anti-TCRγδ antibodies with cytokines. $CD4^+$ T cells were isolated by anti-CD4 labeled I-MAG beads and stimulated with plate-coated anti-CD3 and anti-CD28 with or without cytokines.

2.6. Measurement of STAT5 phosphorylation

DN thymocytes were enriched by depleting thymocytes expressing CD4 and CD8 using MACS beads. Cells were stained with FITC-labeled anti-CD4, anti-CD8, anti-CD3, and anti-TCRγδ. After being washed with RPMI medium without FCS and incubating at 37 °C for 30 min, cells were incubated another 60 min at 37 °C with IL-2 (25 ng/ml), IL-7 (25 ng/ml), or without stimulation. Cells were washed with cold PBS, fixed with 4% paraformaldehyde at RT, and permeabilized with 0.5%TritonX/3 mM EDTA/PBS. Non-specific binding was blocked using 3%BSA/PBS and cells were then stained with PE-conjugated anti-pSTAT5 (BD Biosciences) or Isotype control antibody (eBioscience).

2.7. Real-time quantitative RT-PCR

RNA was extracted from cells using Isogen (Wako Pure Chemical Industries, Ltd.) and reverse transcribed using Reversecript III (Wako Pure Chemical Industries, Ltd.). PCR was performed in triplicate for two to three independent experiments using GoTaq qPCR Master Mix containing SYBR greed on an Applied Biosystems 7500/7500 Fast Real-time PCR Systems. The expression of Foxp3, Egr1, Egr3, and Id3 was measured with the following primer sets: 5′-ccagctctactctgvaccttc-3′ and 5′-gccttgccttttctcatcca-3′ (Foxp3), 5′-gatgtctccgctgcagatctc-3′ and 5′-tgtccatggtgggtgagtga-3′ (Egr1), 5′-caacgacatgggctccattc-3′ and 5′-ggccttgatggtctccagtg-3′ (Egr3), 5′-atcctgcagcgtgtcatagact-3′ and 5′-aggcgttgagttcagggtaagt-3′ (Id3). Data were normalized to the expression of β-actin and are represented as relative mean expression ± SD. Primer sequences of β-actin are 5′-ggctgtattcccctccatcg-3′ and 5′-ccagttggtaacaatgccatgt-3′.

2.8. Statistical analysis

Statistical significance was evaluated by Student's *t*-test; *P* values less than 0.05 are considered significant.

3. Results

3.1. IL-2–dependent differentiation of $Foxp3^+$ thymocytes in TSt-4/Dll-1 co-culture

Precursor cells that are committed to the T-cell lineage diverge into functionally distinct T cell populations within the thymus. This functional differentiation was initiated during the course of TCR gene rearrangement and selection. The development of early thymocytes was analyzed in an *in vitro* co-culture system, where precursor cells differentiate on a stromal cell line expressing delta like-1 (TSt-4/Dll-1 cells) [14]. In this co-culture system pre-committed precursor cells are activated with Notch signals to differentiate along the T cell lineage pathway until they become DP thymocytes.

DN thymocytes were prepared from Foxp3-IRES-EGFP-KI mice by depleting cells that expressed CD4 and CD8 using MACS beads. These cells were sorted using FACS to collect $CD25^+c\text{-}Kit^-$ cells (DN3) by negative gating on differentiation markers (CD3, TCRγδ, CD4, and CD8). DN3 cells were cultured on TSt-4/Dll-1 in the presence or absence of various cytokines. These cells differentiated toward DP thymocytes in 5–7 days. Interestingly, we found that some cells cultured in the presence of TGF-β1 and IL-2 expressed Foxp3 (Fig. 1A). TGF-β1 plus IL-2 stimulation blocked normal differentiation from DN to DP (Fig. 1B), and the $Foxp3^+$ cells were either double negative or $CD8^+$. Although the $Foxp3^+$ cells maintained high expression of CD25, they also expressed CD3 (Fig. 1C). IL-15 but not IL-7 was also able to induce $Foxp3^+$ cells

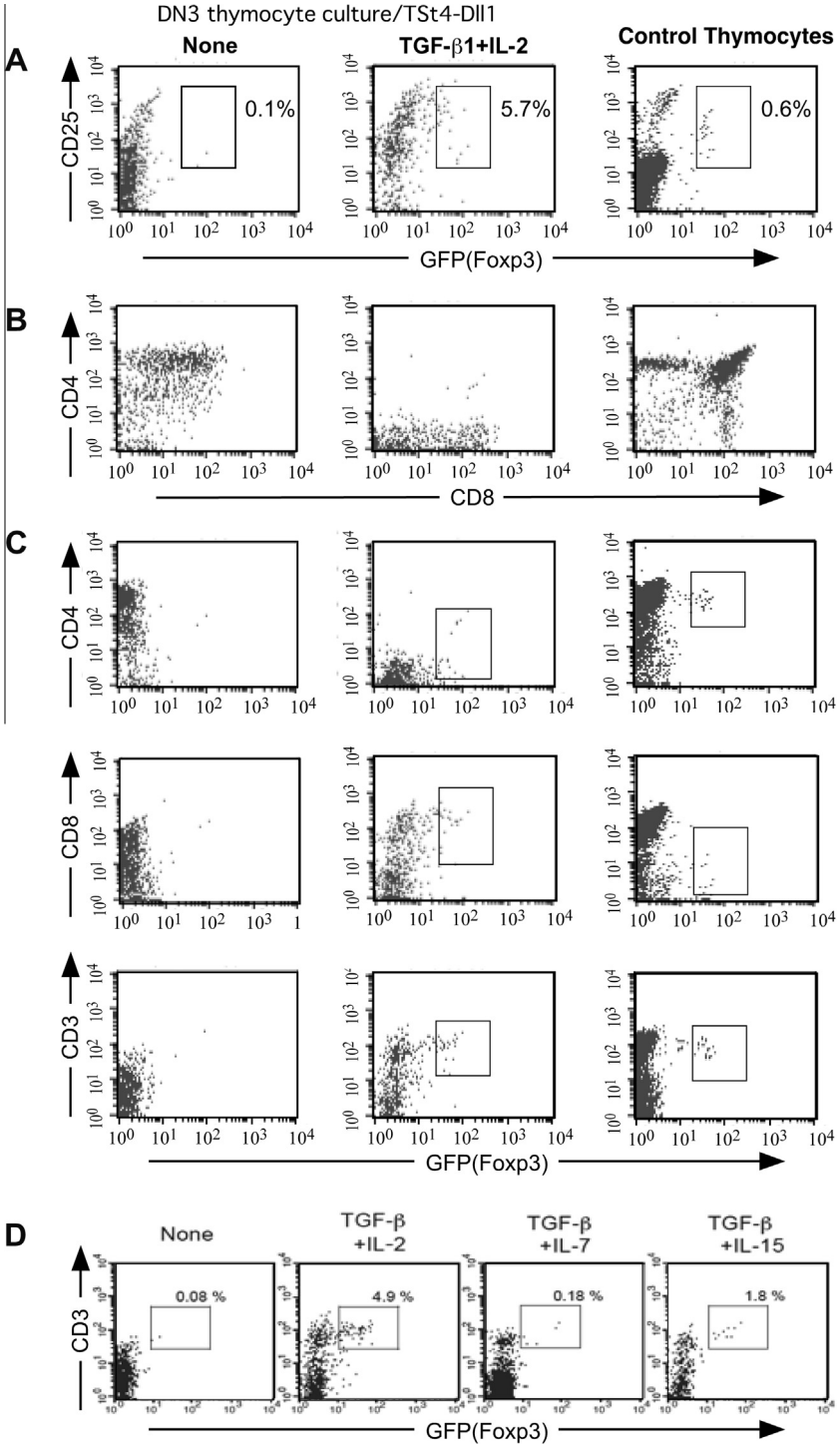

Fig. 1. DN3 thymocytes give rise to Foxp3$^+$ cells on TSt-4/Dll1 stromal cells. (A–C) Sorted DN3 thymocytes were cultured on TSt-4/Dll1 for 1 week with or without TGF-β1 (2 ng/ml) plus IL-2 (25 ng/ml) stimulation. Control thymocytes obtained from adult mice were stained and shown in the right row. (D) IL-7 (25 ng/ml) and IL-15 (25 ng/ml) in addition to IL-2 were used to stimulate DN3 thymocyte as shown in (A)–(C), expression of CD3 and Foxp3 is shown. Representative results from five separate experiments are shown.

(Fig. 1D). These results suggested that IL-2 receptor signaling that involved CD122, commonly used in IL-2R and IL-15R, played a role in Foxp3 expression in DN thymocytes, which is consistent with the previous observations of CD4$^+$Foxp3$^+$ Treg development [11].

TGF-β in concert with IL-2 signaling plays an important role in the induction of Foxp3 in CD4$^+$ naïve T cells [15]. In order to see whether TGF-β also played a role in Foxp3 expression in DN thymocytes, DN3 cells were cultured for 1 week on TSt-4/Dll-1 with or without TGF-β1 in the presence of IL-2. A large proportion of cells differentiated into DP thymocytes in the presence of IL-2; however, the proportion of DP thymocytes was dramatically lower in the presence of TGF-β1. IL-2 was required to induce Foxp3 expression in the thymocytes, and Foxp3 expression was not affected by the presence of TGF-β1 (Fig. 2A). The expression of Foxp3 was also evaluated by semi-quantitative RT-PCR (Fig. 2B) and real-time RT-PCR (Fig. 2C). In order to eliminate TGF-β sup-

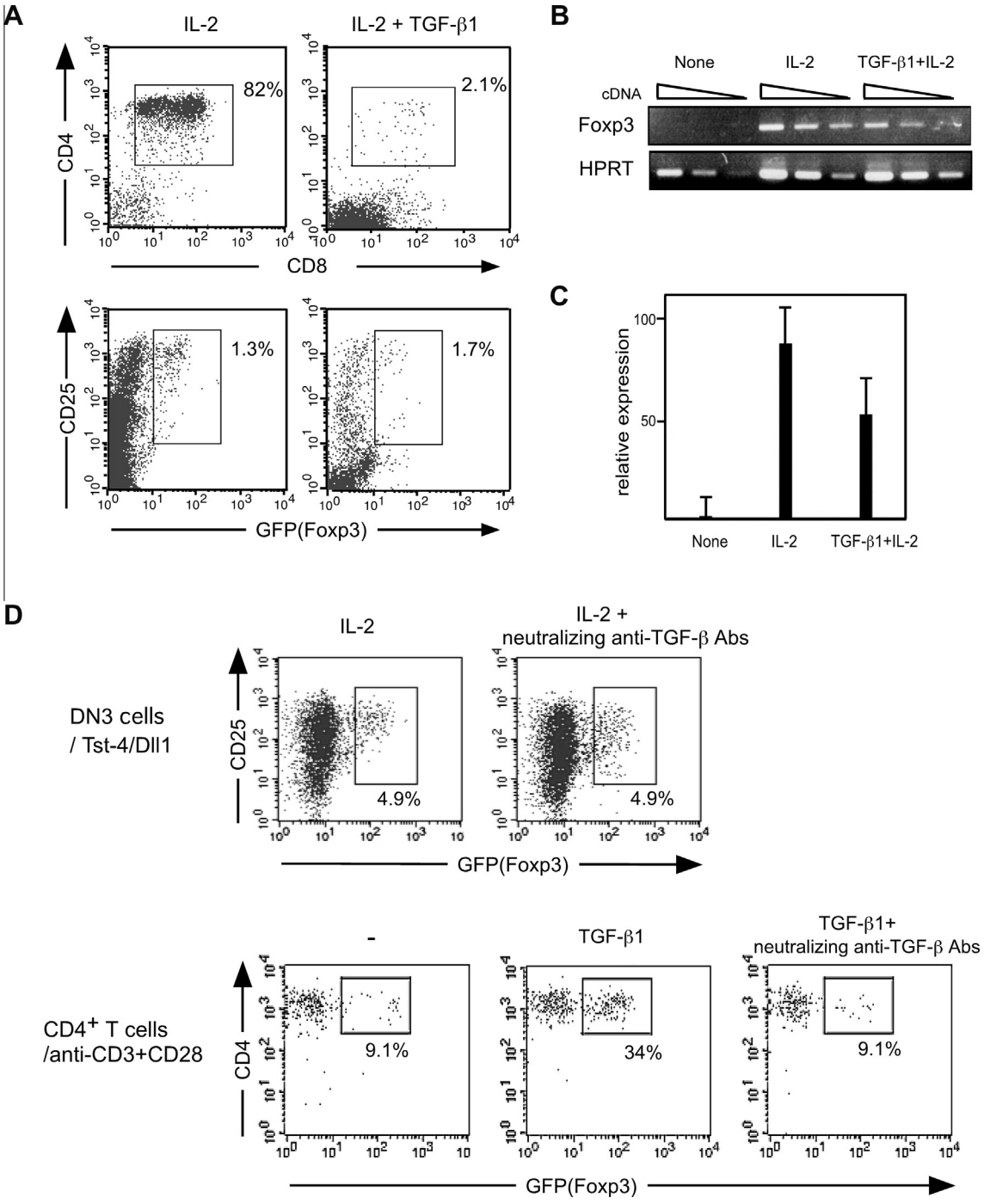

Fig. 2. IL-2 dependent expression of Foxp3 in DN3 thymocytes. (A) Sorted DN3 thymocytes were cultured on TSt-4/Dll1 with IL-2 alone or IL-2 plus TGF-β1. Differentiation of DN3 cells toward CD4/CD8 and Foxp3 expression were analyzed. (B) Cultured thymocytes on TSt-4/Dll1 with or without cytokines were harvested and Foxp3 mRNA expression was analyzed by RT-PCR. (C) Real-time RT-PCR was performed to quantify Foxp3 mRNA expression and relative expression levels normalized with β-actin are shown (mean ± SD). Data are representative of two experiments. (D) Sorted DN3 thymocytes were cultured on TSt-4/Dll1 with IL-2 in the presence or absence of neutralizing anti-TGF-β1 antibodies. As control experiments, sorted CD4 T cells were stimulated with anti-CD3 plus anti-CD28 in the presence or absence of TGF-β1 (3 ng/ml). The same amount of neutralizing antibodies was used to deplete TGF-β1. Data are representative of three experiments.

plied from the stromal cells, neutralizing anti-TGF-β antibodies were added to the culture. The efficiency of Foxp3$^+$ thymocyte development was not changed by TGF-β depletion, while the same amount of neutralizing antibodies abolished the TGF-β1-mediated induction of Foxp3$^+$ CD4 T cells (Fig. 2D). These results suggested that IL-2 signaling induces a subset of thymocytes within the DN3 population to upregulate Foxp3 expression when co-cultured with TSt-4/Dll-1.

3.2. *Foxp3$^+$ thymocytes induced by IL-2 express TCRγδ*

Foxp3$^+$ cells were induced from both DN2 (data not shown) and DN3 thymocytes, which contains precursor cells that can become T cells expressing either TCRαβ or TCRγδ. A large population of Foxp3$^+$ cells induced in this co-culture system expressed TCRγδ, and a small proportion of Foxp3$^+$ cells expressed a TCRβ chain (Fig. 3A). Involvement of TCRβ expression during the development

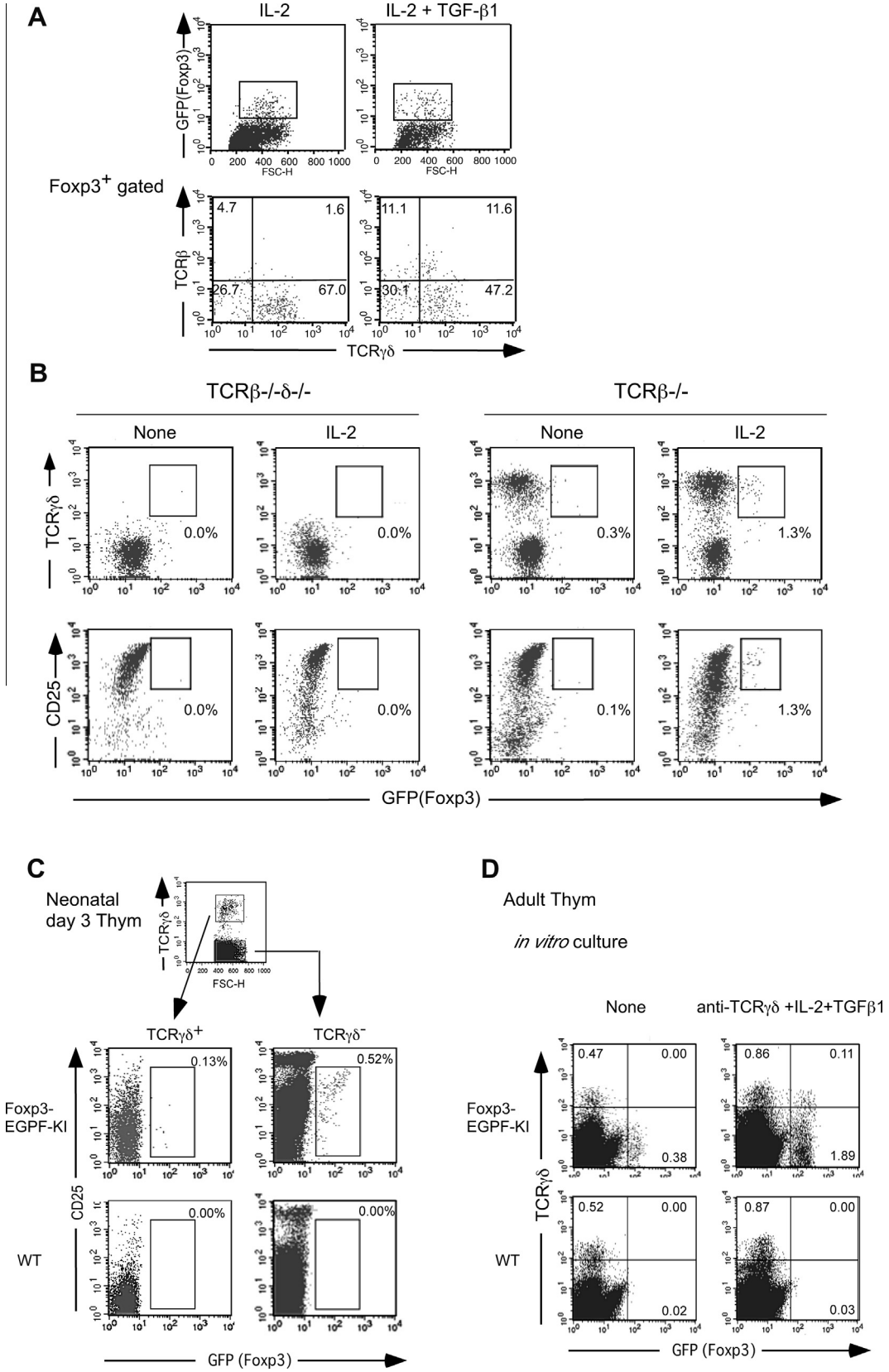

Fig. 3. FoxP3$^+$ cells differentiated from DN3 thymocytes express TCRγδ. (A) DN3 cells were cultured on TSt-4/Dll1 for 1 week in the presence of IL-2 or IL-2 + TGFβ1. Foxp3$^+$ cells were gated and the expression of TCRβ and TCRγδ were analyzed. Data shown are representative of three independent experiments. (B) DN3 thymocytes were sorted from either TCRβ$^{-/-}$δ$^{-/-}$ or TCRβ$^{-/-}$ mice. Cells were cultured on TSt-4/Dll1 in the presence or absence of IL-2. Data are representative of two experiments. (C) Thymocytes were prepared from neonatal day 3 thymuses. Foxp3 (GFP) expression in TCRγδ$^+$ and TCRγδ$^-$ populations from Foxp3-EGFP-KI or wild type (WT) mice was analyzed. Representative data of three experiments are shown. (D) Thymocytes from Foxp3-EGFP-KI or WT mice were cultured for 3 days with or without stimulation (plate-coated anti-TCRγδ + IL-2 + TGF-β1) and Foxp3 (GFP) expression was analyzed. Representative data of two experiments are shown.

of TCR γδ$^+$Foxp3$^+$ cells was tested by culturing DN3 cells isolated from either TCRβ$^{-/-}$δ$^{-/-}$ or TCRβ$^{-/-}$ mice. Foxp3$^+$ cells were not induced in DN thymocytes from TCRβ$^{-/-}$δ$^{-/-}$ mice, while DN3 cells from TCRβ$^{-/-}$ mice gave rise to Foxp3$^+$ thymocytes (Fig. 3B). These results indicated that Foxp3 expression was not dependent on TCRβ or pre-TCRα in conjunction with TCRβ. Furthermore, precursor thymocytes that were committed to the γδ T cell lineage differentiated into γδ T cells expressing Foxp3.

Although we induced Foxp3$^+$ thymocytes expressing TCR γδ *in vitro*, it is not clear whether there is a subset of γδ T cells that express Foxp3 *in vivo*. We analyzed various ages of mice and found Foxp3$^+$ cells in day 3 neonatal thymuses both in TCR γδ$^-$ and TCR γδ$^+$ thymocytes (Fig. 3C). The frequency of GFP$^+$ (Foxp3$^+$) cells in the TCR γδ$^+$ fraction was very low, however, the GFP signals were not artifact because no GFP positive cells were detected in wild-type thymocytes. Undetectable levels of Foxp3$^+$TCRγδ$^+$ cells were found in adult thymuses, however, around 10% of TCRγδ$^+$ thymocytes became Foxp3$^+$ upon stimulation with anti-TCRγδ antibodies in the presence of IL-2 and TGF-β1 (Fig. 3D). These results suggested that some γδ T cells were able to express Foxp3 in response to antigens. In the periphery, low numbers of TCRγδ$^+$ cells were Foxp3$^+$ in mesenteric lymph nodes, and Foxp3 expression was also inducible (Supplementary Fig. 1). Analysis of TCRγδ$^+$ cells from the spleen, intestine, skin, and peritoneal cavity did not detect any Foxp3 expression in γδ T cells.

3.3. IL-2 signals in DN3 thymocytes promotes γδT cell fate

Although DN2 and DN3 thymocytes express the IL-2 receptor α chain CD25, it is not clear whether downstream IL-2/IL-2R signals are activated in DN thymocytes. We analyzed whether phosphorylation of STAT5 was induced in DN3 thymocytes because its activation is required for Foxp3 expression in CD4$^+$ T cells [12]. Phosphorylation of STAT5 was analyzed by flow cytometry after incubating DN thymocytes in IL-2 for 1 h. IL-2 induced STAT5 activation albeit weaker than that of IL-7 (Fig. 4A).

It has been suggested that pre-TCR and γδTCR signaling pathways are differentially activated by extracellular signal-related kinase (ERK)-Egr-Id3, which plays a defining role during the αβ and γδ lineage choice [16,17]. In order to analyze whether IL-2 signals were linked to these pathways, sorted DN3 cells were stimulated for 16 h with either IL-2 or IL-7 and relative expression levels of Id3, Egr1, and Egr3 were analyzed. DN3 cells stimulated with IL-2 or IL-7 expressed similar levels of Id3. Egr1, which acts on thymocytes after β-selection [18], was induced by IL-2 but not by IL-7, while both IL-2 and IL-7 significantly induced the expression of Egr3 (Fig. 4B). It could be possible that higher potential to induce both Egr1 and Egr3 by IL-2 may have supported the differentiation of Foxp3$^+$ γδ T cells.

To determine whether there were additional differences between IL-2 and IL-7, we sorted DN3 thymocytes from TCRβ$^{-/-}$ mice and cultured them in the presence of IL-2 or IL-7 for 3 days. Both IL-2 and IL-7 supported the survival of sorted γδ precursor cells, whereas most of the cells cultured in the normal complete medium died within the 3-day culture period (data not shown). Three days of culture in IL-2 induced the differentiation from precursor cells into TCRγδ$^+$CD25$^-$ cells. In contrast, IL-7 stimulation induced the differentiation of about half of the precursor cells and the other half of the cells maintained an undifferentiated phenotype, characterized by expression of CD25 (Fig. 4C). These results suggested that IL-2 promoted γδ T cell fate in DN3 thymocytes. Foxp3 expression was not induced by IL-2 in DN3 cells that were cultured without the stromal cells, thus suggesting that Foxp3 induction started in uncommitted DN3 cells and required additional signals to differentiate toward T cell lineage.

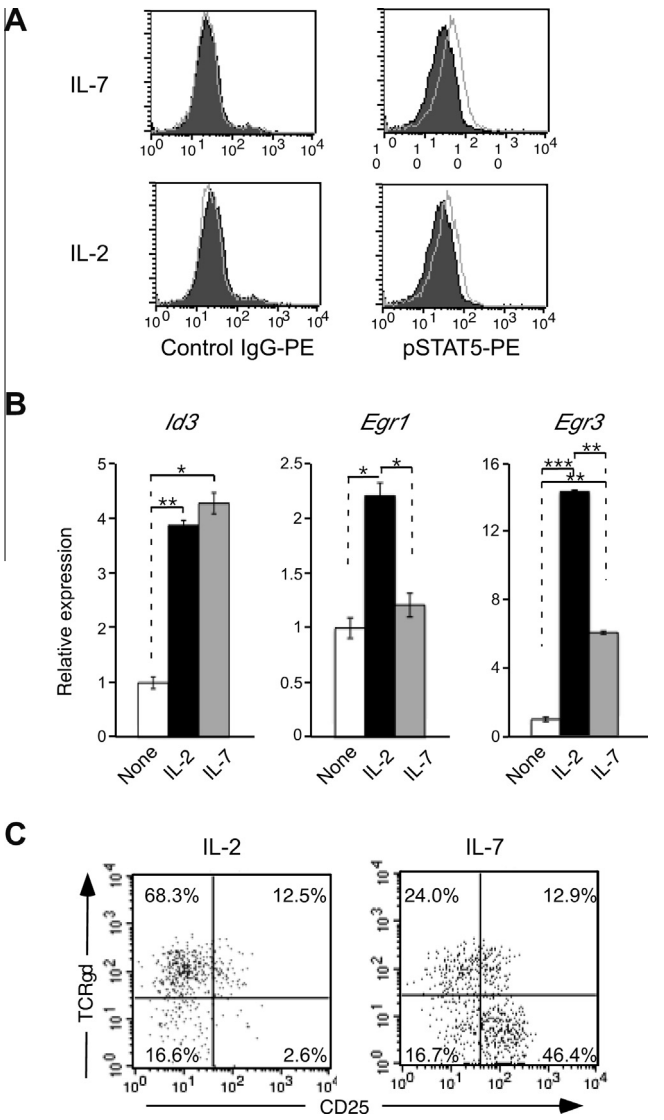

Fig. 4. Distinct downstream signals induced by IL-2 and IL-7 in DN3 thymocytes. (A) Double negative thymocytes were enriched by depleting cells CD4$^+$ and CD8$^+$ by I-MAG sorting, and cells were then stained with FITC conjugated anti-CD4, anti-CD8, anti-CD3, and anti-TCRγδ. Fixed cells were permeabilized and stained with PE-conjugated anti-phosphorylated STAT5 (pSTAT5) or isotype control antibodies (Control). Histograms of FITC negative cells stimulated with either 25 ng/ml IL-2 or 25 ng/ml IL-7 (gray lines) together with no treatment (black shadows) are shown. (B) DN3 thymocytes were sorted from wild type mice and stimulated with either 8 ng/ml IL-2 or 8 ng/ml IL-7 for 15 h. Total RNA was prepared from both stimulated and non-stimulated cells. Real-time RT-PCR analysis of mRNA for Id3, Egr1, and Egr3 was performed. Relative expression levels were normalized to β-actin. A Student's *t*-test was performed: $^*P < 0.05$, $^{**}P \leqslant 0.005$, $^{***}P < 0.001$. (C) DN3 cells were sorted from TCRβ$^{-/-}$ mice and cultured with 8 ng/ml IL-2 or 8 ng/ml IL-7 for 3 days. Differentiation of γδ T cells was analyzed. Results are representative of two experiments.

4. Discussion

In this study, we analyzed the differentiation of early thymocytes that give rise to both αβ and γδ T cells. Foxp3 expression was induced in DN thymocytes cultured on the stromal cell line expressing Dll1 in the presence of IL-2. These Foxp3$^+$ thymocytes were mostly TCRγδ$^+$, suggesting that γδ T cell precursors have a potential to differentiate into Foxp3$^+$ cells in the thymus.

In fetal thymuses, the development of γδ T cells is programmed by unique transcription factors, PLZF, T-bet, or RORγt, which drive the development of precursor γδ T cells into NKT-like γδ T cells,

Th1-like epidermal γδ T cells, or Th17-like γδ T cells, respectively [19]. These γδ T cells are segregated on the basis of distinct γδ TCR Vγ and/or Vδ usages, and they migrate to different tissues [20]. Similarly, Foxp3 is a transcription factor that defines the fate of Th cells into Tregs, however, the role of Foxp3 on the fate of γδ T cells remains undefined. Most γδ T cells do not require positive selection in the thymus for functional maturation, while development of $CD4^+$ T cells requires both positive and negative selection with self-peptide/MHC molecules. Foxp3 expression in $CD4^+$ thymocytes requires stronger interaction with self-peptide/MHC, which suggests that some signals that mimic TCR stimulation could be required for Foxp3 expression in γδ T cells.

Induction of Foxp3 in DN thymocytes was dependent on IL-2. IL-2 is an essential factor for $CD4^+Foxp3^+$ Treg development in the thymus and the periphery. Downstream signaling via the IL-2 receptor is mediated by STAT5 activation and STAT5 binds to the conserved non-coding regions of the Foxp3 gene, which contributes to Foxp3 gene expression [21]. Although IL-7 activated STAT5 in DN3 thymocytes, it did not induce Foxp3 expression, thus suggesting that factors other than STAT5 activation contributed to the expression of Foxp3. Stimulation of sorted DN3 cells with IL-2 or IL-7 for a short time induced Id3 expression, which is a transcription factor involved in γδ T cell development [22]. Both IL-2 and IL-7 induced Egr3 expression, while only IL-2 induced Egr1 in DN3 thymocytes. Since Egr1 and Egr3 are usually up-regulated by TCR stimulation [1], it is possible that *in vitro* stimulation of DN3 thymocytes with IL-2 but not IL7 could mimic γδTCR stimulation by activating downstream signaling molecules. Indeed, IL-2 had higher potential to drive DN3 cells into γδ T cells. Therefore, the enhanced induction of Egr1 and Egr3 gene expression by IL-2 may partly promote γδ T cell development and Foxp3 expression.

Although IL-2 was essential for $Foxp3^+$γδ T cell development, DN3 thymocytes in $RAG2^{-/-}$ mice did not give rise to $Foxp3^+$ thymocytes (data not shown). Similarly, a TSt-4 stromal cell line without Dll1 expression did not support the development of $Foxp3^+$ γδ T cells (data not shown). Taken together these results indicated that IL-2 signals in DN3 thymocytes were not sufficient and the thymocyte developmental program toward specific T cell lineages required additional factors. Despite the activation of similar signaling pathways, IL-2 but not IL-7 induced the expression of Foxp3 in DN3 cells. It is important to note that we cannot exclude the possibility that there were pre-committed thymocytes in DN3 cells that responded to IL-2 and differentiated into $Foxp3^+$ thymocytes.

Previous reports detected γδ T cells expressing Foxp3 in $TCR\beta^{-/-}$ and $preTCR\alpha^{-/-}$ mice. In normal mice, there is an abundance of $CD4^+CD8^+$ thymocytes that regulate γδ T cell progenitors to differentiate in trans, whereas lack of this trans conditioning leads to development of γδ T cells with Foxp3 expression [23]. Our experiments demonstrated that there are precursor cells in early DN thymocytes that have the potential to express Foxp3. These cells express Foxp3 in response to IL-2; however, Foxp3 expression may not be epigenetically stable as has been previously demonstrated in naturally occurring $CD4^+Foxp3^+$ cells [24]. It is also possible that the precursor cells maintain the ability to give rise to $Foxp3^+$ $TCR\alpha\beta^+$ thymocytes. In our *in vitro* culture system, we detected $Foxp3^+$ cells that expressed TCRβ, but these cells failed to differentiate into DP thymocytes. Our results suggested that DN3 cells that expressed Foxp3 were not in the normal differentiation pathway to become $\alpha\beta TCR^+$ thymocytes and CD4 expression in these cells was likely silenced.

$Foxp3^+$ γδ T cells were detectable *in vivo* at low frequencies in the neonatal thymus. Stimulation of adult thymocytes with anti-TCR γδ antibodies in the presence of IL-2 and TGF-β1 induced Foxp3 expression in γδ T cells. It has been shown that human peripheral $V\gamma2^+V\delta9^+\gamma\delta$ T cells express Foxp3 upon antigen stimulation in the presence of TGF-β1 and IL-15. These γδ T cells stably express Foxp3 and possess regulatory functions [25]. Foxp3 expression in the cells developed from DN3 thymocytes was also stable (data not shown). It could be possible that murine $Foxp3^+\gamma\delta$ T cells also exhibit suppressor functions, although it is not clear whether $Foxp3^+$ γδ T cells play physiological roles at this time. These γδ T cells generated after birth express a diverse repertoire of γδ TCRs and respond more like T cells in adaptive immunity [26]. In some autoimmune diseases, where γδ T cells producing IL-17 are involved [27], $Foxp3^+$ γδ T cells could play a regulatory role.

Conflict of interest

The authors have no financial conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.155.

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