

# Thymic Development of CD4<sup>+</sup>25<sup>hi</sup> Foxp3<sup>+</sup> T-Regulatory Cells Echoes their Suppressogenic Capacity in Periphery

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**Abstract:** The CD4<sup>+</sup>25<sup>hi</sup>Foxp3<sup>+</sup> T regulatory (T-reg) cells are naturally-born in thymus and they are critical for maintaining the tolerance to self and non-self antigens. Foxp3 is the master-regulatory gene of development and function of this cell subset. Using two mouse strains that share the same MHC class II (H-2<sup>d</sup>) haplotype, we found that Foxp3 is early expressed in the CD3<sup>+</sup>4<sup>+</sup>8<sup>+</sup>25<sup>+</sup>/44<sup>-</sup> (DN3/4) double negative thymocytes. Furthermore, Foxp3 showed a differential kinetics of expression in the thymus of these two strains that were controlled through the rates of proliferation and apoptosis of T-reg precursors, but not by epigenetic alterations at the Foxp3 gene promoter. Faster T-reg proliferation and lower apoptosis were associated with higher Foxp3<sup>+</sup> thymic cell output. Also, faster proliferating T-reg precursors showed lower expression of CD3/TCR complex, leptin receptor, Bad, and Caspase3 early in the DN stage of differentiation. Despite a differential T-reg thymic output in these two mouse strains, the size of peripheral CD4<sup>+</sup>25<sup>hi</sup>Foxp3<sup>+</sup> T-reg compartment was homeostatically normalized. However, the Foxp3<sup>+</sup>T-reg suppression tested in an autoimmune mouse model for diabetes was stronger in mice with a higher T-reg thymic output. These findings demonstrate a differential thymic development and suppressive capacity of Foxp3<sup>+</sup> T-reg cells in two genetic backgrounds regardless the MHC II haplotype. This raises the question of whether a differential suppressogenic capacity of the T-reg compartment may affect the susceptibility to autoimmune disorders in individuals from different ethnic groups.

**Keywords:** Foxp3, T regulatory cells, Thymic development, Suppressogenicity, Genetic background.

## INTRODUCTION

The “professional” CD4<sup>+</sup>25<sup>hi</sup> T regulatory cells (T-regs) represent a subset of T lymphocytes that differentiate in thymus as the “guardians” of immune system [1]. The T-regs control the immunoreactivity to *self* and foreign antigens, and at the same time maintain homeostasis of T-cell compartment in peripheral lymphoid organs [2-5]. Although the CD4<sup>+</sup>25<sup>hi</sup> T-regs represent only 5-10% of the entire CD4 T-cell population, subtle alterations in their frequency and function associate with higher incidence of autoimmune disorders [6-12].

The CD4<sup>+</sup>25<sup>hi</sup> T-reg cells express high levels of IL-2R $\alpha$  chain (CD25) on the cell surface [13]. They are anergic by themselves and do not secrete IL-2, but they require IL-2 for thymic development and expansion in the peripheral lymphoid organs [14-17]. Several other surface receptors expressed on Tregs like CTLA-4, OX40 (TNFRSF4), glucocorticoid induced TNF receptor super family member (GITR), TNFR2, 4-1BB, PD1, CD103 ( $\alpha$ E $\beta$ 7 integrin), Toll Like Receptors (TLR) and galectin-10, were described [18-22]. However, the Foxp3 gene encoding for the Scurfin (SFN) fork head-winged helix transcription factor is the most reliable marker for CD4<sup>+</sup>25<sup>hi</sup> T-reg cells [23-25]. A mutated SFN variant is responsible for alterations in

the T-reg compartment associated with X-linked autoimmune disorders in *scurfy* mice and IPEX/XLAAD syndrome (Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked) in humans [26].

In general, patients with autoimmune diseases have a reduced number of Foxp3<sup>+</sup> T-reg cells such as those with hepatitis C virus-associated mixed cryoglobulinemia [27], autoimmune liver disease [28], juvenile idiopathic arthritis [29, 30], systemic lupus erythematosus [23, 31], Kawasaki disease [32], and psoriatic arthritis [30]. It is believed that a low frequency of circulating T-reg cells in these patients is the result of their impaired clonal expansion in the peripheral lymphoid organs [28, 33]. In contrast, no significant reduction in the peripheral, circulating T-reg cells was observed in patients with autoimmune diseases like multiple sclerosis [33, 34], autoimmune diabetes [35, 36], and spondyloarthritis [30]. Even more contrasting, patients with Sjogren syndrome showed an increased number of T-reg cells [37], and those with rheumatoid arthritis [38-42] and myasthenia gravis [38, 39] showed either reduced or unaltered number of T-reg cells. However, little is known about the function and size of T-reg compartment in autoimmune diseases in relation to Foxp3 expression and the genetic background.

This study provides evidence that two mouse strains with different genetic backgrounds (BALB/c and B10.D2) and sharing the same MHC class II haplotype (H-2<sup>d</sup>) have different kinetics of CD4<sup>+</sup>25<sup>hi</sup>Foxp3<sup>+</sup> T-reg thymic development resulting in a different Foxp3<sup>+</sup> thymic output and suppress-

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sogenic function in periphery. The rates of T-reg proliferation and apoptosis in thymus were found as a major mechanism leading to the differential Foxp3<sup>+</sup> thymic output in these two mouse strains. Despite a differential Foxp3<sup>+</sup> T-reg thymic output in these strains, the size of peripheral CD4<sup>+</sup> Foxp3<sup>+</sup> T-reg compartment was homeostatically normalized, although the threshold of suppressogenicity was not. Higher suppressogenicity of T-reg cells was strongly correlated with higher amount of Foxp3 protein at the single-cell level.

## MATERIALS AND METHODOLOGY

### Mice

One-month-old, female BALB/c and B10.D2 (H-2<sup>d</sup>) mice were purchased from Jackson Laboratories. The TCR-HA and RAG2 KO, RIP-HA transgenic (Tg) mice used in the adoptive cell co-transfer experiments are currently maintained in our facilities. Mice were housed at USUHS in a pathogen-free facility according to federal and local regulations.

### Cell Purification

The CD4<sup>+</sup> thymocytes and splenocytes from pools of mice (n = 4-6) were negatively sorted on mouse CD4 enrichment columns (R & D Systems, Minneapolis, MN) according to the manufacturer's protocol. A second passage of CD4<sup>+</sup> cells on mouse CD8 enrichment columns (R & D Systems) yielded negatively-sorted CD4<sup>+</sup> CD8<sup>-</sup> DN thymocytes. The purity and viability of thymic populations was more than 90% as determined by FACS and trypan blue exclusion method. The CD4<sup>+</sup> CD25<sup>+</sup> thymocytes and splenocytes were isolated on MS MACS paramagnetic columns (Miltenyi Biotec Inc., Auburn, CA). Briefly, the negatively-sorted CD4<sup>+</sup> CD8<sup>-</sup> single positive (SP4) or CD4<sup>+</sup> CD8<sup>-</sup> double negative (DN) cells were incubated with anti-CD25-PE Ab-dye conjugate for 10 min, washed, and further incubated anti-PE Ab-microbeads for 15 min. The cells were sorted on magnetic columns (Miltenyi Biotec) into a CD25<sup>-</sup> fraction (cell flow through) and a CD25<sup>+</sup> fraction (T-regs, column bound cells). In some experiments, the DN thymocytes were stained with anti-CD44 and CD25 Ab-dye conjugates and sorted by a FACS Aria instrument/FACSDiva software (Becton Dickinson) into DN1-4 or CD4<sup>+</sup> CD25<sup>hi</sup> subpopulations at 97-98% purity.

### RT-PCR and Quantitative Real-Time RT-PCR

Total RNA and cDNA from CD25<sup>+</sup> and CD25<sup>-</sup> thymocytes was prepared using NucleoSpin RNA II kit (BD Biosciences Clontech, Palo Alto, CA) and Qiagen One Step RT-PCR kit (Qiagen Inc., Valencia, CA) respectively, as previously described [43]. The primers for murine Foxp3 were: (forward 5'CAGTGCCTACAGTGCCCTAG3', and reverse 5'CATTGGCCAGCA GTGGGTAG3' [23], Fas (forward CGCTGTTTTCCCTTGCTGCAG, reverse ACAGG TTG GTGTACCCCAT) [44], Fas-L (forward CACTGAC CCCTCTAAAGAAGAA, reverse TTGAATACTGCCCC AGGTA) [45]. The specific primers for Bcl-2, Bcl-X<sub>L</sub>, Bad, Bax, and Caspase 3 were previously described [43]. Four nanograms of total RNA were used to prepare first-strand cDNA using a Qiagen One Step RT-PCR kit (Qiagen, Valencia, CA) at 35 and 40 cycles of amplification following the manufacturer's protocol. 25µL of each PCR product was then electrophoresed in 1.5% agarose gel containing ethid-

ium bromide, and the amount of each amplicon was calculated using the Scion Image software analysis (Scion Corp., Frederick, MD) based on the integration of the percent of pixels and band intensity after normalization against the corresponding β-actin band [43]. To measure the expression level of Foxp3 by quantitative real-time RT-PCR, the RNA was extracted from various thymic populations using Total RNA Purification System (Invitrogen). Some 500 ng RNA was used to synthesize the first cDNA strand using the High Capacity cDNA Archive kit (Applied Biosystems). PCR amplification mixtures containing the Applied Biosystems Universal PCR MasterMix Buffer (25 µl), cDNA template (22.5µl), Foxp3 and 18S rRNA primers (2.5 µl), respectively. Quantitative RT-PCR measurements were performed on an ABI Prism 7700 with SDS 1.9.1 Software (Applied Biosystems). The melting-curve analysis has confirmed the specificity of PCR amplifications. The relative mRNA levels were estimated by the standard method using 18S rRNA as reference.

### Cytofluorimetry

Thymocytes or splenocytes (2x10<sup>5</sup>) were surface, intracellularly, or intra-nuclearly stained with various antibody-dye conjugates, their isotype controls, or with Annexin V-PE conjugate according to the manufacturer's instructions (BD PharMingen, San Diego, CA). Between 200,000 and 500,000 total cell events were acquired by a LSR II Becton-Dickinson instrument for each cell preparation. For accurate measurements of mean fluorescence intensity (MFI) at the single-cell level, the slight difference in the cell size and signal-to-noise cell autofluorescence were compensated with WINlist analysis software 3D5.0 (Verity Software House, Topsham, ME) during the data acquisition. Measured parameters were compared under various experimental conditions using the Sigmaplot analysis software v.8 (Aldrich-Sigma). Overall data were presented for convenience as mean ± SD of pooled samples.

### In Vivo Cell Cycle Division

Mice were injected intravenously (i.v.) with 1 mg of carboxyfluorescein succinimidyl ester (CFSE) in saline, and then one, four, and six days later some 1 x 10<sup>6</sup> gated CD4<sup>+</sup> CD25<sup>hi</sup> T-reg cells isolated from the thymus and spleen were harvested. Data on cell cycle division were acquired using a LSR II instrument (BD Biosciences). The pattern and number of cell cycle divisions based on the CFSE dilution factor were analyzed with the WINlist software 3D 5.0.

### Caspase 3 Activity

Some 25 µg of protein extract from DN thymocytes isolated as described were incubated for 40 min in 96-well plates in 100 µl buffer (100 mM HEPES, 10% sucrose, 0.15% CHAPS, 10 mM DTT, 0.1 mg/ml ovalbumin, pH 7.4) supplemented with 1 µM of substrate (Ac-DEVD-AMC, Peptide Intern., Louisville, KY) at 37°C. The amount of substrate released was measured in a spectrofluorimeter (excitation λ = 360 nm and emission λ = 436 nm) equipped with an XFluor analysis software (Tecan US, Durham, NC). Standard dilutions of the substrate were used in each assay to measure the absolute concentration of released AMC, and the measured fluorescence units were converted into rate of AMC cleavage per mg protein (corresponding to pmole

cleaved DEVD-AMC ( $\text{x min}^{-1} \times \text{mg}^{-1}$ ). Values were compensated for the signal-to-noise background (spontaneous release of AMC in the absence of cell extract)

### Adoptive Cell Transfers

The RAG2 KO, RIP-HA Tg mice (BALB/c x B10D2, H-2<sup>d</sup> background) expressing the hemagglutinin protein (HA) of influenza PR8 virus in the pancreatic  $\beta$ -cells under the rat insulin promoter [10] were used as recipients of splenic CD4<sup>+</sup>25<sup>hi</sup> Foxp3<sup>+</sup> T-reg cells ( $10^5$  cells) isolated from BALB/c or B10.D2 mice, and/or diabetogenic HA-specific CD4<sup>+</sup>25<sup>-</sup> Th cells ( $2.5 \times 10^5$  cells) isolated from BALB/c mice expressing the HA-TCR transgene (TCT-HA Tg) that recognizes the HA110-120 immunodominant CD4 T-cell epitope of HA of PR8 influenza virus in context of MHC class II H-2<sup>d</sup> haplotype [46]. The TCR-HA Tg mouse expresses the TCR-HA transgene on 35-40% splenic CD4 T-cells. The i.p. infusion of diabetogenic CD4<sup>+</sup>25<sup>-</sup> T-cells from TCR-HA Tg mice ( $2.5 \times 10^5$  cells) in RAG2 KO, RIP-HA Tg mice induces a rampant hyperglycemia followed by death within 12 to 14 days. The T-reg cells were infused i.p. 7 days prior infusion of diabetogenic TCR-HA T-cells.

### Diabetes Monitoring

Glycemia in the RAG2 KO, RIP-HA Tg recipients of T-regs and diabetogenic T-cells was monitored by-weekly starting 3 days post transfer of diabetogenic T-cells by using Accu-Check Advantage glucose strips (Boehringer Mannheim, Indianapolis, IN). Mice were considered diabetic when glycemia was two-times consecutively over 200 mg/dl. One week after the transfer of diabetogenic T-cells, 2 pancreases were collected from each group of mice and analyzed histochemically for the presence of lymphocyte infiltration of the pancreatic  $\beta$ -islets using hematoxylin-eosin (HE) staining of paraffin-embedded pancreatic sections. Survival in the RAG2 KO, RIP-HA Tg recipients was also monitored on a daily basis.

### DNA Methylation Assay

Genomic DNA was isolated from purified thymocytes of 1 month-old mice BALB/c and B10.D2 mice using the DNeasy tissue kit (Qiagen, Valencia, California, United States) and following the supplier's recommendations. Sodium bisulphite treatment of genomic DNA was performed as previously described [47] with minor modifications aimed at deamination of unmethylated cytosines to uracil, whereas the methylated cytosines remained unchanged. In a subsequent PCR amplification, uracils were replicated as thymidines. Thus, detection of a "C-element" in sequencing reactions indicated methylation of the genomic DNA. Detection of a "T-element" at the same site indicated instead the absence of a methyl modification of the genomic cytosine. PCRs were performed in a final volume of 25  $\mu$ l containing 13  $\mu$ l PCR buffer, 1-U Taq DNA polymerase (Qiagen), 200 IM dNTPs, 12.5 pmol each of the forward and reverse primers, and 7 ng of bisulphate treated genomic DNA. The amplification conditions were 95<sup>o</sup> C for 15 min and 40 cycles at 95<sup>o</sup> C for 1 min, 55<sup>o</sup> C for 45 sec, and 72<sup>o</sup> C for 1 min with a final extension step of 10 min at 72<sup>o</sup> C. PCR products were purified using Qiagen MinElute gel extraction kit, sequenced in both directions applying the PCR primers and the ABI Big Dye Terminator v1.1 cycle sequencing chemistry (Applied

Biosystems, Foster City, California, United States), and measured by capillary electrophoresis on an ABI 3100 genetic analyzer. For each sample, both the PCR amplification and sequencing analysis were repeated twice. The following primers specific for the murine Foxp3 gene promoter (5' to 3' direction) were used for both PCR amplification of bisulphite converted genomic DNA and sequence reactions: Amp 1 (forward: AGGAAGAGAAGGGGGTAGATA; reverse: AAATAACATTCCAAAACCAAC), Amp 2 (forward: ATTTGAATTGGATATG GTTTGT; reverse: AACCTTAACCCCTCTAACATC).

### Statistic Analysis

Significance of survival of RAG2 KO, RIP-HA Tg recipients of T-regs and diabetogenic T-cells was determined along with that of control groups by Kaplan-Meier test. The significance of individual differences in MFI measured by FACS, and percentage of cell populations in different groups of mice analyzed by FACS was determined by the Student's *t*-test.

## RESULTS

### The DN Stage of Thymic Differentiation is an Early Time-Point of Foxp3 Expression

Studies on the murine thymic development showed that at day 12 of gestation the bone marrow-derived T cell precursors migrate to the thymic cortico-medullary junction as triple negative CD3<sup>-</sup>4<sup>-</sup>8<sup>-</sup> cells [48, 49]. They then differentiate into double negative DN1 (CD4<sup>+</sup>8<sup>+</sup>44<sup>+</sup>25<sup>-</sup>) and DN2 (CD4<sup>+</sup>8<sup>+</sup>44<sup>+</sup>25<sup>+</sup>) stages and enter the subcapsular cortex to differentiate into DN3 (CD4<sup>+</sup>8<sup>+</sup>44<sup>+</sup>25<sup>+</sup>) cells and DN4 (CD4<sup>+</sup>8<sup>+</sup>44<sup>+</sup>25<sup>-</sup>) cells. The DN4 cells migrate further to the cortex to proliferate and differentiate into double positive DP (CD4<sup>+</sup>8<sup>+</sup>) cells expressing a mature  $\alpha/\beta$  heterodimer TCR on the surface. The majority of DP cells undergo a negative selection by apoptosis-induced cell death leading to elimination of *self*-reactive precursors. Few DP cells undergo positive selection to generate single positive (SP) T cells (CD4<sup>+</sup>8<sup>+</sup> or CD4<sup>+</sup>8<sup>-</sup>) with low or no specificity for *self*-antigens [50] that migrate to periphery.

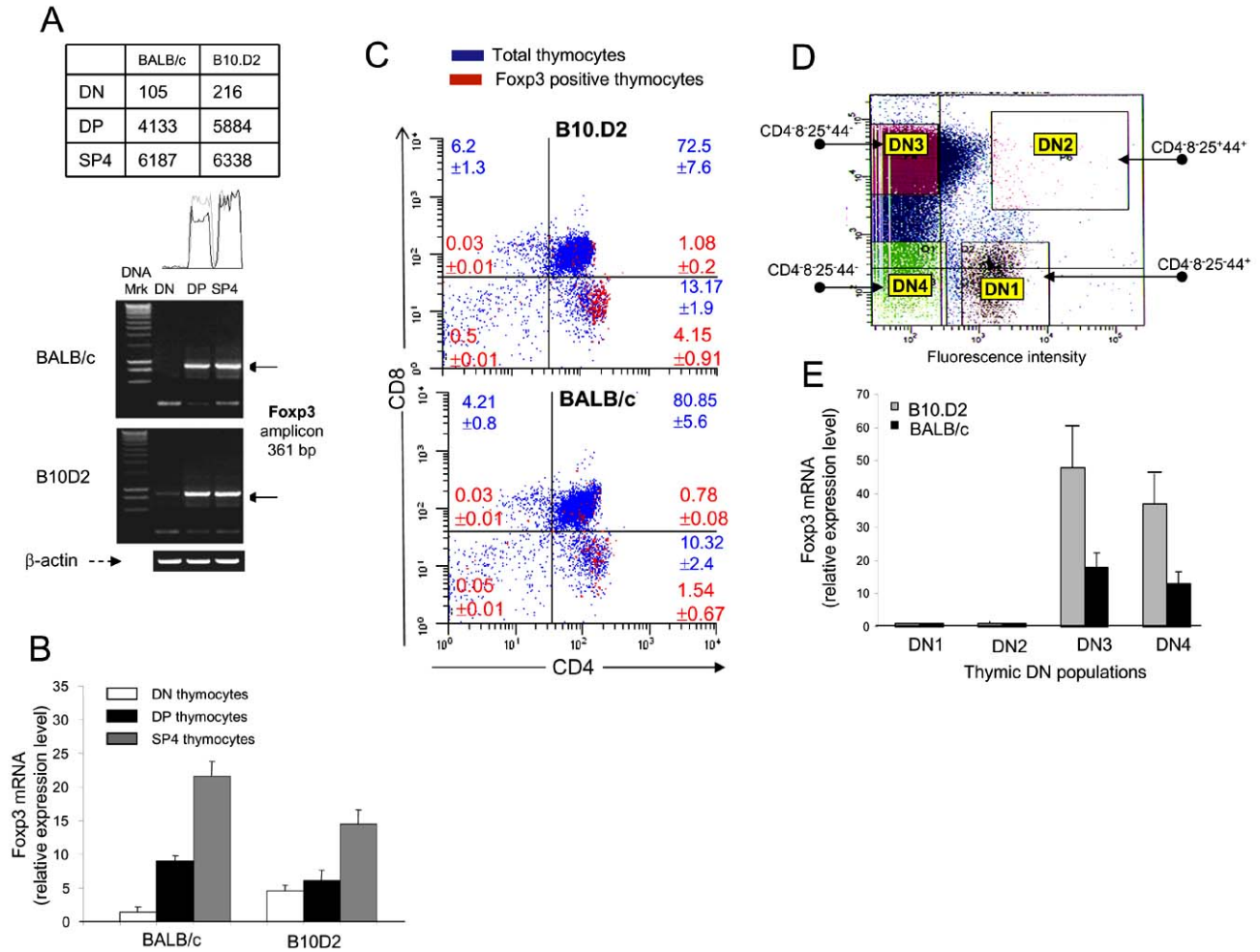
Foxp3 transcripts were previously detected by RT-PCR in CD4<sup>+</sup>8<sup>+</sup> double positive (DP) and CD4<sup>+</sup>8<sup>-</sup> single positive (SP4) thymocytes of BALB/c mice [23]. Herein, we have analyzed the kinetics of Foxp3 mRNA and protein in T-reg precursors from BALB/c and B10.D2 mice during different stages of thymic differentiation. The Foxp3<sup>+</sup> thymocytes are thereafter referred as T-reg precursors.

The B10.D2 inbred mouse strain on a C57BL/6 background shares the same MHC class II haplotype (H-2<sup>d</sup>) with the BALB/c mouse. In both strains, Foxp3 mRNA was barely detected by RT-PCR in DN thymocytes (Fig. 1A), but they were clearly detected by real-time RT-PCR (Fig. 1B). The level of Foxp3 mRNA in DN thymocytes from B10.D2 was higher than that in BALB/c mice and this was paralleled by an increased Foxp3 intranuclear expression as measured by single-cell level FACS (Fig. 1C). We next measured the kinetics of Foxp3 mRNA expression among the FACS-sorted DN1, DN2, DN3 and DN4 thymic populations (Fig. 1D), and by quantitative real-time RT-PCR. The Foxp3 transcripts were detected in the DN3 and DN4 thymocytes in

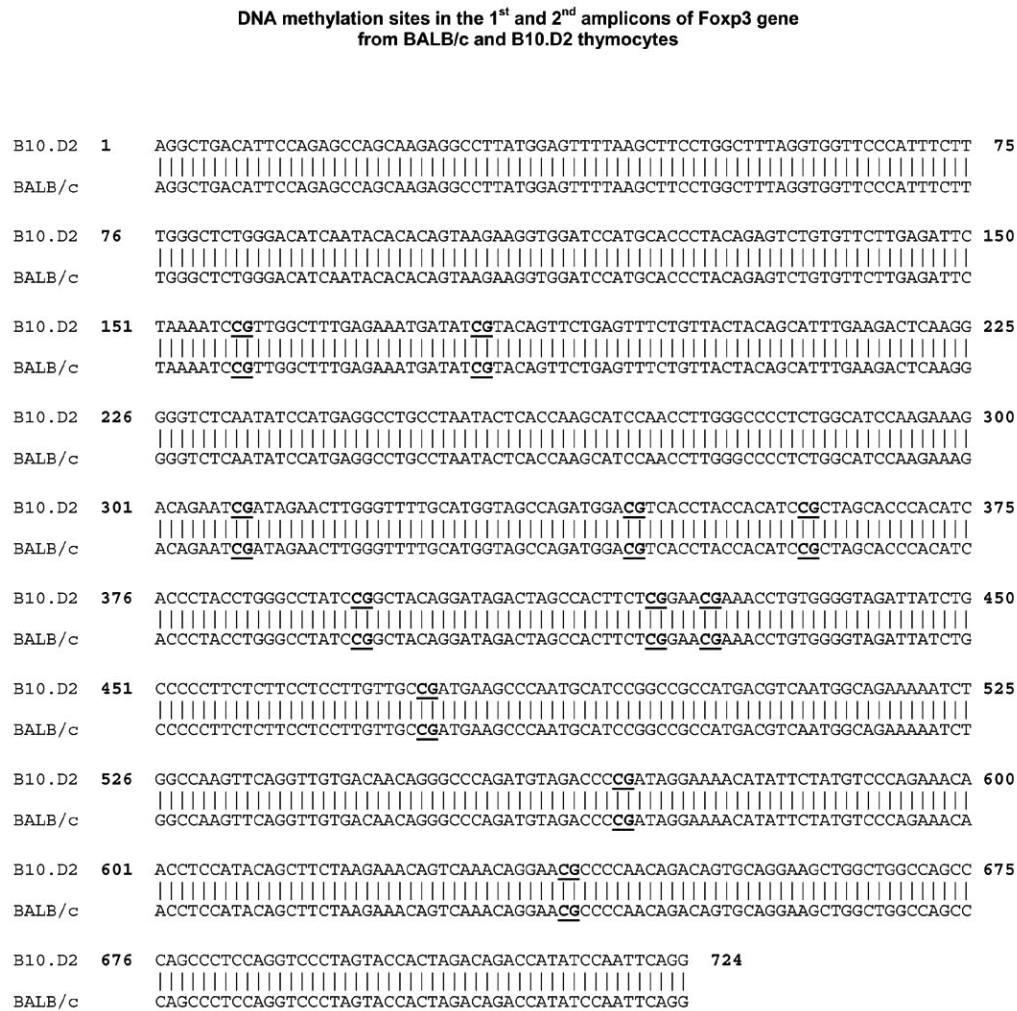
both strains, but not in the DN1 and DN2 thymocytes (Fig. 1E).

A differential Foxp3 expression in the BALB/c and B10.D2 thymocytes raised the question of whether genetic or post-translational events may control the Foxp3 expression in thymus. The 1<sup>st</sup> and 2<sup>nd</sup> amplicon of Foxp3 promoter located upstream of exon 1 is the most susceptible region for DNA methylation [51]. Our bisulfite-mediated DNA methylation assay in the CD4<sup>+</sup>25<sup>hi</sup> T-reg precursors ruled out the possibility of a differential DNA methylation in the Foxp3 gene promoter in T-reg precursors as a possible mechanism responsible for variations in the thymic expression of Foxp3 gene in BALB/c and B10.D2 thymocytes. That is because this region showed 100% homology in T-reg precursors, and an equal number of CpG potential sites of methylation (15 sites) in the 1<sup>st</sup> and 2<sup>nd</sup> amplicon of Foxp3 promoter in both strains (Fig. 2).

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**Fig. (1). Kinetics of Foxp3 expression in T-reg precursors from BALB/c (H-2<sup>d</sup>) and B10.D2 (H-2<sup>d</sup>) mice. (A)** CD4<sup>+</sup>8<sup>-</sup> (DN), CD4<sup>+</sup>8<sup>+</sup> (DP), and CD4<sup>+</sup>8<sup>+</sup> (SP4) thymocytes analyzed by RT-PCR for Foxp3 mRNA expression. Shown are the Foxp3 transcripts at 40 cycles of PCR amplification for a representative BALB/c and B10.D2 mouse. Similar results were obtained in other five mice. Foxp3 transcripts could not be detected in DN thymocytes at 35 cycles of PCR amplification (not shown). Upper panel indicates the absolute values of Foxp3 amplicon in each thymic population from the two strains as measured by ScionImage analysis software. **(B)** Quantitative measurements of Foxp3 transcript in thymic populations as determined by quantitative RT-PCR. The relative expression level of Foxp3 mRNA (Y-axis) measured in DN, DP, and SP4 thymocytes from BALB/c and B10.D2 mice was calculated in relation to the base level of Foxp3 mRNA as found in BALB/c DN thymocytes. Shown are the mean values ± SD from six individual mice in each group. **(C)** Distribution of Foxp3<sup>+</sup>T-reg precursors in thymus. Thymocytes from three individual BALB/c and B10.D2 mice were cell surface stained with CD4-PE-Cy7 and CD8-PerCP, and intranuclear with Foxp3-PE Ab-conjugates. Overlapped forward scatters in each quadrant indicate the percent ±SD of total DN, DP, SP4 and SP8 thymocytes (blue color) vs Foxp3-expressing thymocytes (red color) within the DN, DP, SP4 and SP8 thymic populations. The signal-to-noise background fluorescence of a rat IgG isotype PE conjugate control for Foxp3 was subtracted. Some 850,000 total cell events were acquired by a LSR II instrument and analyzed by WINList software. **(D)** FACS-sorting of DN1-4 thymic subpopulations. Gated DN thymocytes from BALB/c or B10.D2 mice were stained with CD4 and CD8 PerCP-Ab conjugates, and sorted based on CD44-FITC and CD25-APC Ab staining. **(E)** Measurements of Foxp3 transcript in DN1-4 thymocytes by quantitative real-time PCR. Shown are the mean values ± SD for Foxp3 mRNA as extracted from FACS-sorted DN1-4 thymocytes of three BALB/c and B10.D2 mice.



**Fig. (2). Frequency of CpG motifs in the 1<sup>st</sup> and 2<sup>nd</sup> amplicon of Foxp3 gene promoter in T-reg precursors from BALB/c and B10.D2 T-reg precursors.** Individual CpG motifs are marked as bold/underlined base pairs, and the alignment of BALB/c and B10D2 genomic sequences corresponding to the overlapping amplicons #1 and #2 in the Foxp3 gene promoter in T-reg precursors are shown.

These results demonstrated for the first time that the earliest time-point of Foxp3 expression in thymus is the DN3/4 stage of differentiation. A differential Foxp3 expression and Foxp3 thymic output in BALB/c and B10.D2 mice sharing the same H-2<sup>d</sup> (MHC class II) haplotype suggests that other genes that MHC class II genes may control the Foxp3<sup>+</sup> thymic output.

### The T-Reg Precursors have Higher Proliferation Rate in the B10.D2 Mice than in BALB/C Mice

Since the BALB/c and B10.D2 mice showed a differential Foxp3 thymic output, we first compared the *in vivo* proliferation rates of T-reg precursors during the thymic differentiation by measuring the CFSE dilution factor in relation to the number of cell cycle divisions. The BALB/c and B10.D2 thymocytes uptake equal amounts of CFSE per cell *in vivo* as determined 30 min post injection (Fig. 3A, two upper panels, grey peaks). Since the Foxp3 intranuclear staining quenches the CFSE fluorescence, herein we identified T-reg precursors based on their CD25<sup>hi</sup> expression, a reliable biomarker of CD4<sup>+</sup> Foxp3<sup>+</sup> T-regs. More than 95% of CD4<sup>+</sup>Foxp3<sup>+</sup> cells express the CD25<sup>high</sup> marker. The

BALB/c DP (CD25<sup>hi</sup>) and SP4 (CD25<sup>hi</sup>) T-reg precursors exhibited a lower rate of thymic proliferation (4-5 cycles of division on day 4, and 5-6 cycles of division on day 6 post-CFSE-labeling) as compared with their B10.D2 counterparts (5-6 cycles on day 4, and 7-8 cycles on day 6 post-CFSE-labeling) (Fig. 3A, two upper panels). This clearly indicated a differential rate of proliferation of BALB/c and B10.D2 T-reg precursors during their thymic development.

Leptin is a hormone that binds the Obese Receptor (ObR) and regulates cholesterol synthesis. Since we have previously found a differential partitioning of plasma membrane lipid rafts on the thymic T-cell precursors [52], and the leptin hormone was shown to negatively regulate the proliferation of human T-cells [21], we thought to investigate whether the levels of serum leptin and the expression level its receptor (ObR) on T-reg precursors may influence the rate of T-reg proliferation in the thymus of these two mouse strains. The serum leptin concentration measured by ELISA was similar in B10.D2 mice (4.8 ± 0.6 µg/ml) and BALB/c mice (4.5 ± 0.4 µg/ml) (n = 7 mice per group), which ruled out the possibility that the proliferation rates of T-reg precursors is











### Variations in the T-Reg Thymic Output are Homeostatically Normalized in Periphery

The differential T-reg thymic output between the BALB/c and B10.D2 mice raised the question of whether the size of mature CD4<sup>+</sup>25<sup>hi</sup> Foxp3<sup>+</sup> T-reg pool in the peripheral lymphoid organs is affected. While the frequency of terminally differentiated SP4 (CD4<sup>+</sup>8<sup>+</sup>25<sup>hi</sup> Foxp3<sup>+</sup>) T-reg precursors was 3-times lower in BALB/c than in B10.D2 mice ( $1.54 \pm 0.67\%$  vs.  $4.15 \pm 0.91\%$ ), the frequency of mature CD4<sup>+</sup>25<sup>hi</sup> Foxp3<sup>+</sup> T-reg cells in the spleen of both strains was in a close range ( $6.8 \pm 1.4\%$  and  $7.5 \pm 1.2\%$  respectively). Furthermore, the analysis of cell cycle divisions of splenic, mature CD4<sup>+</sup>25<sup>hi</sup> Foxp3<sup>+</sup> T-reg cells from CFSE-labeled mice showed different proliferation rates *in vivo* in these strains. Splenic T-reg cells from both strains went to 4-5 cycles of homeostatic division within a 4-day interval, and to 7 cycles at the end of day 6 post-CFSE injection, but a significant number of splenic B10.D2 T-reg cells (32-35%) were slow-dividing (2-3 cycles of division within 6 days) (Fig. 7A). Noteworthy, all mice investigated in this study were housed in a pathogen-free barrier, which rules out the possibility of opportunistic infections that may alter the T-cell homeostatic proliferation.

Since we found a strong correlation between the ObR partitioning on T-reg precursors and their rate of proliferation in BALB/c and B10.D2 mice, we next analyzed the ObR partitioning on splenic, mature CD4<sup>+</sup>25<sup>hi</sup> Foxp3<sup>+</sup> T-reg cells. The patterns of ObR partitioning on splenic T-reg cells was similar to that on SP4 T-reg thymic precursors, since two major T-reg populations were detected, i.e., ObR<sup>lo</sup> and ObR<sup>hi</sup> T-reg cells (Fig. 7B). However, in contrast to the pattern observed in the thymus, the B10.D2 mice showed a higher number of slow-dividing (ObR<sup>hi</sup>) splenic T-reg cells than the BALB/c mice (53% vs. 33%). This was consistent with the CFSE results showing a larger population of slow-dividing splenic T-reg cells in B10.D2 mice than in BALB/c mice (Fig. 7A). Furthermore, *in vitro* stimulation of splenic ObR<sup>hi</sup> T-reg cells with leptin in the presence of CD3/CD28 Abs significantly inhibited the Cdk4 mRNA expression in both strains, indicating again that signaling of ObR leads to inhibition of T-reg proliferation (Fig. 7C). This may well explain why the size of peripheral T-reg pool in B10.D2 mice having a higher T-reg thymic output was quiet similar to that of BALB/c mice having a lower T-reg thymic output. It thus appears that a switch in the ObR cell density occurs in periphery in the T-reg cells in these two strains that may compensate for the difference in their Foxp3 T-reg thymic output. This is consistent with previous reports showing that the requirements for T-reg clonal expansion in thymus are different than those in peripheral lymphoid organs [53].

Interesting enough, the overall ObR expression on (CD25<sup>hi</sup>) T-reg thymic precursors was not only higher than that on conventional (CD4<sup>+</sup>25<sup>-</sup>) T-cell thymic precursors (Figs. 7B vs. 7D), but also showed a different pattern of partitioning. While the splenic T-reg cells showed two major populations (ObR<sup>lo</sup> and ObR<sup>hi</sup>), the conventional CD4<sup>+</sup> T-cells showed a predominant ObR<sup>lo</sup> population.

Together, these results showed that the number of thymic T-reg immigrants does not affect significantly the size of peripheral T-reg compartment in these two mouse strains due

to a homeostatic normalization. The ObR partitioning on T-reg cells appears to provide a negative signal for proliferation of T-reg precursors in thymus, and can also control their peripheral homeostasis. The cellular and molecular events leading to ObR re-partitioning in mature T-reg cells requires further investigations.

### Differential T-Reg Development in BALB/C and B10.D2 Mice Echoes Their Suppressogenic Capacity in Periphery

Since a differential Foxp3 expression and CD4<sup>+</sup>25<sup>hi</sup> T-reg thymic output did not affect the size of peripheral T-reg compartment in BALB/c and B10.D2 strains, we next investigated the function of mature, splenic T-reg cells in these strains. It has been suggested that the suppressogenic capacity of T-reg cells is much related to the amount of Foxp3 protein per cell. The *in vitro* suppressogenic capacity of splenic CD4<sup>+</sup>25<sup>hi</sup> T-reg cells was tested using a transcriptional approach relying on the mRNA expression level for Th1 and Th2 transcription factors in syngeneic CD4<sup>+</sup>25<sup>-</sup> T helper responder cells in parallel with their ability to secrete cytokines upon CD3/CD28 stimulation. At the 1:1 ratio of T-reg cells to Th responders, the B10D2 T-reg cells inhibited almost 2-times stronger the IL-2 and IFN- $\gamma$  secretion by Th responders than the BALB/c T-reg cells (Fig. 8A, upper panel). Consistently, the Th1 and Th2 transcriptome expression (T-bet, STAT4, NFATc, STAT6, GATA3, and cMAF) was lower upon B10.D2 T-reg-induced suppression (Fig. 8A, lower panel). These two assays indicated that the capacity of B10.D2 T-reg cells to suppress a Th response *in vitro* is stronger than that of BALB/c T-reg cells.

To compare the *in vivo* suppressogenicity of splenic CD4<sup>+</sup>25<sup>hi</sup> T-reg cells from BALB/c and B10.D2 mice, we took advantage of an experimental autoimmune model for type 1 diabetes (T1D). In this system, RAG2 KO, RIP-HA Tg mice (H-2<sup>d</sup>) expressing the influenza HA viral antigen in the pancreatic  $\beta$ -islets under the rat insulin promoter develop a rampant hyperglycemia (some 10-12 days) after infusion with diabetogenic (HA-specific) CD4<sup>+</sup> T-cells from mice bearing a TCR-HA<sub>110-120</sub> transgene (TCR-HA Tg T-cells/H-2<sup>d</sup>) [10]. The suppressogenic capacity of splenic CD4<sup>+</sup>25<sup>hi</sup> Foxp3<sup>+</sup> T-reg cells from BALB/c and B10.D2 mice was tested in relation to their ability to suppress the diabetogenic function of CD4<sup>+</sup>TCR-HA T-cells *in vivo*. At the 1 to 2.5 ratio of T-reg cells to diabetogenic CD4<sup>+</sup> TCR-HA cells co-transferred in RAG2 KO RIP-HA mice, both the B10D.2 and BALB/c T-reg cells were unable to delay significantly the onset of hyperglycemia (Fig. 8B, upper panel). However, only the B10.D2 T-reg cells prolonged the survival of hyperglycemic RAG2 KO, RIP-HA recipients (Fig. 8B, lower panel). Some 80% of hyperglycemic recipients receiving B10D2 T-reg cells survived for 1 month after the onset of hyperglycemia. Prolonged survival occurred in the context of a protective peri-islet pancreatic infiltration with lymphocytes (Fig. 8C). In contrast, only 50% of the hyperglycemic RAG2 KO, RIP-HA mice receiving BALB/c T-reg cells survived two weeks after hyperglycemia onset, and displayed a higher degree of destructive (intra-islet)  $\beta$ -islet infiltration. Thus, the number of heavily infiltrated  $\beta$ -islets in diabetic mice receiving B10.D2 T-reg cells ( $10 \pm 2$  islets of 20 scored per pancreas in 3 individual mice) was lower than in those receiving BALB/c T-reg cells ( $16 \pm 3$  islets of 20 scored per pancreas in 3 individual mice).





clock-regulated gene expression during differentiation of T-cells toward a Th1 or Th2 phenotype [47, 55], similar events may play a role in T-reg thymic differentiation. Our DNA methylation assay ruled out the possibility that epigenetic alterations such as methylation in the *Foxp3* gene promoter in T-reg precursors could be the mechanism responsible for variations in thymic expression of *Foxp3* gene in BALB/c and B10.D2 mice. However, DNA methylation in the 1<sup>st</sup> *Foxp3* intron was recently described to be inversely correlated with the binding of CREB/ATF complex transcription factor and *Foxp3* expression [56], and further investigations are required to find out whether a polymorphism in *Foxp3* non-coding regions may lead to variations in *Foxp3* expression in different genetic backgrounds. Epigenetic alterations may affect not only the *Foxp3* locus, but also loci encoding modulators of cell cycle division and apoptosis in T-reg precursors, as suggested by our experiments revealing a differential expression of Bad, Caspase 3, ObR, and CD3 in T-reg precursors from BALB/c and B10.D2 mice. More than 700 genes have been recently identified to be up- and down-regulated in developing and mature CD4<sup>+</sup>25<sup>hi</sup> T-reg cells from C57BL/6 mice, and several polymorphic genes with unique expression in different genetic backgrounds were also found to affect the thymic differentiation of T-reg cells [57].

The *Foxp3* thymic output was almost 3-times higher in B10.D2 mice than in BALB/c mice. A differential CD4<sup>+</sup>25<sup>hi</sup> thymic output between the B6, DBA/2 and SJL inbred mice bearing different MHC class II haplotypes was previously described [58]. Herein, we found a dual mechanism associated with the differential T-reg thymic output in BALB/c and B10.D2 mice. One arm of this mechanism appears to control the rate of T-reg thymic proliferation. Higher rates of proliferation were associated with a larger number of fast-dividing (ObR<sup>lo</sup>) *Foxp3*<sup>+</sup> thymocytes. In addition, besides the negative signaling of T-reg proliferation through ObR, its high cell density on T-reg precursors and mature CD4<sup>+</sup>25<sup>hi</sup> T-reg cells as compared with conventional T-cells suggests that ObR is a new biomarker for T-reg.

A second regulatory event that contributed to a differential T-reg thymic output in BALB/c and B10.D2 mice was apoptosis of T-reg precursors. Higher rates of T-reg apoptosis in BALB/c mice than in B10.D2 mice occurred in the context of an increased basal level of Bad and Caspase 3 expression. Thus, the DN3/4 transition stage of thymic differentiation appeared to be not only the earliest time-point of *Foxp3* expression in T-reg precursors, but also the earliest checkpoint for T-reg negative selection. Indeed, the thymic cortical area where most of the DN thymocytes reside displays a high extent of cell death [59]. Not surprisingly, the DN thymocytes can receive CD3/TCR signals in the absence of a CD4 receptor, since the p56<sup>lck</sup> phosphorylation and segregation of pre-TCR $\alpha$  in the plasma membrane lipid rafts occurs in DN thymocytes as well [60]. Furthermore, we found that a lower CD3, but not CD28 cell density on B10.D2 T-reg precursors than on their BALB/c counterparts was associated with a lower sensitivity to CD3-induced apoptosis in the DN and DP stages of thymic differentiation. In contrast, the T-reg precursors showed similar CD3 cell density in the SP4 terminal stage of thymic differentiation in both mouse strains, and their sensitivity to CD3-induced apoptosis was negatively correlated with the strength of CD3 signaling. Stronger CD3 stimulation provided better protection against

apoptosis. It thus appears that CD3, but not CD28 stimulation plays an important role in thymic selection of T-reg cells.

Interesting enough, a differential T-reg thymic output between the BALB/c and B10.D2 mice did not affect the size of splenic CD4<sup>+</sup>25<sup>hi</sup> T-reg pool due to a regulatory homeostatic mechanism. Previous data showed that the T-reg thymic output in various mouse strains lacks correlation with the size of T-reg compartment in periphery [58]. The T-reg homeostatic proliferation in the spleen of BALB/c mice was more accelerated than in B10.D2 mice, and this occurred in the context of a larger number of fast-dividing (ObR<sup>lo</sup>) T-reg cells than in B10.D2 mice. However, the T-reg suppressogenic capacity was different in these two strains, since the splenic T-reg cells from B10.D2 mice were more suppressogenic in the context of a higher expression of *Foxp3* protein per cell.

## CONCLUSION REMARKS

In summary, this study revealed a differential tuning of key regulatory proteins for thymic proliferation and apoptosis of CD4<sup>+</sup>*Foxp3*<sup>+</sup> T-reg cells that appear to be under the genetic control unrelated to the MHC class II genes. This was associated with variations in the T-reg thymic output in two different mouse strains. A dual mechanism responsible for differential rates of proliferation and apoptosis in T-reg precursors in two different mouse strains seemed to rely on the cell density of several receptors and signaling molecules such as the leptin receptor (ObR), CD3/TCR complex, Bad, and Caspase 3. The differential T-reg thymic output was homeostatically normalized in periphery, whereas their suppressogenic capacity was not. A stronger suppressogenic capacity of peripheral, mature T-reg cells was paralleled by a higher *Foxp3* expression per cell. The differential thymic development and function of CD4<sup>+</sup>*Foxp3*<sup>+</sup> T-reg cells in different genetic backgrounds may explain at least in part the variations in susceptibility to infections and autoimmune disorders reported in individuals from different ethnic groups. Recently, correlates between the genetic polymorphism at (GT)<sub>n</sub>, (TC)<sub>n</sub>1, DXS573, and DXS120B loci in *Foxp3* gene, and variations in susceptibility to autoimmune thyroiditis between Caucasian and Japanese ethnic groups, have been reported [61].

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