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Research paper

Epigenetic and transcriptional analysis supports human regulatory T cell commitment at the CD4+CD8+ thymocyte stage

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ABSTRACT

The natural CD25+ FOXP3+ regulatory T cell (Treg) population is generated as a distinct lineage in the thymus, but the details of Treg development in humans remain unclear, and the timing of Treg commitment is also contested. Here we have analyzed the emergence of CD25+ cells at the CD4+CD8+ double positive (DP) stage in the human thymus. We show that these cells share T cell receptor repertoire with CD25+ CD4 single-positive thymocytes, believed to be committed Tregs. They already have a fully demethylated FOXP3 enhancer region and thus display stable expression of FOXP3 and the associated Treg phenotype. Transcriptome analysis also grouped the DP CD25+ and CD4 CD25+ thymocytes apart from the CD25− subsets. Together with earlier studies, our data are consistent with human Treg commitment already at the DP thymocyte stage. We suggest that the most important antigens and signals necessary for human Treg differentiation may be found in the thymic cortex.

1. Introduction

The best-characterized population of regulatory T cells is defined by the stable expression of the forkhead/winged-helix transcription factor FOXP3, which promotes and sustains the Treg cell phenotype and function [1–3]. The importance of FOXP3 and Treg cells to the maintenance of immunological tolerance is provided by immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX), an early and severe form of autoimmunity caused by loss-of-function mutations in FOXP3 [4,5]. The natural Tregs develop in the thymus, and the current data suggest that they form a lineage distinct of conventional T cells. The epigenetic stabilization of FOXP3 expression is also imprinted in the thymus, and provides a key distinguishing marker of stable Treg phenotype in the periphery [6]. The human FOXP3 locus contains the promoter region and three conserved non-coding regions (CNSs) that regulate the FOXP3 expression. CNS2 also known as Treg-specific-demethylated region (TSDR) is a CpG-rich intronic enhancer region that is completely demethylated in natural Tregs but methylated in conventional T cells and activated T cells that can transiently upregulate FOXP3 [7].

Most of the data on thymic Treg development is based on murine models. Extensive literature suggests that autoreactive thymocytes escaping clonal deletion can be diverted to the Treg lineage, and Tregs would thus form a suppressive population specific to self-antigens [8–12]. In support of this scenario the TCR repertoire of conventional T cells and Tregs has been reported to be largely distinct [13], with a recent study on the murine thymus reporting only a 6% overlap [14]. However, Tregs can also be specific to non-self-antigens, and using TCRs obtained from Treg cells as transgenes can result in inefficient thymic Treg generation, indicating that TCR affinity alone is not enough to determine commitment to Treg lineage [15,16]. Indeed, it is possible the Treg differentiation requires a succession of suitable TCR signals, instead of a unitary contact [12]. The current scenario holds that TCR-mediated signals, probably modified by costimulatory molecules, first lead to upregulation of the high-affinity IL-2 receptor CD25. Interleukin-2 then provides an essential and largely nonredundant second signal for the upregulation and stabilization of FOXP3 expression [17,18].

The timing of Treg commitment remains unclear. It has been suggested that in mice the commitment takes place only in medulla, at the CD4 single-positive stage. The great majority of FOXP3+ thymocytes are confined to the medulla and in intrathymic transfer experiments the SP cells showed the greatest propensity for Treg differentiation [19,20]. Moreover, IL-2 availability may also present limitations, depending on

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localization of IL-2 sources [21,22]. For example, it has been reported that paracrine production of IL-2 by the most mature conventional SP thymocytes may be important, and these cells are not found in the thymic cortex [23]. Thus it may be that Treg commitment may be dependent on a limited number of suitable medullary niches.

The existing data in humans, although scarce, is consistent with commitment already at the cortical DP stage [24,25]. FOXP3 expression is first found already at the CD4-CD8- stage, but becomes more pronounced at the DP stage [26,27]. The cortical FOXP3+ cells are mainly found in the most mature, $CD3_{high}$ CD4+CD8+DP population, and they express high levels of CD69, a marker of positively selected cells [28,29]. The level of FOXP3 expression is as high as that found in the medullary CD4 SP cells, and the FOXP3+ DP cells also express several Treg-associated molecules and are capable of suppressive function in vitro [24,28,30–32]. Moreover, linear regression analysis reported by Nunes-Cabaco et al points to the FOXP3+ DP population as the major contributor to the FOXP3+ CD4 SP subset [24].

In the present report we show that the FOXP3+ represent a distinct lineage already at the DP stage, with strong similarity to the FOXP3+ CD4 SP subset in terms of TCR repertoire, TSDR demethylation and transcriptomics. These data provide further support for the DP stage as the crucial point of divergence in human Treg cell development.

2. Materials and methods

2.1. Patient samples

Thymic tissue was obtained from 13 otherwise healthy children (3 males, 10 females) undergoing corrective cardiac surgery. The children had no known immunological or genetic abnormalities. The tissue is routinely removed for improved exposure during cardiac surgery. The children were from 0.0 to 9.3 years old (mean 1.2 year, median 0.3). The pediatric ethics committee of Helsinki University Hospital approved the study, and an informed consent was obtained from the parents of the children. The study was performed in accordance of the Declaration of Helsinki.

2.2. Cell isolation and cell sorting

Thymocytes were released within 6 h of the thymectomy from the thymus tissue sample by mechanical homogenization. The antibodies used in the flow cytometry were direct fluorochrome conjugates: CD3-PE, CD8-APC, CD3-FITC, CD69-APC (Immunotools, Friesoythe, Germany), CD25-PE (clone M-A251), CD4-APC-Cy7, CD8-PE-Cy7 (Beckton Dickinson, San Jose, CA), CD25-PE-Cy7 (clone BC96) and CD4-Pacific Blue (eBioscience, San Diego, CA). Since permeabilization cannot be used with the cell sorting, Treg-associated marker CD25 was used instead of FOXP3. Previous studies have shown that at the DP stage roughly half of FOXP3+ cells express CD25, while most of $CD25_{high}$ thymocytes are FOXP3+ [25,28].

Flow cytometry and sorting was performed using the FACSAria II instrument. Fluorescence compensation settings were optimized by using BD Bioscience CompBeads (Beckton Dickinson, San Jose, CA). During sorting we performed a strict doublet exclusion using flow cytometric height and width parameters [33].

2.3. Genomic DNA extraction and sequencing

Frozen sorted samples were analyzed by Adaptive Biotechnologies (Seattle, USA). Genomic DNA was extracted using the QIAsymphony system, according to the manufacturer's instructions (Qiagen, Germany). The amount of DNA and the quality of samples were verified before sequencing. The TCRβ chain was sequenced using the ImmunoSEQ assay (Adaptive Biotechnologies) and Illumina platform as previously described [34]. Repertoire data analysis was done using the ImmunoSEQ analyzer toolset.

2.4. Methylation analysis

Demethylation status of the TSDR was determined by bisulfite conversion of demethylated cytosines, followed by quantitative PCR, as previously described [35]. Bisulfite conversion was performed with Qiagen Epitect, according to the manufacturer's instructions. Real-time quantitative PCR was performed with Bio-Rad iCycler (Hercules, CA).

2.5. RNA sequencing

The transcriptome analysis was based on the Drop-seq protocol [36]. RNA was mixed with Indexing Oligonucleotides (Integrated DNA Technologies) and cDNA synthesized using the Maxima H- RTase (all ThermoFisher Scientific). After PCR amplification the amplicons were tagged using the Nextera XT reaction (Illumina) and again amplified. The concentration of the libraries was measured using a Qubit 2 fluorometer (Invitrogen) and the Qubit DNA HS Assay Kit (ThermoFisher Scientific). The quality of the sequencing libraries was assessed using the LabChip GXII Touch HT electropheresis system (PerkinElmer), with the DNA High Sensitivity Assay (PerkinElmer). Sequencing of the libraries was performed at the Functional Genomics Unit of the University of Helsinki, Finland, using the Illumina NextSeq 500. Subsequently, the original pipeline suggested in Macosko et al. was used [36]. Briefly, reads were additionally filtered to remove polyA tails of length 6 or greater, then aligned to the human (GRCh38) genome using STAR aligner [37] with default settings. Uniquely mapped reads were grouped according to the 1–8 barcode, and gene transcripts were counted by their Unique Molecular Identifiers (UMIs) to reduce the bias emerging from the PCR amplification. Digital expression matrices (DGE) reported the number of transcripts per gene in a given sample (according to the distinct UMI sequences counted).

3. Results

3.1. Sample preparation and sequencing of TCR genes

To analyze the relationship between FOXP3+ DP and CD4SP subsets we used flow cytometric sorting to isolate them, with CD25 as a surrogate cell-surface marker for the intracellular FOXP3. The CD25- counterparts were isolated, as well. Altogether 5 thymus samples were analyzed (samples A-E), obtained from otherwise healthy children undergoing corrective cardiac surgery. To exclude any major thymic abnormalities we measured the frequency of the main thymocyte subsets. All samples showed the typical distribution of CD4/CD8 double negative (mean 1.2%, range 0.6–1.5%), double-positive (79.9%, range 75.1–83.3%), CD4+ single-positive (9.8%, range 6.3–16.5%), and CD8+ single-positive cells (9.0%, range 7.1–11.7%). As expected, most double-negative thymocytes were CD3- (data not shown), double-positive cells showed a distribution ranging from CD3- to $CD3_{high}$, and the single-positive subsets were almost completely $CD3_{high}$ (Fig. 1). The mean frequency of CD25+ cells was 0.7% (range 0.5–0.9%) in the DP population and 14.2% (range 11.9–15.7%) in the CD4SP population.

In addition to the CD25+ subsets, in samples A and B the DP subset was also divided into $CD3_{high}$ and $CD3_{low}$ cells, while in samples C-E the CD25− DP subset was isolated as one. The purity of CD25+ subsets was on average 96.9% ± 5.8% in DP population and 95.4% ± 2.5% in CD4SP population. The number of cells obtained varied according to the frequency of the subset in question (Table 1). Genomic DNA was extracted from the isolated subsets and the complementarity determining region 3 (CDR3) sequences of TCRβ amplified and sequenced. The number of TCRβ reads obtained ranged from as low as 2000 in the DP CD25+ population (sample D) to as high as 3.5×10^6 in the DP $CD3_{high}$ population (sample A), again largely dictated by the frequency of the subset in question. The number of unique productive TCRβ sequences ranged from 600 to 50 000 (see Table 1).

Consistent with the previously reported selection for shorter CDR3

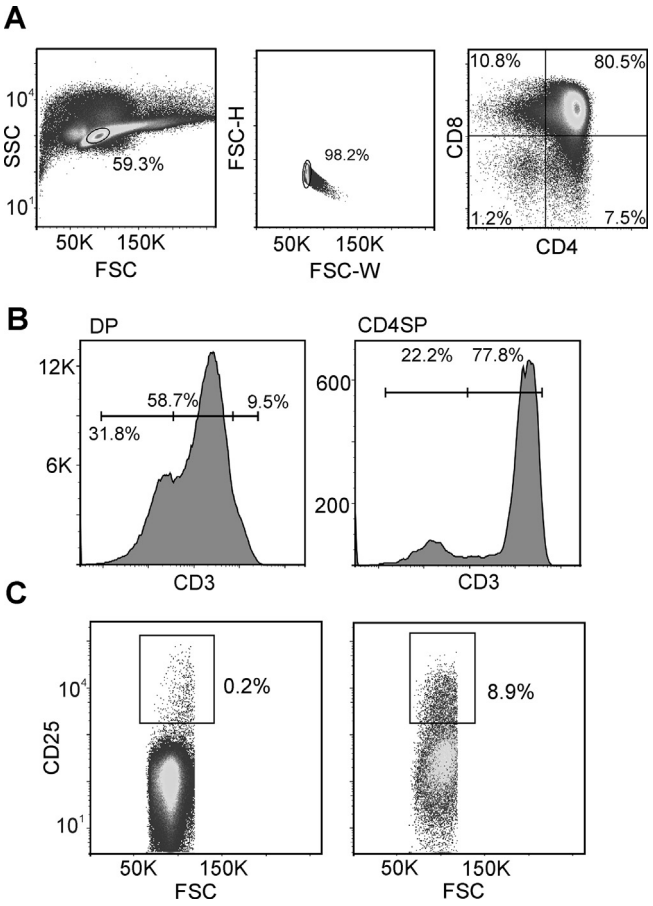

Fig. 1. Flow cytometric analysis of a representative thymus sample (C). A. Thymocyte gating and distribution of main thymocyte subsets. The data is shown on logarithmic scale, except for forward scatter (FS). The frequency of each main subset is indicated in the figure. B. CD3 expression pattern in DP and CD4SP thymocytes. C. CD25 expression pattern in DP and CD4SP thymocytes. DN: CD4/CD8 double-negative, DP: CD4/CD8 double-positive.

Table 2
Average CDR3 length in the thymus samples A-E.

Nonproductive	46.2 ± 1.0
DP $CD3_{low}$	46.5 ± 1.1
DP $CD3_{high}$	44.2 ± 0.5
DP CD25−	45.6 ± 1.2
DP CD25+	43.4 ± 0.6
CD4 CD25-	43.7 ± 0.5
CD4 CD25+	44.3 ± 0.1

lengths during thymic selection, the CDR3 was shorter in productive than in nonproductive rearrangements, and in more mature subsets. At the DP stage the mean length of productive CDR3 recombinations was 45.6 bp, while in the CD4SP stage the length was 43.7 bp and 44.3 bp in CD25− and CD25+ cells, respectively. Within the DP population, the $CD3_{low}$ stage had a CDR3 length of 46.5 bp, the $CD3_{high}$ stage 44.2 bp. Interestingly, the shortest CDR3 was found in the DP CD25+ subset (43.4 bp) (Table 2, Fig. 2). Since TCR-dependent selection in the thymus has been shown to drive CDR3 shortening, the fact that DP CD25+ and CD4 SP CD25+ subsets had a similar CDR3 length suggests the absence of major TCR-dependent selection events between these subsets.

3.2. DP CD25+ and CD4 CD25+ thymocytes have overlapping TCR repertoire

To track the developmental pathway of the regulatory T cells at the DP stage we then measured the relative overlap in TCR repertoire between the isolated subsets. As a determinant of overlap we used the overlap coefficient, obtained by dividing the number of shared sequences by the smaller of the size of the two sets. The data were analyzed at both nucleotide and amino acid level. In particular, overlap in identical nucleotide sequences is likely to reveal cells of common clonal background, whereas identical amino acid sequences encoded by different nucleotide sequences may also indicate convergent recombination or selection.

Given the small number of sequences obtained from some of the isolated subsets, especially the relatively infrequent DP CD25+ cells, the overlaps were generally small. However, analysis of unique productive nucleotide sequences showed that strongest affinity of the DP

Table 1
Sequencing results of TCRβ genes in the samples A-E.

	Age and gender	Population	Cell count	Total reads	In-frame	In-frame %	Unique in-frame
A	3 years 3 months, female	DP CD25+	27 000	482 609	387 339	80.3	7660
		CD4+ CD25+	100 000	1 250 729	1 037 326	82.9	24 958
		DP CD3high	300 000	3 557 300	2 921 600	82.1	50 047
		DP CD3low	1 million	113 693	81 455	71.6	581
		CD4SP	1 million	111 568	78 940	70.8	616
		CD8SP	1 million	129 274	96 899	75.0	974
B	2 months, female	DP CD25+	10 000	176 460	132 387	75.0	2412
		CD4+ CD25+	120 000	2 309 773	1 882 086	81.5	28 828
		DP CD3high	190 000	1 830 345	1 554 601	84.9	26 782
		DP CD3low	1 million	84 453	68 927	81.6	1252
		CD4SP	1 million	63 764	45 319	71.1	735
		CD8SP	1 million	4 443 726	3 723 236	84.0	99 631
C	1 year 4 months, female	DP CD25+	32 000	3416	2611	76.4	2289
		DP CD25−	1 million	40 632	36 236	79.1	28 661
		CD4+ CD25+	132 000	15 207	12 086	79.5	10 721
		CD4+ CD25−	108 000	13 500	10 719	79.4	9669
D	2 weeks, male	DP CD25+	21 000	2642	1891	71.6	1751
		DP CD25−	1.2 million	67 610	51 826	76.7	47 456
		CD4+ CD25+	182 000	19 091	14 279	74.8	13 293
		CD4+ CD25−	164 000	13 541	10 185	75.2	9482
E	7 months, male	DP CD25+	21 000	2212	1698	76.7	1571
		DP CD25−	520 000	30 090	24 585	81.7	22 808
		CD4+ CD25+	93 000	7741	6254	80.8	5889
		CD4+ CD25−	112 000	10 261	8412	82.0	7885

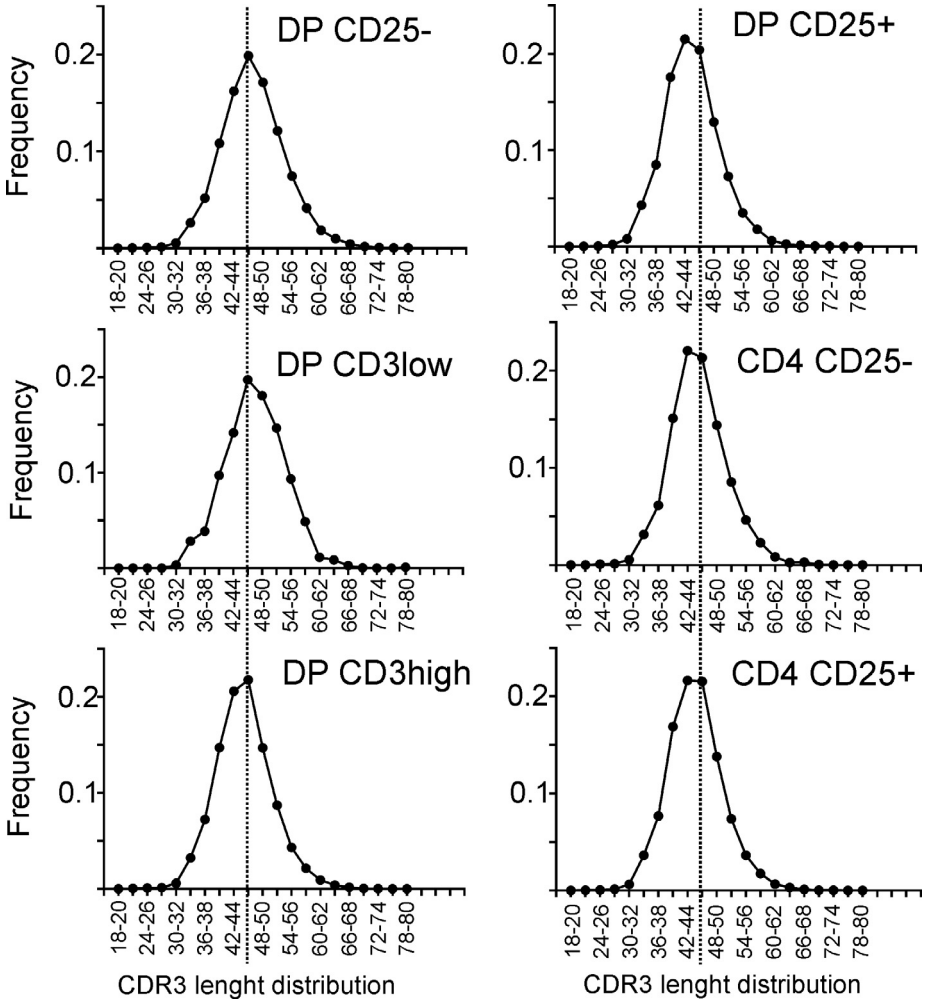

Fig. 2. CDR3 length distribution in the thymic subsets. CDR3 length distribution of productive TCRβ rearrangements. Shorter CDR3 recombinations are detected during thymic selection in more mature populations.

CD25+ subset was with the CD4SP CD25+ subset (0.016), followed by the DP CD25- subset (Fig. 3). When the number of reads was taken into account, providing information on the clonal abundance, the overlap between DP CD25+ and CD4SP CD25+ subsets was even more pronounced (0.039). Notably, the two CD25+ populations were also closest to each other in nonproductive nucleotide repertoire. Since the nonproductive sequences do not result in an expressed protein, they are not subject to thymic selection and reflect the original recombination process. The fact that the CD25+ subsets shared identical nonproductive nucleotide sequences thus further suggests that they have a shared clonal origin.

Analysis of amino acid sequences were largely similar to the nucleotide repertoire, with the biggest overlap found between the DP CD25+ and CD4SP CD25+ populations in both unique amino acid sequences and in total reads. However, the overlap between CD25+ and CD25- DP subsets was somewhat larger than observed in the nucleotide sequences.

We then looked separately at the DP $CD3_{low}$ subset, isolated from thymus samples A and B. At this stage no CD25+ cells were observed, and we found no shared nucleotide sequences between DP CD25+ and DP $CD3_{low}$ cells. Together, these data suggest a chain of clonal maturation from DP $CD3_{high}$ to DP CD25+ and then CD4SP CD25+ population.

3.3. Methylation analysis of DP and CD4SP CD25+ thymocytes

We have previously shown that on a single-cell level the expression of FOXP3 is equally high in DP and CD4SP thymocytes [28,38]. However, the epigenetic status of the TSDR has been shown to be essential in stabilizing Treg phenotype, and perhaps even more important than FOXP3 expression as such [39]. To test the demethylation status of the developing Tregs at the DP and CD4SP stage in the human thymus, we sorted four thymus samples (thymus samples F-I, all donors were female) to obtain DP CD25+ and CD4SP CD25+ populations and their CD25- counterparts. We then isolated genomic DNA for bisulfite conversion of demethylated cytosines to uracil and used methylation-dependent primers to amplify the TSDR. Quantitative real-time PCR showed high methylation state in both CD25- subsets (mean ΔCt of 6.2 ± 0.2 in the DP CD25- and 6.0 ± 0.1 in CD4 CD25- cells) (Fig. 4). The CD25+ subsets, in contrast, showed a clear pattern of demethylated TSDR, with clearly lower mean ΔCt values. Most importantly, there was no difference between the DP CD25+ (0.8 ± 0.2) and CD4SP CD25+ (0.8 ± 0.6) subsets. Thus, a stable Treg phenotype can be detected already at the DP stage.

3.4. Differential gene expression analysis of DP and CD4SP Tregs

To further track the developmental pathway of thymic regulatory populations we compared the transcriptome of CD25+ and CD25− cells in the DP and CD4SP subsets, isolated from further four thymus

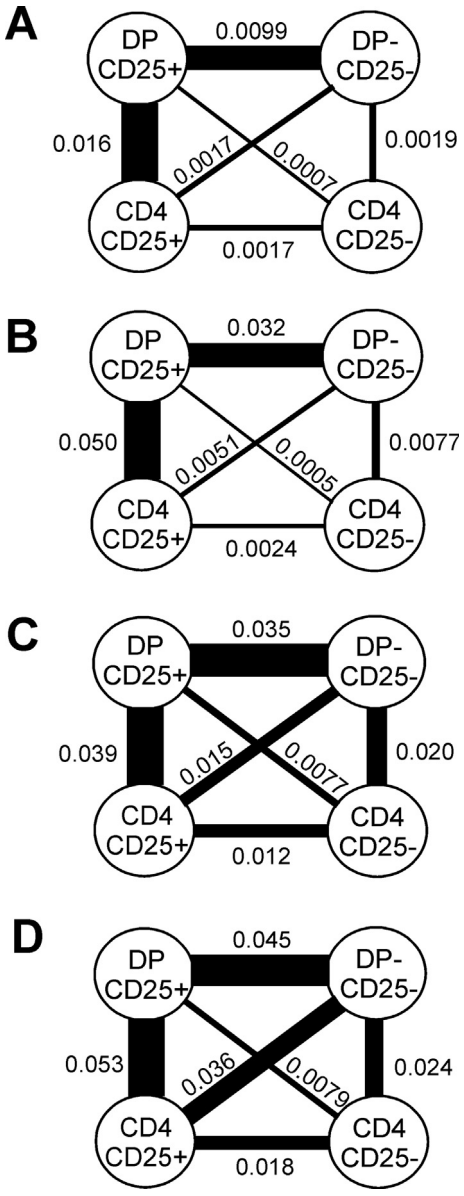

Fig. 3. TCRβ repertoire overlap between the main thymocyte subsets. The overlap is shown as overlap coefficient of productive sequences. A. unique nucleotide sequences. B. total nucleotide sequence reads. C. unique amino acid sequences. D. total amino acid sequence reads. The thickness of each connecting bar indicates the degree of overlap relative to the overlap between CD25+ DP and CD25+ CD4 SP subsets, which is shown as a fixed thickness. The overlap coefficients are shown as numbers beside the bars. n = 5.

samples (J-M). We first used edgeR Bioconductor analysis package to look for differential gene expression between the populations, and calculated the difference as Log2fold change. Strikingly, no statistically significant difference between DP CD25+ and CD4SP CD25+ cells was found in any of the genes identified. We then used the expression data to construct a phylogenetic table and a principal components analysis (PCA) of the relationship between the 16 samples (Fig. 5). Six of the CD25+ samples grouped together, while one DP CD25+ (thymus M) and one CD4SP CD25+ (thymus L) grouped among the CD25- samples. Notably, there was no clear distinction between the DP CD25+ and CD4SP CD25+ subsets. In the PCA the CD25+ samples were likewise interspersed, while clearly different from the CD25- subsets. These similarities were based on overall gene expression pattern; no specifically Treg-associated genes were detected, most likely because of the small size of the samples.

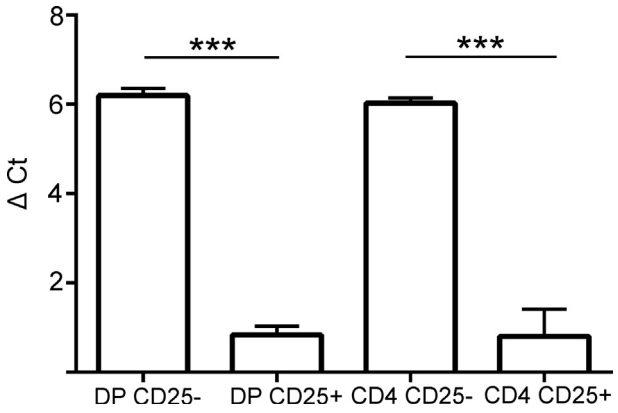

Fig. 4. The methylation status of TSDR in the CD25+ and CD25− thymocyte populations. The degree of methylation was measured using methylation-sensitive primers and real-time quantitative PCR. The data are shown on a relative scale as change of Ct: ΔCt = Ct (demethylated DNA) – Ct (methylated DNA). Each bar indicates mean value, the error bars SD (n = 4). ***p < 0.001.

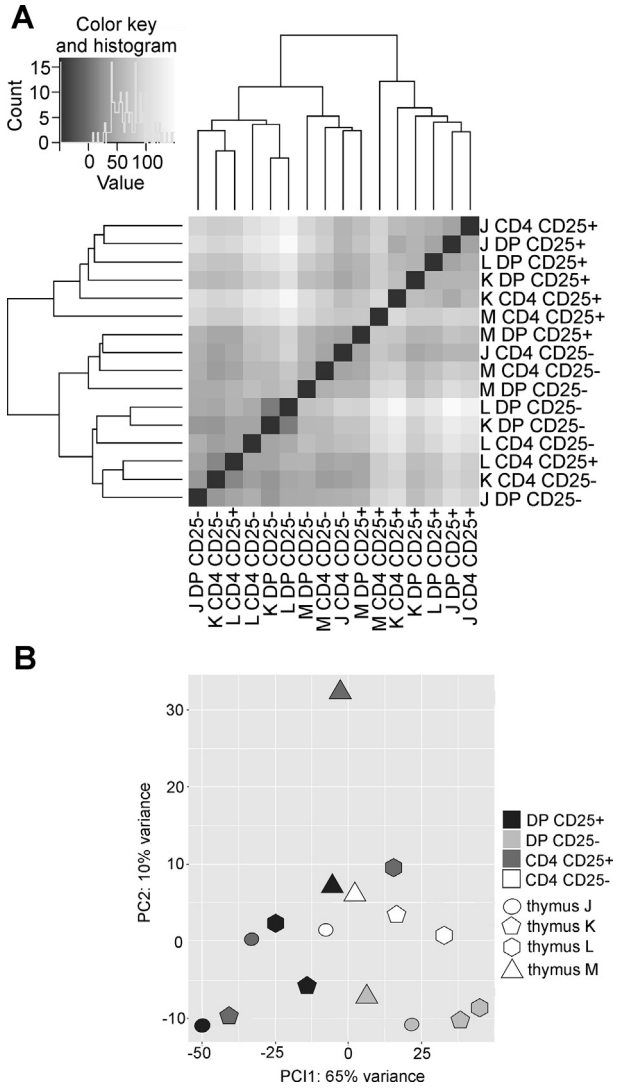

Fig. 5. Phylogenetic transcriptome analysis of the relationship between the CD25+ and CD25− thymocyte populations. A. The data are shown as a heatmap and cladogram indicating the closeness of the transcriptome. The color key is shown in the figure. The letters before the subset designations refer to thymus samples J-M. B. A principal components analysis (PCA) of the relationship between the thymus samples J-M.

4. Discussion

Details of the developmental pathway of regulatory T cells remain debated, and particularly little is known of human thymus. The preponderance of data in both species suggests that natural Tregs and conventional T cells form distinct lineages, and our repertoire analysis indicates that in humans this distinction is made at the DP $CD3_{high}$ stage. Thereafter, the DP CD25+ thymocytes possess the hallmarks of stable Treg lineage: They express high levels of FOXP3 as well as other Treg-associated markers [24,30,32], share transcriptome features with medullary CD4SP CD25+ Treg cells, display capability for suppressive function [31], and their FOXP3 TSDR is fully demethylated. It should be noted that our data do not exclude the possibility that part of the mature natural Treg population is converted at the CD4SP stage. Also, it is possible that some of the CD4SP cells actually represent mature re-entrants from the periphery. However, previous work by Nunes-Cabaco identified DP CD25+ thymocytes as the main source of CD4SP CD25+ population [24], and our data show much higher sequence overlap between DP CD25+ and CD4SP CD25+ cells than between the medullary CD4SP subsets. It should also be noted that the DP FOXP3+ population is quite substantial, accounting for 18% of the FOXP3+ thymocyte population [27].

Similarly to murine Treg cells, it is likely that the divergence to Treg lineage is linked to TCR-mediated signalling. The FOXP3+ population expresses high levels of CD5 and CD69, markers associated with positive selection, and they are also $CD3_{high}$ [28,29]. Our data on CDR3 length provides another approach to identify the role of TCR-mediated selection. Previous studies in both mice and humans have shown that during thymic selection the CDR3 gets progressively shorter, and this shortening has been linked to MHC-TCR interactions [40–42]. It thus reveals mainly the impact of positive selection. Our results show a major decrease during the DP stage, and the CDR3 of DP CD25+ cells was actually shorter than that of CD4SP CD25+ cells. This suggests that positive selection of and commitment to the Treg lineage mostly takes place at the DP stage. Whether the events leading to Treg commitment start already earlier remains unclear. In humans FOXP3+ cells are found already at the CD4-CD8- stage of thymic development [26], and murine studies have shown that Treg phenotype can be established even before FOXP3 upregulation [43].

It is clear that cytokines are also essential to Treg development. The existing data from human thymus show that IL-2 promotes Treg differentiation both at the DP and CD4 SP stage [25,44]. Given the high expression of CD25 IL-2 is likely to be the most important second signal, following TCR-mediated positive selection and CD25 upregulation. Other common γ chain cytokines also have an impact [45]. We and others have shown that IL-7 signalling leads to FOXP3 upregulation in human thymocytes, and at the DP stage induces BCL-2 expression and promotes the selective survival of FOXP3+ cells [38]. Interleukin-15 is also likely to have similar effects, but may affect Treg differentiation already at an earlier stage than IL-2 and IL-7 [25].

Together with earlier studies, our data are consistent with a developmental pathway of human Tregs that starts already at the DP stage, if not earlier. We have suggested in the earlier study that divergence to the Treg lineage takes place at the DP $CD3_{low}$ to $CD3_{high}$ transition, driven by TCR-mediated positive selection [25]. Nothing as yet is known of the Treg-selecting antigens in the human thymus, however, and likewise there is no direct evidence that human Tregs are autoreactive. Similarly to that reported in mice, homeostatic cytokines provide a crucial second signal, as well as promote Treg survival, and the role of IL-2 at least is probably nonredundant [25,44]. At the CD4 SP stage the number of Tregs increases, and this increase is possibly also augmented by further conversion of FOXP3- CD4SP thymocytes. However, if the human Treg commitment indeed takes place at the cortical DP stage, then the antigens and signals necessary for it may also be found in the thymic cortex.

Footnotes

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CRediT authorship contribution statement

Reetta Vanhanen: Conceptualization, Methodology, Investigation, Writing - original draft. **Katarzyna Leskinen:** Methodology, Investigation, Software. **Ilkka P. Mattila:** Resources. **Päivi Saavalainen:** Writing - review & editing. **T. Petteri Arstila:** Conceptualization, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.104026.

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