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Transcriptome Profiling of Human FoxP3⁺ Regulatory T Cells

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Abstract

The major goal of this study was to perform an in depth characterization of the “gene signature” of human FoxP3⁺ T regulatory cells (Tregs). Highly purified Tregs and T conventional cells (Tconvs) from multiple healthy donors (HD), either freshly explanted or activated *in vitro*, were analyzed via RNA sequencing (RNA-seq) and gene expression changes validated using the nCounter system. Additionally, we analyzed microRNA (miRNA) expression using TaqMan low-density arrays. Our results confirm previous studies demonstrating selective gene expression of *FoxP3*, *IKZF2*, and *CTLA4* in Tregs. Notably, a number of yet uncharacterized genes (*RTKN2*, *LAYN*, *UTS2*, *CSF2RB*, *TRIB1*, *F5*, *CECAM4*, *CD70*, *ENC1* and *NKG7*) were identified and validated as being differentially expressed in human Tregs. We further characterize the functional roles of *RTKN2* and *LAYN* by analyzing their roles *in vitro* human Treg suppression assays by knocking them down in Tregs and overexpressing them in Tconvs. In order to facilitate a better understanding of the human Treg gene expression signature, we have generated from our results a hypothetical interactome of genes and miRNAs in Tregs and Tconvs,

Keywords

Suppressive cells; RNA sequencing; Gene signature; miRNA; Differentially expressed; nCounter analysis; *RTKN2*; *LAYN*; *FoxP3*; miR-146a; miR-146b; miR-21; PNA

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1. Introduction

Tregs are indispensable for immune homeostasis, suppression of inflammatory responses and maintenance of immune tolerance [1]. In humans, mutations in the *FOXP3* gene result in the immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome [2, 3]. *Foxp3* expression is regarded as the most suitable marker for distinguishing Tregs from Tconvs [4–6]. However, in human T cells *FOXP3* is also expressed following activation of Tconvs and expression of *FOXP3* may not be sufficient to confer immunosuppressive functions [7]. The most commonly used surrogate markers for isolation and characterization of human Tregs are co-expression of high levels of IL-2R α (CD25) and low levels of IL-7R α (CD127). Analysis of microarray studies for genes differentially expressed between Tregs and Tconvs has identified the “Treg gene signature”. Of interest is that several of the differentially expressed genes include transcription factors and cell surface molecules, such as *IKZF2*, *IKZF4*, *LRRC32*, *CTLA4*, *IL1R1*, *CD39*, *CD73* and *TIGIT*, which have been implicated in the suppressive function of Tregs [8–10].

The major objective of this study was to better characterize the “gene signature” of human Tregs. The other objective was to identify genes that may encode cell surface markers or genes that mediate Tregs suppressor function. We have used poly-A⁺ RNA sequencing (RNA-seq) to comprehensively profile mRNA expression in Tregs and Tconvs. In the mouse, deletion of Treg-specific genes required for miRNA biogenesis results in a scurfy like syndrome, so in addition to RNA-seq of mRNA, we analyzed microRNA (miRNA) expression using TaqMan low-density array technology [11–16]. Some studies have attempted to characterize the human Treg miRNA signature, but major differences exist between these studies [9, 10, 17–20]. Understanding the expression patterns of mRNAs and miRNAs in human Tregs should be highly relevant for furthering our functional understanding of the role of Tregs in immune responses.

2. Materials & Methods

2.1 Cell purification, in vitro expansion, and characterization

Leukapheresis products for the study were obtained from healthy adult donors (HD), at the Department of Transfusion Medicine (Clinical Center, NIH). Umbilical cord blood (CB) was collected from term placentas at Shady Grove Adventist Hospital (Gaithersburg, MD). The acquisition of blood products was approved according to the Institutional Review Boards (IRBs) of these two institutions and in accordance with the Declaration of Helsinki. CD4⁺CD25^{hi}CD127⁻ Tregs and CD4⁺CD127⁺CD25^{lo} Tconvs were isolated from peripheral blood (PB) and CB samples as previously described [21, 22]. To evaluate the purity of Tregs isolated by this cell-sorting scheme, they were stained with anti-FoxP3 (Clone 259D/C7, eBioscience) in FoxP3 staining buffer (eBioscience). Sorted populations were expanded in RPMI (Lonza) supplemented with 10% heat inactivated FBS (Lonza) in the presence of recombinant human IL-2 (rIL-2, 100U/ml, Roche) in plates coated with anti-CD3 (clone OKT3, 10ug/ml, eBioscience) and anti-CD28 (clone 28.2, 2ug/ml, eBioscience) for 6hr, 24hr. Cells were expanded for longer time periods under similar conditions with changes in medium every third day. Flow cytometry data were acquired on an LSRII or FACS Caliber

(Becton Dickinson); FACS Diva software (BD) and FlowJo version 9.4.10 software (Treestar) were used for analysis.

2.2 RNA Purification and RNA-seq analysis

Total RNA was isolated using mirVana (Applied Biosystems) isolation kit. RNA-seq libraries were prepared from 4 µg of total RNA using the TruSeq RNA sample preparation kit following manufacturer's protocol (Illumina). Briefly, oligo-dT purified mRNA was fragmented and subjected to first and second strand cDNA synthesis. cDNA fragments were blunt-ended, ligated to Illumina adaptors, and PCR amplified to enrich for the fragments ligated to adaptors. The resulting cDNA libraries were verified and quantified on Agilent Bioanalyzer and RNA-seq was conducted using the GAIIX Genome Analyzer (Illumina). Reads from RNA-seq were mapped to the human genome (GRCh37, hg19) using the splice-aware aligner TopHat with option `--mate-inner-dist 160 --coverage-search --microexon-search --max-multi hits` [23]. Aligned reads were then visualized on a local mirror of the UCSC Genome Browser [24]. Quantification of gene expression was performed by counting aligned reads on each gene, for each condition in Tregs and Tconvs for same HD. DEGseq was then applied to identify differentially expressed genes between Tregs and Tconvs with *p* value $1e^{-5}$ [25]. For qRT-PCR, cDNA was prepared using TaqMan Reverse Transcription Reagents (Applied Biosystems) with random hexamers.

2.3 Nanostring nCounter expression and analysis

Custom-designed code-set included probes for 136 genes (Supplementary table 4), including housekeeping genes, were analyzed using the nCounter analysis system as per manufacturer's instructions [26, 27]. The normalization factor was calculated from the geometric mean of the reference genes and further normalized with geometric mean of 5 housekeeping genes. Probe sets were produced in a single batch, and several sets of sorted Tregs and Tconvs were run together. Differential gene expression was calculated by Log2Fold change of Treg/Tconv for the same HD.

2.4 miRNA expression and validation

TaqMan low-density arrays (Applied Biosystems) for human miRNAs were used for expression profiling of Tregs and Tconvs sorted from multiple HD per manufacturer's instructions. Individual TaqMan assays were used for validations. A 7900HT Fast Real Time PCR Systems (Applied Biosystems) was used, and normalized using U6 expression; the comparative C_T method was used to calculate relative miRNA expression.

2.5 siRNA knockdown in Tregs

Rhotekin-2 (*RTKN2*) and Layilin (*LAYN*) siRNAs were purchased from Invitrogen (Stealth Select RNAi). Scrambled oligonucleotide was used as a non-silencing siRNA control. To transfect the Tregs, 300pmol of siRNA was mixed with 100µL of human T-cell Nucleofector (Lonza) solution and $0.5-1.0 \times 10^6$ cells were resuspended in this mixture. The sorted Treg single cell suspension was immediately electroporated by the Nucleofector II instrument (Amaxa Biosystems) and transfected Tregs were rested in culture medium containing rIL-2 (50 IU/mL) for 24hr before being stimulated with anti-CD3/anti-CD28 and

rIL-2. Quantitative PCR was performed on a 7900HT Fast Real Time PCR Systems (Applied Biosystems)

2.6 Retroviral overexpression in Tconvs

Gene sequences for human *RTKN2* or *LAYN* were cloned into bicistronic retroviral vector pRetrozX-IRES-ZsGreen1 (Clontech) and retroviral particles expressing *RTKN2* or *LAYN* were generated using HEK293T cells with retroviral packaging plasmids. Sorted Tconvs were stimulated for 12–18hr with anti-CD3/anti-CD28 and rIL-2 and then were transduced by spinoculation at $600 \times g$ for 1h, 30°C on retronectin coated plates ($5\mu g/cm^2$, Takara Shuzo). Cells were harvested 48hr post transduction and sorted for ZsGreen positive and ZsGreen negative cells to analyze *RTKN2* or *LAYN* expression by qRT-PCR and functional analysis.

2.7 Peptide nucleic acid (PNA) inhibition of miRNA

100nM of PNA inhibitors conjugated to a cell penetrating peptide for miR-146a, miR-146b and miR-21 (PANAGENE Inc.) were added to sorted Tregs on day 1 and day 3 of culture stimulated with anti-CD3/anti-CD28 and rIL-2 in 24 well plates.

2.8 Tregs suppression assays

CD4⁺CD127⁺CD25^{lo} T cells (50,000) sorted by FACS were stimulated with irradiated (40 Gy or 4000 rad) autologous MACS sorted CD3-depleted PBMCs (50,000) and anti-CD3 (1 $\mu g/ml$, UCHT1, eBioscience) alone or titrated with different numbers of Tregs. Cell proliferation was assayed by thymidine incorporation as previously described [21].

2.9 Mouse dendritic cell (DC)-human Treg suppression assay

Mouse CD11c⁺ DC cells were purified by MACS using CD11c beads and co-cultured with human Tregs (50,000) at 1:1 ratio in a 96 well plate for 12hr. Expression of CD86 mean fluorescence intensity (MFI) in gated mouse CD11c⁺ DC was determined by FACS analysis. Purified anti-human CD18 (eBioscience) antibody was used as positive control to reverse Treg suppression [28]. The MFI for CD86 from mouse CD11c⁺ DC cultured alone was used as reference and set at 100%; percentage suppression was calculated based on the difference in presence of Tregs.

2.10 Statistical Analysis

All groups are expressed as mean $\pm$ SD unless stated otherwise. The statistical comparisons of data were calculated with unpaired Student's *t*-test (GraphPad Prism software).

3. Results

3.1 Unbiased analysis of FoxP3⁺ Tregs transcriptome

We performed RNA-seq from donor matched freshly FACS-sorted Tregs (CD4⁺CD127^{lo}CD25^{hi}) and Tconvs (CD4⁺CD127^{hi}CD25^{lo}) [29] to compare both the protein-coding and non-coding transcriptomes from three different HD. A typical FACS-sort is shown in Supplementary Fig. 1A. The log expression of raw reads from genes expressed

by Tregs and Tconvs from the three HD are plotted (Fig. 1A) and certain key genes (*FOXP3*, *IKZF2*, *CTLA4*, *LRRC32* and *IL1R1*) are highlighted. This analysis demonstrates that in each HD >5000 genes were differentially expressed ($\text{Log}_2\text{FC} \pm 0.5$) between Tregs and Tconvs. Importantly, 2041 differentially expressed genes were shared in all three HD (Fig. 1B, Supplementary Table 1). Among this group of shared differentially expressed genes, we identified several genes that have not been previously reported as being preferentially expressed in Tregs or Tconvs including *LAYN*, *UTS2*, *RTKN2*, *CCR3*, *CSF2RB*, *CD80*, *ENC1*, *ZEB2* and *NKG7* (Supplementary Table 1). We also identified 36 differentially expressed un-annotated transcripts that were also shared among all three HD (Fig. 1C) and listed their transcript loci in Supplementary Table 2.

Since Treg suppressor function is only be observed *in vitro* following TCR activation [21, 30, 31], we extensively analyzed gene expression from Tregs and Tconvs of one donor (3HD) at freshly sorted (0hr), 6hr and 24hr after *in vitro* activation. We identified several differentially expressed genes (Fig. 1D). We then compared these time point changes in gene expression between the Tregs and Tconvs with *in vitro* activation at after 7 days of expansion (Fig. 1E). *In vitro* activation strongly influenced gene expression. We identified 321 genes that were differentially expressed in freshly explanted cells at all times points after activation. This group of 321 genes contained most of the genes known to be differentially expressed in Tregs vs Tconvs (*FOXP3*, *IKZF2*, *LRRC32*, *CTLA4*, *IL7R*, *IFNG*) and many other genes that were not previously characterized (*RTKN2*, *LAYN*, *TRIB1*, *F5*, *CSF1*, *CSF2RB*, *PLC1*, *FAM164A*, *CCR1*, *GPPR56*, *CCR4*, *VIPR*, *CD80*). Not surprisingly, 1283 genes differentially expressed in freshly sorted cells, were no longer differentially expressed after *in vitro* activation. The majority of this group of genes was highly expressed in Tconvs and only a few were preferentially expressed in Tregs (*TNFRSF13B*, *VAV3*, *SEMA3G*, *CCNG2*, *FCRL1*, *CCR9*). The log expression of genes differentially expressed after 7 days are plotted in Supplementary Fig. 1B and are listed in Supplementary Table 3.

3.2 Validations of differentially expressed genes

We used multiple strategies to select genes that could be accurately validated. First, we preferentially selected genes that were differentially expressed under all activation conditions, and then selected membrane-associated genes as determined by the Gene Ontology (GO) term “membrane”, which describes locations for cellular components. We also included several genes that were not identified as “membrane” because they appeared to be preferentially expressed in either Tregs or Tconvs. In addition, several genes previously reported as preferentially expressed in Tregs (e.g, *FOXP3*, *IL2RA*, *IL7R*) were included. Using these criteria, 136 genes (Supplementary Table 4) were selected and validated for their expression with the nCounter system. We performed nCounter gene expression profiling on up to eight HD, using freshly sorted Tregs and Tconvs, as well as Tregs and Tconvs that had been activated *in vitro* for 6hr and 24hr. Furthermore, we also incorporated sorted Tregs and Tconvs from human CB samples (n=5) in these validation studies.

The expression of raw reads using RNA-seq and nCounter from Tregs and Tconvs for selected genes (*FOXP3*, *IL2RA*, *IKZF2*, *LRRC32*, *IL7R*, *CSF2RB*, *RTKN2*, *LAYN* and *UTS2*) is illustrated in Fig. 2A. The expression patterns of genes from RNA-seq were comparable

to nCounter expression. Tregs and Tconvs from HD were analyzed when freshly sorted (0hr, n=6) and after stimulation for 6hr (n=3) or 24hr (n=8). In Fig. 2B, we illustrate the Log2fold change (Log2FC) between Tregs and Tconvs paired from a HD for each differentially expressed gene analyzed by nCounter and RNA-seq. Both methods of analysis demonstrated unique trends of gene expression for Tregs and Tconvs at 0hr and after 6hr and 24hr of activation. The reliability and accuracy for the analysis by nCounter system was established by the similar trend in gene expression as RNA-seq gene expression for previously characterized genes (*IL2RA*, *FOXP3*, *IKZF2*, *IL7R*, *LRRC32*, *IL1R1* and *TIGIT*). The nCounter analysis also verified differential expression of genes that had not been previously characterized as being selectively expressed in human Tregs: *RTKN2*, *LAYN*, *UTS2*, *CSF2RB*, *CD80* and *IL12RB2*. The expression of genes (*GPR55*, *CCR4*, *CCR6*, *CSF1*, *SELP*, *CDKN2B*, *SPP1*, *SOCS2*, *GPR56*, *CD40LG*, *IKZF4*, *ENTPD1*, *TRIB1*, *FCRL1* and *CTLA4*) previously described was also verified. Comparable expression of housekeeping genes (*GAPDH*, *G6PDH*, *B2M*, *ACTB* and *CD4*) was observed among Tregs and Tconvs (Supplementary Fig. 3, Supplementary Table 5). The results of the nCounter analysis of for several genes (*RTKN2*, *LAYN* and *UTS2*) were also validated by qRT-PCR in samples from multiple HD (Supplementary Fig. 4).

3.3 Gene signature correlation among donors

To determine correlation among donors for the set of 136 genes (log2FC DE Treg/Tconv) analyzed by nCounter, we created a heatmap using a non-parametric Spearman matrix (Fig. 3) of their respective significant p values (Supplementary Table 6). Interestingly, there was a very high correlation ($r^2=0.81-0.90$) among the CB and HD samples for the set of genes analyzed at all time points. There was also high inter donor correlation within each CB and HD sets, as detailed in the supplementary figure 6 with respective p values in Supplementary Table 7.

3.4 Functional analysis of genes differentially expressed in Tregs

RTKN2 and *LAYN* have not been characterized previously in Tregs and were prominently differentially expressed in our studies. We initially carried out siRNA knockdown experiments to determine whether decreased expression of these genes would alter the suppressive capacity of Tregs *in vitro*. Gene specific knockdowns were performed with three different *RTKN2* and *LAYN* specific siRNA oligos using Amaxa transfection (Fig. 4A). The transfected cells were then stimulated for an additional 24 hr *in vitro* with anti-CD3, CD28 and rIL-2 and analyzed for knock down efficiency on the third day. All three oligos for *RTKN2* and *LAYN* resulted in ~60% knockdown of their respective target gene (Fig. 4B) without effecting FOXP3 expression (Supplementary Fig. 5A). We used *RTKN2*-A siRNA and *LAYN*-A siRNA for further functional assays. The siRNA-treated Tregs were then used in the standard Treg suppression of proliferation assay in which they are titrated against Tconvs in presence of antigen presenting cells and soluble anti-CD3. *RTKN2* or *LAYN* siRNA-transfected Tregs enhanced the suppression of Tconvs proliferation when compared to scrambled oligo siRNA transfected Tregs or untreated Tregs (Fig. 4C). The data are representative of three suppression assays performed with cells from different donors. We also analyzed the effects of the *RTKN2* or *LAYN* siRNA-treated Tregs in another Treg suppression assay, in which the Tregs are co-cultured with mouse CD11c⁺ dendritic cells

(DC) and the change in the expression of CD86 (MFI) on the mouse DC in presence of Tregs analyzed after 12hr [28]. Both the *RTKN2* and *LAYN* siRNA-treated Tregs suppressed mouse CD86 expression on mouse DC to the same extent as the scrambled oligo siRNA-treated or the untreated Tregs control (Fig. 4D).

We also explored an alternative approach to search for genes that may modulate Treg function by overexpressing them in Tconvs using retroviral transfection. Tconvs were first stimulated with anti-CD3, CD28 and rIL-2 and then transfected with either *RTKN2* or *LAYN* expressed in the pRetroX-IRES-ZsGreen vector. After 48hr of transduction, GFP⁺ve and GFP⁻ve fractions were sorted and rested for 12hr prior to further analysis (Fig. 5A). GFP⁺ve Tconvs expressed 5–6 fold higher levels of either target gene (either *RTKN2* or *LAYN*) compared to the GFP⁻ve Tconvs fraction (Fig. 5B). To determine the functional potential of these populations, the sorted transfected (GFP⁺ve) cells were titrated with donor matched Tconvs in the suppression of proliferation assay. Tconvs that overexpressed either *RTKN2* or *LAYN* retained their proliferative potential and did not gain suppressive functions (Fig. 5C). We show representative plots from data of two different HD.

3.5 miRNA expression in Tregs

In parallel to gene expression, we also performed a large-scale analysis of human miRNA expression to identify miRNAs that are differentially expressed in Tregs and Tconvs. We observed increased expression for miR-21, miR-146a, let-7a, let-7g and decreased expression for miR-125a in Tregs compared to Tconvs (Fig. 6A). To validate the results of the miRNA microarray studies, qRT-PCR studies were performed on fresh-sorted Tregs and Tconvs. There was high expression of miR-146a, miR-146b, let-7g, miR-21, miR-660, miR-28 3p and miR-598 in Tregs compared to Tconvs normalized to U6 expression; while miR-125a, miR-31, miR-99a and miR-100 was highly expressed in Tconvs compared to Tregs normalized to U6 expression (Fig. 6B and Supplementary Fig. 4b).

3.6 Effect of inhibition of differentially expressed miRNAs on Tregs function

We selected several of the miRNAs (miR-21, miR-146a and miR-146b) that were up regulated in Tregs for functional studies. We activated sorted Tregs with anti-CD3, CD28 and rIL-2 and treated the cells on day 1 and day 3 with PNA inhibitors [32] (Fig. 7A). When analyzed on day 5 after *in vitro* culture, the combined use of PNA miR-146a and miR-146b resulted in >90% reduction in both miR-146a and miR-146b compared to scrambled non-specific PNA inhibitors. The use of the combined inhibitors more efficiently knocked down their respective miRNA compared to individual PNA inhibitors (data not shown). The PNA miR-21 resulted in >90% reduction of miR-21 expression. The suppressive potential of PNA-inhibited Tregs remained unchanged when they were used to suppress Tconvs proliferation (Fig. 7C). The PNA-inhibited Tregs also had no change in their ability to suppress CD86 expression on mouse DC (Fig. 7D). PNA-inhibited Tregs also retained their non-responsive state as they failed to proliferate to anti-CD3 when cultured in the absence of responder T cells (Fig. 7C). PNA inhibition of miR-21, miR-146a and mi-146b in Tregs did not alter FoxP3 expression (Supplementary Fig. 5b).

4. Discussion

Previous gene profiling studies have determined a human Treg gene “signature” based on the analysis of either bead purified PB Tregs or FACS sorted CB Tregs using microarray technology [9, 10, 19, 20, 33]. A number of genes up or down regulated in Tregs were identified and many of those may play important roles in Treg biology. These studies also attempted to determine potential cell surface antigens to serve as surrogate markers for characterization of Tregs. The availability of such a marker would greatly facilitate the isolation of purified Tregs and would also be extremely useful for the definitive identification of Tregs in inflammatory conditions, in which *Foxp3* expression may be induced in activated Tconvs.

We have utilized RNA-seq for comprehensive mRNA profiling and low-density arrays for miRNA profiling. Although immunomagnetic bead separation technology can be used to obtain enriched populations of human Tregs, cell sorting is required to achieve highly purified Tregs with expression of *Foxp3* >90–95% [7, 21, 22, 28, 29, 34]. We are confident of the appropriateness of our purification approach, as we observed reproducible gene signatures even with low read counts in multiple HD. We validated gene expression by using the nCounter system. We identified genes selectively over expressed in Tregs (*LAYN*, *UTS2*, *RTKN2*, *FCRL1*, *CCR3*, *CSF2RB*, *CECAM4*) and several genes selectively over expressed in Tconvs (*FCRL6*, *ZEB2* and *NKG7*). In one other study [19], RNA-seq was used for transcriptome profiling of resting and activated human Tregs, however they used magnetic bead purified cells and the validation of differentially expressed genes was not reported. Moreover, gene expression was only analyzed in freshly sorted cells and at single time point (16hr) following activation, and cell populations from multiple HD were not analyzed separately. The donor-to-donor variation in differential gene expression seen in freshly isolated Tregs and Tconvs increased with *in vitro* activation.

After multiple unsuccessful attempts to stain Tregs with monoclonal antibodies commercially available for various cell surface genes, we focused our attention on two of the unique genes *RTKN2* and *LAYN* that were highly differentially expressed in Tregs under all activation conditions. *RTKN2* belongs to the Rhotekin family of proteins [35]. Initial characterizations of *RTKN2* revealed dominant expression in lymphoid subsets with highest expression in CD3⁺ T cells [35]. Previous studies of *RTKN2* demonstrated an association with the resistance to apoptosis in HL60 cells exposed to 25-hydroxy-cholesterol [36] and an association with rheumatoid arthritis susceptibility [37]. *LAYN*, a transmembrane glycoprotein homologous to C-type lectins [38,39] is expressed in human articular chondrocytes and synoviocytes [40]. One preliminary report demonstrated in a mouse tumor xenograft model, an association of downregulation of *LAYN* expression with decreased tumor growth and metastatic potential [41]. *RTKN2* and *LAYN* were not yet characterized for their roles in human Tregs to the best of our knowledge.

Studies of human Treg function are limited to *in vitro* assay for Treg suppressor function. A major limitation of this approach is that it remains unclear if *in vitro* assays of Treg function are correlated with Treg function *in vivo*. In a number of murine models [18, 42], deletion of a given gene can markedly impair Treg function *in vivo* yet have no effect on the capacity of

the deficient cells to suppress T cell activation *in vitro*. We find that siRNA treated Tregs with 60% knockdown of both *RTKN2* and *LAYN* behaved differently in the two *in vitro* human Treg suppression assays. They had enhanced Treg suppression in standard Treg suppression of Tconvs examined by thymidine incorporation, but had no effect in downregulating the expression of CD86 on mouse DC. This could be due to different mechanisms for Treg suppression, which might suggest that *RTKN2* and *LAYN* may selectively interact with distinct components of the Treg suppression pathways.

Overexpression of either *RTKN2* or *LAYN* did not have any effect in introducing suppressor function in Tconvs. This result is not unexpected as it has been shown that Treg expression of Treg signature genes and Treg suppressor functions are dependent on expression of *Foxp3* [10, 18–20]. Furthermore, optimal expression of the Treg signature requires expression of at least one of a group of other transcription factors (Eos [*IKZF4*], *STAT3*, *IRF4*, *SATB1*, *BACH2* and *GATA-1*) in addition to *Foxp3* [18, 43]. A detailed understanding of the potential functions of *RTKN2* and *LAYN*, and the other human Treg-specific genes may require an analysis of their expression in the mouse and selective deletion in mouse Tregs.

In parallel studies of miRNAs in Tregs and Tconvs using TaqMan technology, we observed increased expression of miR-146a, miR-146b, let-7g, miR-21, miR-660 and miR-598 in Tregs and increased expression of miR-125a in Tconvs. It is difficult to compare our results with other studies of human miRNA expression [33,44] because very different cell populations and separation techniques were used. Several studies [14, 45] have demonstrated that miR-146 is upregulated in human Tregs [33, 46] and a few studies have noted that miR-21 is also upregulated in human Tregs [47]. Many miRNAs bind to complementary sequences in the 3'UTR of their target genes. miR-21 expression was postulated to be a marker of memory cells and not a Treg-specific marker [33]. Of note, one study has demonstrated that miR-146a is essential for maintaining the cell fate of Tregs by targeting STAT1 [14].

We were quite successful in our attempts to downregulate expression of the miRNAs selectively expressed in Tregs using PNA inhibitors [32], but the decrease in expression of these miRNAs had no effect in the standard Tregs suppression of Tconvs proliferation assay. Dual inhibition of miR-146a and miR-146b in human Tregs had no effect in our hands, whereas germline deletion of miR-146a in the mouse resulted in a dramatic phenotype in Tregs. Thus, gene targeting studies in the mouse will be required to determine the role, if any, of these miRNAs in Treg function. Overall, there appears to be relatively few miRNAs that are exclusively expressed in Tregs and a great deal of overlap in miRNA expression between Tregs and activated Tconvs or T memory cells.

We performed predictive analysis using Ingenuity Pathway, miRecords, Ingenuity Expert findings and Ingenuity Expert assist findings (experimentally observed, highly predicted or moderately predicted, Supplementary Table 8) to generate a hypothetical circus plot “interactome”. To selectively display only the most significant interactions in this interactome, we limited plotting expression to only the most differentially expressed miRNAs and paired them with their respective differentially expressed mRNAs to generate

111 interactions. The interactomes predict pairing of previously reported miRNA and mRNA to reveal possible cross talk between networks. These predictions, for example in Treg, suggest pairing of certain target genes (*IL10*, *IL1R1*, *TNFRSF1*, *DUSP8*, *CCL4*, *CD109* and *IGFB2BP2*) with the low expression of miR-125a; and pairing of target genes (*IL12R*, *TGFB1*, *ITGB8*, *PEL1*, *FASLG* and *SATB1*) with high expression of miR-21. These potential interactions between miRNAs and mRNA could facilitate the functional understanding of the gene signatures in Tregs. While it was challenging for us to establish functional significance for *RTKN2* and *LAYN* in Tregs, monitoring their expression may nonetheless prove useful in future studies dealing with the therapeutic manipulation of human Tregs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CB	umbilical cord blood
DC	dendritic cell
DE	differentially expressed
FACS	fluorescence activated cell sorter
GO	Gene Ontology
HD	healthy donor
miRNA	micro RNA
PB	peripheral blood
PNA	peptide nucleic acid
RNA-seq	poly A ⁺ RNA sequencing
Tconv	T conventional cells
Tregs	T regulatory cells
rIL-2	recombinant Interleukin-2
Log2FC	Log2 Fold Change
RTKN2	Rhotekin-2

LAYN

Layilin

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Fig. 1.

Unbiased FoxP3⁺ Tregs transcriptome profiling: (A) Scatter plots show pairwise global gene expression between sorted Tregs and Tconvs from 3HD, non-manipulated *in vitro* for RNA-seq transcripts. Gene expression values are plotted in log scale and dashed red lines indicate a log 2 expression; specific genes validated further are highlighted. (B) Venn diagram of known differentially expressed transcripts among freshly explanted Tregs and Tconvs (+/-0.5Log2FC). A detailed list of genes is available in Supplementary Table 1. (C) Venn diagram of unannotated transcripts differentially expressed among freshly sorted Tregs and Tconvs. (D) Scatter plots showing genes differentially expressed between Tregs and Tconvs from 3HD that had been activated *in vitro* for 6hr and 24hr with anti-CD3 and anti-CD28 and rIL-2. Gene expression values are plotted in log scale and dashed red lines indicate a log 2 expression; specific genes validated further are highlighted. (E) Venn diagram of

activation induced genes differentially expressed among Tregs and Tconvs (Log2FC >0.5genes). A detailed list of genes is available in Supplementary Table 3.

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Figure:2A

Figure:2B

Fig. 2.

Validation of differentially expressed genes by nCounter analysis. (A) Raw reads for genes *FOXP3*, *IL2RA*, *IKZF2*, *LRRC32*, *IL7R*, *CSF2RB*, *RTKN2*, *LAYN*, and *UTS2* that are uniquely expressed in either CB or PB sorted Tregs and Tconvs as shown by RNA-seq and nCounter analysis. Each dot represents raw read for the respective gene in Tregs and Tconvs by RNA-seq at the respective activation time point. Each triangle represents raw read for the respective gene in Tregs and Tconvs by nCounter analysis at the respective activation time point. (B) Log2FC of genes *FOXP3*, *IL2RA*, *IKZF2*, *LRRC32*, *CTLA4*, *CSF2RB*, *LGALS3*, *CD80*, *RTKN2*, *LAYN*, *UTS2*, *CCR4*, *ENTPD1*, *GPR55*, *IFNG*, *NKG7*, *IL7R*, *ENCI*, *CD40LG* and *ZEB2* uniquely expressed in CB, sorted Tregs and Tconvs shown by RNA-seq and nCounter analysis. Each dot represents paired (Tregs and Tconvs) log fold change for the respective gene from an individual CB and PB sample.

	CB 0hr (n=5)	PB 0hr (n=6)	PB6hr (n=3)	PB 24hr (n=6)
CB 0hr (n=5)	1.00	0.90	0.84	0.90
PB 0hr (n=6)	0.90	1.00	0.81	0.87
PB6hr (n=3)	0.84	0.81	1.00	0.88
PB 24hr (n=8)	0.90	0.87	0.88	1.00

Fig. 3.

Gene signature correlation among donors by nCounter analysis. Heat map matrix plotted as Spearman non-parametric correlation (r^2 value) of 136 genes validated by nCounter analysis for Log2FC of sorted Tregs and Tconvs from CB (n=5) and PB freshly isolated (0hr, n=6), 6hr activated (n=3), and 24hr activated (n=8) cells. Detailed p values and r values for each population are provided in Supplementary Table 6.

Fig. 4.

Loss of *RTKN2* or *LAYN* in Tregs partially enhances Treg suppressor function. (A) Schematic representation of the protocol used for siRNA transfection of *RTKN2* and *LAYN* in fresh-sorted Tregs. (B) Relative expression of *RTKN2* and *LAYN* normalized by *HPRT1* using qRT-PCR in Tregs transfected by three different siRNA oligos (300nM) for each *RTKN2* and *LAYN* 48hr after transfection. (C) Treg suppression assay performed by titrating Tregs with a fixed number of Tconv; irradiated PBMCs and soluble anti-CD3 were added to all samples. ³H-Thymidine incorporation was determined after 72hr of culture. Four different sets of Tregs (Tregs alone, Tregs with non-specific siRNA, Treg-*LAYN* siRNA and Treg-*RTKN2* siRNA) were used. Results are representative of three different experiments. Statistical significance calculated between unaltered Tregs and indicated groups by paired t test, * represents $p < 0.05$, **represent $p < 0.005$. (D) Percentage suppression of CD86 mean fluorescence intensity on CD11c gated mouse DC following co-culture with human Tregs. Co-culture of Tregs with anti-CD11a reversed suppression of CD86 expression by Tregs.

Fig. 5.

Overexpression of *RTKN2* or *LAYN* does not confer suppressive functions to Tconvs. (A) Schematic representation of protocol used for retroviral overexpression of *RTKN2* and *LAYN* in fresh-sorted Tconvs. (B) Relative expression of *RTKN2* and *LAYN* normalized by *HPRT1* using qRT-PCR in Tconvs. (C) Treg suppression assay as described in Fig. 4. Four different sets of cells (Tregs alone, Tconvs GFP-ve, Tconvs-*LAYN* and Tconvs-*RTKN2*) were used. Results are representative of three different experiments.

Figure:6 A Tregs miRNA signature by Taqman low density array

Figure:6B Validations of DE miRNA

Fig. 6.

Tregs miRNA signature by Taqman low-density array. (A) Heat map for microarray of selected miRNAs expressed by unbiased clustering from multiple HD sorted for Tregs (n=6) and Tconvs (n=4). Red shows positive expression and green shows negative expression compared to relative total expression. (B) Relative expression of human miR-146a, miR-146b, Let7g, miR-21, miR-660, miR-598, miR-10a and miR-125a by qRT-PCR of sorted Tregs (n=6) and Tconvs (n=4). miRNA expressions were normalized to U6 snRNA levels from respective samples. Statistical significance was determined using paired two-tailed students's t test, * represents $p < 0.05$.

Fig. 7.

PNA inhibition of miRNA function in Tregs. (A) Schematic representation of the protocol used for PNA inhibition for miR-146 combined (miR-146a + miR-146b) or miR-21 in fresh-sorted Tregs. (B) Relative expression of miR-146a, miR-146b and miR-21 normalized by snU6 using qRT-PCR after inhibitions either in combined PNA miR-146 or PNA miR-21 sets. (C) Treg suppression assay as described in Fig. 4. Three different sets of Tregs (Treg-PNA-scrambled miRNA, Treg-PNA-miR-21 and Treg-PNA-miR146a and b) were used. (D) Percentage suppression of CD86 mean fluorescence intensity on CD11c gated mouse dendritic cells in co-cultures with Tregs. Co-culture of Tregs with anti-CD11a reversed Tregs suppression of CD86 expression.

are preferentially expressed in Tregs, while mRNAs and miRNAs in green are preferentially expressed in Tconvs.

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