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Transcriptome analysis of the Th17/Treg axis reveals multiple pathways that ensure distinct differentiation patterns

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The Th17/ Treg axis is a crucial regulator of anti-inflammatory and proinflammatory pathways that play fundamental roles in cancer and autoimmune diseases. The alteration of the Th17/Tregs axis is highly significant for developing novel therapeutic approaches. Th17 and Treg cells share a large portion of their transcriptome, albeit with distinct functions. The mechanisms governing the bifurcation of Th17 /Treg axis differentiation from naïve T cells are not yet understood. This study aims to identify the differentially expressed genes (DEGs) and gene-enriched pathways that contribute to the distinction between these closely aligned phenotypes. We analyzed RNA sequencing data of CD4+ T cells differentiated in vitro into Th0, Th17, and Tregs phenotypes and built gene enrichment networks. We studied the network rewiring of Th17 and Tregs from Th0 cells. In addition to common pathways and genes that are equally expressed between these two distinct phenotypes, we identified key genes and pathways that contribute to their distinctiveness.

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For example, the Keap1 gene and several of its interactors such as MCC1 were upregulated in Tregs indicating that Tregs possess mechanisms that prevent the over-activation of antioxidants pathways. Tregs also possess several genes that are involved in the hydrolysis of ATP to ADP as RAB37, ATP1A2, ATP1B, and PDE2A to ensure Treg anti-inflammatory capabilities. Additionally, one of the pathways that ensure Tregs differentiation and function seems to point toward an insulin role mediated by APOL9B and GOLM. Taken together our findings shed light on various essential regulators of the Th17/Treg axis and pave the way for treatment strategies that selectively target one phenotype while sparing the other.

KEY WORDS: Th17 cells / Treg cells / differentiation

The Th17/Treg axis plays multiple roles in various pathologies [Kubick *et al.* 2020, Mickael *et al*. 2021, Bhaumik *et al*. 2023]. Th17 can take a pro-inflammatory phenotype (e.g., Th1-like), where they produce IFNγ and IL1β [Mickael *et al*. 2022, Bhaumik *et al*. 2021]. This proinflammatory phenotype is supportive of auto-immune disease development. For example, we have found that in the gut-brain axis, Th17 cells from the gut can infiltrate the brain where they cause over-activation of astrocytes, demyelination, and support the appearance of lesion-like structures [Mickael *et al.* 2021, 2022]. This pattern is also mirrored in other diseases where Th17(Th1-like) cells have been shown to increase inflammation in multiple scoliosis, Alzheimer's diseases, depression, and colitis. Tregs are capable of inhibiting Th17 and creating more favorable anti-inflammatory conditions [Bhaumik *et al.* 2017]. Treg therapy is gaining momentum in the last decade where using Treg has been shown to alleviate at least in part the proinflammatory effect of Th17. Conversely, in cancer immune therapy, the effect of inhibiting Th17 by Tregs has been shown to negatively influence cancer survival rates. Thus, regulating the Th17/Treg axis has become one of the forefronts of fighting several diseases [Bhaumik *et al*. 2021].

Th17 and Treg share a large section of their transcriptome. Although Tregs and Th17 cells fundamentally differ in function, they also share several characteristics [Omenetti *et al*. 2015]. Th17 and Tregs seem to be highly co-localized in the periphery, especially in the intestine. They also share several lineage-specific transcription factors, including RORγt and Foxp3, that were thought to be mutually exclusive. We and others have shown the existence of other intermediate phenotypes, such as RORγt Foxp3+ Tregs [Bhaumik *et al*. 2021]. Th17/Tregs also share specific cytokines that support their differentiation [Kubick *et al*. 2021]. For example, TGFβ is necessary for both cell types' induction. High concertation of Transforming growth factor beta (TGFβ) is required for the generation of Tregs. However, at low concentrations, TGFβ acts in combination with IL6 and IL21 to promote IL23 receptor (IL23R) expression, supporting Th17 polarization [Ivanov *et al*. 2006, Korn *et al*. 2007, Zhou *et al.* 2008, Das *et al.* 2009]. In the case of differentiating naïve T cells under IL1B, IL6, and TGFβ conditions, the resulting CD4+ T cells have been reported to have a highly proinflammatory phenotype known as Th17 (Th1-like cells). IL6 downstream targets include STAT3. IL1 supports Th17 polarization by activating p38 mitogen-activated protein kinase (MAPK) and the Akt/mTOR pathway. IL1 also induces IRF4, which

both activates RORc and STAT3 and enhances IL21 production. Several other proteins can also influence the balance between Th17 and Tregs [Mucida *et al*. 2007]. For example, the aryl hydrocarbon receptor (AhR), which is expressed by both Th17 and Tregs, can promote the induction of both cell types based on environmental stimuli.

In this report, we used a data analysis approach for alienating the networks controlling Th17 cells and Tregs differentiation. To that end, we analyzed bulk RNA-seq of CD4+ T cells differentiated in vitro under Th0, pathogenic Th17, and Tregs conditions. We investigated network rewiring to investigate the unique pathways that ensure the uniqueness of the Th17 and Tregs phenotypes. From these phenotypes, MCC1, a previously unknown regulator of Treg activation, seems to be performing a distinct function in inhibiting Th17 cells induction. We also discovered that the enrichment of purinergic metabolism that aims to hydrolyze Inflammatory ATP to anti-inflammatory ADP is a key hallmark of Treg differentiation. Our investigations also showed that Treg differentiation is associated with several anti-inflammatory pathways such as nicotine, and oxytocin.

Material and methods

We analyzed the Th17/Treg axis by comparing a bulk RNA-seq data set to pinpoint the key regulators of Th17 and Treg that ensure the stability of their phenotypes. To that end, we examined the public data (GSE140443) which covers the differentiation of naive T cells (Th0) into Th17 and Tregs in vitro after 3 days. Then, we built pathway enrichment networks by utilizing GeneTrail and Bingo. We studied the wiring of enriched pathways from Th0 to either Th17 and Tregs using Dynet in Cytoscape. This approach allowed us to dissect the key pathways behind the uniqueness of these two cell types (e.g., Th17 vs Treg).

Data and data expression analysis

A transcriptomic dataset "Transcriptome analysis of WT and TRIM28 KO CD4 T cells, naïve or stimulated with anti-CD3 (plate-bound) and anti-CD28 (soluble) in Th0, Th1, Th2, Th17 or Treg conditions" (GSE140443), was obtained from Gene Expression Omnibus [Gehrmann *et al*., 2019]including helper and regulatory cells (Th and Treg, respectively. Flow cytometry was used to separate naive mice CD4 T cells, which were then stimulated for 72 hours with anti-CD3 (plate-bound) and anti-CD28 (soluble) antibodies in Th17 or Treg conditions. T cell differentiation tests. Th17: 10ng/ml IL1b, 20ng/ml IL6, 1ng/ml TGFβ1, 10ug/ml IFN, 10ug/ml IL4; Treg: 10ng/ml TGFβ1, 100ug/ml rhIL2, 10ug/ml IFN, 10ug/ml IL4. Microarray data were processed using R (version 3.1.0) and Bioconductor libraries. The raw data was preprocessed using the oligo package's RMA (Robust Multi-array Average) approach. The linear models for microarray data (Limma) R package analyzes gene expression data derived from experiments such as microarrays and RNA-seq. The Limma R package was used to find the genes that are differentially expressed (DEGs) [Kubick *et al*. 2020, where

patients suffer from pathological disturbances associated with a proinflammatory M1 macrophage phenotype. Presently, the most commonly used antidepressants such as Zoloft and Citalopram can reduce inflammation, but suffer from dangerous side effects without offering specificity toward macrophages. We employed a new strategy for drug repurposing based on the integration of RNA-seq analysis and text mining using deep neural networks. Our system employs a Google semantic AI universal encoder to compute sentences embedding. Sentences similarity is calculated using a sorting function to identify drug compounds. Then sentence relevance is computed using a custom-built convolution differential network. Our system highlighted the NRF2 pathway as a critical drug target to reprogram M1 macrophage response toward an anti-inflammatory profile [M2 Ritchie *et al*. 2015] limma has been a popular choice for gene discovery through differential expression analyses of microarray and high-throughput PCR data. The package contains particularly strong facilities for reading, normalizing and exploring such data. Recently, the capabilities of limma have been significantly expanded in two important directions. First, the package can now perform both differential expression and differential splicing analyses of RNA sequencing (RNA-seq. Limma using the adjusted p-value was also performed. We also built Self-organizing maps using the Kohonen library in R [Wehrens *et al*. 2007].

Investigation of Molecular Pathways Related to Th17/ Treg differentiation

We employed two different algorithms to investigate pathways upregulated in three sets (i) Th17/Th0 (ii) Treg/Th0 and (iii) Th17/Treg namely GeneTrial3 and Bingo [Maere *et al*. 2005, Minadakis *et al*. 2019, Gerstner *et al*. 2020]. First, the statistically significant DEGs were used for gene set enrichment analysis (GSEA) based on gene ontology biological process using the GeneTrail3 software using GO (Biological Process, Cellular Component, and Molecular Function), KEGG (Pathways), Reactome (Pathways) and WikiPathways databases. In this investigation, upregulated genes from each set were uploaded to the server. Gene enrichment analysis was done using the over-representation. Over-representation analysis (ORA) is a tool for determining whether a set of known or suspected biologically relevant variables, such as a gene set or pathway, is more prominent in a set of variables of interest than we would anticipate by chance [Pomyen *et al.* 2015]. The default setting for the ORA was used including the Benjamini Yekutieli which was used to control the False discovery rate. A dependency wheel was used to visualize the results, different enriched pathways are laid as nodes of a circle. The size of the nodes and the width of links between them is proportional to the flow quantity or weight of each link. Secondly, we applied BiNGO in Cytoscape to identify pathways that are over represented from our investigated datasets. BiNGO is implemented as a plugin for Cytoscape, which is an open-source bioinformatics software platform for visualizing and integrating molecular interaction networks [Shannon *et a*l. 2003].

Pathways Re-Wiring

The network rewiring approach identifies and visualizes how molecular interaction networks change in connectivity and composition in response to cellular signals. DyNet is a Cytoscape plug-in that performs network rewiring [Shannon *et al*. 2003]. DynNet allows for the visualization and analysis of large-scale multi-state dynamics molecular interaction networks [Goenawan *et al*. 2016]. We employed the pairwise analysis mode that compares two sets of networks. The input for Dynet was the network produced by Bingo for two main cases (i) Treg/Tho vs Th17/Th0. (ii) Treg (enriched pathways) vs Th17 (enriched pathways). This analysis mode aids in the visualization of node and edge variations based on their presence, absence, or the value of a selected numeric attribute, such as node abundance and edge weight. When comparing numeric attributes between the two biological networks in the pairwise mode, DyNet calculates the log2 fold change of the numeric attribute.

Results and discussion

We performed differential gene expression using Limma in R, between different subsets Treg/Th0, Th17/Th0, and Th17/Tregs (Fig. 1). Overall the number of upregulated genes in Tregs compared to Th0 was 307 genes (log2>1.4), while for Th17 vs Th0, it was 697. In the case of Th17 and Treg analysis, a lower number of genes was upregulated in Treg (e.g., 599) in comparison to Th17 (e.g., 1077 genes). We discovered several transcription factors, such as FoxP1, in addition to FoxP3, were upregulated in Tregs. We also found several cytokines and cytokine receptors, such as IL3 and IL10RA. Additionally, our analysis revealed the upregulation of multiple CD-associated genes, including CD7, CD96, CD46M, CD55M, CD200RM, CD27, CD40, and CD97. One of the interesting genes that we discovered to be upregulated in Tregs is MCC1 which seems to be particularly upregulated in all the combinations we investigated. On the other hand, we found several cytokines upregulated in the Th17 set but not in Tregs, such as the well-known IL21, IL17F, IL12RRB2, IL17A, and IL9. Several CD genes were also upregulated in Th17, such as CD109, CDD74, and CD80. Numerous chemokines and chemokine receptors were upregulated in the Th17 groups, such as CCR5, CCR2, CCL20, CCL5, and CCL22. These results were reciprocated in the Th17/Th0 and Treg/Th0 groups with few exceptions. Paradoxically, IL6Ra, the main receptor of IL6 was not upregulated in the Th17 vs Th0 but slightly upregulated in Th17/Tregs (log2 FC of 1.3 and adjusted p-value of 0.0001). Additionally, STAT3 one of the main downstream targets of IL6 was not upregulated in Th17 as expected. Conversely, IL1R2 was upregulated in the Th17 groups investigated, together with its downstream targets such as IRF4, NFAT5, and BATF3. Additionally, while AHR plays an important role in Th17, our results seem to show it is performing a considerable role in Treg as its fold change value in Tregs/Th0 was 2.3, while in Th17/Th0 3.3. AHR was not significantly differentially expressed between Th17 and Tregs.

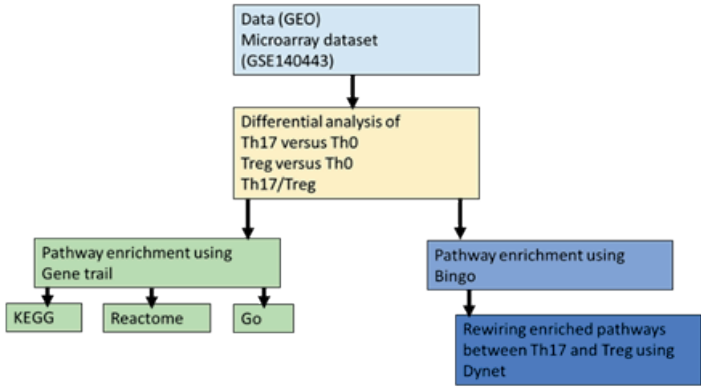

Fig. 1. Workflow of the analysis. We investigated the principle genes controlling the Th17/Treg axis by analyzing a key public data repository (e.g., GSE 140443). This dataset includes the microarray sequencing results of three different datasets, namely Th17, Treg, and Th0, with each dataset consisting of three samples. We computed differentially expressed genes by comparing various combinations of this data in R. Then, we used GeneTrail and Bingo to explore enriched pathways in each comparison. Following that, we constructed rewiring networks that explored the progress of Th0 toward Th17 and Tregs and investigated the rewiring between Th17 and Tregs.

Using Gene Trail, we found that the number of enriched Reatcome processes for Treg compared with Th0 was 21 processes, while for Th17 it was 52 processes. Of these processes that are upregulated in Tregs 10 were also common in Th17. This observation confirms the similarity of the transcriptomes profile of Th17 and Treg. However, several significant differences still exist between the two cell groups. One of the strongest examples is the upregulation of nicotine metabolism in Tregs hinting at a role of nicotine in Treg differentiation (Fig. 3). The main gene that is associated with nicotine metabolism is CD38, with a p-value< 1.396e-3. Another important pathway that is uniquely in Tregs but not Th17 is Insulin-like Growth Factor regulation and is primarily mediated by APOL9B (p-value < - 3.278e-2) and GOLM1(p-value < - 3.278e-2). Moreover, another pathway that is especially upregulated in Tregs is oxytocin. Interestingly morphine associated pathways were also upregulated in the Treg KEGG pathways analysis but not in Th17.

We compared upregulated genes' molecular functional ontology between different populations. We used Bingo implemented in Cytoscape using genes upregulated in each dataset investigated (log2>2) using two approaches (i) Th17/Th0 versus Treg/Th0 and (ii) Th17/Treg. In the case of the first comparison, Treg had 112 pathways, while genes upregulated in Th17 had 140 pathways (Fig. 4). The number of pathways that was common among Th17 and Treg was 28 pathway. One of the most important pathways that seems to be different between Treg and Th17 is the calcium pathways, where the gene GLP1R which is known to increase intracellular free calcium concentration is upregulated in Tregs [Gromada *et al*. 1995]. The gene XCL1, which has been linked to increased intracellular calcium in peripheral blood lymphocytes, is also upregulated in

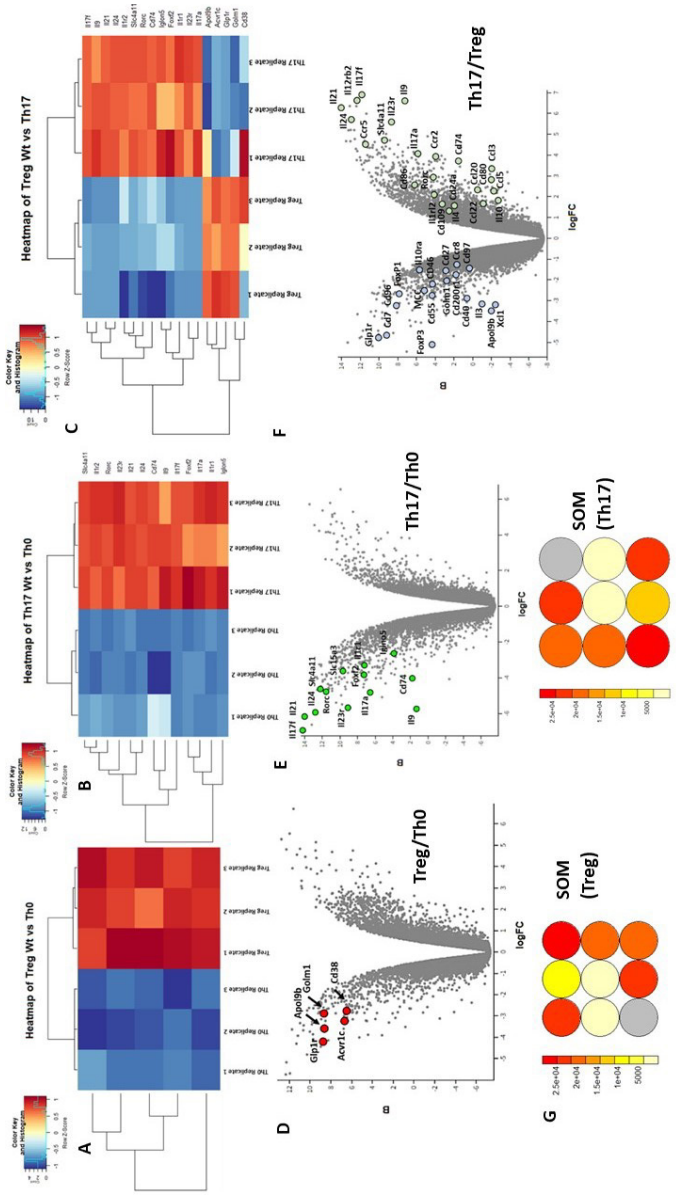

Fig. 2. Transcriptome analysis of Th17, Treg, and Th0. Data was downloaded from GO for a single experiment setting (GSE140443). All the analyses were done using the 72 hours post-seeding datasets. A and D) Microarray analysis of Tregs and Th0. B and E) Microarray analysis of differentially expressed between Th17 and Th0. C and F) Analysis of Tregs versus Th17 microarray at 72 hours. G and H) We have built self-organizing maps (SOM) comparing Th17 and Treg datasets. We found a significant degree of mutual exclusivity between the Th17 and Tregs respective datasets.

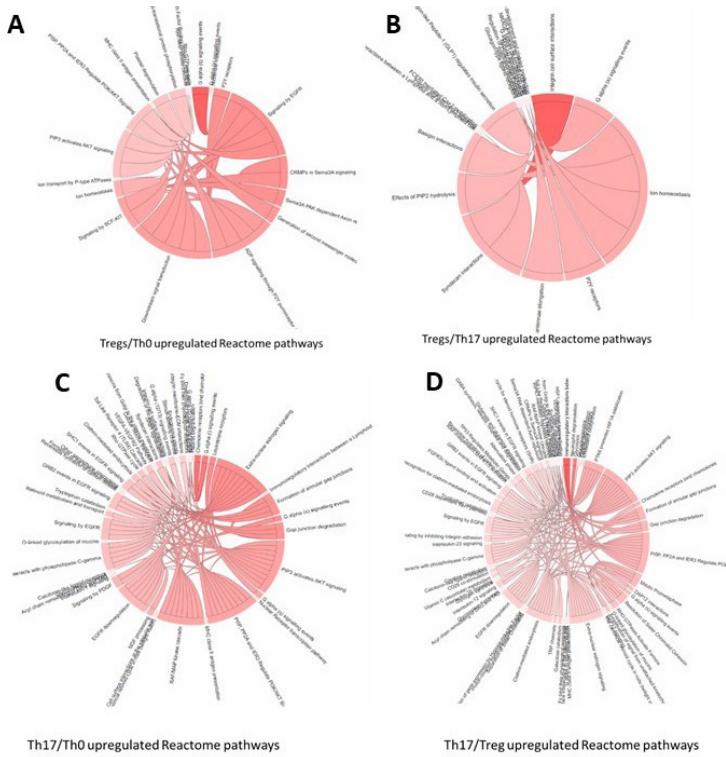

Fig. 3. Cross comparison between different Th17/Treg analyses. A) Treg/Th0 B) Treg/Th17 C) Th17/Th0 c) and d) Th17/Treg. Overall high similarity between each Treg and Th17 respective comparison. For example, Treg/Th0 and Treg/Th17 share G alpha signaling effect, P2Y receptors pathways, and ion homeostasis. Similarly in case of Th17/Th0 and Th17/Tregs, PIP3 activation pathways, EGFR-associated signaling, and formulations of annular gap junctions.

Tregs. On the other hand calcium pathways upregulated in Th17 include a pathway that aims to increase calcium ion concentration which are involved in G-protein signaling coupled to IP3 second messenger pathway. This is done by the upregulations of two genes PLCD1 and TGM2. PLCD1 hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). It has been shown that Treg is capable of inhibiting this pathway. Also upregulated in the Th17 group but not in the Treg group is TGM2 (transglutaminase 2). TGM2 catalyzes cross-linking of extracellular matrix proteins such as FN1 and SPP1, leading to scaffold formation [Mian *et al*. 1995]. Another pathway that is also different between Tregs and Th17 is the regulation of insulin secretion and this is primarily shown through the upregulations of two genes namely ACVR1C and ABCC8 in Tregs but not Th17. Interestingly, ACVR1C is a type I receptor for the TGFβ family of signaling molecules. In particular, ACVR1C transduces signals of Nodal. Nodal binds to ACVR2B and

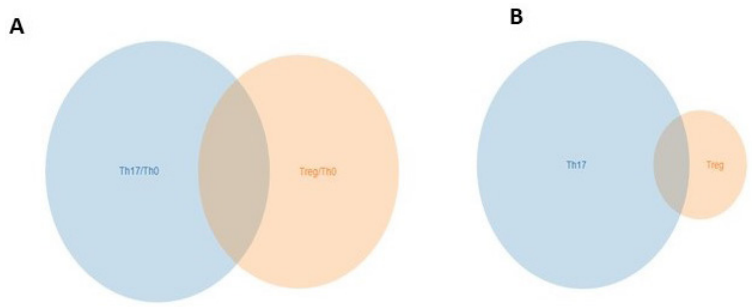

Fig. 4. Bingo results reveal a complex landscape of pathway enrichment among Th17 and Treg datasets. A) comparison of Th17/Th0 versus Treg/Th0 B) comparison of The Th17/Treg pathways. High similarity between Th17/Th0 and Th17/Treg with 102 common enrichment pathways. Similarly, most of the upregulated pathways in the Treg/Th17 dataset were also upregulated in Treg/Th0 (e.g., 24 pathways). However, Treg/Th0 was far larger than Treg/Th17 with an additional 86 pathways. This might indicate that while there is full concurrency between Th17 genes needed for differentiation and functionalization, this might not be the case for Treg.

ACVR1C to recruit SMAD2 or SMAD3. SMADs regulate insulin secretion. Notably, GLP1R and ACVR1C play an important role in response to glucose stimulus. Both genes are upregulated in Treg but not Th17 when compared with Th0. Moreover, one of the upregulated genes in Treg but not in Th17 is IL3. IL3 is known to play an important role in mast cell proliferation and it has been recently shown to inhibit Th17 differentiation [Rani *et al*. 2022]. Thus, Th17 differentiation must possess a mechanism that inhibits IL3 [Rani *et al*. 2022]. IL3 pathway was also unique when comparing the Th17/Treg datasets. Comparing Th17/Treg revealed that Th17 enriched molecular pathways are 196. Of the Th17 specific pathways, 12 at least are also repeated in Tregs. Tregs' unique pathways are 22. Other pathways that were upregulated in Tregs and not in Th17 in this specific comparison include the IL33 pathway and its receptor. Purinergic nucleotide receptor activity was also highlighted in Tregs but not in Th17. On the other hand, various pathways were upregulated in th17 including cytokines pathways such as IL1. Additionally, known pathways were also upregulated such as retinoid acid, vitamin A, chemokines bindings, and various other pathways. This might include that at 72 hours, Th17 cells are fully functional. Interestingly, we observed a difference between pathways upregulated in Treg/Th0 in comparison with Treg/Th17. These pathways could be playing a more significant role in Treg differentiation. These pathways include semaphorin receptor activity, ceramide kinase activity, glutamine-fructose-6-phosphate transaminase (isomerizing) activity, ciliary neurotrophic factor receptor activity, and xylosyltransferase activity among others.

We conducted pathway network rewiring analysis using the Dynet plugin in Cytoscape. The input for this analysis was the enriched pathways for each respective analyzed group. We performed two main analyses (i) Treg/Th17 and (ii)Treg/Th0 vs Th17/Th0. The first analysis showed several common pathways that Th17 and Tregs share. These pathways include metabolism regulation, cell cycle regulation response

to stress, signal transduction, and supporting organ development. On the other hand, the comparison between Th17/Th0 and Treg/Th0 revealed multiple specific pathways that were unique among Th17 and Tregs. For example, two pathways that seem to be enriched in Tregs are purine metabolism and cellular anion homeostasis. On the other hand, several specific Th17 pathways were unrevealed including retinoic acid and vitamin A processes, cell adhesion, and cellular amino acid catabolic processes.

We compared Th17 and Treg and Th0 after 72 hours of in vitro differentiation, using more than one combination. We and others have shown previously that Th17 and Tregs cells share a large section of their transcriptome as well as several intermediary phenotypes such as RORγt^{+} Tregs and proinflammatory Tregs [Mickael *et al.* 2021, Bhaumik *et al*. 2023, the time of Treg-mediated suppression mechanisms' emergence has not been identified. It is not yet known whether Treg suppression mechanisms diverged from a single pathway or converged from several sources. We investigated the evolutionary history of Treg suppression pathways using various phylogenetic analysis tools. To ensure the conservation of function for investigated proteins, we augmented our study using nonhomology-based methods to predict protein functions among various investigated species and mined the literature for experimental evidence of functional convergence. Our results indicate that a minority of Treg suppressor mechanisms could be homologs of ancient conserved pathways. For example, CD73, an enzymatic pathway known to play an essential role in invertebrates, is highly conserved between invertebrates and vertebrates, with no evidence of positive selection ($w = 0.48$, p-value < 0.00001 Bhaumik *et al*. 2021]. However, the function of conventional FoxP3+Tregs and pathogenic Th17 seems to be mutually exclusive. The main question we were aiming to answer is: what are the sets of genes that ensure the specificity of the phenotype of Tregs and Th17? Interestingly it seems that the number of genes upregulated in the case of Tregs is half of those upregulated in the case of Th17 (Fig. 2). This observation could be attributed to the in vitro nature of the experiment. In addition to anti IFNγ and anti IL4 that were added to suppress Th1 and Th2 phenotypes, TGFβ and IL2 were added to naïve T cells to acquire a Treg phenotype. In the case of Th17 conditions, a similar set of cytokines were used albeit with higher concertation of TGFβ as well as the addition of IL1 and IL6. However, we did not find IL6RA upregulated in Th17 compared to Treg or Th0. Similarly, IL6RA downstream target STAT3 did not seem to be upregulated. However, IL1R and its downstream targets including IRF4, BATF, and RORα, were upregulated in Th17 (Fig. 2). Intriguingly IL6 is known to be fundamental for Th17 and function [Bhaumik *et al*. 2021]. One of the answers to this riddle would be in the dynamic nature of Th0 to Th17 differentiation, specifically, IL6R upregulation could be a function in time. However, time-set analysis studies would be needed to confirm this hypothesis.

Our findings indicate that nicotine might be causing an increase in Treg differentiation (Fig. 3). There seems to be an agreement with previous reports that suggest that nicotine could be playing an important role in stabilizing Treg phenotype and inhibiting th17 phenotypes [Galitovskiy *et al*. 2011, Zhang *et al*. 2022,]. The role

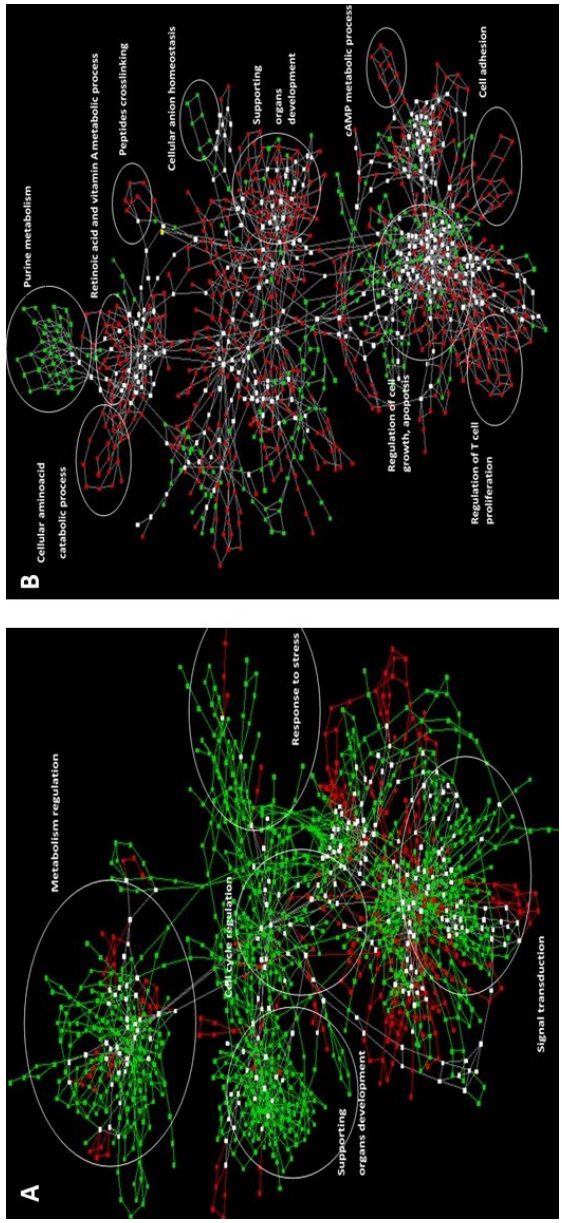

Fig. 5. Network rewiring between Th17 and Treg. We performed two network rewiring analyses using Dynet in Cytoscape A) Th17verus Treg B) Treg/Th0 versus Th17/Th0. Our analysis shows multiple pathways that are enriched equally between Th17 and Tregs including cell cycle regulation, and metabolism regulation among others. However, it also shows specific enriched pathways such as purine metabolism in the case of Tregs and cell adhesion in the case of Th17.

of Th17 in Parkinson's disease is still in its infancy; however, it seems that Th17 could be increasing brain inflammation by infiltrating the brain during the disease. Tregs, on the other hand, have been proposed to improve Parkinson's disease prognosis. Interestingly, nicotine intake constitutes a lower risk of developing Parkinson's disease but not Alzheimer's disease [Nicholatos *et al.* 2018]. The reason for this observation could be related to the complexity of these two sibling diseases as well as the disease's stage and amount of nicotine. Future research analyzing the excretory nicotine concentration for optimal Treg differentiation in vitro and in vivo could be important to shed more light on this point.

Our results highlighted the uniqueness of several Tregs specifically enriched pathways such as purine metabolism (Fig. 5). One of the main molecules regulating intracellular energy homeostasis and nucleotide synthesis are purines and their derivatives, in particular adenosine and ATP [Huang *et al*. 2021]. This specific pathway is enriched based on the upregulation of various genes including RAB37, ATP1A2, ATP1B, and PDE2A. These genes seem to contribute to regulating the balance between proinflammatory ATP and anti-inflammatory adenosine. For example, ATP1A2 is a component of an active enzyme, which catalyzes the hydrolysis of ATP. Similarly, we observed that Treg cells express high levels of the ectonucleotidases CD39 and CD73 (Fig. 2). To reduce the negative effects of extracellular ATP and enhance suppressive function, CD39 and CD73 convert excessive amounts of ATP into immunosuppressive adenosine [Shi *et al*. 2019]. This suggests that purine metabolism driving the balance between ATP and adenosine is playing a crucial role in regulating immune responses and manipulating this balance could have therapeutic potential in boasting Treg function in inflammatory diseases.

Conclusions

One of the main intriguing aspects behind the Th17/Tregs axis is the close similarity in the transcriptome of both cells. Network rewiring analysis revealed that they share many enriched pathways. However, we were also able to identify key features that promote Treg uniqueness such as purine metabolism, ATP hydrolysis, enrichment of oxytocin, and nicotine among other previously not known pathways. Our analysis also hints toward a dynamic role for IL6 in the differentiation of Th17. Additional analysis of the two cell transcriptomes at different time points pre the 72-hour threshold would be beneficial to decipher the development of the unique pathways identified in this report.

Conflict of interest

The authors declare no conflicts of interest.

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