

ORIGINAL ARTICLE

Upregulation of miR-9 and miR-193b over human Th17 cell differentiation

Fahimeh Shirani¹ | Masoud Baghi^{1,2} | Mahsa Rostamian Delavar² |
Alireza Shoaraye Nejadi¹ | Amir Eshaghiyan³ | Mohammad Hossein Nasr-Esfahani¹ |
Maryam Peymani^{1,4}  | Kamran Ghaedi² 

¹Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

²Department of Cell and Molecular Biology and Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran

³Department of Genetics, Arsanjan Branch, Islamic Azad University, Arsanjan, Shiraz, Iran

⁴Department of Biology, Faculty of Basic Sciences, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

Correspondence

Kamran Ghaedi, Department of Cell and Molecular Biology and Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Hezar Jerib Ave., Azadi Sq., P.O. Code 81746-73441, Isfahan, Iran.
Email: kamranghaedi@sci.ui.ac.ir

Funding information

National Institute for Medical Research Development, Grant/Award Number: NIMAD's project no. 942792

Abstract

Background: Th17 cells are a newly discovered subset of CD4⁺ T cells known as key participants in various immune responses and inflammatory conditions including autoimmune diseases. Mi(cro)RNAs are a family of non-coding RNAs that regulate numerous critical immune functions. Immuno-miRNAs modulate cell biological processes in T cells, such as differentiation and function of Th17 cells. The aim of the present study is to investigate the expression of miR-9-5p, miR-193b-3p, and autoimmunity-related genes during human Th17 cells differentiation.

Methods: Human naïve CD4⁺ T cells were purified from peripheral blood mononuclear cells (PBMCs) by magnetic cell sorting system (MACS) and their purity was checked by flow-cytometric analysis. Naïve CD4⁺ T cells were cultured under Th17-polarizing condition for 6 days. IL-17 secretion was determined by means of enzyme-linked immunosorbent assay (ELISA). Next, the expression levels of miRNAs and putative targets genes were assessed by qRT-PCR at different time points of differentiation.

Results: Our result showed dramatic downregulation of TCF7, MAP3K1, ENTPD1, and NT5E genes during human Th17 differentiation. Polarization also had a significant inducible effect on the expression of miR-9 and miR-193b over differentiation of human Th17 cells. According to our results, miR-9-5p and miR-193b-3p may contribute to Th17 differentiation probably by inhibiting the expression of negative regulators of Th17 differentiation.

Conclusion: This study confirmed deregulation of TCF7, MAP3K1, ENTPD1, and NT5E genes in Th17 differentiation process and introduced miR-9 and miR-193b as Th17 cell-associated miRNAs, making them good candidates for further investigations.

KEYWORDS

autoimmune diseases, microRNAs, miR-193b-3p, miR-9-5p, T helper 17 cells

Masoud Baghi and Mahsa Rostamian Delavar contributed equally to this article.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2020 The Authors. *Molecular Genetics & Genomic Medicine* published by Wiley Periodicals LLC.

1 | INTRODUCTION

The immune system triggers defensive responses following any infection or injury and preserves homeostasis by recruiting an integrated network of innate and adaptive immune cells under normal physiological circumstances (Antonioli et al., 2013; Crimeen-Irwin et al., 2005). Although the immune system is a strictly regulated network, its inappropriate activation results in development of disparate pathophysiological conditions such as autoimmunity, allergic diseases, and tissue damage (Antonioli et al., 2013; Crimeen-Irwin et al., 2005). As an indispensable part of immune system, naïve T cells are capable of differentiating into several subsets of T helpers including Th1, Th2, as well as Th17 in response to antigen stimulation. Decreased or increased potential for a particular subtype's forming can culminate in immunodeficiency or autoimmunity since T helper subsets have specific, sometimes opposite functions (Ma et al., 2011). Th17 is an effective lineage of pro-inflammatory T helpers differentiated from naïve CD4⁺ T cells characterized by secreting distinct inflammatory cytokines such as Interleukin (IL)-17 (Anwar, 2013; Honardoost et al., 2015; Zhang, et al., 2018). Th17 cells can be generated *in vitro* by activating naïve CD4⁺ T cells in the presence of transforming growth factor-beta (TGF- β), IL-6, and IL-23 cytokines over a matter of days (Majd et al., 2018; Montoya et al., 2017). Whereas Th17 cells protect the host against bacterial and fungal infections, inappropriately exaggerated Th17 response is closely associated with development of several autoimmune inflammatory disorders, including multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel disease (IBD), as well as experimental allergic encephalomyelitis (EAE). (Honardoost et al., 2015; Majd et al., 2018; Zhang, et al., 2018; Zhang, et al., 2018). Accordingly, considerable research is being devoted to elucidate the precise molecular mechanisms and signaling pathways inducing pathogenic Th17 differentiation in the hope of finding the best therapeutic targets for suppressing Th17 cell-associated autoimmune inflammation (Honardoost et al., 2015; Zhang, et al., 2018).

T cell factor 1 (TCF-1), also known as TCF7 (gene name), is a transcription factor enriched in hematopoietic T cells which plays a crucial role in both T cell development and differentiation (Ma et al., 2011; Mazzola et al., 2015). TCF-1 promotes Th2 differentiation, while Th1 and Th17 differentiation are negatively regulated by TCF-1 (Mazzola et al., 2015). IL-17 gene locus is maintained epigenetically in repressive state by TCF-1 to restrain Th17 responses and that's why TCF-7 deletion culminates in enhanced Th17 differentiation (Ma et al., 2011; Zhang, et al., 2018). Signal transduction by mitogen-activated protein kinases (MAPKs) emerged as a potential mechanism of regulation for T lymphocyte development and effector responses (Anwar, 2013). Increasing number of studies have revealed the implication of MAP3K1 in

Th17 cell signaling regulation and IL-17 expression (Anwar, 2013). MEKK1, encoded by the MAP3K1, also regulates cell cycle inhibitor genes such as Cdkn1b over Th17 differentiation process (Suddason & Gallagher, 2016). According to (Suddason & Gallagher, 2016) MAP3K1 deletion in T cells leads to increased IL-17 production while differentiating to Th17. CD39 is an immune system enzyme hydrolyzing extracellular ATP or ADP to AMP which is expressed on cells of both the innate and adaptive immune systems, including various T cells subtypes (Borsellino et al., 2007; Friedman et al., 2009). CD73 is another ectonucleotidase present on the lymphocytes' surface which converts AMP, the product of CD39-mediated hydrolysis, to adenosine, a nucleoside with direct immunosuppressive impacts (Borsellino et al., 2007). Therefore, ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1) and 5'-nucleotidase ecto (NT5E) encoding CD39 and CD73, respectively, are considered to be immune suppressive genes preserving immune balance (Borsellino et al., 2007; Friedman et al., 2009). Disruption in the CD39/CD73 machinery has been shown to impair complex pathways underlying immune tolerance to self-antigens, resulting in the development of multiple autoimmune disorders (Antonioli et al., 2013).

Mi(cro)RNAs are single-stranded noncoding RNAs consisting of 21-23 nucleotides which regulate gene expression in a post-transcriptional manner. Through binding to the 3'-untranslated region (3'-UTR) of target mRNAs, miRNAs mostly suppress the expression of target genes (Baghi et al., 2020; Zhang, et al., 2018; Zhao et al., 2014). A growing body of research demonstrates that miRNAs are involved in the regulation of an extensive diversity of cellular processes such as cell cycle, differentiation, and apoptosis (Honardoost et al., 2015). Several studies linked aberrant expression of miRNAs to the pathogenesis of autoimmune diseases including MS, with a number of these miRNAs accounting for differentiation and pathogenesis of Th17 cells (Honardoost et al., 2015; Majd et al., 2018; Murugaiyan et al., 2015; Zhang, et al., 2018; Zhao et al., 2014). miR-9 is a highly conserved microRNA previously reported to be deregulated in inflammation and numerous autoimmune diseases including MS and IBD (Boldin & Baltimore, 2012; Honardoost et al., 2015; Majd et al., 2018). Altered expression of miR-193b has also been detected in some autoimmune diseases such as MS and psoriasis (Honardoost et al., 2015; Zhao et al., 2014).

Although the associations between miR-9-5p and miR-193b-3p with inflammation and autoimmune diseases have been identified, no studies have ever investigated their roles in human Th17 differentiation process. Therefore, we aimed to evaluate the expression of TCF7, MAP3K1, ENTPD1, and NT5E as well as their potential targeting miRNAs (miR-9 and miR-193b) during human Th17 differentiation to characterize novel deregulated miRNAs and autoimmunity-related genes involved in differentiation of human Th17 cells.

2 | MATERIALS AND METHODS

2.1 | In silico methods

A list of genes strongly related to autoimmune diseases was prepared using DisGeNET6.0 database (Piñero González et al., 2020). KEGG (Kanehisa et al., 2017), Reactome (Jassal et al., 2020), and NCI-Nature (Krupa et al., 2007) were employed to identify the important pathways associated with genes. Interactions of four selected genes with other critical genes (55 strongly associated genes with autoimmunity with gda score >0.2 obtained from DisGeNET) as well as Th17 differentiation genes (116 genes participating in Th17 cell differentiation identified through literature mining in our recent study (Teimuri et al., 2018)) were assessed by STRING 11 (Szklarczyk et al., 2019) and Cytoscape 3.7.2 (Shannon et al., 2003) software was used to visualize gene networks. Three databases miRWalk 3.0 (Sticht et al., 2018), TargetScan 7.1 (Agarwal et al., 2015), and miRmap (Vejnar et al., 2013) were recruited to predict miRNAs targeting MAP3K1, ENTPD1, NT5E, and TCF7 genes. Literature mining was performed to select miRNAs whose implication in immune responses and disorders has been shown by previous studies. In addition, miRNAs participating to autoimmunity were identified through using HMDD version 3.0 (Huang et al., 2019). Moreover, DIANA-miRPath v3.0 (Vlachos et al., 2015) was used to visualize the involvement of candidate miRNAs in various signaling pathways. Using TargetScan7.1 and miRmap, the potential binding sites of miR-9 and miR-193b within mRNAs 3'UTR were retrieved.

2.2 | Isolation of naïve CD4⁺ T Cells and Th17 differentiation

Peripheral blood mononuclear cells (PBMCs) were isolated from human whole blood by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich). PBMCs were washed twice and suspended in Hank's balanced salt solution (HBSS; Thermo-Fisher Scientific) to isolate CD4⁺ T cells, after which naïve CD4⁺ T cells were purified from PBMCs by magnetic cell sorting system (MACS; Miltenyi Biotech) according to the manufacturer's instructions. The culture plates were coated overnight with 5 μ g/ml anti-CD3 antibody (R&D Systems) at 2–8°C, and then, cells were cultured under Th17 cell-polarizing condition using the CellXVivo Human Th17 Cell Differentiation Kit (R&D Systems) at 37°C in a humidified atmosphere containing 5% CO₂ for 6 days (Hakemi et al., 2011, 2014). The protocol of study to use human samples was confirmed by both the Bioethics Committee of University of Isfahan and ROYAN institute review board under the bioethical code number: IR.ACECR.ROYAN.REC.1396.111.

2.3 | Flow cytometry

Single cell suspensions were prepared to perform surface staining of naïve CD4⁺ T cells. Naïve CD4⁺ T cells were incubated with PE-conjugated anti-human CD4, FITC-conjugated anti-human CD45RA, and Mouse IgG2b K Isotype control (BD Biosciences, Franklin Lakes, NJ) for 30 minutes at room temperature in the dark. Finally, the fluorescence intensity was quantified by FACSCalibur flow cytometer (Becton Dickinson) and results were analyzed using CellQuest Pro software.

2.4 | Enzyme-linked immunosorbent assay

Culture supernatants were collected 5 hours, 2 days, 4 days, and 6 days after suspending naïve CD4⁺ T cells in human Th17 differentiation media to check IL-17 cytokine content. Enzyme-linked immunosorbent assay (ELISA) was performed in duplicate using Quantikine ELISA Human IL-17 Immunoassay kit (R&D) according to the manufacturer's protocols. Optical density values were measured by ELISA microplate reader (Awareness) at a wavelength of 450 nm.

2.5 | Quantitative real-time PCR

Cells were collected on day 0, 2, 4, and 6 of differentiation and total RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer's instructions. Isolated RNAs were treated with DNaseI (Ferments) and their purity was evaluated by NanoDrop spectrometer (Biochrom WPA, Biowave). Complementary DNA (cDNA) synthesis for mRNAs was performed by using RT reagent Kit (Takara). Real-time PCR was carried out on ABI PRISM 7500 instrument (Applied Biosystems) employing specific primers synthesized by Macrogen Company, South Korea (Table S1). Gene expression levels were normalized to transcript amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control. Universal cDNA synthesis kit (Exiqon) was applied to synthesize cDNAs for miR-9 and miR-193b. miRNAs' expressions were quantified by SYBR green method using ABI PRISM 7500 instrument. U6 small nuclear RNA was considered as an internal reference to normalize miRNAs expression. All reactions were carried out in triplicate to confirm reproducibility of the results. Real-time PCR data were analyzed based on the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

2.6 | Statistical Analyses

Student's *t*-test was used to analyze the disparity between two means. The results are presented as mean \pm SEM of three

independent experiments and P values below 0.05 were considered to be statistically significant. All statistical analyses were conducted by GraphPad Prism 8 software.

3 | RESULTS

3.1 | In silico results

According to the data collected from DisGeNET, MAP3K1, ENTPD1, NT5E, and TCF7 are categorized as autoimmune genes playing important roles in autoimmune diseases such as MS, and systemic lupus erythematosus (SLE). Pathways analysis of four selected genes indicated that these genes are mainly enriched in pathways concerned with immune system (e.g. JNK signaling in the CD4+ TCR pathway, IFN-gamma pathway, Immune System, TNF receptor signaling pathway) and Th17 differentiation (e.g. Ca2+ pathway, MAPK signaling pathway, Toll-Like Receptors Cascades, HIF-1-alpha transcription factor network; Table 1). As illustrated in Figure 1, there are a large numbers of important connections between MAP3K1, ENTPD1, NT5E, TCF7, and other genes highly involved in autoimmune diseases and Th17 differentiation. Accordingly, four Th17-associated genes, including

MAP3K1, ENTPD1, NT5E, and TCF7 were taken into account to select putative targeting miRNAs.

Our selection criteria for identifying miRNAs targeting MAP3K1, ENTPD1, NT5E, and TCF7 genes included two conditions: (a) There must be strong predicted interactions between the miRNA and candidate genes. (b) miRNA's involvement in immune system disorders must be previously reported. Among several miRNAs with the strong possibility to interact with selected mRNAs, miR-9, and miR-193b whose association with autoimmune disorders have been validated in our literature review have been chosen. HMMD 3.0 confirmed participation of these two miRNAs in several autoimmune disorders including MS, RA, allergic asthma and psoriasis. Interaction information related to each miRNA-mRNA pair obtained from three databases including miRWalk, TargetScan, and miRmap is shown in Figure 2a. The potential matching positions of miR-9 and miR-193b within the 3'-UTR of target genes are depicted in Figure 2b, which are gained from TargetScan 7.1 and miRmap. To shade light on the importance of this miRNAs in crucial cellular processes, miRNA versus pathways heatmaps were depicted. The signaling pathways in which miR-9-5p and miR-193b-3p are involved were represented on the heat maps (Figure 3). miR-9-5p is related to HIF-1 signaling pathway,

Gene	Database	Pathway	P-value
TCF7	KEGG	Wnt signaling pathway	.007900
	REACTOME	Ca2+ pathway	.003050
		Signaling by Wnt	.01475
MAP3K1	KEGG	RIG-I-like receptor signaling pathway	.003500
		MAPK signaling pathway	.01475
	REACTOME	TRAF6-mediated NF-kB activation	.001200
		RIG-I/MDA5-mediated induction of IFN-alpha/beta pathways	.003950
		Toll-Like Receptors Cascades	.00700
	NCI-Nature	Immune System	.07735
		JNK signaling in the CD4+ TCR pathway Homo sapiens	.002797
		Regulation of cytoplasmic and nuclear SMAD2/3 signaling Homo sapiens	.003396
		IFN-gamma pathway Homo sapiens	.007977
		TNF receptor signaling pathway	.009169
ENTPD1	KEGG	Epstein-Barr virus infection	.01005
NT5E	NCI-Nature	HIF-1-alpha transcription factor network Homo sapiens	.003300

TABLE 1 Signaling pathways relevant to TCF7, MAP3K1, ENTPD1, and NT5E genes obtained from multiple databases

Note: Selected genes appear to be play roles in many critical signaling pathways concerned with immune system as well as Th17 differentiation.

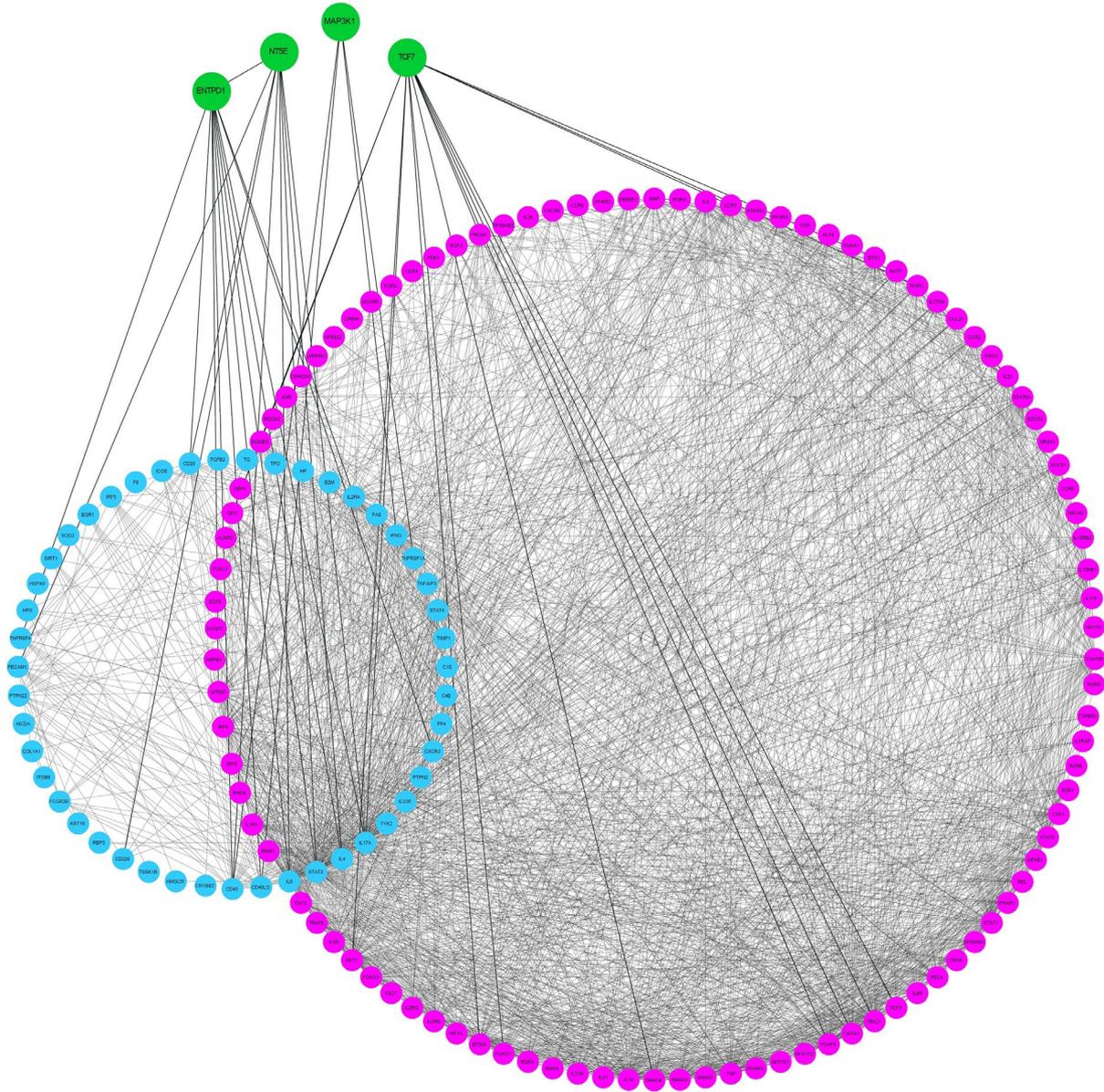


FIGURE 1 Associations between TCF7, MAP3K1, ENTPD1, NT5E, and 55 genes involved in autoimmune diseases and 116 Th17 differentiation genes. TCF7, MAP3K1, ENTPD1, and NT5E are strongly associated to Th17 differentiation genes shown in purple. Additionally, there are a large number of interactions between selected genes and autoimmune disease-relevant genes depicted in blue.

TGF- β signaling pathway, NF-kappa B signaling pathway, and MAPK signaling pathway all of which play indispensable roles in differentiation of Th17 cells. There were also relations between miR-193b-3p and critical Th17 differentiation pathways such as Toll-like receptor, and Notch signaling pathways.

3.2 | Purity of isolated naïve CD4⁺ T cells

To determine purity of naïve CD4⁺ T cells isolated from PBMCs, surface proteins were stained with CD45RA and

CD4 antibodies and approximately, 50% purity was confirmed via flow cytometric analysis (Figure 4b).

3.3 | Th17 differentiation characterization

Naïve CD4⁺ T cells were cultured in vitro for 6 days under Th17-polarizing condition. Cells started aggregating to small clumps on day 2. Cell aggregates grew from several cells to dozens of cells and large clumps were formed on day four. On day 6, cell suspensions were full of large clumps and majority of the cells were in large cell

(a)

miRNA	Gene	TargetScan		miRWalk		miRmap	
		Context++ score	Context++ score percentile	Score	Energy	Score	ΔG open
has-miR-9-5P	MAP3K1	-0.20	94	0.961	-20.7	74.26	88.52
	NT5E	-0.04	67	0.846	-18	37.54	67.82
	TCF7	-0.17	92	N/A	N/A	19.50	34.56
	ENTPD1	-0.13	88	0.846	-18	99.83	99.89
hsa-miR-193b-3p	MAP3K1	-0.10	67	0.846	-26.3	94.24	70.21
	NT5E	-0.67	99	0.846	-25	96.51	39.25
	TCF7	N/A	N/A	0.923	-24.8	N/A	N/A
	ENTPD1	-0.16	77	0.923	-25.5	81.53	60.89

(b)

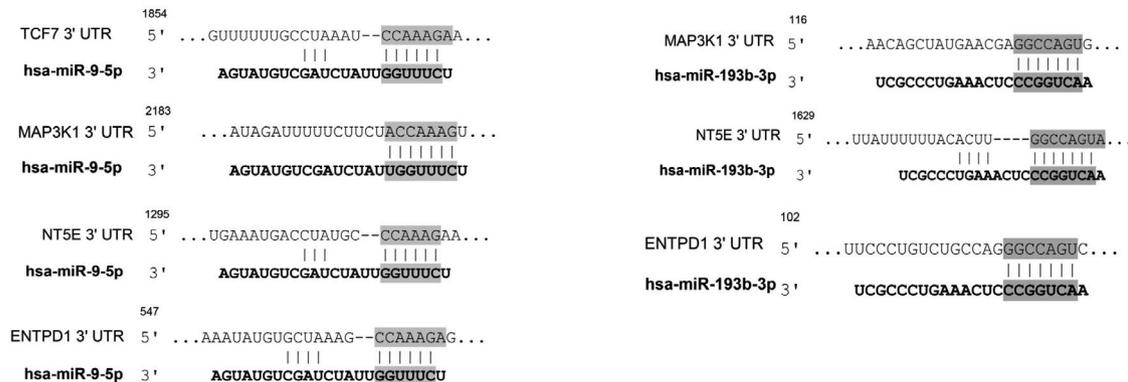


FIGURE 2 Predicted interactions of mRNA-miRNA pairs. (a) Features of predicted miRNA-mRNA interactions gained from three reputable databases; miRwalk 3.0 and TargetScan 7.1, and miRmap. (b) The potential matching sites of miR-9 and miR-193b within 3'-UTR of TCF7, MAP3K1, ENTPD1, and NT5E genes obtained from TargetScan 7.1 and miRmap databases. N/A, not applicable.

aggregates (Figure 5a). To confirm efficiency of differentiation process, concentrations of IL-17 as a characteristic marker of Th17 were examined, indicating a substantial increase in its secretion during differentiation, reaching a peak of 750 pg/ml on day 6 (Figure 5b).

3.4 | Downregulation of TCF7, MAP3K1, ENTPD1, and NT5E over human Th17 differentiation

In order to evaluate expression alterations of selected genes, qRT-PCR analysis was carried out, demonstrating downregulation of TCF7, MAP3K1, ENTPD1, and NT5E during differentiation of human Th17 cells. TCF7, MAP3K1, and ENTPD1 genes showed exactly the same expression pattern. Their expression dropped around 90% over day 1 and 2 in comparison with day 0, reaching their lowest levels on day 4, before increasing slightly on day 6 (Figure 6a-c). There was also a constant decline in the level of NT5E during human Th17 differentiation. Polarization caused a significant decrease of about 60% in NT5E expression level over first two days compared with day 0. NT5E mRNA level showed a downward trend over the next days, with its expression reaching the lowest level on day 6 (Figure 6d).

3.5 | Upregulation of miR-9 and miR-193b during human Th17 differentiation

Quantitative real-time PCR was used to assess changes in miRNAs levels, showing upregulation of miR-9 and miR-193b over human Th17 differentiation. Although the expression of miR-9 increased in cells differentiated for 2 days in comparison with naïve CD4⁺ T cells, it was not statistically significant. The expression level of miR-9 increased markedly on day 4 compared to day 0 of differentiation, before decreasing slightly on day 6 (Figure 7a). The expression pattern of miR-193b was slightly different during human Th17 differentiation. A dramatic growth has occurred in miR-193b expression during four first days compared with day 0, reaching a peak on day 6. (Figure 7b).

4 | DISCUSSION

IL-17-producing CD4⁺ T cells known as Th17 cells have emerged as a crucial pathogenic cell type that produce inflammatory cytokines mediating various immune responses (Fletcher et al., 2009; Ma et al., 2011). Th17 cells not only provide protection against extracellular pathogens such as bacteria and fungi, but also contribute to pathogenesis of several autoimmune and inflammatory diseases, comprising

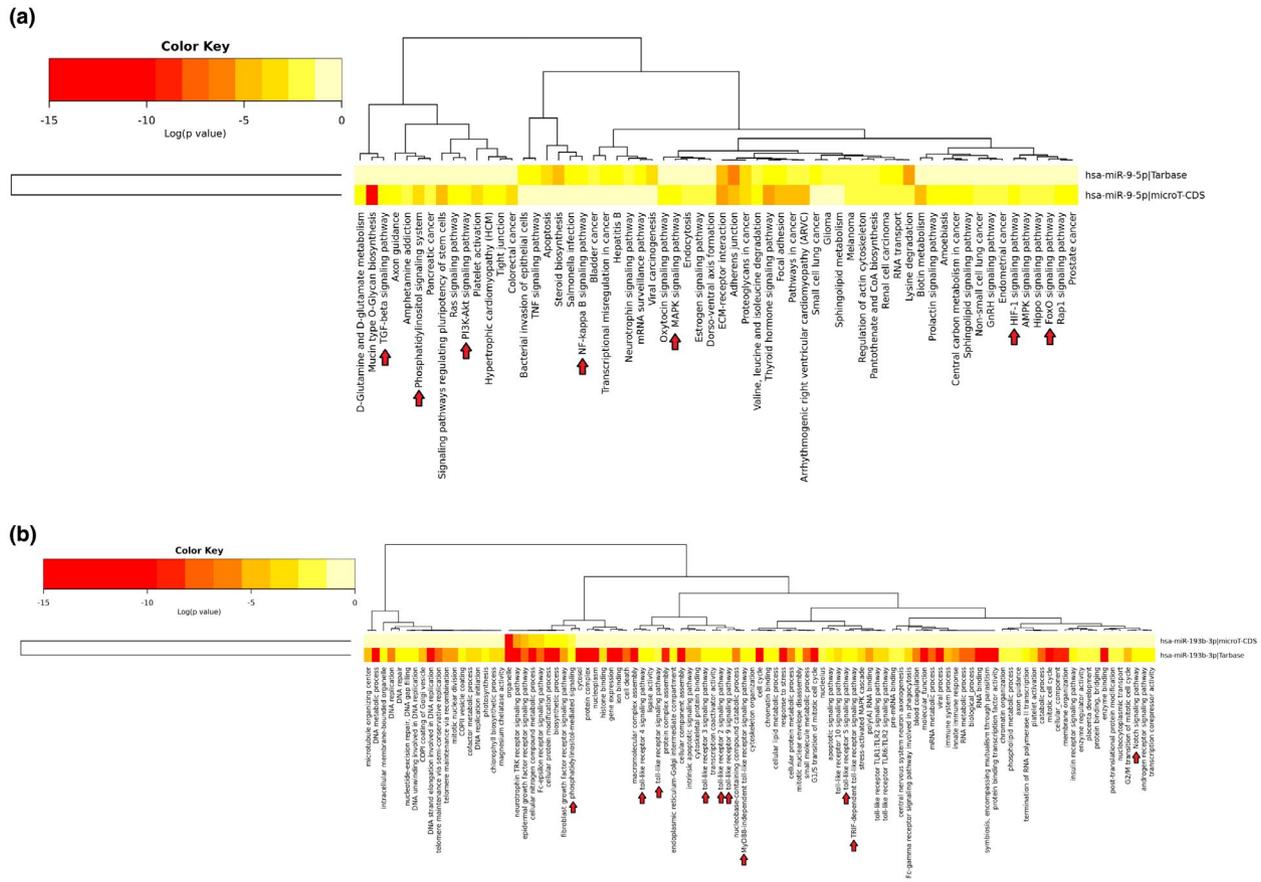


FIGURE 3 Heatmap of miR-9-5p and miR-193b-3p related signaling pathways. (a) miR-9-5p involvement in multiple Th17 differentiation pathways such as TGF- β signaling, NF- κ B signaling, MAPK signaling, HIF-1 signaling. (b) Association of miR-193b with critical pathways regulating Th17 differentiation including Toll-like receptor, and Notch signaling pathways. Color gradient demonstrates pathway values with pale yellow, as the lowest and red, as the highest importance.

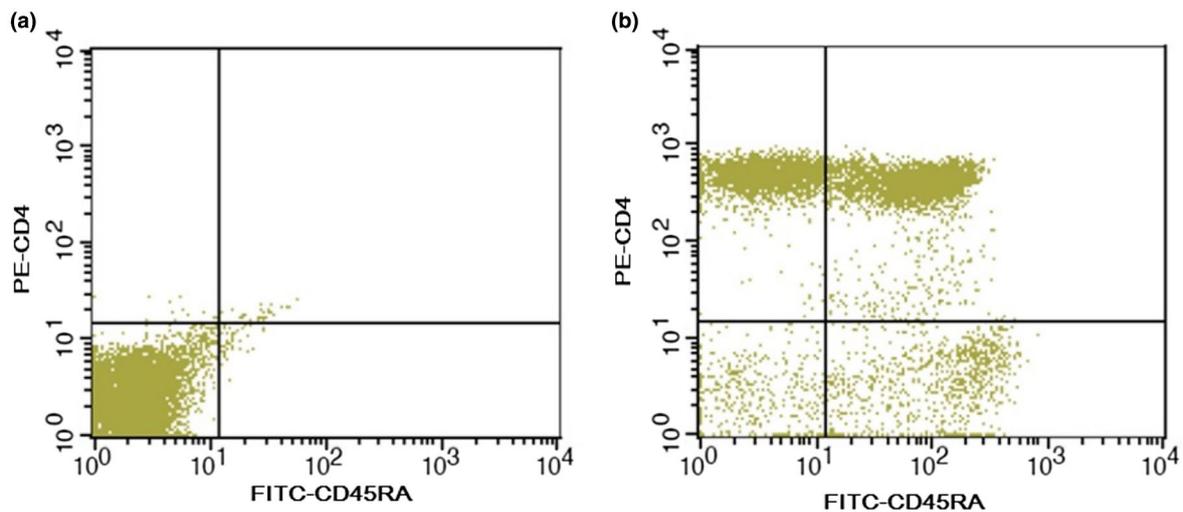


FIGURE 4 Flowcytometric analysis of isolated naive CD4⁺ T cells. (a) Isotype control. (b) Isolated human naive CD4⁺ T cells were labeled by CD4 and CD45RA antibodies which mark CD4⁺ and naive T cells, respectively. Dot plot diagram indicates approximately 50% purity of CD4⁺/CD45RA⁺ T cells in cell samples obtained from human naive CD4⁺ T cell isolation column.

MS, psoriasis, RA, IBD, SLE, asthma, and allergy (Liu et al., 2016; Montoya et al., 2017; Murugaiyan et al., 2015). Since discovery of Th17 cells, research efforts have been devoted

to identifying the factors responsible for differentiation and functions of this pathogenic T cell population as finding modulatory factors involved in their differentiation provides a

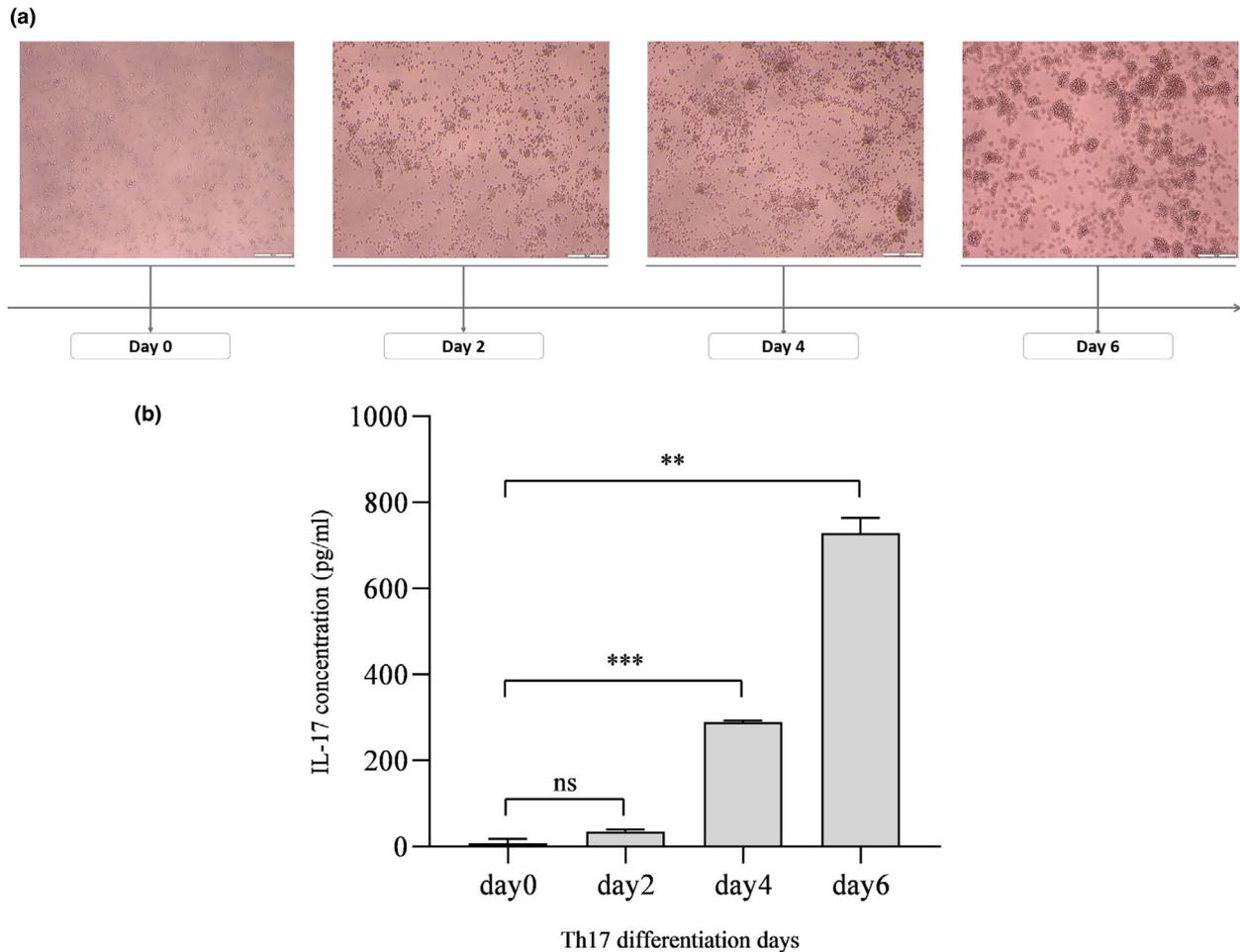


FIGURE 5 Differentiation of Th17 cells. (a) Representative images of differentiating human naïve CD⁺ T cells on day 0 to 6. The number and size of cellular aggregations increased during differentiation, promoting differentiation process by increasing cell-cell contacts. (b) Concentration of IL-17 as a characteristic marker of Th17 cells during differentiation. IL-17 secretion rose substantially during differentiation, reaching a peak on day 6. (** $p < 0.01$, *** $p < 0.001$, students t -test; Scale bar = 100 μ M).

potential avenue for targeting autoimmune diseases (Burgler et al., 2009; Hakemi et al., 2011; Majd et al., 2018; Zhang, et al., 2018). However, the greater part of our existing knowledge on Th17 cells results from mouse model studies, while only a few studies have assessed differentiation pathways of human Th17 cells (Hakemi et al., 2011). Thus, here, naïve CD4⁺ T cells isolated from human blood were differentiated to Th17 subtype over several days.

Each T helper subtype produces a distinct set of cytokines and IL-17 is a pro-inflammatory cytokine specifically secreted by the Th17 that induces secretion of other pro-inflammatory cytokines and chemokines, such as TNF- α , and IL-6 (Burgler et al., 2009; Hakemi et al., 2011). Accumulating evidence suggests a significant association between inflammation severity and IL-17 concentrations in tissue fluids and serum of human and mouse models (Hakemi et al., 2011). Enhanced expression of IL-17 has been also detected in cerebrospinal fluid of MS patients (Fletcher et al., 2009). MS patients-derived CD4⁺ T cells also secrete higher levels of

IL-17 compared to healthy controls (Fletcher et al., 2009). As Th17 cells are known as IL-17-producing cells, we assessed level of IL-17 secreted by the cells as a characteristic marker of Th17 cell over 6 days (Burgler et al., 2009; Hakemi et al., 2011). Naïve CD4⁺ T cell polarization to Th17 cells led to a considerable rise in IL-17 protein secretion especially on day 6, confirming the efficiency of differentiation process. After validating differentiation process of human Th17 cells, expressions of selected genes and miRNAs were examined every other day from day 0 to 6 over differentiation.

TCF-1 is known as a negative regulator of Th17 immunity whose deletion increases IL-17 gene expression by opening the IL-17 locus during T cell development and leads to enhanced Th17 differentiation (Ma et al., 2011; Zhang, et al., 2018). Here, expression levels of TCF7 gene decreased substantially from day 0 to 6 of human Th17 differentiation, with the day 4 showing the lowest level of TCF7 expression. Consistently, T cells from multiple sclerosis patients showed a significant decrease in TCF-1 expression

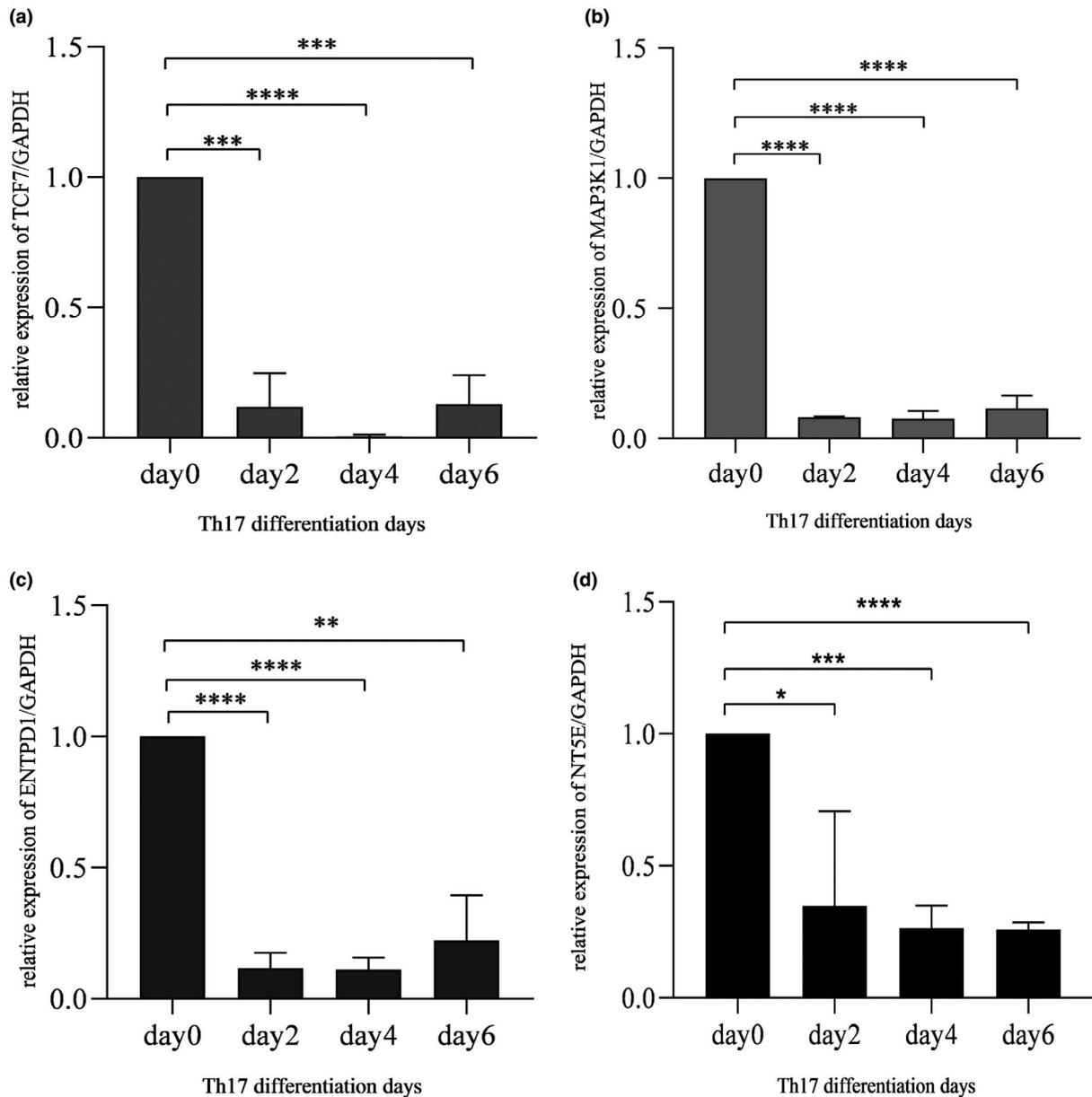


FIGURE 6 RT-qPCR analysis of TCF7, MAP3K1, ENTPD1, and NT5E expression levels during human Th17 differentiation. The expression levels of TCF7 (a), MAP3K1 (b), ENTPD1 (c), and NT5E (d) declined dramatically during Th17 differentiation. Expression of genes was normalized to GAPDH level as an internal control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, students *t*-test).

in comparison with those from healthy individuals, revealing the association between TCF-1 expression in human CD4⁺ T cells and multiple sclerosis (Mazzola et al., 2015). There was also a remarkable reduction in the expression of TCF7 in patients with ileal Crohn's disease (CD) compared with healthy controls, contributing to the dysfunction of Paneth cell and ileal CD development (Patman, 2014). TCF7 was also among significantly underexpressed genes in PBMCs of SLE patients (Crow & Wohlgemuth, 2003). MAP3K1 is another gene participating in the regulation of IL-17 expression and signaling of Th17 cells. (Anwar, 2013; Suddason & Gallagher, 2016). Overproduction of IL-17 was observed in MAP3K1-deficient invariant natural

killer T (iNKT) cells correlating with the enhanced expression of Th17-related genes including RORC, MMP9 and IL17RB (Anwar, 2013). This upregulated expression of Th17-related genes following MAP3K1 deletion suggests an association between the Th17 differentiation pathway and the MAP3K1 (Anwar, 2013). MAP3K1 single nucleotide polymorphisms (SNPs) also showed evidence of association to treatment response in RA patients (Bowes et al., 2009). MAP3K1 is also involved in genetic risk to CD (Hancock et al., 2008). In this study, we assessed the MAP3K1 expression level from day 0 to day 6 of human Th17 differentiation and detected a dramatic decline in its expression during differentiation, confirming previous

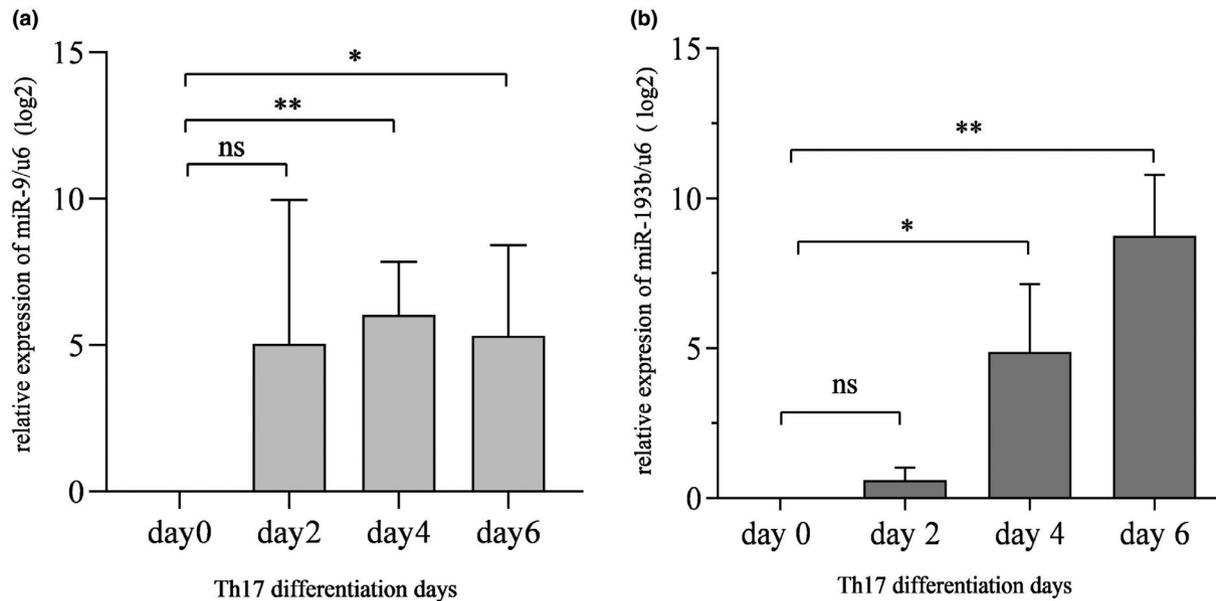


FIGURE 7 RT-qPCR analysis of miR-9, and miR-193b expression levels over human Th17 differentiation. (a) miR-9 expression increased significantly over human Th17 differentiation. (b) There was a substantial rise in miR-193b level during human Th17 differentiation. Results were normalized to U6 snRNA expression (* $p < 0.05$, ** $p < 0.01$, students t -test).

finding suggesting negative role of MAP3K1 in Th17 differentiation.

Suppression of pathogenic Th17 cells can be intensified by adenosine generated as a product of ectonucleotidases CD39 and CD73 activities (Fletcher et al., 2009). Morianos et al., (2020) revealed that upregulation of the anti-inflammatory CD73 and CD39 ectonucleotidases induced by activin-A contributes to the suppression of pathogenic Th17 responses in animal MS model. CD39 provides protection against IBD and there are significant correlation between its polymorphisms and IBD in humans (Friedman et al., 2009). CD39 induced by IL-27 signaling of dendritic cells remarkably decreases extracellular ATP levels, leading to the reduced differentiation of pathogenic Th17 cells and suppressed autoimmunity (Mascanfroni et al., 2013). In the present study, expression level of ENTPD1 fell dramatically over 6 days of being cultured under Th17-polarizing conditions. Similarly, Th17 polarization caused a significant reduction in NT5E mRNA levels, with the day 6 displaying the lowest level of NT5E expression. Consistently, expression of CD73 was considerably lower in male MS patients in comparison with male controls (Kobarfard et al., 2019). NT5E participates in the response of MS patients to treatments like IFN, which acts through enhancing NT5E expression and endogenic levels of adenosine. Several studies reported abnormal NT5E activity in monocytes of patients with active MS (Kobarfard et al., 2019). Collectively, TCF7, MAP3K1, ENTPD1, and NT5E are immunoregulatory genes which negatively modulate Th17 pathogenesis and have previously shown suppressed expression in various autoimmune disorders. In the current

study, we observed declined level of these genes over Th17 differentiation, confirming their possible inhibitory effects on the human Th17 pathogenicity.

It has been estimated that miRNAs regulate the expression of over one-third of the total human genes, comprising genes implicated in immune system (Chen et al., 2018). MiRNAs have been recognized as critical modulators of T helper cell differentiation, plasticity as well as function. That is why miRNA deficiency in T cells causes impairment in Th17 cell differentiation (Montoya et al., 2017). miRNAs regulate Th17 differentiation either indirectly by acting on other cell types and affecting Th17 lineage commitment or directly through influencing cell-intrinsic signaling, inducing Th17 cell programming and effector function (Montoya et al., 2017). Furthermore, it has been revealed that the viability, proliferation, and differentiation of T helper cells are affected following deletion of miRNA biogenesis factors such as microprocessor complex subunit DGCR8, Dicer, and Drosha, indicating vital role of miRNAs in T helper cells fate decisions and functions (Baumjohann & Ansel, 2013). In spite of many efforts allocated to decipher the molecular mechanism of Th17 differentiation, in recent years, the exact role of miRNAs in differentiation of Th17 cells is still unknown. Additionally, introducing new miRNAs related to Th17 cell differentiation might prepare the basis for developing therapeutic strategy for Th17-mediated inflammatory diseases. Thereby, two miRNAs including miR-9-5p and miR-193b-3p were selected here to evaluate their expression levels over human Th17 differentiation.

Recent reports strongly suggest that miR-9 is highly implicated in immunity and inflammatory diseases (Gao

et al., 2013). Deregulation of miR-9 has been identified in several types of cancers and there is a strong relationship between miR-9 and inflammation in cancer (Gao et al., 2013). miR-9 is induced in monocytes and neutrophils during Lipopolysaccharides (LPS) or cytokine-induced immune responses (Bazzoni et al., 2009; Boldin & Baltimore, 2012; Gao et al., 2013). Furthermore, activation of CD4+ T cells upregulates miR-9 which represses Blimp-1, leading to enhanced production of IL-2 and IFN- γ (Gao et al., 2013). Elevated secretion of these pro-inflammatory cytokines in turn induces immune responses in inflammatory disorders such as MS and Asthma bronchiale (Gao et al., 2013). In the present study, expression pattern of miR-9 was checked over human Th17 differentiation, showing a substantial rise in its expression during 6 days. Similarly, miR-9 was among upregulated miRNAs in IBD patients (Honardoost et al., 2015; Majd et al., 2018). In our previous study, a significant rise in miR-9 expression was identified in CD4+ T cells of MS patients in relapsing phase compared to controls, proposing an inducing role of miR-9 in Th17 differentiation in MS pathogenesis (Majd et al., 2018). miR-9 is induced in other pathological events such as brain inflammation as two separate studies showed that inflammatory stimuli (LPS) induces elevation of miR-9 in both microglia and monocytes (Yao et al., 2014). Accordingly, miR-9 mediates microglial activation and inflammatory response through NF- κ B pathway and specific blockage of miR-9 has been suggested as potential therapeutic strategy for treating neuroinflammatory conditions (Yao et al., 2014). One study showed an association between miR-193b overexpression and inflammation and introduced it as a predictive biomarker for patients developing chronic kidney disease (CKD) following radical nephrectomy (RN; Trevisani et al., 2016). In addition, Zhao et al., (2014) compared the difference in miRNAs expression between psoriasis vulgaris (PV) and healthy subjects, finding upregulated levels of miR-193b in PV patients' PBMCs. An increased level of miR-193-3p was also detected in MS patients (Honardoost et al., 2015). Consistently, we detected constant remarkable upregulation of miR-193b during human Th17 cells differentiation, confirming possible involvement of this miRNA in inflammatory response and Th17 function. It is worth noting that miR-9 expression rose substantially over first 2 days, with its level remaining constant until day 6. Based on the expression pattern miR-9 seems to play a role in promotion and continuity of Th17 differentiation throughout the whole process, while miR-193b expression increased constantly during polarization reaching a peak on day 6, suggesting that this miRNA probably contributes more to the accomplishment of Th17 differentiation process.

5 | CONCLUSION

Overall, downregulation of TCF7, MAP3K1, ENTPD1, and NT5E genes over polarization reconfirms their significance in autoimmune disorders, highlighting them as possible negative regulators of Th17 pathogenesis. To our knowledge, this is the first study to reveal significant upregulation of miR-9 and miR-193b during human Th17 cells differentiation, suggesting that these miRNAs may be involved in Th17 cell differentiation possibly through affecting levels of their putative target genes. The opposite expression pattern observed between miRNAs and their putative targets can confirm our bioinformatics predictions although further experiments have to be conducted to clarify exact mechanisms of miR-9 and miR-193b' actions in human Th17 differentiation and validate their interactions with selected genes.

ACKNOWLEDGMENTS

This project was supported by the National Institute for Medical Research Development (NIMAD's project no. 942792).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

F. SH. and M.D. designed the experiments, drafted sections of the manuscript, and performed in silico study, cell culture and real-time PCR analyses. M. R. D. performed flow cytometry. A.SH.N. and A.E. edited the revised version and incorporated at the final stage of manuscript preparation and data analyses. M. H. N.-E and K. G. were responsible for the supervision of project and wrote the manuscript and approved the final version of manuscript. All authors read and approved the final version of manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Maryam Peymani  <https://orcid.org/0000-0001-5075-5661>

Kamran Ghaedi  <https://orcid.org/0000-0002-9790-5644>

REFERENCES

- Agarwal, V., Bell, G. W., Nam, J.-W., & Bartel, D. P. (2015). Predicting effective microRNA target sites in mammalian mRNAs. *Elife*, 4, e05005.
- Antonoli, L., Pacher, P., Vizi, E. S., & Haskó, G. (2013). CD39 and CD73 in immunity and inflammation. *Trends in*

- Molecular Medicine*, 19(6), 355-367. <https://doi.org/10.1016/j.molmed.2013.03.005>
- Anwar, S. (2013). Analysis of Map3k-dependent molecular mechanisms regulating signaling in iNKT and B cells. Molecular Biology Master's Thesis (60 Cr). Imperial College London.
- Baghi, M., Rostamian Delavar, M., Yadegari, E., Peymani, M., Pozo, D., Hossein Nasr-Esfahani, M., & Ghaedi, K. (2020). Modified level of miR-376a is associated with Parkinson's disease. *Journal of Cellular and Molecular Medicine*, 24(4), 2622-2634.
- Baumjohann, D., & Ansel, K. M. (2013). MicroRNA-mediated regulation of T helper cell differentiation and plasticity. *Nature Reviews Immunology*, 13(9), 666-678.
- Bazzoni, F., Rossato, M., Fabbri, M., Gaudiosi, D., Mirolo, M., Mori, L., Tamassia, N., Mantovani, A., Cassatella, M. A., & Locati, M. (2009). Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proceedings of the National Academy of Sciences*, 106(13), 5282-5287.
- Boldin, M. P., & Baltimore, D. (2012). MicroRNAs, new effectors and regulators of NF- κ B. *Immunological Reviews*, 246(1), 205-220.
- Borsellino, G., Kleinewietfeld, M., Di Mitri, D., Sternjak, A., Diamantini, A., Giometto, R., Höpner, S., Centonze, D., Bernardi, G., Dell'Acqua, M. L., Rossini, P. M., Battistini, L., Röttschke, O., & Falk, K. (2007). Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: Hydrolysis of extracellular ATP and immune suppression. *Blood, the Journal of the American Society of Hematology*, 110(4), 1225-1232.
- Bowes, J. D., Potter, C., Gibbons, L. J., Hyrich, K., Plant, D., Morgan, A. W., Wilson, A. G., Isaacs, J. D., Worthington, J., & Barton, A. (2009). Investigation of genetic variants within candidate genes of the TNFRSF1B signalling pathway on the response to anti-TNF agents in a UK cohort of rheumatoid arthritis patients. *Pharmacogenetics and Genomics*, 19(4), 319-323.
- Burgler, S., Ouaked, N., Bassin, C., Basinski, T. M., Mantel, P.-Y., Siegmund, K., Meyer, N., Akdis, C. A., & Schmidt-Weber, C. B. (2009). Differentiation and functional analysis of human TH17 cells. *Journal of Allergy and Clinical Immunology*, 123(3), 588-595. e587.
- Chen, C., Zhou, Y., Wang, J., Yan, Y., Peng, L., & Qiu, W. (2018). Dysregulated MicroRNA involvement in multiple sclerosis by induction of T helper 17 cell differentiation. *Frontiers in Immunology*, 9, 1256.
- Crimeen-Irwin, B., Scalzo, K., Gloster, S., Mottram, P., & Plebanski, M. (2005). Failure of immune homeostasis-the consequences of under and over reactivity. *CURRENT DRUG TARGETS-IMMUNE ENDOCRINE AND METABOLIC DISORDERS-*, 5(4), 413.
- Crow, M. K., & Wohlgemuth, J. (2003). Microarray analysis of gene expression in lupus. *Arthritis Research & Therapy*, 5(6), 279.
- Fletcher, J. M., Lonergan, R., Costelloe, L., Kinsella, K., Moran, B., O'Farrelly, C., & Mills, K. H. (2009). CD39+ Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *The Journal of Immunology*, 183(11), 7602-7610.
- Friedman, D. J., Kunzli, B. M., A-Rahim, Y. I., Sevigny, J., Berberat, P. O., Enyoji, K., Csizmadia, E., Friess, H., & Robson, S. C. (2009). CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. *Proceedings of the National Academy of Sciences*, 106(39), 16788-16793.
- Gao, F., Zhao, Z.-L., Zhao, W.-T., Fan, Q.-R., Wang, S.-C., Li, J., Zhang, Y.-Q., Shi, J.-W., Lin, X.-L., Yang, S., Xie, R.-Y., Liu, W., Zhang, T.-T., Sun, Y.-L., Xu, K., Yao, K.-T., & Xiao, D. (2013). miR-9 modulates the expression of interferon-regulated genes and MHC class I molecules in human nasopharyngeal carcinoma cells. *Biochemical and Biophysical Research Communications*, 431(3), 610-616.
- Hakemi, M. G., Ghaedi, K., Andalib, A., Hosseini, M., & Rezaei, A. (2011). Optimization of human Th17 cell differentiation in vitro: Evaluating different polarizing factors. *Vitro Cellular & Developmental Biology-Animal*, 47(8), 581.
- Hakemi, M. G., Ghaedi, K., Homayouni, V., Andalib, A., Hosseini, M., & Rezaei, A. (2014). Positive and negative regulation of Th17 cell differentiation: Evaluating the impact of RORC2. *Cell Journal (Yakhteh)*, 16(3), 343.
- Hancock, L., Beckly, J., Geremia, A., Cooney, R., Cummings, F., Pathan, S., & Ahmad, T. (2008). Clinical and molecular characteristics of isolated colonic Crohn's disease. *Inflammatory Bowel Diseases*, 14(12), 1667-1677.
- Honardoost, M. A., Naghavian, R., Ahmadinejad, F., Hosseini, A., & Ghaedi, K. (2015). Integrative computational mRNA-miRNA interaction analyses of the autoimmune-deregulated miRNAs and well-known Th17 differentiation regulators: An attempt to discover new potential miRNAs involved in Th17 differentiation. *Gene*, 572(2), 153-162.
- Huang, Z., Shi, J., Gao, Y., Cui, C., Zhang, S., Li, J., & Cui, Q. (2019). HMDD v3. 0: A database for experimentally supported human microRNA-disease associations. *Nucleic Acids Research*, 47(D1), D1013-D1017.
- Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., & Haw, R. (2020). The reactome pathway knowledgebase. *Nucleic Acids Research*, 48(D1), D498-D503.
- Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., & Morishima, K. (2017). KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Research*, 45(D1), D353-D361.
- Kobarfard, F., Noroozi, R., Beirami, A. D., Taheri, M., & Ghafouri-Fard, S. (2019). Dys-regulation of peripheral transcript levels of ecto-5'-nucleotidase in multiple sclerosis patients. *Human Antibodies*, 27(3), 161-165.
- Krupa, S., Anthony, K., Buchoff, J., Day, M., Hannay, T., & Schaefer, C. (2007). The NCI-Nature Pathway Interaction Database: A cell signaling resource. *Nature Precedings*, 1ss.
- Liu, M., Gao, W., van Velkinburgh, J. C., Wu, Y., Ni, B., & Tian, Y. (2016). Role of Ets Proteins in Development, Differentiation, and Function of T-Cell Subsets. *Medicinal Research Reviews*, 36(2), 193-220.
- Ma, J., Wang, R., Fang, X., Ding, Y., & Sun, Z. (2011). Critical role of TCF-1 in repression of the IL-17 gene. *PLoS One*, 6(9), e24768.
- Majd, M., Hosseini, A., Ghaedi, K., Kiani-Esfahani, A., Tanhaei, S., Shiralian-Esfahani, H., & Nasr-Esfahani, M. H. (2018). MiR-9-5p and miR-106a-5p dysregulated in CD4+ T-cells of multiple sclerosis patients and targeted essential factors of T helper17/regulatory T-cells differentiation. *Iranian Journal of Basic Medical Sciences*, 21(3), 277.
- Mascanfroni, I. D., Yeste, A., Vieira, S. M., Burns, E. J., Patel, B., Sloma, I., & Efroni, S. (2013). Interleukin-27 acts on dendritic cells to suppress the T-cell response and autoimmunity by inducing the expression of ENTPD1 (CD39). *Nature Immunology*, 14(10), 1054.

- Mazzola, M. A., Raheja, R., Murugaiyan, G., Rajabi, H., Kumar, D., Pertel, T., Regev, K., Griffin, R., Aly, L., Kivisakk, P., Nejad, P., Patel, B., Gwanyalla, N., Hei, H., Glanz, B., Chitnis, T., Weiner, H. L., & Gandhi, R. (2015). Identification of a novel mechanism of action of fingolimod (FTY720) on human effector T cell function through TCF-1 upregulation. *Journal of Neuroinflammation*, *12*(1), 1-12.
- Montoya, M. M., Maul, J., Singh, P. B., Pua, H. H., Dahlström, F., Wu, N., Huang, X., Ansel, K. M., & Baumjohann, D. (2017). A distinct inhibitory function for miR-18a in Th17 cell differentiation. *The Journal of Immunology*, *199*(2), 559-569.
- Morianos, I., Trochoutsou, A. I., Papadopoulou, G., Semitekolou, M., Banos, A., Konstantopoulos, D., Manousopoulou, A., Kapasa, M., Wei, P., Lomenick, B., Belaidi, E., Kalamatas, T., Karageorgiou, K., Doskas, T., Sallusto, F., Pan, F., Garbis, S. D., Quintana, F. J., & Xanthou, G. (2020). Activin-A limits Th17 pathogenicity and autoimmune neuroinflammation via CD39 and CD73 ectonucleotidases and Hif1- α -dependent pathways. *Proceedings of the National Academy of Sciences*, *117*(22), 12269-12280.
- Murugaiyan, G., da Cunha, A. P., Ajay, A. K., Joller, N., Garo, L. P., Kumaradevan, S., Yosef, N., Vaidya, V. S., & Weiner, H. L. (2015). MicroRNA-21 promotes Th17 differentiation and mediates experimental autoimmune encephalomyelitis. *The Journal of Clinical Investigation*, *125*(3), 1069-1080.
- Patman, G. (2014). TCF1 regulates Paneth cell α -defensins. *Nature Reviews Gastroenterology & Hepatology*, *11*(9), 517.
- Piñero González, J., Ramírez Anguita, J. M., Saüch Pitarch, J., Ronzano, F., Centeno, E., Sanz, F., & Furlong, L. I. (2020). The DisGeNET knowledge platform for disease genomics: 2019 update. *Nucleic Acids Research*, *48*(D1):D845-D855.
- Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., & Ideker, T. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Research*, *13*(11), 2498-2504. <https://doi.org/10.1101/gr.1239303>
- Sticht, C., De La Torre, C., Parveen, A., & Gretz, N. (2018). miR-Walk: An online resource for prediction of microRNA binding sites. *PLoS One*, *13*(10), e0206239. <https://doi.org/10.1371/journal.pone.0206239>
- Suddason, T., & Gallagher, E. (2016). Genetic insights into map3k-dependent proliferative expansion of t cells. *Cell Cycle*, *15*(15), 1956-1960.
- Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N. T., Morris, J. H., Bork, P., Jensen, L. J., & Mering, C. (2019). STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Research*, *47*(D1), D607-D613. <https://doi.org/10.1093/nar/gky1131>
- Teimuri, S., Hosseini, A., Rezaenasab, A., Ghaedi, K., Ghoveud, E., Etemadifar, M., Nasr-Esfahani, M. H., & Megraw, T. L. (2018). Integrative analysis of lncRNAs in Th17 cell lineage to discover new potential biomarkers and therapeutic targets in autoimmune diseases. *Molecular Therapy-Nucleic Acids*, *12*, 393-404. <https://doi.org/10.1016/j.omtn.2018.05.022>
- Trevisani, F., Ghidini, M., Larcher, A., Lampis, A., Lote, H., Manunta, P., Alibrandi, M. T. S., Zagato, L., Citterio, L., Dell'Antonio, G., Carezzi, C., Capasso, G., Rugge, M., Rigotti, P., Bertini, R., Cascione, L., Briganti, A., Salonia, A., Benigni, F., ... Valeri, N. (2016). MicroRNA 193b-3p as a predictive biomarker of chronic kidney disease in patients undergoing radical nephrectomy for renal cell carcinoma. *British Journal of Cancer*, *115*(11), 1343-1350. <https://doi.org/10.1038/bjc.2016.329>
- Vejnar, C. E., Blum, M., & Zdobnov, E. M. (2013). miRmap web: Comprehensive microRNA target prediction online. *Nucleic Acids Research*, *41*(W1), W165-W168. <https://doi.org/10.1093/nar/gkt430>
- Vlachos, I. S., Zagganas, K., Paraskevopoulou, M. D., Georgakilas, G., Karagkouni, D., Vergoulis, T., & Hatzigeorgiou, A. G. (2015). DIANA-miRPath v3. 0: Deciphering microRNA function with experimental support. *Nucleic Acids Research*, *43*(W1), W460-W466.
- Yao, H., Ma, R., Yang, L. U., Hu, G., Chen, X., Duan, M., Kook, Y., Niu, F., Liao, K. E., Fu, M., Hu, G., Kolattukudy, P., & Buch, S. (2014). MiR-9 promotes microglial activation by targeting MCPiP1. *Nature Communications*, *5*(1), 1-12. <https://doi.org/10.1038/ncomms5386>
- Zhang, J., He, Z., Sen, S., Wang, F., Zhang, Q., & Sun, Z. (2018). TCF-1 Inhibits IL-17 gene Expression to restrain Th17 immunity in a stage-specific manner. *The Journal of Immunology*, *200*(10), 3397-3406. <https://doi.org/10.4049/jimmunol.1800193>
- Zhang, Z., Xue, Z., Liu, Y., Liu, H., Guo, X., Li, Y., Yang, H., Zhang, L., Da, Y., Yao, Z., & Zhang, R. (2018). MicroRNA-181c promotes Th17 cell differentiation and mediates experimental autoimmune encephalomyelitis. *Brain, Behavior, and Immunity*, *70*, 305-314. <https://doi.org/10.1016/j.bbi.2018.03.011>
- Zhao, M., Wang, L.-T., Liang, G.-P., Zhang, P., Deng, X.-J., Tang, Q., Zhai, H.-Y., Chang, C. C., Su, Y.-W., & Lu, Q.-J. (2014). Up-regulation of microRNA-210 induces immune dysfunction via targeting FOXP3 in CD4+ T cells of psoriasis vulgaris. *Clinical Immunology*, *150*(1), 22-30. <https://doi.org/10.1016/j.clim.2013.10.009>

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1

How to cite this article: Shirani F, Baghi M, Rostamian Delavar M, et al. Upregulation of miR-9 and miR-193b over human Th17 cell differentiation. *Mol Genet Genomic Med.* 2020;8:e1538. <https://doi.org/10.1002/mgg3.1538>