miR-141 and miR-200a are involved in Th17 cell differentiation by negatively regulating RARB expression

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Research

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Abstract

Background: Among T helper (Th) lineages differentiated from naïve $CD4^+$ T cells, interleukin (IL)-17-producing Th17 cells are highly correlated with the pathogenesis of autoimmune disorders. Although a series of microRNAs (miRs) that modulate autoimmunity progress have been reported, further studies on microRNAs particularly involved in Th17 lineage commitment are still warranted.

Results: In this study, to clarify the involvement of miR-141-3p and miR-200a-3p in Th17 cell differentiation, as well as to explore their potential target gene involved, purified human naïve $CD4^+$ T cells were cultured under Th17 cell polarizing condition. Differentiation process was confirmed applying ELISA method to measure IL-17 secretion and RT-qPCR to assess Th17 cell-defining genes expression during differentiation period. miR-141 and miR-200a expression pattern as well as expression alteration of their predicted downstream genes, which were identified via consensus and integration *in-silico* approach, was then evaluated by RT-qPCR. Finally direct interaction between both microRNAs and their common predicted target sequence was approved by dual-luciferase reporter assay. Highly increased IL-17 secretion and Th17 lineage-specific genes expression confirmed Th17 cell differentiation.

Conclusions: Results demonstrated that miR-141 and miR-200a were Th17 cell-associated microRNAs and their expression levels upregulated significantly during Th17 cell induction. We also found that *retinoic acid receptor beta (RARB)* gene, whose product has been reported as a negative regulator of Th17 cell generation, was a direct target of both microRNAs and its downregulation could affect the transcriptional level of JAK/STAT pathway genes. Overall, our results introduced two novel Th17 lineage-associated microRNAs and provided a rationale to further clarify the *RARB*-dependent mechanism of miR-141 and miR-200a to promote Th17 cell differentiation and hence Th17-mediated autoimmunity.

Background

Autoimmune diseases are a class of more than 80 chronic, often debilitating and, in some cases, life-threatening illnesses. For reasons not yet understood, the incidence of autoimmunity is growing around the world and thousands of people worldwide are being diagnosed with autoimmune disorders each year. Some ascribe this surge to environmental factors such as exposure to bacterial or viral pathogens and others believe that it stems from genetic deficiencies. Whatever the cause, $CD4^+$ T cell–mediated autoimmunity is one of the most important aspects of autoimmune disease pathogenesis.

After antigen stimulation, naïve $CD4^+$ T cells differentiate into several subsets of effector helper cells with distinct functions [1]. T helper (Th) 17 cells have been identified as a proinflammatory Th cell lineage that plays important role in host defense and is involved in various autoimmune and inflammatory diseases mainly by secreting interleukin (IL)-17 [2]. IL-6 in conjunction with IL-1ß initiates Th17 cell differentiation, whilst IL-23 serves as a pivotal factor in the continuous differentiation as well as recruitment of Th17 cells to sites of inflammation upon binding to its receptor (IL23R) [3]. Th17 differentiation is directed by *retinoic acid-related orphan receptor (ROR) C* gene product called RORγt as Th17 lineage-defining

transcription factor, and is controlled by delicate balanced activity of a set of positive and negative regulators [4].

Retinoic acid receptor (RAR) ß is a transcription factor that contributes negatively to Th17 differentiation process [5]. Retinoic acid (RA) which influences various immune cell fates and a range of immunological functions, needs to bind to heterodimers of nuclear receptors (RARα, RARß and RARγ) prior to its function [6, 7]. It has been shown that in vivo RA treatment suppresses Th17-mediated immunopathology in various autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE), rheumatoid arthritis and type 1 diabetes [8–10]. Although the past few years have seen substantial progress in defining the inhibitory function of RARs particularly RARα in autoimmunity, *RARB* gene expression pattern, as well as the possible underlying mechanism of its expression alterations during Th17 cell differentiation are largely unknown and need to be clarified.

MicroRNAs (miRs) constitute a large family of small, approximately 22-nucleotide-long, noncoding RNAs that have emerged as key post transcriptional regulators of gene. By base pairing to complementary sequences on untranslated region (UTR) of target mRNAs, microRNAs mediate translational repression, mRNA degradation and destabilization [11]. In immune system, it has been found that microRNAs can play a role as critical regulators of T cell differentiation, so that deregulated expression of microRNAs leads to development of autoimmune disorders [12–14]. Therefore, microRNAs can potentially serve as good diagnostic markers or even therapeutic targets. Regarding the role of microRNAs in Th17 lineage induction, Cobb et al. reported that IL-17A production from Th17 cells was reduced in cells with conditional deletion of Dicer, a RNase III enzyme which is indispensable for the maturation of microRNAs [15]. Furthermore, miR-155, miR-181c, miR-21 and miR-326 have been shown to promote Th17 differentiation [16–19]. The miR-200 family consists of five members classified as two functional groups based on the similarity of their seed sequences. Two functional groups only differ one nucleotide in the seed region (AAUACUG for miR-200b/200c/429 and AACACUG for miR-141/200a) [20]. Microarray analyses of transfected cells and target prediction algorithms reveal a high degree of similarity in target sequences of miR-141-3p and miR-200a-3p, suggesting that these two microRNAs may target a large number of common genes to improve the efficiency of gene regulation [21]. Studies on miR-141 and miR-200a have shown that deregulation of these microRNAs have been correlated with the development of different autoimmune inflammatory diseases such as psoriasis, systemic lupus erythematosus (SLE) and inflammatory bowel disease (IBD) [22–24].

Through a systematic literature mining of studies related to microRNA expression profiling in different immune system-related disorders, a list of microRNAs which had been reported to be deregulated in autoimmune diseases was prepared by our previous study [25]. Expression levels of some of listed microRNAs were then measured in $CD4^+$ T lymphocytes of patients with autoimmunity [26, 27]. Although according to increase in the percentage of $ROR\gamma t^+$ $CD4^+$ T cells as well as upregulation of miR-141-3p and miR-200a-3p in $CD4^+$ T cells of multiple sclerosis (MS) patients we suggested that miR-141 and miR-200a could be key microRNAs in Th17 cell induction, whether and how these microRNAs participate in

In present study, in order to extend our knowledge of the involvement of miR-141-3p and miR-200a-3p in Th17 cell differentiation, we aimed to screen for changes in both microRNAs expression level over human Th17 cell induction. Furthermore, based on our *in-silico* experiments, we planned to evaluate expression pattern and direct interaction of *RARB*, as potential target gene of miR-141 and miR-200a. Finally, we intended to detect expression changes of downstream genes of *RARB* to explore the plausible mechanism of miR-141 and miR-200a to promote Th17 cell polarization and maybe Th17-mediated inflammatory and autoimmune diseases.

Results

1. miR-141-3p and miR-200a-3p target analysis

To clarify how miR-141-3p and miR-200a-3p may involve in Th17 cells differentiation, we applied three microRNA target prediction algorithms TargetScan, miRDB and microT-CDS with the aim of identification of the potential downstream target of both microRNAs (Supplementary Excel file sheet 1 and 2). In that context, by common-target screening with strict thresholds mentioned above in methods and using Venn diagrams, we found *RARB* as the only common target gene which is regulated by miR-141 and miR-200a (Fig. 1A and B). Applying miRTarBase and TarBase, we also realized that the interaction between these microRNAs and *RARB* gene had not been validated before through strong experimental tests including RT-qPCR, reporter assay and western blotting. The potential matching positions of miR-141-3p and miR-200a-3p within the 3'UTR of RARB are depicted in Fig. 1C. Interaction information of each microRNA-RARB interaction demonstrates that *RARB* is one of the highest ranking predicted genes with context + + score = 0.39, miRDB score = 97 and miTG score ~ 0.98 (Fig. 1D).

2. Molecular signaling pathway enrichment analysis of RARB

Molecular signaling pathway enrichment analysis was conducted for further evaluating our *in-silico* prediction approach. Statistically meaningful pathways associated with *RARB* were acknowledged using three online databases, Reactome, KEGG and BioPlanet (Table 1). Functional analysis of *RARB* indicated that this gene is mainly enriched in pathways correlated with cancer and development and interestingly in immune respond- and Th17 differentiation- related pathways (RXR/VDR, TGFß, calcium and retinoic acid signaling pathways).

Table 1

Signaling pathways relevant to RARB gene obtained from various databases. Selected genes appear to be play roles in many key signaling pathways concerned cancer as well as Th17 differentiation.

Database	Pathway	p value
Reactome	Signaling by retinoic acid	0.00210
	Nuclear receptor transcription pathway	0.00255
	Activation of HOX genes	0.00445
	Developmental biology	0.03930
	Generic transcription pathway	0.04060
KEGG	Non-small cell lung cancer	0.00330
	Small cell lung cancer	0.00465
	Gastric cancer	0.00745
	Pathways in cancer	0.02650
	TGFß signaling pathway	0.03988
	Calcium signaling pathway	0.04877
BioPlanet	RXR/VDR pathway	0.00138
	Retinoic acid receptor-mediated signaling	0.00150
	Nuclear receptors in lipid metabolism	0.00170
	Vitamin A and carotenoid metabolism	0.00195
	Non-small cell lung cancer	0.00270
	Small cell lung cancer	0.00420
	Pathways in cancer	0.01625
	Generic transcription pathway	0.01885

3. Interaction analysis of *RARB*

For a higher confidence in our predicted target gene, we also explored the interaction between RARB and genes closely associated with Th17 differentiation or autoimmunity via STRING database and visualized the results using cytoscape software (Supplementary Excel file sheet 3). Interestingly, as illustrated in Fig. 2, there are significant interactions between RARB and SIRT1 (from autoimmunity-related genes) and AKT1, EP300, RXRA, FOXO3, CREM, VDR, RARG and RARA (from Th17-related genes). The combined

ed in Th17 differentiation or immune response are indicated

in supplementary Fig. 1 in which pathways and genes associated with RARB are indicated with red stars. Collectively, RARB was taken into account as putative target gene.

4. Naïve CD4$^+$T cell purification and Th17 cell differentiation

Naïve CD4$^+$ T cells were isolated from human peripheral blood sample and more than 65% purity was confirmed via flow cytometric analysis of surface proteins stained with CD45RA and CD4 antibodies (Fig. 3A and B). Purified naïve cells were then cultured under Th17 cells differentiation condition for six days. Within the first 48 h of differentiation, cells were attracted to the bound anti-CD3, causing creation of the Th17 lineage in the presence of appropriate cytokines and additional antibodies. Therefore positive indications of Th17 cells differentiation including visible clumping of cells around the coated antibody and increased signs of proliferation could be seen in all cultured wells (Fig. 3C). To confirm the efficiency of Th17 differentiation process, transcriptional levels of master markers of Th17 cells including *IL-17*, *IL-23R* and *RORC* genes were measured by RT-qPCR and the protein level of IL-17 was evaluated using ELISA method on day 0, 2, 4 and 6 of differentiation. All assessed gene expressions displayed constant increase over Th17 differentiation period. To be precise, based on real-time PCR analysis, the expression levels of IL-17, IL-23R and RORC mRNAs on day 6 reached 17.25 ($P = 0.0102$), 2.99 ($P = 0.0052$) and 6.85 ($p = 0.0082$) folds higher than on day 0 respectively (Fig. 3D-F). Moreover, there was a substantial increase in IL-17 secretion during Th17 cell polarization, reaching a peak of 905 pg/ml on day 6 (Fig. 3G).

5. Upregulation of miR-141 and miR-200a during human Th17 cell differentiation

In order to explore the participation of miR-141-3p and miR-200a-3p in Th17 cell induction, we evaluated changes in both microRNAs expression over Th17 differentiation. Quantitative analysis of the microRNA expression applying RT-qPCR revealed that miR-141 and miR-200a were significantly overexpressed during differentiation of human naïve CD4$^+$ T cells into Th17 lineage. Both microRNAs showed consistent expression alteration pattern in that their expression levels were constantly upregulated until reaching their highest level on day 6 compared to day 0 by mean factors of 8.23 ($P = 0.0077$) and 9.11 ($P = 0.0053$) for miR-141 and miR-200a respectively (Fig. 4A and B).

6. Confirmation of *RARB* as a direct target gene of miR-141 and miR-200a

To evaluate our hypothesis that *RARB* is a downstream target of miR-141-3p and miR-200a-3p, we used reporter assays in HEK 293T cells with luciferase report vectors that contained the putative wild type or mutant microRNA binding sites within the RARB-3´UTR (Fig. 5A). Both microRNAs inhibited the luciferase activity of the reporter containing the wild type RARB-3´UTR whereas luciferase activity did not change in cells transfected with control vacant pBud-EGFP vectors in which microRNA precursor had not been ligated. However, neither of pBud-EGFP vectors influenced the luciferase activity of the reporter with a mutant 3´UTR that was unable to bind to microRNAs (Fig. 5B and C). Because of the ability of microRNAs to silence genes by mRNA destabilization, we next researched the impact of both microRNAs on the levels of RARB mRNA. For this purpose, we measured *RARB* expression on day 0, 2, 4 and 6 of Th17 cells induction using RT-qPCR. Interestingly, the *RARB* showed an opposite changing trend compared to that of

microRNAs. Our statistical analysis confirmed markedly lower expression of *RARB* on days 2, 4 and 6 of differentiation in the way that its transcriptional level dropped around 70, 90 and 95 percent in comparison with day 0 respectively (Fig. 5D).

7. Downregulation of downstream genes of *RARB*

To further understanding of possible pathway affected by miR-141 and miR-200a in Th17 differentiation, we investigated the expression level of *STAT6* and *GATA3* as downstream genes of *RARB*. These genes were chosen based on Zhu et al. study which stated binding of RARß to IL4Ra promoter contributes negatively to Th17 differentiation [5]. By KEGG pathway analysis, we found *STAT6* and *GATA3* as downstream genes of IL4Ra in JAK/STAT signaling pathway (data not shown). Interestingly, expression level of *STAT6* and *GATA3* displayed similar pattern to *RARB* in which their transcriptional level approximately halved until day 6 of differentiation. ($P = 0.0040$ for *STAT6* and $P = 0.0053$ for *GATA3*) (Fig. 6A and B).

Discussion

Inappropriate mobilization of Th17 cells against a person's own tissues occurs in almost all autoimmune disorders. Th17 lineage is a subpopulation of T helper cells which is differentiated from naïve $CD4^+$ T cells and secretes proinflammatory cytokines [28]. These cells have high abilities to stimulate acute and chronic inflammation and several studies confirmed their pivotal role in pathogenesis of human autoimmune diseases [29–31]. Since it is revealed that microRNAs are pivotal controllers of autoimmunity, over recent years, increasing attention has focused on finding and nominating different microRNAs involved in Th17 differentiation with the aim of introducing novel biomarkers or even therapeutic targets to the world of autoimmune disorders.

As only mere studies have assessed human Th17 differentiation, here, naïve $CD4^+$ T cells isolated from human peripheral blood were cultured under Th17 polarizing condition in order to directly test the association of miR-141 and miR-200a with human Th17 lineage commitment. IL-17 is one of the most specific cytokines of Th17 cells expressing RORγt as their master transcription factor [2, 4]. In humans, microarray analysis of MS plaques demonstrated increased level of IL-17 mRNA compared with the healthy controls [32]. Like MS patients, enhanced expression of IL-17 has been detected in inflamed mucosa from patients with IBD and in synovial fluid from patients with rheumatoid arthritis [33, 34]. It has been also approved that IL-23 took a prominent role in Th17 cell function, as IL-23-deficient mice was reported to lack IL-17-producing T cells and to be resistant to induction of EAE [28]. For evidence mentioned, noticeable increase in IL-17 protein secretion along with elevated mRNA level of RORC, IL23R and IL-17A were considered as strong confirmation of human Th17 differentiation in our study.

In current study, we focused on miR-141 and miR-200a, since these microRNAs have been proved to contribute to the development of some autoimmune disorders [22–24, 26, 27]. We discovered that human Th17 cell differentiation involves miR-141-3p and miR-200a-3p so that the mRNA level of both

microRNAs was constantly upregulated during six-day differentiation of Th17 cells from human naïve $CD4^+$ T cells. Several studies evaluated miR-141 and miR-200a expression levels in PBMCs, B cells, serum and/or other tissues of autoimmune patients to explored the correlation between these microRNAs with autoimmunity. In 2011, Olaru et al. demonstrated the significant increase of both microRNAs in IBD-dysplasia [35]. Also a study on brain tissue of EAE mice displayed miR-141 overexpression suggesting the potential role of this microRNA in MS [36]. In 2018, two separate studies by our colleagues showed upregulation of miR-141 and miR-200a in MPP+-treated differentiated PC12 cells as a model of Parkinson's disease [37, 38]. In our previous study it has been also revealed that both microRNAs expression level and frequency of Th17 cells were higher in MS patients compared to healthy groups [27]. Despite the lines of evidence described above, direct involvement of miR-141 and miR-200a in human Th17 differentiation as well as the plausible mechanism behind their contribution has not been established. We report in this study, to our knowledge for the first time, the involvement of miR-141and mir-200a in human Th17 cell differentiation as our RT-qPCR analysis clearly shows dramatic increase in both microRNAs expression over Th17 cell induction until they reached peak level on day 6 of differentiation. There are a few number of studies reporting reduction of miR-141 and/or miR-200a in B lymphocytes, PBMCs, serum and urine of autoimmune patients [23, 39, 40]. Considering the fact that the participation and expression alteration of both microRNAs were directly investigated during Th17 differentiation process in this study, we can vindicate our results.

Generally, microRNAs are thought to function by targeting 3´UTRs of genes, which leads to mRNA degradation or inhibition of mRNA translation [11].We documented in the present study that *RARB*, a negative regulator of Th17 generation, is a potential common target of both microRNAs. The immune system is profoundly influenced by a range of effects mediated by RA, not only for the development of lymphoid tissues but also for the functional fate of different immune cells [7, 41]. By promoting regulatory T cell (Treg) differentiation and suppressing Th17 cell generation, RA and its receptors including RARα, RARß and RARγ play significant roles in maintaining the balance between inflammatory and tolerogenic responses [42]. In 2007, Mucida et al. reported that generation of Treg cells, which are in charge of suppressing deleterious activities of Th cells, was inhibited by RAR antagonists [42]. Conversely, in another study, diet supplementation of RA led to increased differentiation of Tregs and amelioration of inflammation in a mouse model of Crohn’s disease [43]. Th17 differentiation drastically reduced in mouse models of EAE and arthritis upon RA or RA agonist treatment [8, 44]. RA counteracts the Th17 differentiation mainly via its suppressive effect on IL-6R expression. This results in less IL-23R expression, which further negatively influences maturation and stabilization of Th17 cells [45]. As a retinoic acid receptor, RARß doubtlessly involves in many cellular processes, including immune responses. Remarkable impact of RARß on the inhibition of Th17 differentiation was identified in Zhu et al. study [5]. They reported that Il-4 plus RA-treated inflammatory dendritic cells markedly reduced the secretion of proinflammatory cytokines and strongly suppressed Th17 differentiation. Mechanistically, RARß mediate these effects through close interaction with IL4Ra that is also a negative controller of Th17 differentiation and inducing aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2) which is a rate limiting enzyme for RA synthesis. In fact, only a few studies provided preliminary clues that RARß is

a participant in the Th17 cell inhibition. However, no further studies have been performed to reveal the *RARB* expression profile and its potential regulating factors during Th17 polarization. Our bioinformatics analysis applying prediction algorithms suggested that *RARB* might be a downstream target of miR-141-3p and miR-200a-3p. To indicate direct interaction between our microRNAs and RARB-3´UTR, we performed a luciferase assay. In support of dual luciferase assay results, quantitative analysis of RARB mRNA level revealed its significant decrease during Th17 differentiation process. Therefore, our study examined the previously untouched area of the participation of miR-141 and miR-200a in Th17 differentiation by targeting *RARB* gene.

While characterization of the mechanisms behind these findings is out of the scope of this study, we examined *STAT6* and *GATA3* expression level during six-day differentiation process in order to explore possible pathway affected by miR-141 and miR-200a to promote Th17 differentiation. Zhu et al. study specified RARß binding to the Il4Ra promoter which based on our *in-silico* research could cause high expression of *STAT6* and *GATA3* genes in JAK/STAT pathway. This leads ultimately to the suppression of *RORC* expression and therefore Th17 differentiation. Interestingly, our RT-qPCR results illustrated downregulation of *STAT6* and *GATA3* over Th17 induction. Considering the fact that *STAT6* and *GATA3* are master regulators of Th2 cells, lessened expression level of *RARB* and then *STAT6* and *GATA3* may help explain the probable mechanism of our microRNAs to take part in Th17 induction.

Conclusion

In conclusion, by differentiation of human naïve T cells into Th17 cells and using RT-qPCR, we indicated that expression level of miR-141 and mir-200a increased over Th17 differentiation process and therefore we were able to nominate two novel microRNAs involved in Th17 generation. In addition, our target prediction and luciferase reporter assay results revealed that these microRNAs interfere directly with a negative regulator of Th17 differentiation, RARß. Finally, based on declined expression level of *STAT6* and *GATA3*, we proposed that miR-141 and miR-200a could involve in Th17 differentiation by affecting JAK/STAT signaling pathway. Collectively, the present work is a preliminary study on the identification of two deregulated microRNAs and understanding of their underlying molecular mechanisms involved in Th17 cell differentiation, which could be indispensable for developing novel strategies aimed at diagnosing or even treating Th17-mediated autoimmune diseases. However, in-vitro and in-vivo studies are still required for further confirmation.

Materials And Methods

MicroRNA target analysis

Three microRNA target prediction databases including TargetScan (release 7.2), miRDB (release 6.0) and microT-CDS (release 5.0) were used to identify potential miR-141-3p and miR-200a-3p target sites in genomic sequences [46–48]. Moreover, 115 genes strongly correlated with Th17 cell differentiation identified through literature mining in our earlier study [49]. The common predicted targets for both

microRNAs from Th17-related gene list, TargetScan with a total context + + score ≤ − 0.3, miRDB with a score ≥ 90 and microT-CDS with a miTG score ≥ 0.9 were retrieved using VENNY 2.1 tool [50]. These strict thresholds were chosen to achieve high-confidence target genes and reduce the incidence of false positives. Experimentally validation of predicted microRNA-mRNA interactions was also checked from miRTarBase (release 7.0) and TarBase (release 8.0) [51, 52]

Molecular Signaling Pathway Enrichment Analysis

Reactome, KEGG and BioPlanet were employed to obtain a comprehensive view of statistically significant signaling pathways and molecular networks related via predicted target gene (p value < 0.05) [53–55].

Interaction Network Construction

For further evaluating our candidate target gene, we also constructed a gene-based network which included two sets of 55 genes highly associated with autoimmune diseases (gda score > 0.2) and 115 genes strongly involved in Th17 cell differentiation obtained from DisGeNET database (release 7.0) and our earlier study respectively [49, 56]. Interactions of candidate target gene with the genes of both groups were assessed by STRING-db and visualized by Cytoscape software (release 3.8.0) [57, 58].

$CD4^+$ naïve T cell purification

Human peripheral blood mononuclear T cells (hPBMCs) were separated from 20 mL freshly collected, heparinized peripheral blood via Ficoll– Hypaque density gradient centrifugation (Sigma-Aldrich, USA). Purification of naïve $CD4^+$ T cells was performed using magnetic cell sorting system (MACS) according to Naïve $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec, Germany) instruction. Briefly, non-naïve T cells were magnetically labeled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads. Isolation of pure naïve $CD4^+$ T cells was achieved by depletion of magnetically labeled non-target cells.

Flow Cytometry

Purification analysis of isolated naïve $CD4^+$ T cells was carried out using flow cytometry. After single cell preparation, FITC-conjugated antiCD4 and PE-conjugated anti-CD45RA antibodies (all antibodies were purchased from eBioscience, USA) were added to cell suspension and resulting mixture incubated for 30 min at 4 °C. Flow cytometry analysis was performed by Canto II (BD FACS Calibur) and analyzed using BD CellQuest Pro software.

Th17 Cell Differentiation

For Th17 cell differentiation, the CellXVivo Human Th17 Cell Differentiation Kit (R&D Systems, USA) was used. Purified naïve $CD4^+$ T cells were cultured at a density of 2×10^5 cell per well in 12-well plates with beads coated with anti-CD3 and containing X-VIVO™ 15, Serum-free hematopoietic cell medium (Lonza, Switzerland), under Th17 cell polarizing condition prepared according to the kit manufacturer's

instruction. Cells were maintained in a humidified incubator containing 5% CO2 at 37 °C for six days and differentiation media were refreshed every other day.

Enzyme linked immunosorbent assay (ELISA)

Th17 cells differentiation was confirmed using Quantikine ELISA Human IL-17 Immunoassay kit (R&D, USA) according to the manufacturer's protocols. IL-17A content of supernatant culture media was measured 5 hours, 2 days, 4 days, and 6 days after induction of Th17 differentiation. Results were read by microplate reader (ELX 800) at 450 nm in duplicate.

RNA extraction, cDNA synthesis and real-time quantitative PCR (RT-qPCR)

Cells were lysed by TRIzol (Invitrogen, USA) for RNA isolation on day 0, 2, 4 and 6 of Th17 differentiation process. The RNA quality and quantity was determined via a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA) and the 260/280 nm absorbance ratio was evaluated. In addition, to eliminate any potential genomic DNA contamination, the total RNA samples were treated with DNase I (Fermentas, USA). For gene expression analysis, one microgram of total RNA from each sample was reverse-transcribed using cDNA Synthesis Kit (TaKaRa, Japan) and examined employing SYBR Green Master Mix (TaKaRa, Japan) and specific primer sets synthesized by Macrogen Company, South Korea (Table 2). To test miR-141 and miR-200a expression, 100 ng total RNA from each sample was performed using a miR-CURY LNA Universal RT microRNA PCR kit (Exiqon, Denmark). Relative expression of mRNAs and microRNAs was evaluated by the $2^{-\Delta\Delta Ct}$ method on ABI PRISM 7500 instrument (Applied Biosystems, USA) and normalized to the expression of *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and U6 small nuclear RNA as internal references respectively.

Table 2
RT-qPCR primer pairs.

Gene Name	Forward/Reverse	Primer Sequence	Amplicon Size (bp)
RARB	Forward	5´TACAAACCCTGCTTCGTC 3´	141
	Reverse	5´ACACAGTTCTTATCTCGGTGA 3´	
IL17A	Forward	5*CA* ⊤ *GGTGTCACTGCTACTGC*3	201
	Reverse	5 ∀ *GGTGAGGTGGATCGG* ⊤ *G*3	
RORC	Forward	5*TG*ℂ*AG* ∀ *TGA*ℂ*AGA* ⊤ *GTGC* ⊤ 3	132
	Reverse	5*G* ∀ *CAGCT*ℂ*ATG*ℂ*A*ℂ*GTA*3	
IL23R	Forward	5*CACATGG* ∀ ⊤ *CTGGGCT* ∀ *CAG*3	111
	Reverse	5*AGC* ∀ *AGACGATCA* ⊤ ℂ*C* ∀ *T*3	
GAPDH	Forward	5ℂ*ACT*ℂ*T*ℂ*A*ℂ ⊤ *TGACG*3	107
	Reverse	5ℂ*A*ℂ*A*ℂ*CTG* ⊤ *GCTGTAG*3	

Plasmid Construction And Dual-luciferase Reporter Assay

To specify direct interaction between microRNAs and 3´UTR of predicted target gene, sequenced purified PCR products of microRNA precursor were digested with XbaI and HindIII and ligated into the pBud-EGFP vector (Promega, USA). Synthesized wild type (wt) and mutant (mut) target gene–3´UTR were also cloned into the psiCHECK2 vector (Promega, USA). Both vectors were then co-transfected into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen, USA) following the instructions. Cells were harvested at 48 h post-transfection, assayed for the firefly and Renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, USA). Each experiment was repeated in triplicate and changes in Renilla luciferase activity were calculated relative to firefly luciferase enzyme activity (internal control).

Statistical analysis

The results are expressed as the means ± standard error of the mean (SEM) of three independent experiments and analyzed by and GraphPad Prism 8 software. Independent sample t tests were used to evaluate the differences between groups. A *P* value of 0.05 or less was considered significant.

Declarations

Ethics approval and consent to participate

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.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declares that there is no competing interests regarding the publication of this article.

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Authors' contributions

I. B. and M. B. designed the experiments, drafted sections of the manuscript, and performed in silico study, cell culture and real-time PCR analyses. M. P. performed flow cytometry. A. J. and K. G. were responsible for the supervision of project, wrote the manuscript and approved the final version of manuscript. All authors read and approved the final version of manuscript.

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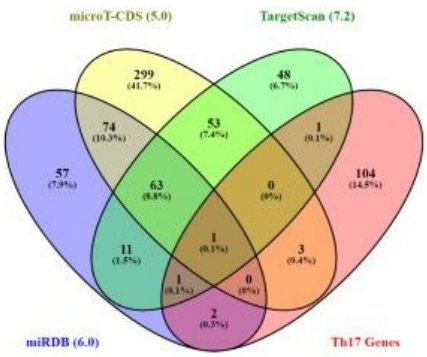
Figures

A



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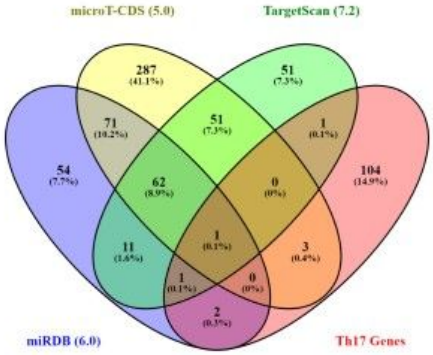

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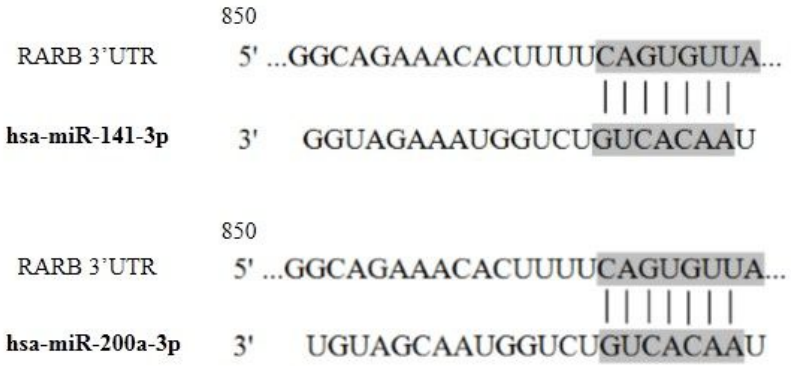


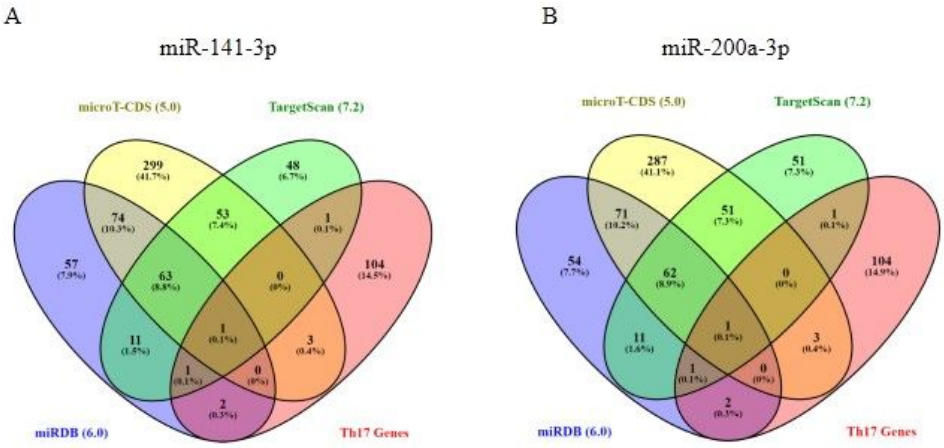
D

Gene	microRNA	Target Scan context++ score	miRDB Target score	microT-CDS miTG score
RARB	hsa-miR-141-3p	-0.39	97	0.98
	Has-miR-200a-3p	-0.39	97	0.97

Figure 1

Results of miR-141-3p and miR-200a-3p target analysis. (A and B) Identification of the common predicted target gene of both microRNAs from Th17-related genes list, TargetScan, miRDB and microT-CDS with strict thresholds based on Venn diagrams. RARB was the only common target gene. (C) The potential binding sites of both microRNAs within RARB-3'UTR obtained from TargetScan. (D) Interaction

information of predicted microRNAs-RARB interactions gained from three utilized prediction databases. RARB was one of the highest scoring potential target genes.



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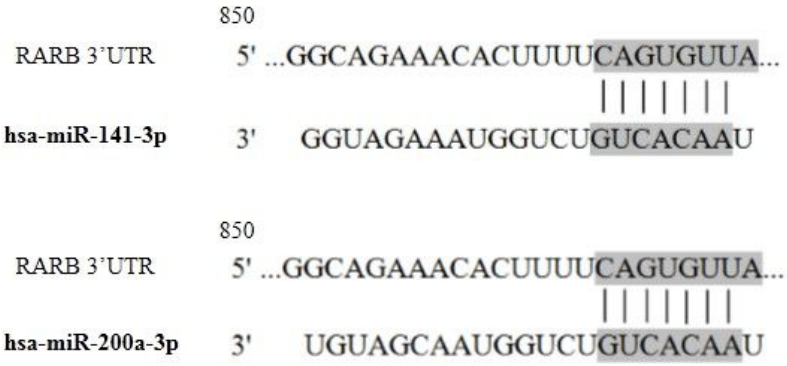

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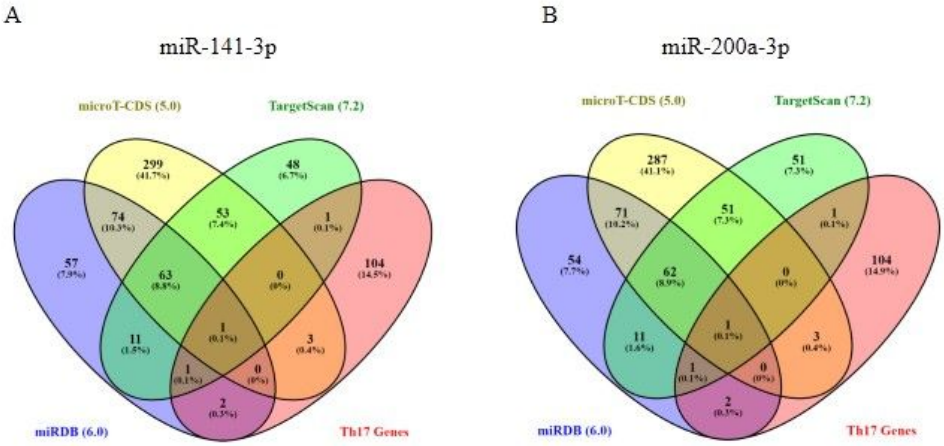

C

```
                    850
RARB 3'UTR      5' ...GGCAGAAACACUUUUCAGUGUUA...
                                    |||||||
hsa-miR-141-3p  3'    GGUAGAAAUGGUCUGUCACAAU

                    850
RARB 3'UTR      5' ...GGCAGAAACACUUUUCAGUGUUA...
                                    |||||||
hsa-miR-200a-3p 3'    UGUAGCAAUGGUCUGUCACAAU
```

D

		Target Scan	miRDB	microT-CDS
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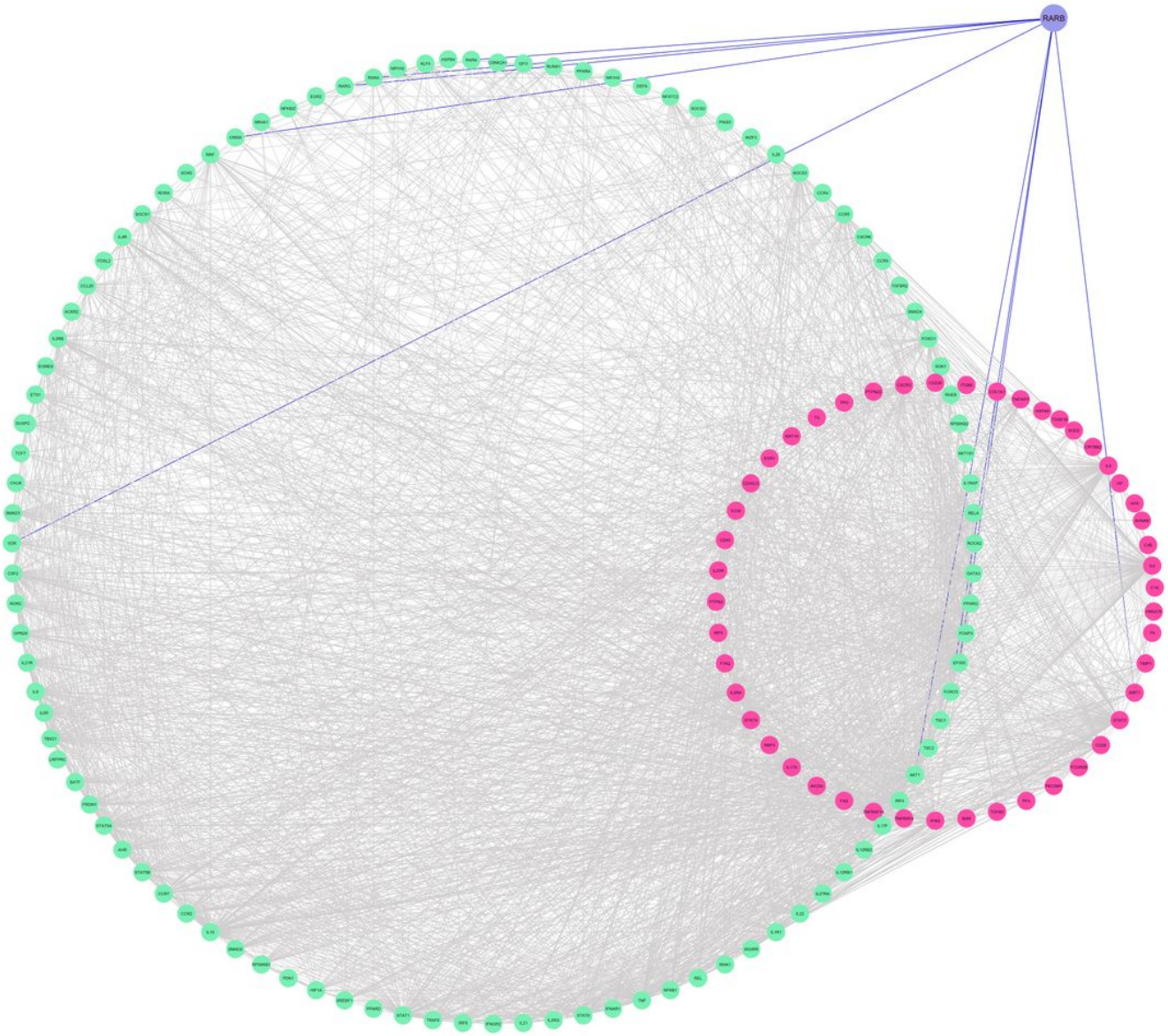
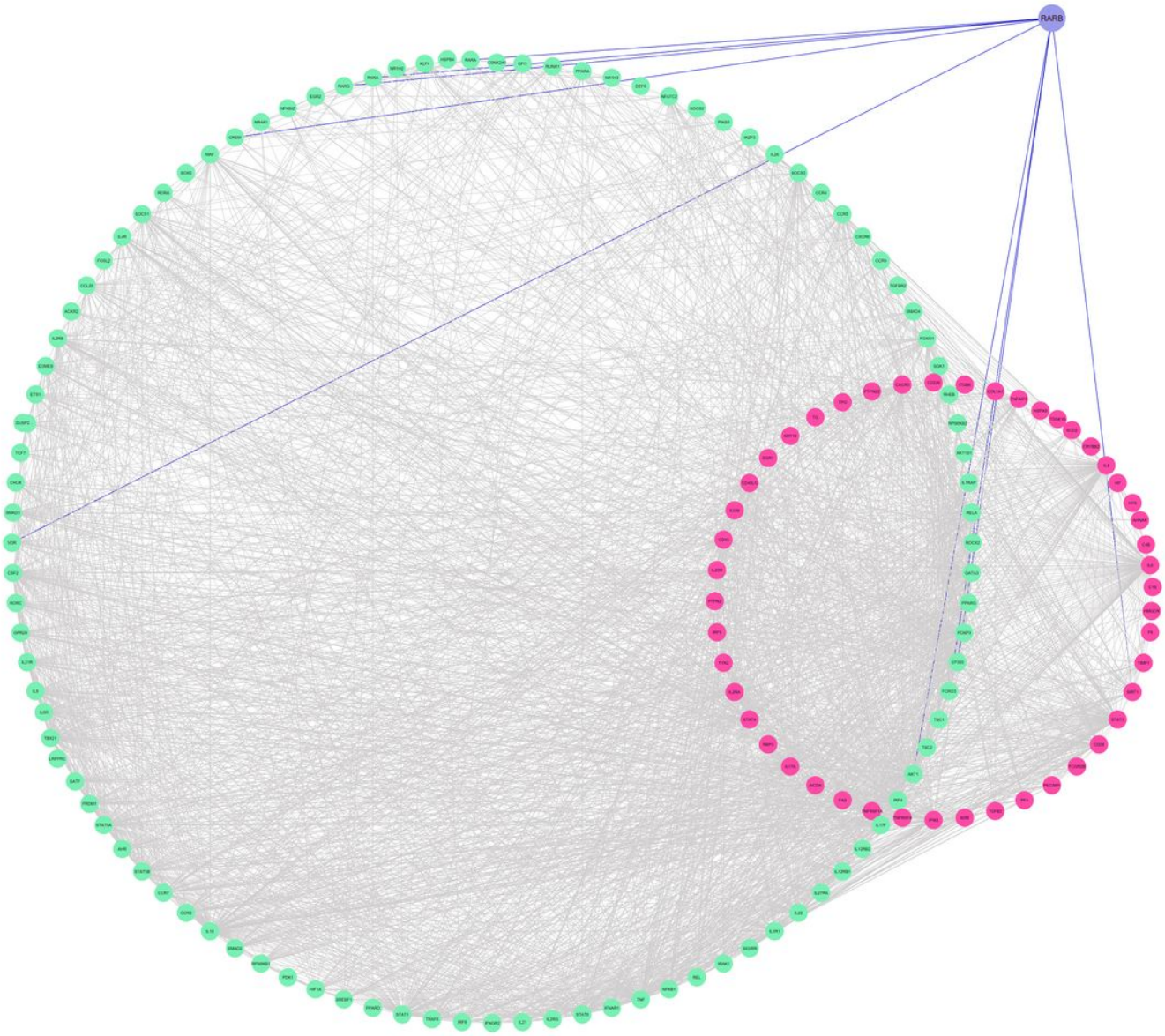


Figure 2

RARB interaction network. Official symbols of 115 Th17- and 55 autoimmunity-related genes were transferred into STRING-db and Interactions between RARB and genes belong to both groups were then visualized in Cytoscape. RARB-interactome genes are shown in different color. There are important interactions between RARB and genes correlated with Th17 generation or those involved in autoimmune



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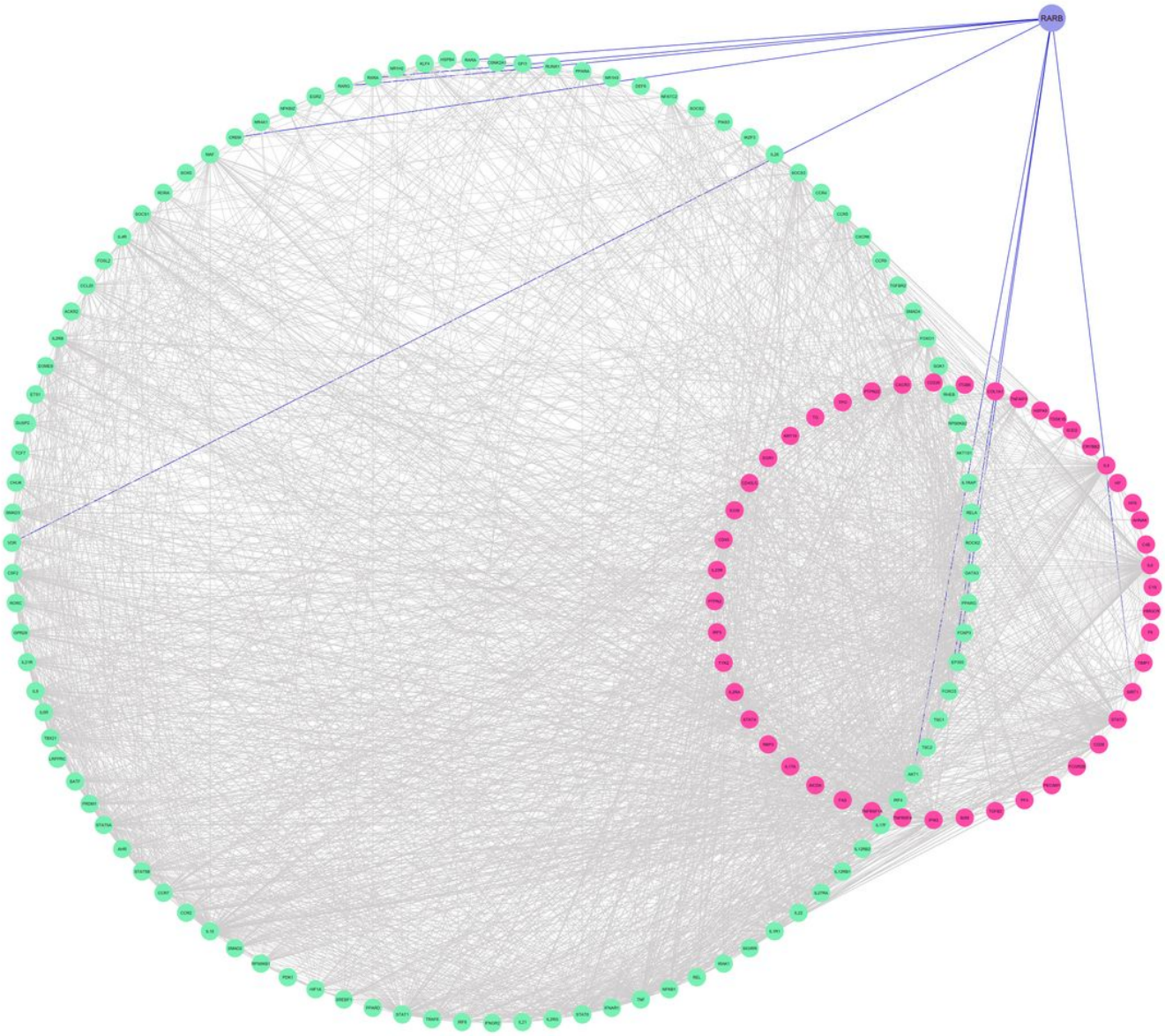

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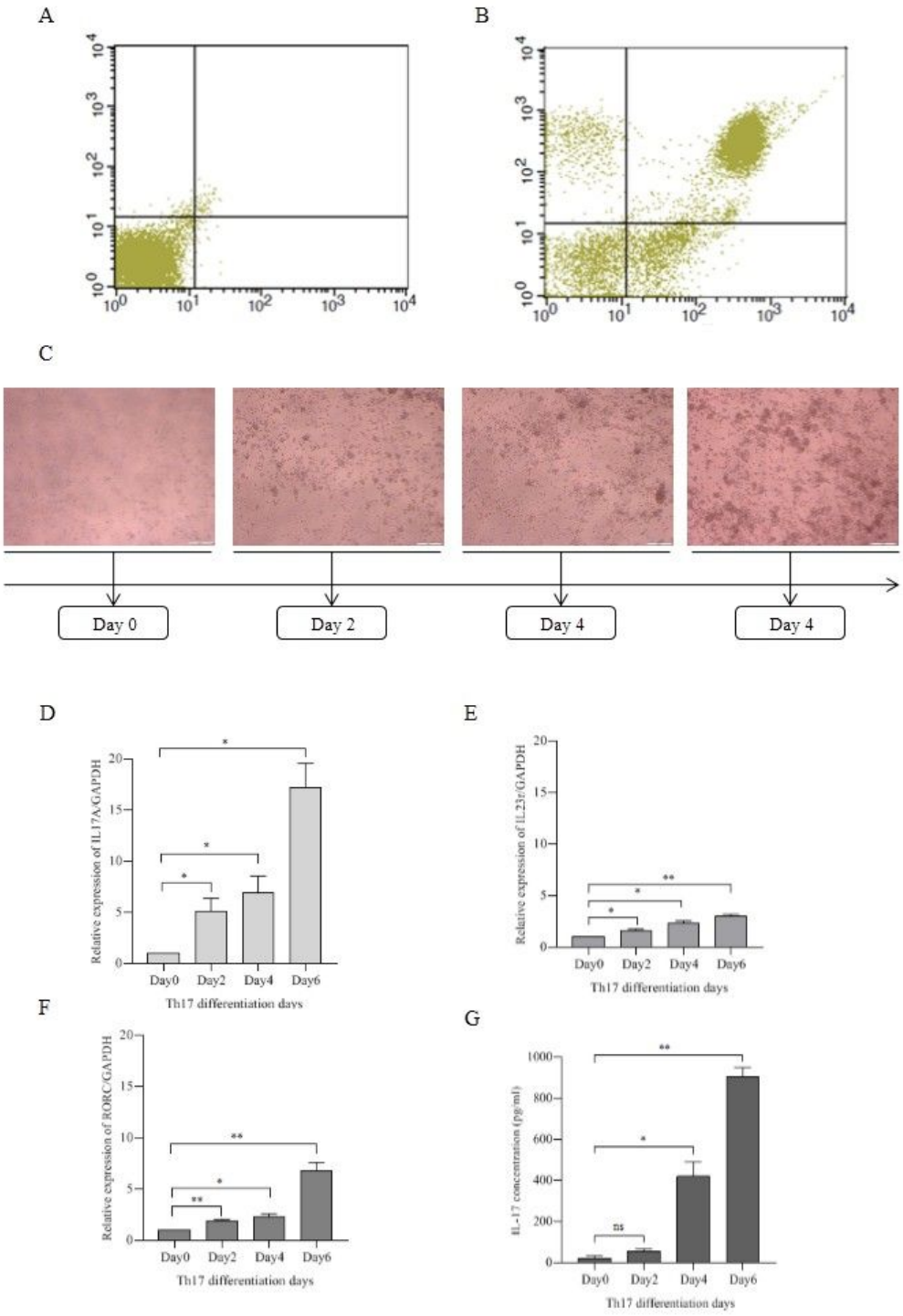

Figure 3

Confirmation of naïve CD4+ T cell purification and Th17 cell differentiation. (A and B) Flow cytometric analysis of naïve CD4+ T cells population purified from human peripheral blood samples. Dot plot A shows three distinct cell populations exist in isolated hPBMCs before applying human naïve CD4+ T cell isolation kit. Dot plot B indicates more than 65% purity of CD4+/CD45RA+ T cells in cell samples ation column. (C) Brightfield images of differentiating human

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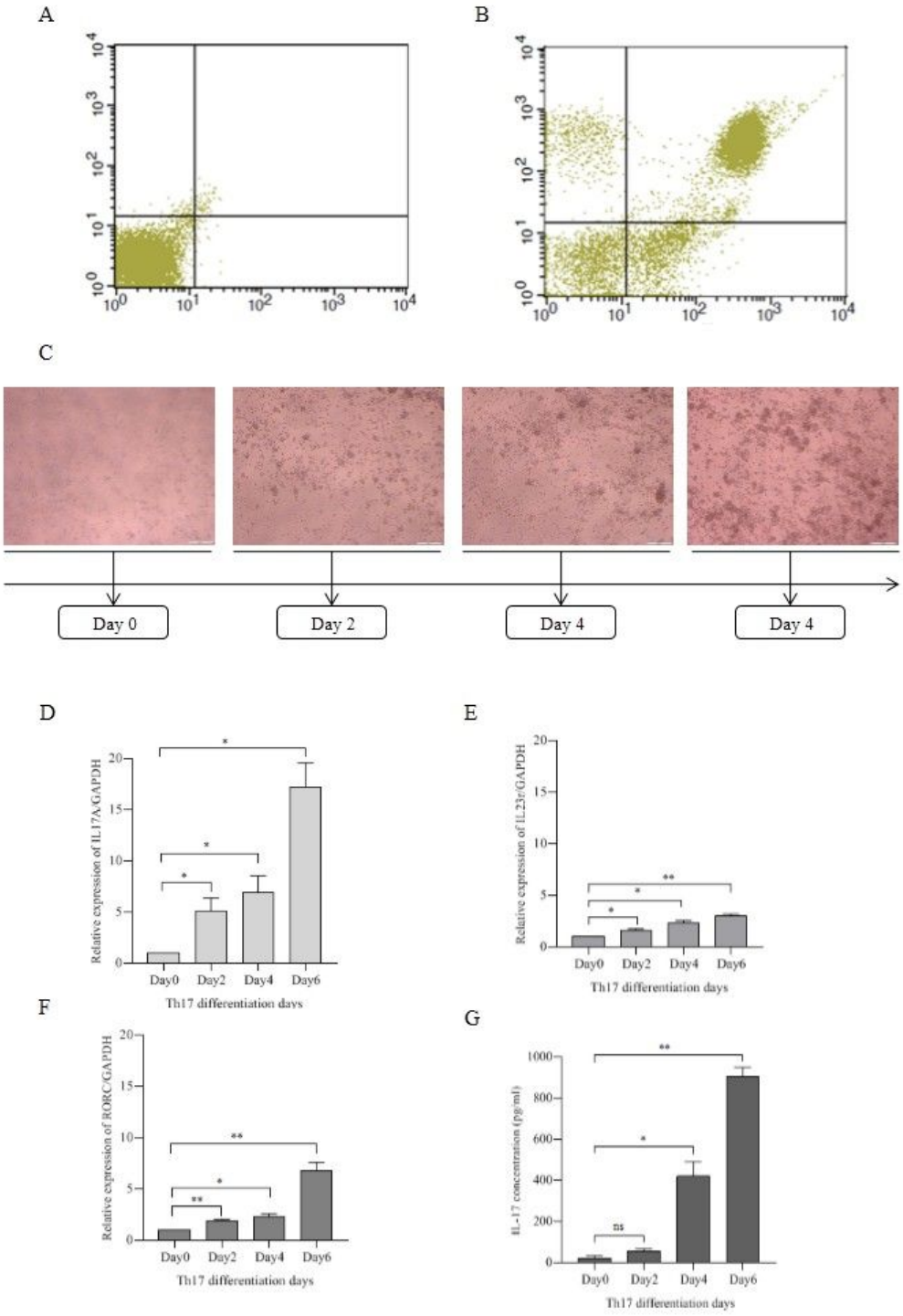

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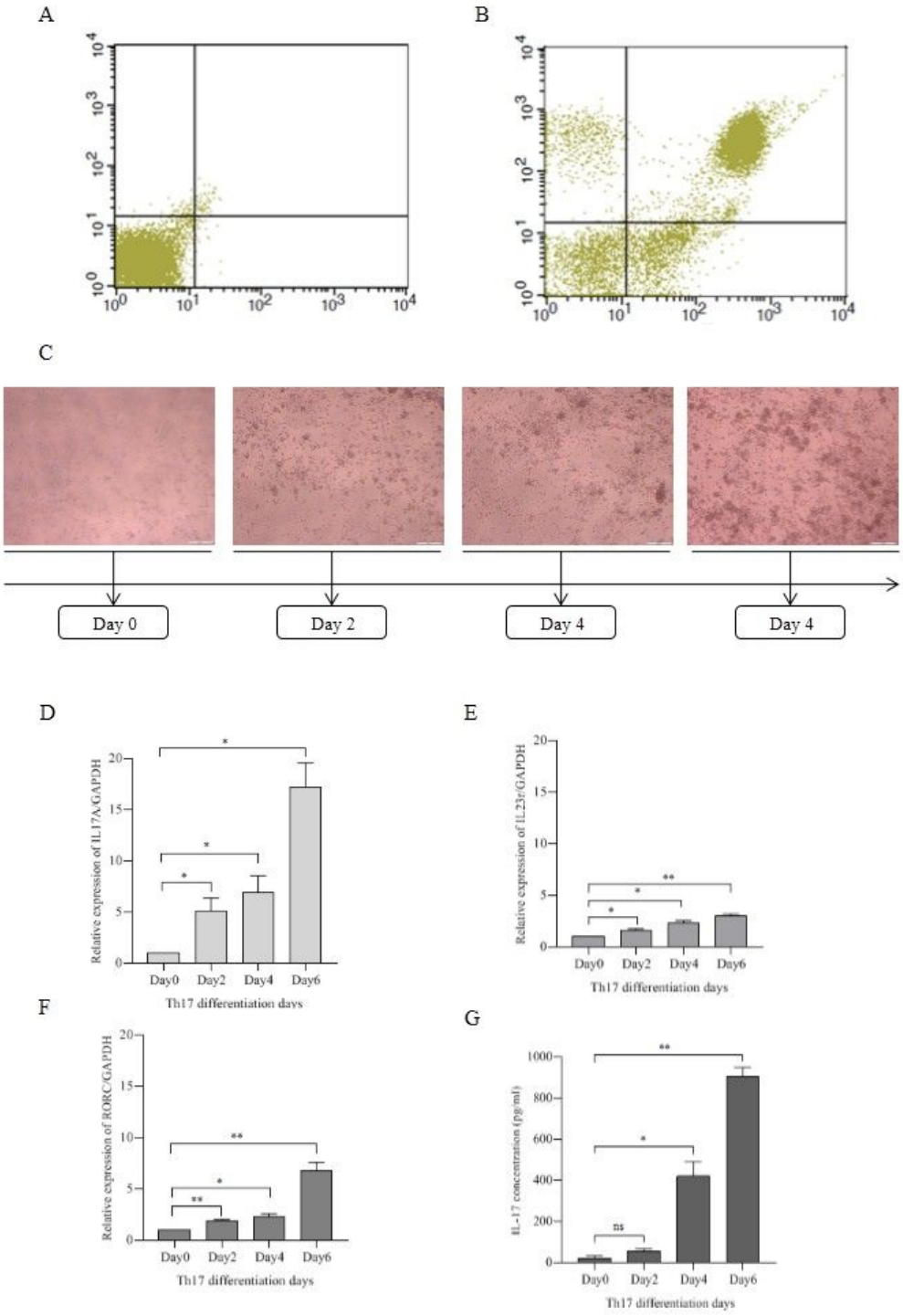

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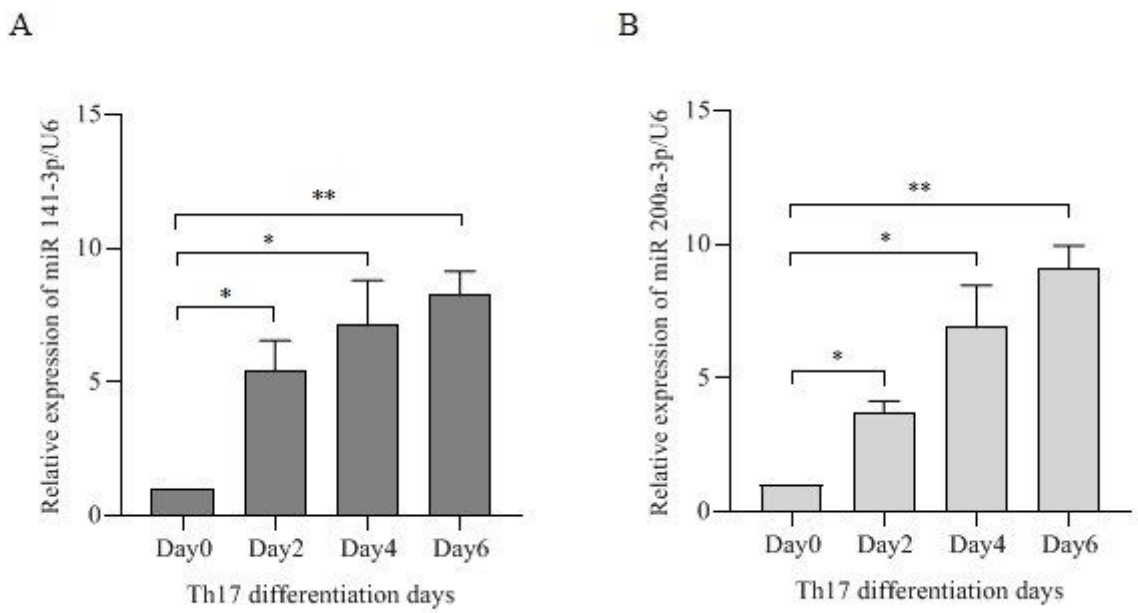
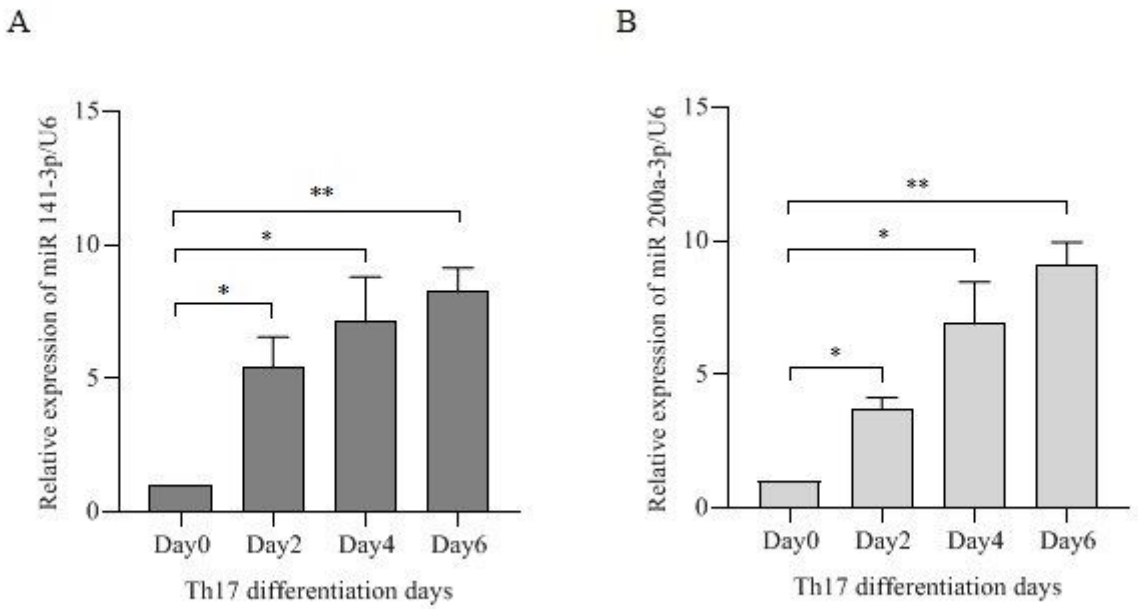


Figure 4

Assessment of the relative expression levels of microRNAs over Th17 differentiation period. (A) miR-141 expression pattern (B) miR-200a expression pattern. Both microRNAs upregulated significantly from day o to day 6 of differentiation. Results were normalized to U6 snRNA expression (* $p < 0.05$, ** $p < 0.01$, students t-test)



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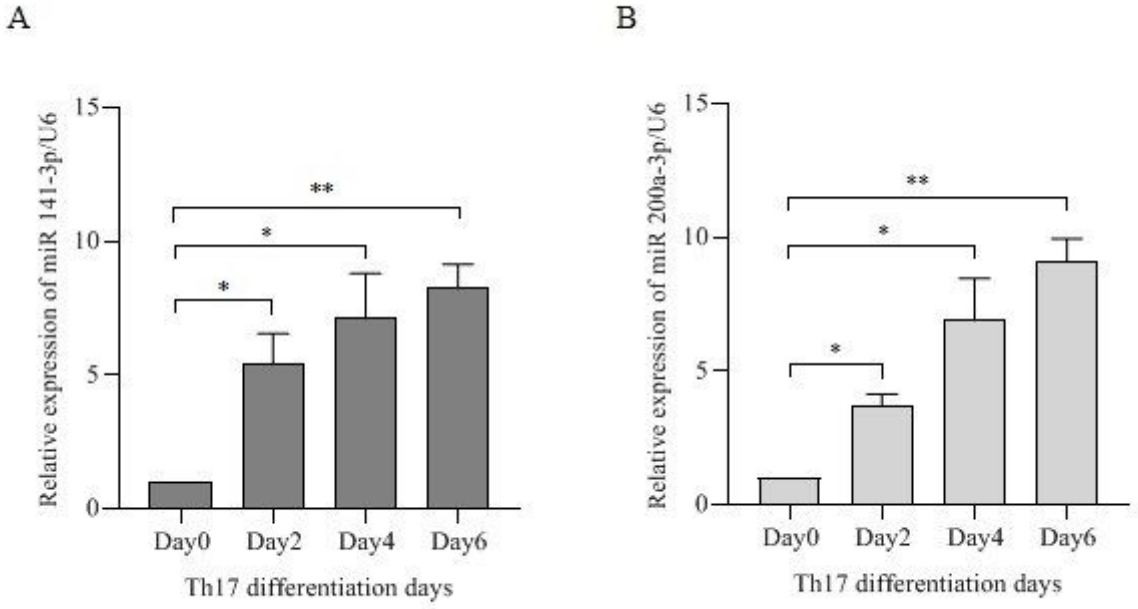

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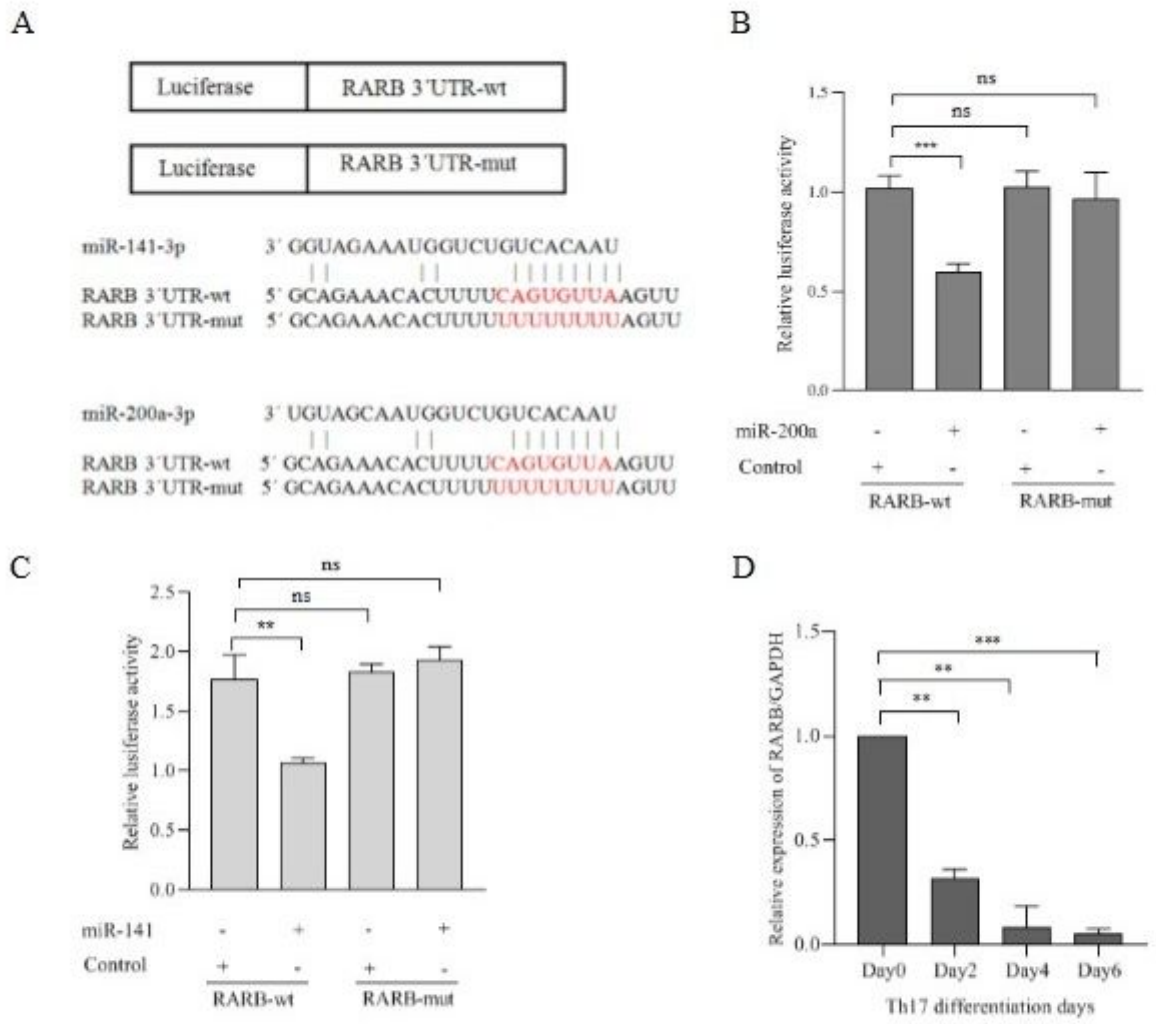

Figure 5

Identification of microRNAs-RARB direct interaction (A) Luciferase reporter constructs for synthesized wt and mut of RARB 3´UTR. Sequences of wt and mut target sites for both microRNAs in RARB 3´UTR are also shown. (B and C) Luciferase reporter assay results. HEK 293T cells were cotransfected by vacant or microRNA precursor containing pBud-EGFP vectors and psiCHECK2 vector including wt or mut RARB 3´UTR. Both microRNAs directly targeted RARB (**$p < 0.01$, ***$p < 0.001$, students t-test). (D) RARB

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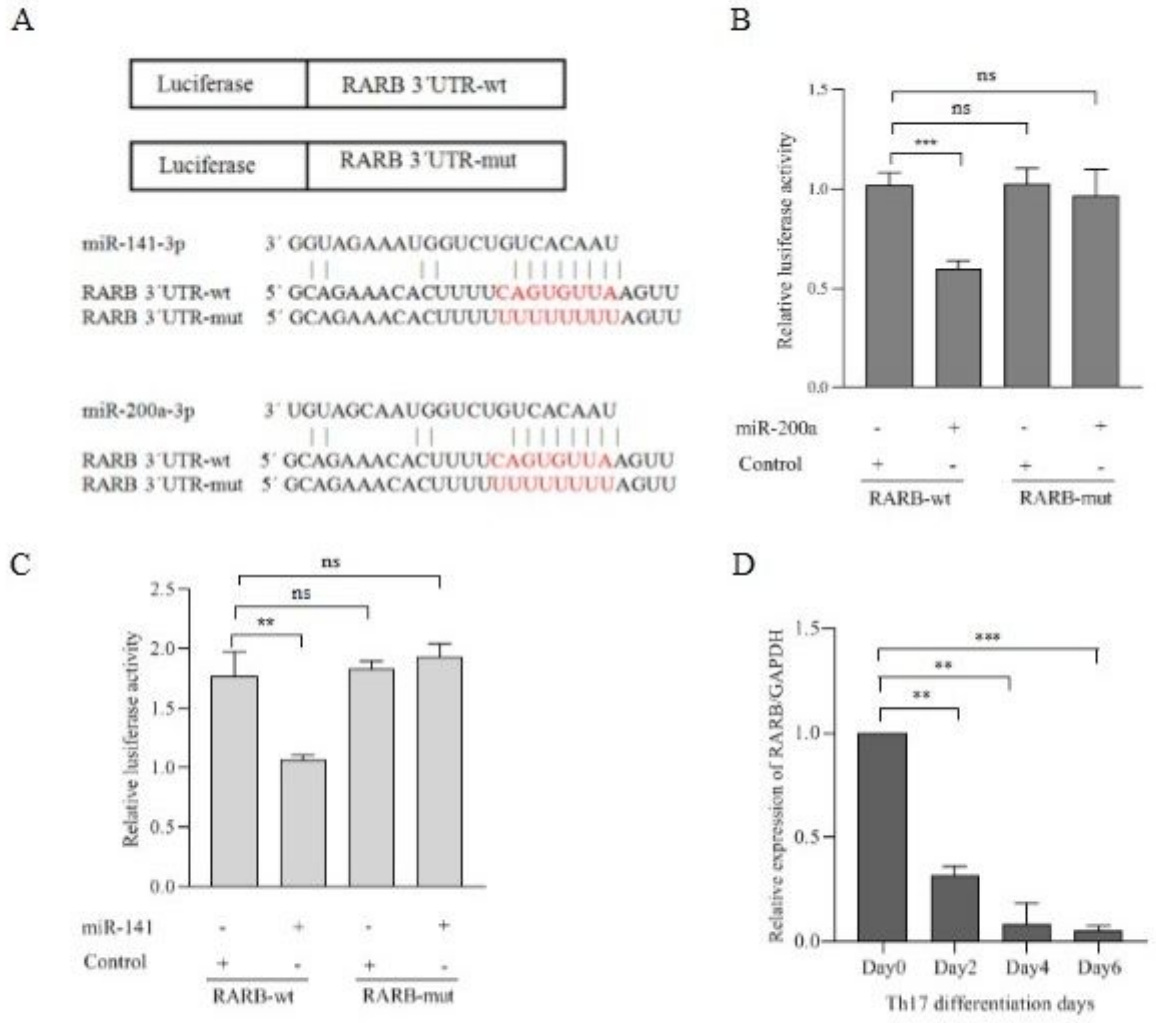

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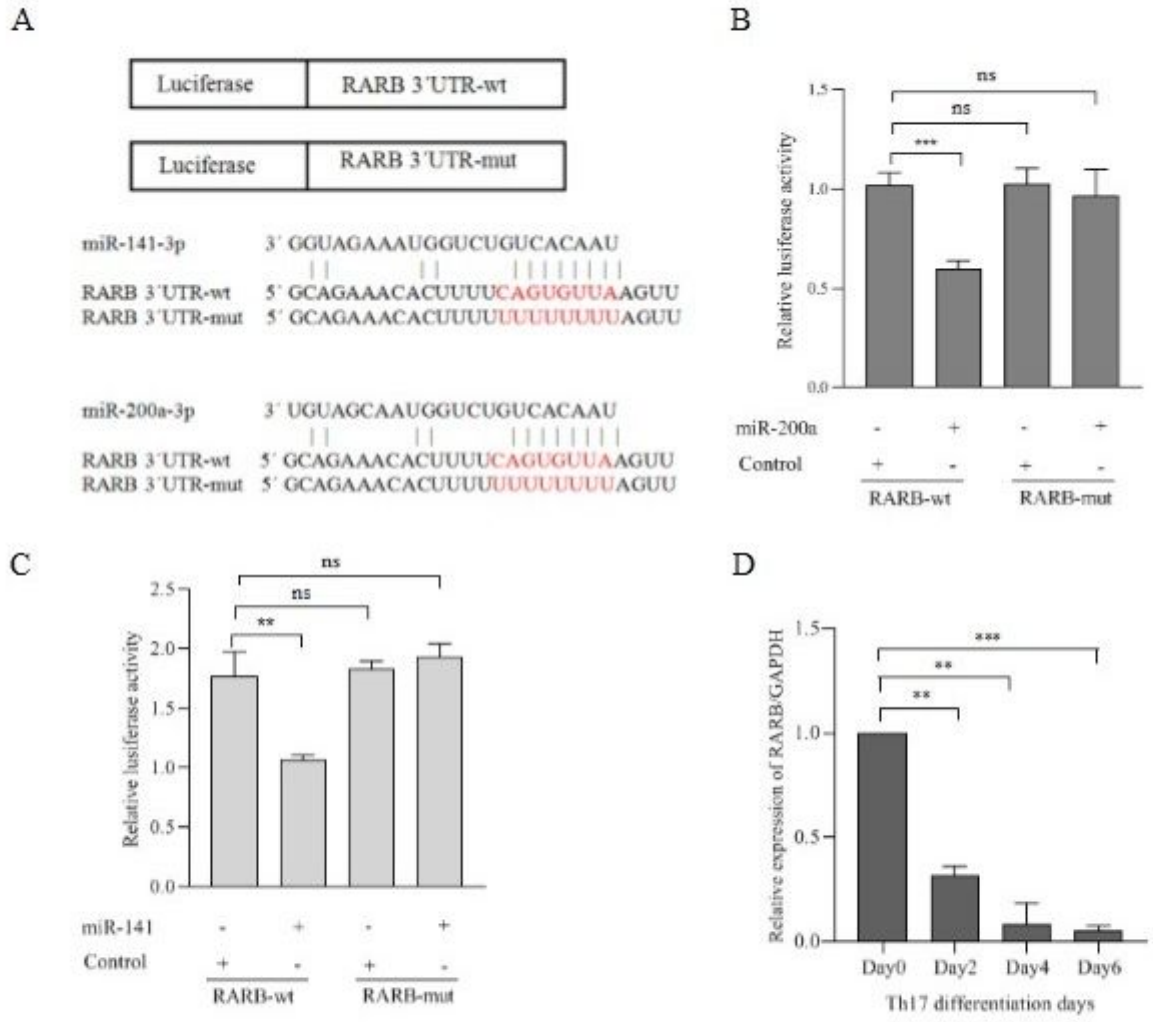

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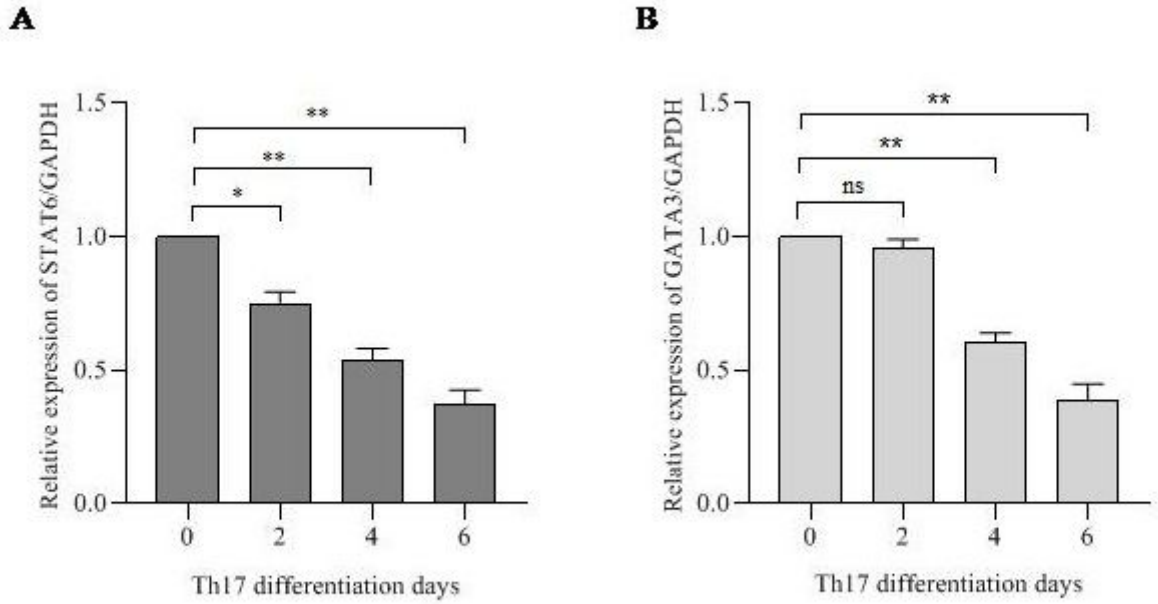
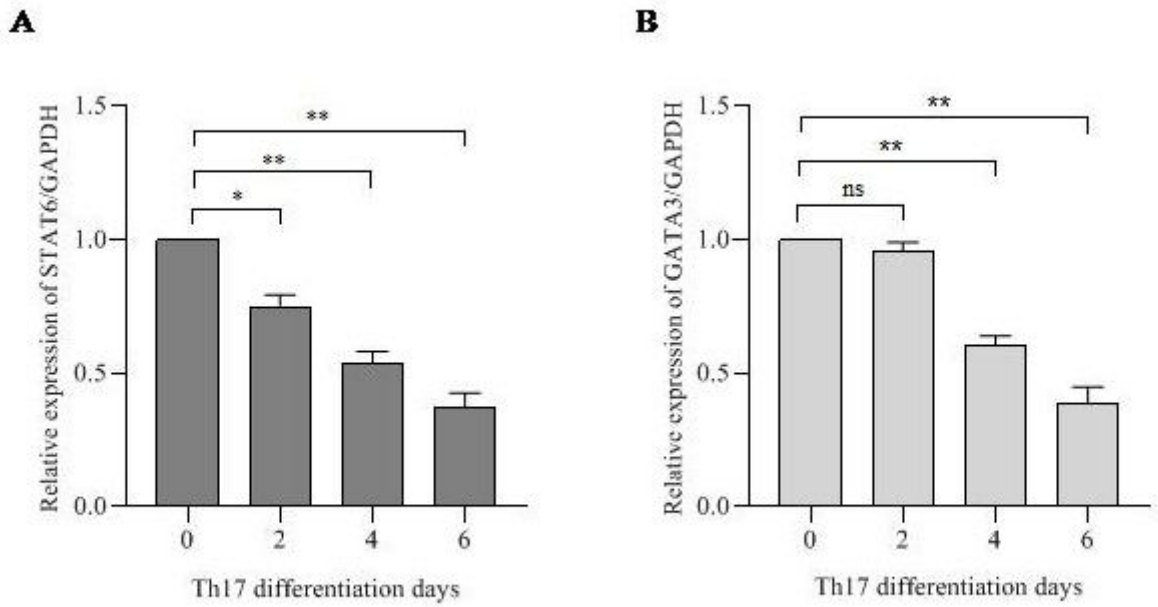


Figure 6

Expression pattern evaluation of RARB downstream genes. (A) STAT6 expression pattern. (B) GATA3 expression pattern. RT-qPCR results displayed downregulation of STAT6 and GATA3 over Th17 cell differentiation. Expression levels were normalized to GAPDH level as an internal control . (* p < 0.05, ** p < 0.01, students t-test). ns stands for non-significance.



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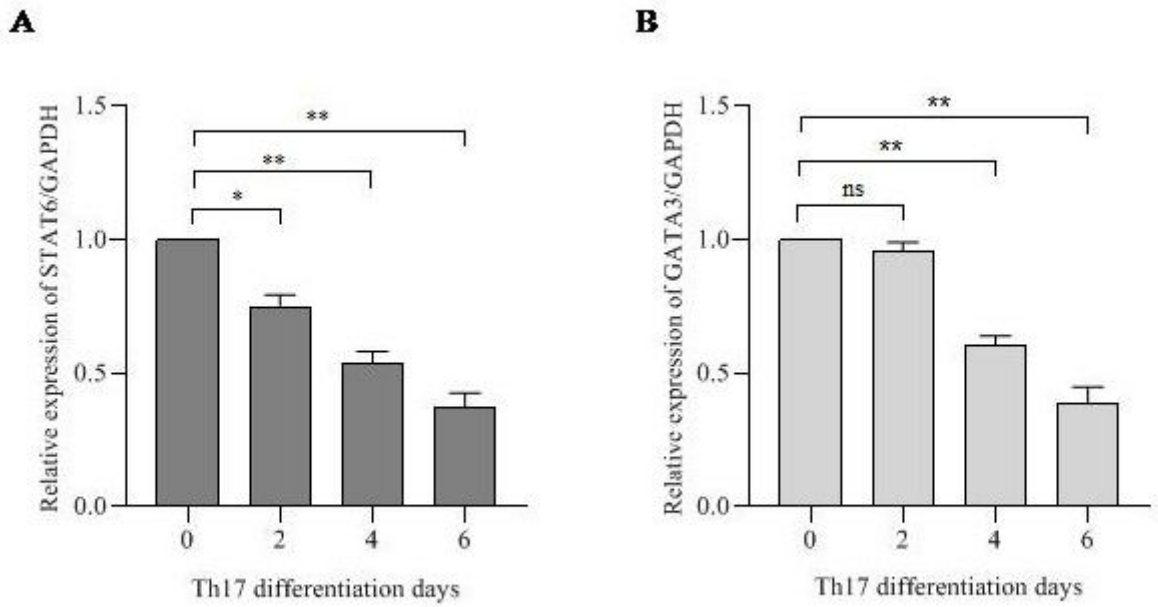

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