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Analyzing the Role of CircSnx5 in an Animal Model of Multiple Sclerosis

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ABSTRACT

Circular RNAs (circRNAs) are endogenous non-coding RNA molecules that form covalently closed molecular loops. By regulating gene expression, circRNAs are known to play crucial roles in the development and progression of various diseases, including autoimmune, neoplastic, and neurological disorders.

In this study, we examined the expression of circSnx5 in inflamed CNS tissue at different stages of experimental autoimmune encephalitis (EAE), an animal model for multiple sclerosis (MS), as well as in T cells that were activated and differentiated into different T helper phenotypes (Th1, Th17, Treg). EAE was induced and spinal cord tissues were isolated at different time points following disease induction. $CD4^+$ T cells were isolated from mouse splenocytes and differentiated toward Th1, Th17, and Treg phenotypes, followed by the analysis of circSnx5 expression.

Compared with control mice, enhanced expression of both circular and linear forms of Snx5 was detected in EAE lumbar spinal cords at the peak and post-peak phases of the disease. However, the ratio of the circular to linear forms (CLR) was decreased in EAE mice compared with controls. Expression of circSnx5 was highly correlated with the levels of inflammatory cytokines in the spinal cord tissue. Significant decreases were observed in circSnx5 expression levels following polyclonal activation of splenocytes. The expression of circSnx5 was also downregulated in differentiated T cells directed toward Th1, Th17, and Treg.

Our findings suggest a potential role of circSnx5 in autoimmune neuroinflammation. The altered expression of circSnx5 during activation and differentiation may offer valuable insights into potential strategies for regulating inflammation in multiple sclerosis (MS).

Keywords: Circular RNAs; Experimental autoimmune encephalomyelitis; Multiple sclerosis; Neuroinflammation; SNX5

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INTRODUCTION

Multiple sclerosis (MS), a chronic inflammatory autoimmune disorder that affects millions worldwide,[1] is characterized by demyelinating plaques in the central

nervous system (CNS).[2] Research has demonstrated that the onset and progression of multiple sclerosis (MS) are influenced by a variety of factors, e.g. viral infections, genetic predisposition and environmental elements. These factors give rise to autoimmune processes that result in the infiltration of immune cells into the CNS with subsequent myelin and neural cell damage.[3,4]

CircRNAs are RNA molecules with a covalently closed loop structure that are formed by the back splicing of exons or introns from precursor RNAs.[5,6] CircRNAs are rich in miRNA binding sites, hence they can act as 'molecular sponges' for miRNAs which regulate the expression of other target genes.[7-9] With advancements in high-throughput sequencing and innovative bioinformatics analyses, numerous circRNAs have been identified as playing crucial roles in regulating gene expression across a variety of diseases.[10]

Evidence indicates that circRNAs play essential roles in regulating the activity of the immune system in physiological conditions, as well as in immune-related disorders, e.g. autoimmune diseases. However, there is limited information about the role of these RNA molecules in autoimmune diseases of the nervous system, including multiple sclerosis.[11]

Sorting nexins (SNX) are a family of PX domain-containing proteins that play vital roles in the sorting and transport of intracellular proteins. Dysfunction in the SNX family genes is associated with various neurological diseases, including Alzheimer's disease and Parkinson's disease.[12-14] One important sorting nexin is SNX5, a 404-residue protein that is comprised of a central PX domain and a large C-terminal domain, which is thought to be involved in membrane curvature and tubulation.([15, 16]) SNX5 was first discovered due to its homology with another SNX protein, SNX1, and was subsequently shown to associate with early endosomes.[17]

CircSnx5 is a circRNA that is derived from Snx5 precursor RNA. There is limited information about the role of circSnx5 in the immune system. Recent studies have reported that ectopic circSnx5 expression suppresses DC activation and induces T cell hypo responsiveness as well as antigen-specific Tregs. Studies have shown that circSnx5 regulates miR-544/SOCS1 axis and PU.1 nuclear translocation, thereby significantly inhibiting the inflammatory differentiation of DCs and inducing immunological tolerance.[18]

Given the available data regarding the effects of circSnx5 in immune cell function, we investigated the potential contribution of this circRNA in the pathogenesis of autoimmune demyelination. We examined CNS tissues from EAE animals at different time points following disease induction. Additionally, we assessed the expression of circcSnx5 in activated lymphocytes and differentiated T cells.

MATERIALS AND METHODS

Mice and EAE Induction

Eight-week-old female C57BL/6 wild-type (WT) mice were obtained from the Pasteur Institute of Iran. All of the experiments were performed in accordance with the Animal Care Committee of Tehran University of Medical Sciences guidelines (IR.TUMS.SINAHOSPITAL.REC.1400.023).

Twelve-week-old female mice (n=20) were immunized subcutaneously with 100 µg of MOG35–55 (MEVGWYRSPFSRVVHLYRNGK) (EK-2110, Hooke Kit™ MOG35–55/CFA Emulsion PTX-, EK-2110, USA) in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) at two different sites. Each mouse received an intraperitoneal (i.p.) injection of 200 ng of pertussis toxin (Sigma-Aldrich, USA) on the day of immunization and again 24 hours later. Clinical assessment of EAE was performed daily for 30 days, following a scoring scale of 0 to 15 points.[19]

The lumbar spinal cord tissues were extracted from EAE mice at two time points following disease induction, including the acute phase (at the peak of the disease) and a late phase referred to as the post-peak phase (days 24–30 post-immunization). The tissues were suspended in Qiazol (Qiagen,79306 Germany), and stored at − 80°C for later processing. After the complete tissue set was collected, total RNA was extracted using miRNeasy Mini kits (Qiagen Biosystems-217004 Germany).

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated using miRNeasy Mini kits (Qiagen Biosystems-217004 Germany) according to the manufacturer's protocol. 500ng of total RNA was used for first-strand cDNA synthesis. Total RNA was incubated for 15 min at 37°C with DNase I (Qiagen-79254 Germany). cDNA was prepared using Yekta Tajhiz cDNA synthesis kit (YT4500, Iran) followed by real-time PCR (Yekta Tajhiz SYBR Green PCR kit, YT2551, Iran).

Divergent primers which crossed the back-splicing junction (BSJ) of the circRNA and convergent primers for the linear form of the RNA were designed using circPrimer software (Figure 1A, B). Sequences of the primers used for PCR are listed in Supplementary Table 1. PCR reactions were performed using a StepOne Plus real-time PCR machine (ABI systems). To ensure the reliability and comparability of the RT-PCR results, PCR efficacy for each reaction was calculated using LinReg software. Livak method was used to calculate the relative fold change (RFC) in gene expression. GAPDH expression levels were used to normalize the data. In addition to melt curve analysis, the purity and length of the PCR-amplified products were examined by 1.5% (wt/vol) agarose gel electrophoresis (Figure 1C).

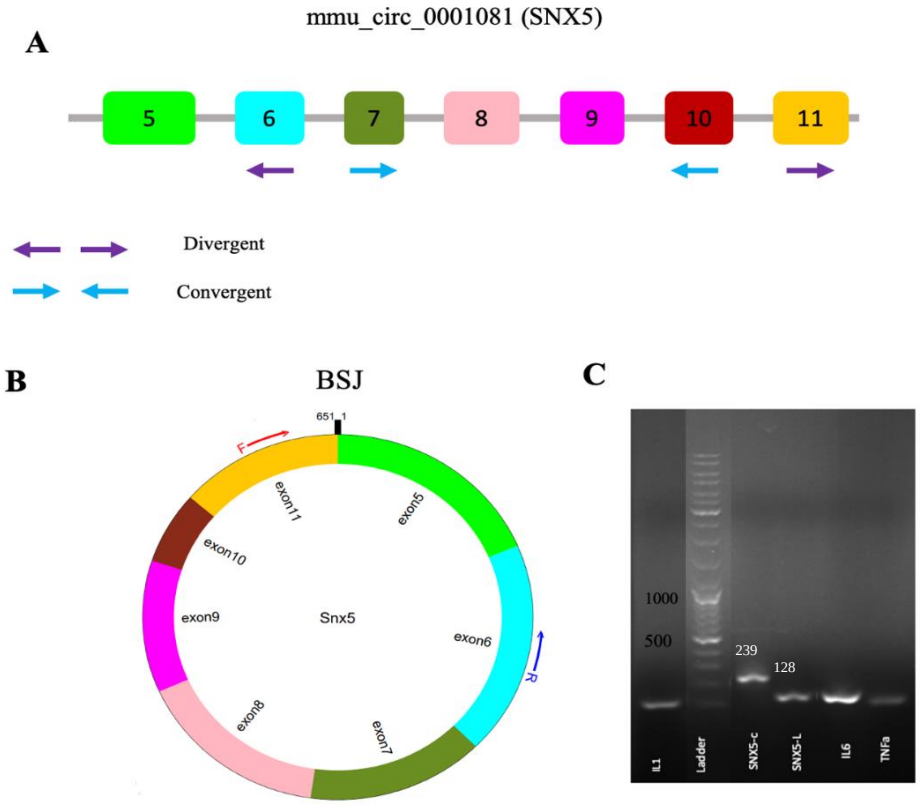


Figure 1. A, Schematic figure illustrates the structure of mmu_circ_0001081 (circSnx5). The binding sites of PCR primers used to detect circSnx5 (blue arrows) and linear Snx5 mRNA (purple arrows) are shown. B, the back spliced site is shown in the circular form. C, Agarose gel electrophoresis of RT-PCR products.
CLR was calculated by determining the ratio of circular to linear transcripts using the formula. $\frac{Circular\ form\ RFC}{Linear\ form\ RFC}$

Splenocyte culture and Stimulation

To perform in vitro stimulation experiments, splenocytes were obtained from 6- to 8-week-old C57BL/6 mice. Mice were sacrificed, and spleens were removed and homogenized in PBS. Splenocytes were isolated using Ficoll-Hypaque density gradient centrifugation. 2×10^6 cells were seeded in RPMI 1640 medium (Gibco USA, B11031) supplemented with 5% FBS (Gibco USA, 1027-106) and subsequently, stimulated with anti-CD3 (1 µg/ mL) (BioLegend USA, 155702) and anti-CD28 (1 µg/mL) (BioLegend USA, 122002) antibodies (eBioscience) for different time points (8-24 hour) followed by gene expression analyses.

$CD4^+$ Tn Cell Purification and T Cell Differentiation

Naïve T cells (Tn cells) were purified from splenocytes using magnetic cell sorting (MACS) according to the manufacturer's protocol (Miltenyi Biotec,130-104-453, Germany). Briefly, $CD4^+$ T cells were magnetically labeled with a cocktail of biotin-conjugated antibodies and anti-biotin microbeads, while memory T cells were

magnetically labeled with CD44 microbeads. Isolation of highly pure naïve T cells was achieved by depleting magnetically labeled non-target cells.

For Th1 induction, purified $CD4^+$ Tn cells were cultured for 96 hours under Th1 cell-polarizing conditions in RPMI-1640 containing 10% FBS, anti-CD3 (1 μg/mL), anti-CD28 antibodies (0.5 μg/mL), Il-2 (20 ng/mL) (BioLegend USA, 575402), Il-12 (50 ng/mL) (BioLegend USA, 577002), and anti-Il-4 antibody (10 ng/mL) (Biloegend, 504108).

To induce Th17 cells, purified $CD4^+$ Tn cells were cultured for 96 hours under Th17 cell-polarizing conditions in RPMI-1640 containing 10% FBS, anti-CD3 (1 μg/mL), anti-CD28 antibodies (0.5 μg/mL), Tgf-β (5 ng/mL) (BioLegend USA, 763102), Il-6 (100 ng/mL) (BioLegend USA, 575702), anti- Ifn-γ (10 ng/mL) (BioLegend USA, 505812), anti-Il-4 (10 ng/mL) (Biloegend USA, 504108), and Il-23 (50 ng/mL) (BioLegend USA, 589002).

For Treg induction, purified CD4+ Tn cells were cultured for 96 hours under Treg cell-polarizing conditions in RPMI-1640 containing 10% FBS, anti-CD3 (1 μg/mL), anti-CD28 antibodies (0.5 μg/mL), Il-2 (20 ng/mL) (BioLegend USA, 575402), and Tgf-β1 (50 ng/mL) (BioLegend USA, 763102).

To produce Th0 cells, transfected cells were cultured in the presence of Il-2 (20 ng/mL) (BioLegend USA, 575402), anti-Ifn-γ (10 ng/mL) (BioLegend USA, 505812), and anti-Il-4 (10 ng/mL) (Biloegend, 504108) for 96 hours. Th0 cells were used as controls.

Statistical Analyses

Statistical analyses were performed using Excel for basic computations and GraphPad Prism for data visualization and statistical tests. ANOVA was used to assess differences across multiple groups, followed by relevant post-hoc analyses. In cases of binary comparisons, Student's t-test was employed. Data are presented as mean±SEM, and a *p*-value of<0.05 was considered statistically significant.

RESULTS

Circular and Linear Forms of Snx5 Are Differentially Expressed in EAE

To investigate the expression of Snx5 circular and linear isoforms in the CNS during autoimmune neuroinflammation, real-time RT-PCR analyses were performed on tissues obtained from 30 mice, stratified into three groups: a control unimmunized group (n=10), an EAE group at the peak of the disease (days 18 to 20 post-induction, n=10), and an EAE group after the peak of the disease (day 25 post-induction, n=10).

As detailed in the Methods section, divergent and convergent primers were used to measure the relative expression levels of mmu_circ_0001081 (circSnx5) and its linear counterpart (NM_001199188). The RT-PCR results showed a significant upregulation in the expression of both linear and circular forms of Snx5 at the peak phase of the disease compared with the control group (Figure 2A, B). In the post-peak phase of the disease, the expression of the linear form was still slightly higher compared with the control group, while the levels of the circular form returned to normal. Interestingly, the analysis of the circular-to-linear ratio (CLR) at the peak and post-peak phases revealed a significant decrease compared with the control group. The CLR ratio was also lower in the post-peak phase compared with the peak phase, highlighting the dynamic changes in RNA expression that occur at different stages of the disease (Figure 2C).

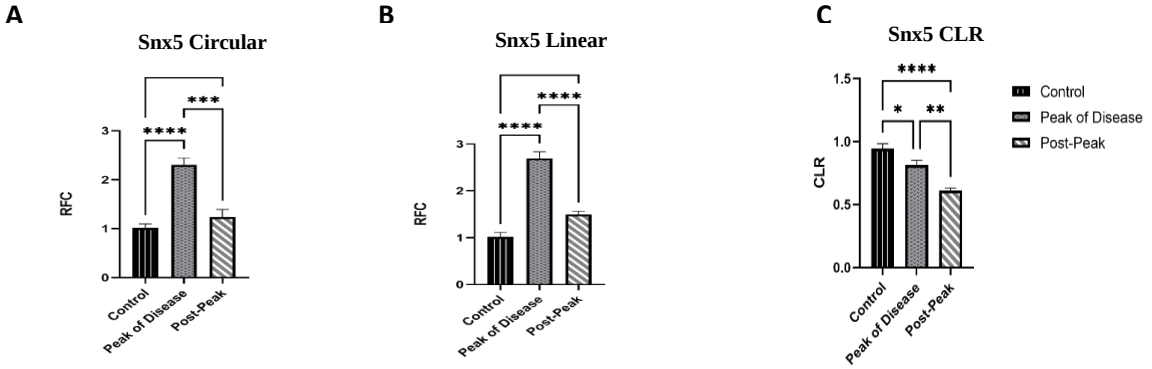
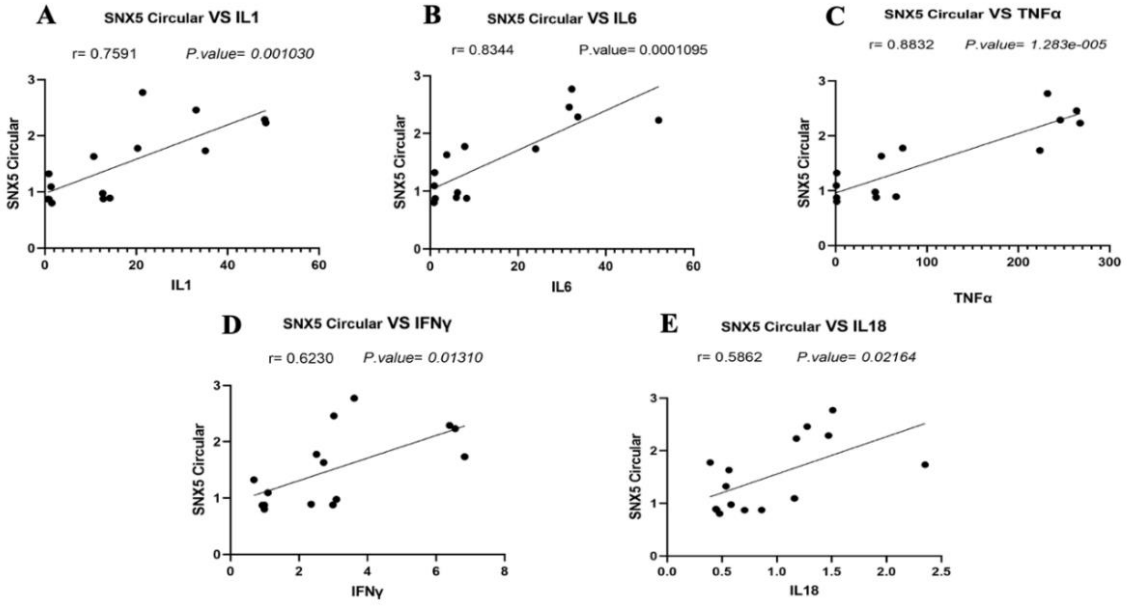


Figure 2. Expression of circular and linear forms of Snx5 and its CLR ratio in EAE spinal cords. Data are presented as mean±SEM. * *p*<0.05, ** *p*<0.01, * *p*<0.001, **** *p*<0.0001, The number of mice in each group was n=10. Mann-Whitney and Kruskal–Wallis tests were used for statistical analysis. RFC: Relative Fold Change, CLR: Circular to Linear Ratio, ns: Not Significant**

Circular and Linear Forms of Snx5 Are Correlated with Inflammatory Markers in EAE

Next, we investigated the relationship between the expression of circular and linear forms of Snx5 and the expression of inflammation-related cytokines, including *Il1, Il6, Il18, Ifn-γ*, and *Tnf-α*, in the spinal cord tissues of EAE mice. Our data demonstrated a positive correlation between circSnx5 expression and the following cytokines*:* *Il1* (r=0.759, *p*<0.001), *Il6* (r=0.834, *p*<0.0001), *Tnf-α* (r=0.883, *p*<1.283e-005), *Il18* (r=0.586, *p*<0.021) and *Ifn-γ* (r= 0.623, *p*<0.013) (Figure 3A-E).

We also observed a significant correlation between the linear form of Snx5 with *Il1* (r=0.826, *p*<0.0001), *IL6* (r=0.870, *p*<2.416e-005), *Tnf-α* (r=0.948, *p*<7.495E-008), *Il18* (r=0.691, *p*<0.004) and *Ifn-γ* (r=0.732, *p*<0.0019) (Fig 3F-J). However, the CLR ratio did not exhibit any significant correlations with these cytokines (Figure 3K-O).



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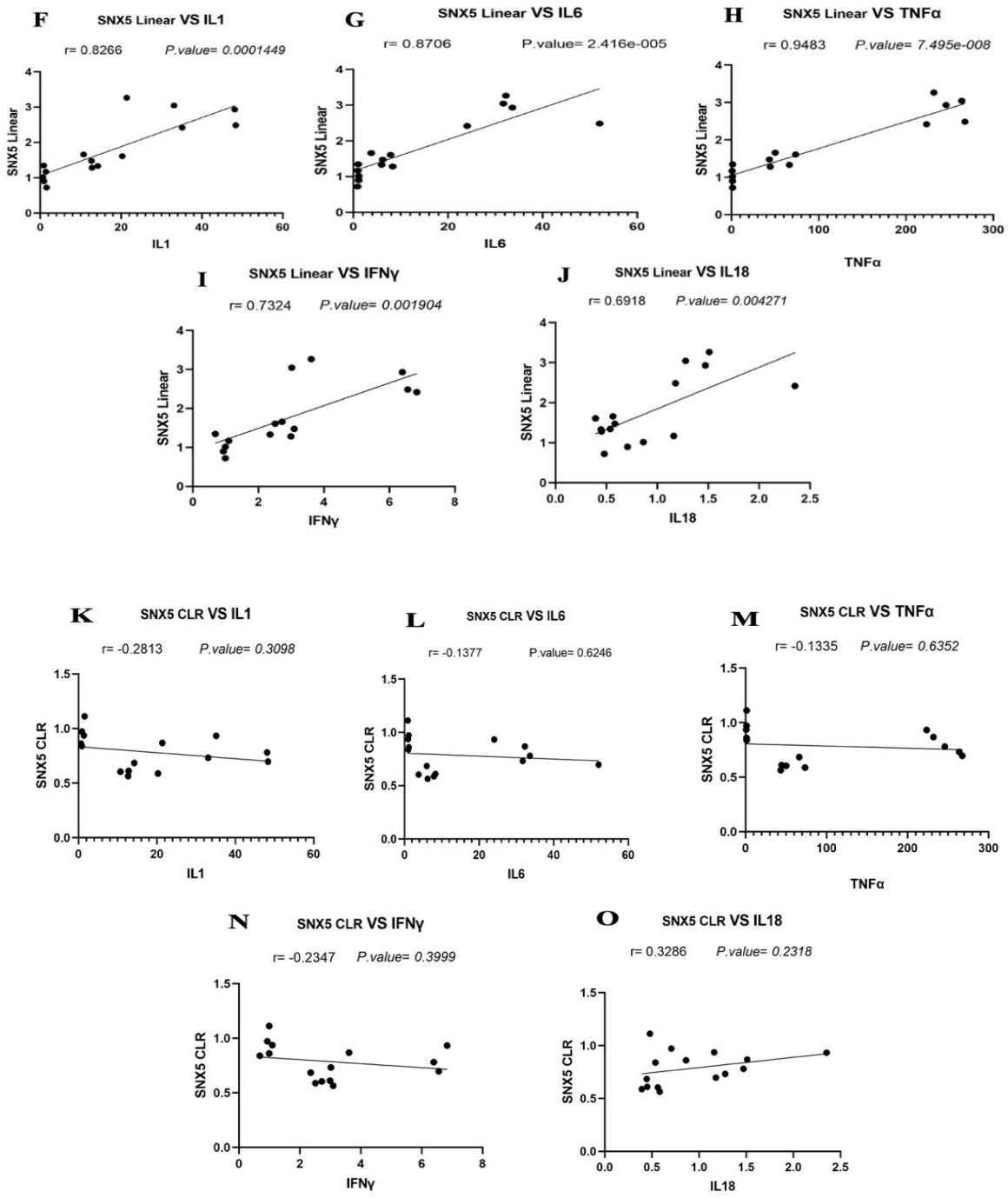

Figure 3. Correlation of circular and linear forms of Snx5 and CLR ratio with inflammatory cytokines in EAE.
Spearman's analysis was conducted to assess the correlation between the expression of circular and linear forms of Snx5 and CLR ratio with inflammatory cytokines in EAE. A-E: Correlation of circSnx5 with *Il1*(A), *Il6* (B), *Tnf-α* (C), *Ifn-γ* (D), *Il18*(E). F-J: Correlations of Linear Snx5 with *Il1*(F), *Il6* (G), *Tnf-α* (H), *Ifn-γ* (I), Il18(J). K-O: Correlations of CLR ratio with *Il1*(K), *Il6* (L), *Tnf-α* (M), *Ifn-γ* (N), *Il18*(O). $p<0.05$ was considered statistically significant.

Circular and Linear Forms of Snx5 Are Downregulated in Activated Splenocytes

To investigate the correlation between Snx5 expression and T cell activation, we activated T cells polyclonally and measured Snx5 expression in both linear and circular forms at different time points following activation, as described in the Methods section. Real-time RT-PCR results displayed a significant decrease in the expression of both circular and linear forms of Snx5, at 8 and 24 hours compared to the control cells that were not subjected to polyclonal activation (Figure 4A, B). Additionally, the CLR ratio showed a significant decrease at both the 8-hour and 24-hour time points compared with the control (Figure 4C).

Circular and Linear Forms of Snx5 Are Altered in Differentiated T Cells

To further investigate the role of Snx5 in T cell biology, we differentiated naïve $CD4^+$ cells (Tn cells) into Th1, Th17, and Treg subgroups in vitro and measured the expression of both circular and linear forms of the transcript in each subgroup. Our results revealed a significant decrease in the expression levels of both circular and linear forms of Snx5 as the T cells differentiated into Th1, Th17, and Treg subgroups (Figure 5A and 5B).

Additionally, the CLR ratio of Snx5 was lower in Th1, Th17, and Treg subgroups compared with Th0 cells (Figure 5C).

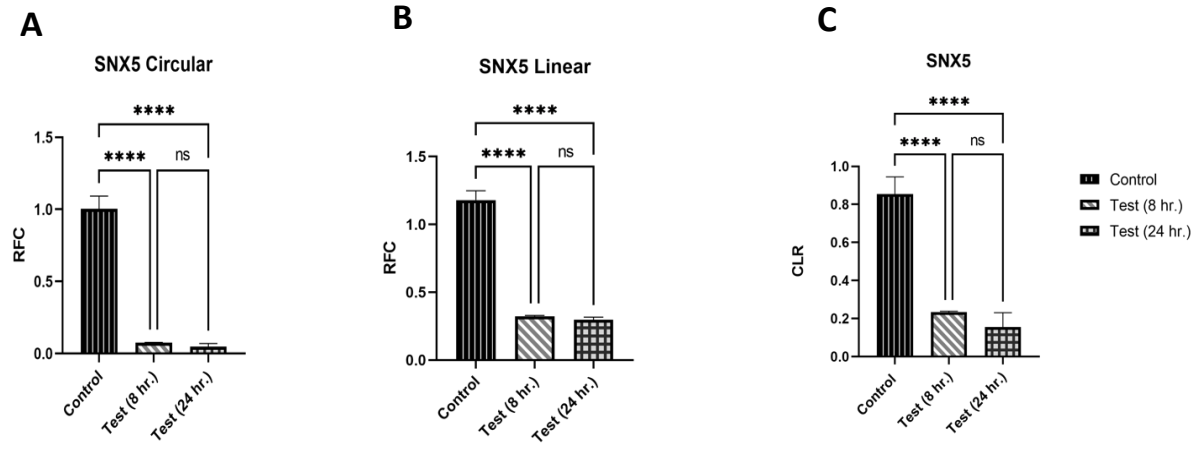

Figure 4. Expression of circular and linear forms of Snx5 and CLR ratio in splenocytes stimulated with anti-CD3 and anti-CD28 for indicated time points. Data are shown as mean±SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, Number of mice in each group=10, Mann-Whitney and Kruskal–Wallis tests.

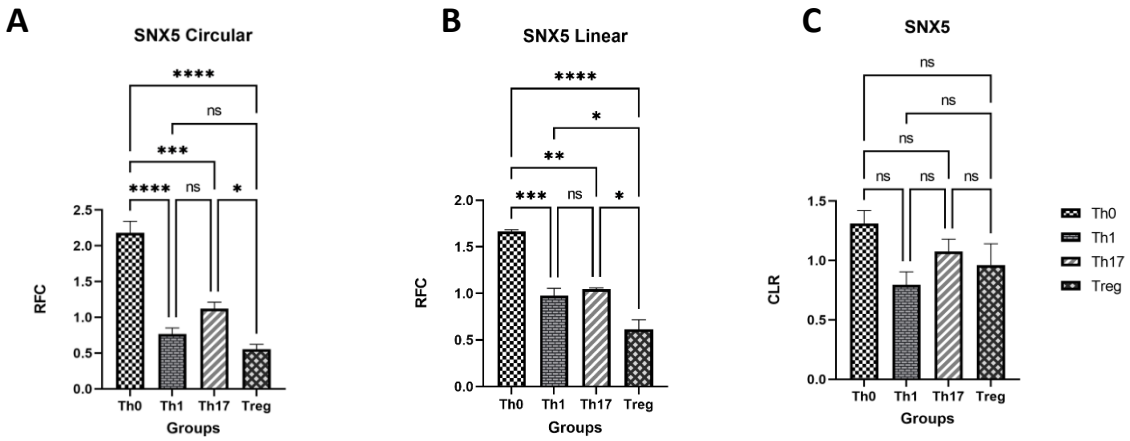

Figure 5. Expression of circular and linear forms of Snx5 and the CLR ratio in T cells differentiated into Th1, Th17, and Treg subgroups. Data are shown as mean±SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, Number of mice in each group=10, Mann-Whitney and Kruskal–Wallis tests.

DISCUSSION

The rapid advancements in RNA sequencing technology and bioinformatics analyses are uncovering the roles of novel classes of noncoding RNAs in human diseases.[20] Over 10,000 candidate circRNAs have been identified in the human transcriptome and studies have demonstrated that these molecules may be involved in various disease process through regulating cell proliferation, differentiation, and apoptosis. Moreover, circRNAs have gained attention as promising biomarkers for diagnosing tumors and inflammatory diseases.[10] Evidence suggests that these molecules are associated with the onset and progression of autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis and MS.[21-23]

In this study, we investigated the role of circSnx5 in autoimmune neuroinflammation, by analyzing its expression in mice CNS tissue at different stages of EAE, as well as in T cells following activation and differentiation. We hypothesized that circSnx5 might be involved in the pathogenesis of EAE, likely through the regulation of T cell function.[10] Our results demonstrated elevated expression of both linear and circular forms of circSnx5 during the peak phase of EAE, suggesting its potential involvement in the inflammatory stage of the disease. However, the circular to linear proportion was lower at the peak of the disease, compared with control tissues. These findings were in line with prior research highlighting the involvement of SNX5 in neurodegenerative conditions like Alzheimer's and Parkinson's disease.[12] The enhanced expression of the linear and circular forms was reduced to near control levels during the post-peak phase of the disease.

In recent years various research groups have investigated the role of circRNAs in MS. Cardamone et al. reported several differentially expressed circRNAs in MS patients compared to controls, including distinct expression patterns of circRNAs in MS subtypes.[24] Paraboschi et al observed significant enrichment of circRNAs in linkage disequilibrium blocks harboring MS-associated single nucleotide polymorphisms SNPs, suggesting that MS-associated SNPs influence the levels of specific circRNAs in the surrounding areas. This influence could modify the ratio of circular to linear RNA isoforms, likely contributing to disease process.[25] Zurawska et al reported that circRNAs expression were dysregulated in immune cells of MS patients, with specific circRNAs potentially participating in the disease's progression by regulating the polarization of Th17 cells and the expression of STAT3, a key transcription factor mediating the inflammatory response.[10] These results propose a potential mechanism by which MS-associated SNPs affect circRNA expression levels, thereby contributing to the disease process.[26] Furthermore, bioinformatic methods have been used to investigate the role of circRNAs and their associated networks in MS. Notably, Huang et al analyzed circRNA/miRNA/mRNA networks in MS patients using bioinformatics approaches and identified four networks that affect B-cell proliferation and B-cell receptor signaling pathways. These findings suggest the potential implications of circRNAs in the pathophysiology of MS.[27]

SNX5 is known to regulate several biological processes related to innate immunity. Of interest, SNX5 is known as a crucial regulator of macrophage micropinocytosis.[28] CircSnx5 also plays a central role in regulating DC-driven immunity and tolerance. It has been reported that circSnx5 sponges miR-544, mitigating the miRNA-mediated inhibition of cytokine signaling 1 (SOCS1). This interaction leads to the inhibition of DC maturation and simultaneously accelerates the formation of Treg. In addition to miRNA sponging, circRNAs also mediate post-transcriptional regulation by sponging proteins. For instance, circSnx5 can sponge PU.1 transcription factor, suppressing its nuclear translocation and inhibiting the transcription of MHC class II in DCs. Studies have shown that overexpression of circSnx5 inhibits DC activation and promotes the development of DC tolerance, whereas circSnx5 knockdown promotes the activation of DCs and the associated inflammatory processes.[18]

In the current study we saw a significant positive correlation between the linear and circular forms of Snx5 and various innate and adaptive inflammatory cytokines, including *Tnf-α*, *Il1*, *Il6*, *Il18*, and *Ifn-γ*, during EAE. These results are consistent with existing research that highlights the involvement of circSNX5 in cytokine signaling pathways.[18] Additionally, our findings indicated a reduction in the expression of both linear and circular SNX5 during activation and differentiation of naive T cells into Th1, Th17 and Treg subtypes. To the best of our knowledge, no prior studies have investigated the role of SNX5 in T cell activation/differentiation. Nonetheless, one study has revealed an inverse correlation between

SNX5 and CD44, a marker that is upregulated following T cell activation, which may provide a potential link to SNX5 role in T cell activation.[29]

Our data highlight the role of circSnx5 in autoimmune neuroinflammation. The altered expression of circSnx5 during EAE, along with its connection to T cell activation and differentiation, could shed light on the mechanisms involved in regulating inflammation in MS. However, understanding of the precise molecular mechanisms underlying circSnx5 function in MS/EAE requires further investigation.

STATEMENT OF ETHICS

The study protocol was approved by the ethics committee of Tehran University of Medical Sciences (Ethical code: IR.TUMS.SINAHOSPITAL.REC. 1400.023).

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

Not applicable.

DATA AVAILABILITY

The data supporting the findings of this study are available from the first authors upon reasonable request (Lkhosroshahi@bccrc.ca).

AI ASSISTANCE DISCLOSURE

Not applicable.

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