

Long non-coding RNA RP11-340F14.6 promotes a shift in the Th17/Treg ratio by binding with P2X7R in juvenile idiopathic arthritis

NA HUANG¹, ZHIDAN FAN¹, LE MA¹, HUIHUI MA¹, HUI HUANG¹, HAIGUO YU¹ and XIAOYU ZHOU²

Departments of ¹Rheumatology and Immunology, and ²Neonatology, Children's Hospital of Nanjing Medical University, Nanjing, Jiangsu 210008, P.R. China

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Abstract. Long non-coding RNA (lncRNAs) have been identified to play important roles in multiple human diseases via the regulation of cell proliferation, cell invasion, or cell death. However, little is known about the role of lncRNAs in the process of shifts in the Th17/Treg ratio during the progression of juvenile idiopathic arthritis (JIA). The aim of the present study was to determine the role of lncRNA RP11-340F14.6 in the shifting of the Th17/Treg ratio in JIA. The distribution of the T cell subgroup was detected by flow cytometry in peripheral blood mononuclear cells from patients with JIA and healthy controls. It was found that the expression of lncRNA RP11-340F14.6 was upregulated, and to positively correlate with that of retinoic acid-related orphan receptor gamma t (ROR γ t), and to negatively correlate with Foxp3 expression in patients with JIA. RP11-340F14.6 induced the expression of its neighbor, P2X7R. Through a P2X7R-independent approach, this lncRNA was also found to play a pivotal role in stimulating Th17 differentiation and simultaneously suppressing Treg distribution. Taken together, the findings of the present study demonstrate that RP11-340F14.6 specifically binds to P2X7R, which results in the continuous activation of P2X7R. Thus, RP11-340F14.6 may serve as a promising therapeutic target for the treatment of JIA.

Introduction

Juvenile idiopathic arthritis (JIA) is the most common childhood rheumatic disease and is highly debilitating. The disease often persists into adulthood, causing obvious functional disabilities, including joint deformation, abnormal growth and development, osteoporosis, pain, psychological abnormalities and difficulty with self-care (1-3). According to the International League of Associations for Rheumatology criteria, there are seven types of JIA. One of these specific types is rheumatoid factor-positive poly-arthritis (RF-positive pJIA), which is defined by the presence of arthritis in >4 joints and a positive rheumatoid factor (4-6). RF-positive pJIA and adult rheumatoid arthritis (RA) have similar clinical manifestations, as well as serological and immunogenetic profiles. Patients with RA exhibit an activation of cells secreting pro-inflammatory interleukin (IL)-17 cytokine (Th17), the activation of which is normally suppressed by regulatory T lymphocytes (Tregs) (7,8). An imbalance in the Th17/Treg cell ratio along with disruptions in the cytokine environment have been reported to be involved in synovial hyperplasia and joint destruction in patients with RA (9,10). A number of *in vivo* studies have demonstrated that primary CD4⁺ T cells can differentiate into different subtypes of helper T (Th) cells under the regulation of various antigens, cytokines and other factors. Alterations in the Th cell subgroup ratio plays a key role in the immunopathology of JIA (11-15). However, the process surrounding the shift in the ratio of Th17/Treg cells is complex and dynamic, and the specific mechanisms involved remain unclear.

Long non-coding RNAs (lncRNAs) play an important role in biological processes and disease development by regulating chromosome recombination, gene modification, gene transcription, post-transcriptional modification and other mechanisms (16-18). The importance of lncRNAs has been studied in the immune system. Specifically, lncRNA insulin receptor precursor (INSR) has been shown to function through an INSR-independent mechanism to enhance Treg differentiation and promote immune suppression in the immune microenvironment of pediatric acute lymphoblastic leukemia (ALL) (19). The involvement of lncRNAs in the development and differentiation of CD4⁺ T cells has also been reported (20).

Correspondence to: Dr Haiguo Yu, Department of Rheumatology and Immunology, Children's Hospital of Nanjing Medical University, 72 Guangzhou Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: yuhaiguo73@yeah.net

Dr Xiaoyu Zhou, Department of Neonatology, Children's Hospital of Nanjing Medical University, 72 Guangzhou Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: xyzhou161@163.com

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For example, lncRNA Tmevpg1 has been reported to be specifically expressed by the Th1 phenotype via T-bet, a T-box transcription factor (21). linc-MAF-4 has also been confirmed to be a chromatin-associated lncRNA that is specific to the Th1 subtypes (22). However, the specific mechanisms through which lncRNAs mediate immune abnormalities and promote the development of JIA remain unclear.

In the present study, sequence-based screening was conducted in patients with JIA and healthy volunteers to explore potential interactions between lncRNAs and mRNAs. A specific lncRNA, RP11-340F14.6, was identified. It was reported that this lncRNA induced the expression of P2X7R and may promote the immune microenvironment that is associated with JIA.

Materials and methods

Clinical samples. Blood samples were obtained through the Department of Children's Healthcare from 30 healthy volunteers with no personal or family history of chronic autoimmune, cancer, metabolic, or infectious diseases. The volunteers included 9 males and 21 females, with an average age of 8.82 ± 3.77 years. Blood samples from 30 RF-positive patients with JIA were obtained between May, 2017 and May, 2019 including 11 males and 19 females, with an average age of 8.64 ± 3.58 years at the Children's Hospital of Nanjing Medical University. Peripheral lymphocytes were isolated from blood samples. In brief, peripheral blood was collected from all patients before receiving any therapeutic drugs, and blood was collected from the healthy controls during a physical examination. Peripheral lymphocytes were isolated by adopting the Ficoll-Hypaque density gradient centrifugation method. Children who had previously received disease-modifying anti-rheumatic drug (DMARD) therapy or steroid therapy were excluded. Clinical characteristics were classified according to the detailed diagnostic information obtained from the medical records and physical examinations. All experiments were performed in compliance with government policies and the Helsinki Declaration. All patients or healthy controls had the consent of their legal guardians or parents who signed an informed consent form before collecting blood samples. The present study was approved by the Ethics Committee of the Children's Hospital of Nanjing Medical University.

Cell culture. Human T cells were filtered through a $75 \mu\text{m}$ strainer and separated by Ficoll centrifugation ($800 \times g$ for 20 min at 4°C). The mononuclear cells were resuspended in RPMI-1640 supplemented with 10% FBS. The anti-CD4 magnetic Dynabeads (Invitrogen; Thermo Fisher Scientific, Inc.) were applied to sort the T cells.

Microarray detection. Total RNA was isolated from 1×10^6 T cells and used for the lncRNA/mRNA integrated microarray analysis (CapitalBio). Each group included 3 samples. Sample preparation and microarray hybridization were performed according to the manufacturer's instructions with minor modifications. Briefly, mRNA was purified from total RNA following the removal of rRNA (using the mRNA-ONLY™ Eukaryotic mRNA Isolation kit, EPICENTRE Biotechnologies), amplified and transcribed into fluorescent cRNA along the entire length

of the transcripts without 3' bias utilizing a random priming method. The arrays were scanned using an Agilent Scanner (Agilent Technologies, Inc.). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.0 software package (Agilent Technologies, Inc.). Following quartile normalization of the raw data, lncRNAs which had flags in present or marginal ('All Targets Value') were selected for further analysis. lncRNA expression patterns were revealed via Hierarchical analysis using Cluster 3.0 software (Stanford University).

Mutagenesis of lncRNA and P2X7R, and lentiviral packaging. The full-length construct of P2X7R, as well as the full-length and mutant constructs of RP11-340F14.6 were synthesized and cloned into pGC-LV plasmid purchased from GenScript Co. Ltd (Nanjing, China). shRNA technology was employed to knockdown the target genes or lncRNA. shRNAs targeting RP11-340F14.6 or P2X7R were designed constructed by GenScript Co. Ltd. and were cloned into the PLL.3.7 vector purchased from GenScript Co. Ltd. and further packaged to produce lentiviral particles, as previously described (23).

Transfection. The target vectors ($20 \mu\text{g}$) were mixed with lentiviral packaging $15 \mu\text{g}$ $\Delta 8.91$ (GenScript Co. Ltd.) and envelope expressing $10 \mu\text{g}$ VSV-G (GenScript Co. Ltd.) plasmids to generate lentiviral particles in $1.2 \times 10^7/20$ ml 293T cells (ATCC) using $100 \mu\text{l}$ Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Viral particles were concentrated by ultracentrifugation and expression vector titers were determined. The plasmids were constructed and transfected using lentivirus. Cells were cultured with $100 \mu\text{g/ml}$ of human-derived IL-2. The 24-well plates were placed in a cell incubator at 37°C 5% CO_2 and cultured for 48 h. Cells increased in volume after 24 h of stimulation, indicating activation, and were transfected with the lentivirus after 48 h of stimulation. Transfection was performed at a multiplicity of infection (MOI) of 100, using polybrene at a concentration of $5 \mu\text{g/ml}$. When the cells reached the optimal transfection state 72 h later, to transfected cells were screened using puromycin. The transfection efficiency was determined by fluorescence intensity and RT-PCR assay.

Flow cytometry. Following CD4^+ T cell enrichment, the cells were incubated with human anti-CD3-FITC (cat. no. 557832) and anti-CD4-PerCP monoclonal antibodies (mAbs, cat. no. 564419) in 4°C for 30 min (BD Pharmingen). Cells were fixed and permeabilized with Cytofix/Cytoperm (cat. no. 56422 Human Fc Block from BD Pharmingen) and then intracellularly stained with IL-17A-Phycoerythrin (IL-17A-PE) or IgG-PE as an isotype control. To detect the Treg cell frequency, cells were labeled with anti-CD4-FITC and anti-CD25-APC antibodies (cat. no. 555434, BD Pharmingen). Following fixation and permeabilization, the cells were stained using anti-forkhead box protein 3 (Foxp3)-PE mAb (cat. no. 560046, BD Pharmingen) or IgG-PE control at 4°C for 30 min and were then analyzed using a BD FACSCanto II flow cytometer (BD Pharmingen). The data were analyzed using Cell Quest analysis software, version 5.1 (BD Pharmingen).

RNA immunoprecipitation (RIP) assay. RIP assay was carried out using the Magna RIP RNA-Binding Protein Immunoprecipitation kit (EMD Millipore), as previously described (24). Anti-HA antibodies (1:50, ab9110; Abcam) were used for RIP. T cells were either transduced with fixed or varying doses of lentivirus containing RP11-340F14.6 along with lentivirus containing P2X7R. The coprecipitated RNAs were detected by reverse transcription PCR and quantitative (real-time) PCR. Total RNA (input control) and IgG were assayed simultaneously.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the cells using TRIzol reagent (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc.) and purified using the RNeasy MinElute Clean up kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the random priming method using the One step PrimeScript kit (RR064A, Takara Bio, Inc.). Transcript levels were measured in duplicate by qPCR (ABI 7900; Life Technologies; Thermo Fisher Scientific, Inc.). The primer for RP11-340F14.6 was as follows: Forward, 5'-GCCAAGCTTCTTGAAAGGCC-3' and reverse, 5'-TTCCACGGAGTAGAGCGAGTC-3'. Primer sequences synthesized by GenScript Co. Ltd. The amplification procedure was 95°C for pre-denaturation for 30 sec; 95°C for 5 sec, 60°C for 31 sec (45 cycles); dissolution curves 95°C for 15 sec, 60°C for 60 sec, 95°C 15 sec. The relative expression of lncRNA and mRNA was normalized to GAPDH and was calculated using the $2^{-\Delta\Delta C_q}$ method as previously described (25). The primer sequences for GAPDH were as follows: Forward, 5'-AAGGTG AAGGTCCGAGTCAAC-3' and reverse, 5'-GGGGTCATT GATGGCAACAATA-3'.

Western blot analysis. Whole cell lysates were prepared as previously described (24). Protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was determined by the BCA method (Beyotime Institute of Biotechnology). Equal amounts of proteins (20 μ g) were boiled, separated on 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% (w/v) non-fat dry milk, the membranes were probed with the primary antibody overnight at 4°C. The secondary antibodies were both horseradish peroxidase (HRP)-conjugated IgG including anti-mouse IgG (ab97040), anti-rabbit IgG (ab7090) and anti-sheep IgG (ab6747) (all from Abcam) and used at a dilution of 1:1,000 and incubation at room temperature for 1 h. Signals were detected by the chemiluminescence procedure (Pierce; Thermo Fisher Scientific, Inc.) with BioMax films (Kodak) and visualized using an ECL kit (EMD Millipore). GAPDH was applied as the reference protein. Antibodies, including P2X7R (ab48871), GAPDH (ab181602), HA-tag (ab18181) were purchased from Abcam and used at a dilution of 1:1,000.

Statistical analysis. Data are presented as the means \pm SEM. Differences between 2 groups were analyzed using the Student's t-test. ANOVA was performed to evaluate differences between multiple groups followed by Tukey's post hoc test. Expression experiments were repeated at least 3 times with samples in triplicates. Pearson's correlation analysis was

used to analyze the correlation between the expression of RP11-340F14.6 and that of associated factors [such as retinoic acid-related orphan receptor gamma t (ROR γ t), Foxp3 and P2X7R]. Statistical analysis was performed using STATA 10.0 software and presented using GraphPad Prism software (GraphPad Software). In all cases, $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Abnormal shift in Th17/Treg in ratio in immune microenvironment in JIA. It has been previously demonstrated that an imbalance in the immune microenvironment is highly associated with the risk of developing JIA, particularly as regards T cell reprogramming. In the present study, peripheral blood T cells were first extracted from patients with JIA and healthy controls. The distribution of human Th17 and Treg immune cells sorted from JIA blood samples was first analyzed. Using CD4 and IL-17 as markers, a marked increase was identified in the percentage of Th17 cells among the total number of T cells in the JIA samples compared to those from healthy children (Fig. 1A). On the contrary, there was a reduced percentage of Tregs labeled with CD25 and Foxp3 presented in the children with JIA compared to the healthy controls (Fig. 1B). These findings suggest an increase in the Th17/Treg ratio in the immune microenvironment of children with JIA.

Transcriptome landscape of lncRNA in the JIA immune microenvironment. Peripheral blood mononuclear cells (PBMCs) were isolated from the JIA samples and matched with the healthy controls. Using anti-CD4 magnetic beads, T cells were sorted in PBMCs that were extracted from 6 pools of paired samples. A high-throughput microarray of lncRNAs was applied to screen for differential expression profiles between the JIA and control samples. The aberrant expression of lncRNA was presented by hierarchical clustering using a heatmap. The profile of the differential expression of lncRNAs in T cells of children with JIA was obtained (Fig. 2A). Among these, lncRNAs were further filtered using the following criteria: i) A fold-change cut-off of 4/0.25; ii) Cq value > 25 for PCR detection; iii) detection of at least 75% in all samples. There were 138 lncRNAs that met these criteria. Finally, 20 of these 138 lncRNAs with the most significant P-values and q values were labeled as candidates. A larger sample size including 20 JIA and 20 paired controls was used for further validation. Among the 20 candidate lncRNAs, LINC01225 presented no expression and therefore the data column for this candidate was removed (Fig. 2B). A total of 4 lncRNAs (LINC00471-001, ZPAS1-002, NEAT1 and RP11-340F14.6) exhibited a significantly altered expression in the JIA samples compared to the normal controls, with RP11-340F14.6 expression exhibiting the most significant difference.

To further investigate the aberrant expression of RP11-340F14.6 in JIA, a case-control experiment as performed with 30 RNA samples extracted from children with JIA and 30 paired control samples. An RT-qPCR assay was conducted and the increased expression of RP11-340F14.6 in patients with JIA was validated (Fig. 2C). Furthermore, a higher presentation of ROR γ t combined with a decreased Foxp3 expression were

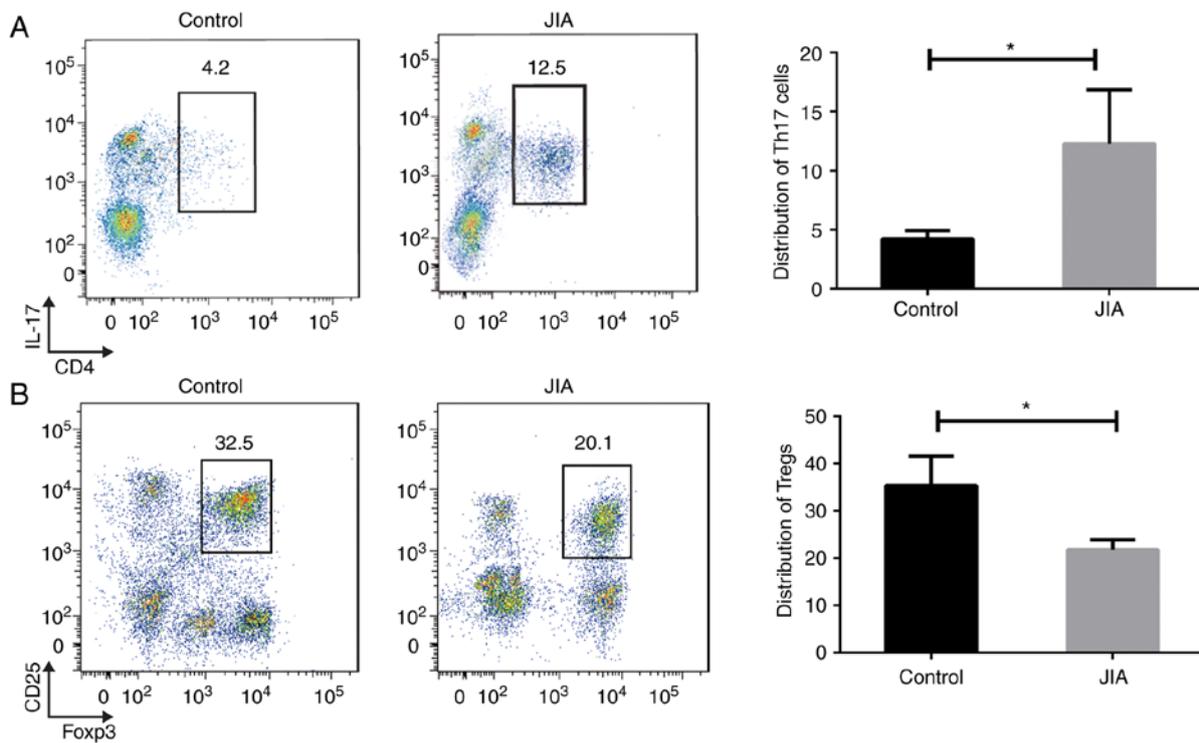


Figure 1. Abnormal distribution of Th17 and Tregs in the circulating immune environment of JIA. (A) Percentage of Th17 cells in the CD4⁺ T cells extracted from PBMCs of patients with JIA determined by flow cytometry; n=30 for each group. (B) Percentage of Tregs in the CD4⁺ T cells extracted from PBMCs of patients with JIA determined by flow cytometry; n=30 for each group. Data are presented as the means \pm SEM; *P<0.05. JIA, juvenile idiopathic arthritis; PBMCs, peripheral blood mononuclear cells.

observed in the JIA group (Fig. 2D and E). Pearson's correlation analysis also revealed that RP11-340F14.6 expression positively correlated with ROR γ t expression and negatively correlated with Foxp3 expression (Fig. 2F). Based on the preliminary data, it was thus hypothesized that RP11-340F14.6 may be associated with the increase in the Th17/Treg ratio in the immune microenvironment of JIA.

RP11-340F14.6 expression increases the Th17/Treg ratio in the JIA immune microenvironment. Subsequently, a series of *in vitro* experiments were conducted to investigate the function of RP11-340F14.6 in CD4⁺ T cells. CD4⁺ T cells were sorted by flow cytometry from PBMCs of patients with JIA and RP11-340F14.6 was overexpressed using a lentivirus in these cells. It was found that the overexpression of RP11-340F14.6 induced the expression of IL-17 and increased the percentage of Th17 cells, which is defined by a CD4-positive cell subgroup (Fig. 3A). The ectopic expression of RP11-340F14.6 also resulted in a decreased expression of Foxp3 and a decrease in the number of Tregs which were labeled with CD25 (Fig. 3B). In the RP11-340F14.6^{high} CD4⁺ T cells from PBMCs, endogenous RP11-340F14.6 expression was silenced using an shRNA lentivirus. The cells in which RP11-340F14.6 was silenced exhibited a lower percentage of Th17 cells and a greater distribution of Tregs (Fig. 3C and D).

RP11-340F14.6 increases the Th17/Treg ratio by specifically binding with P2X7R. According to the results described above, a functional role of RP11-340F14.6 in JIA was identified. However, since RP11-340F14.6 has been poorly investigated

in human diseases, the detailed mechanisms of action of this lncRNA remain unclear. Thus, in the present study, detailed information on RP11-340F14.6 was obtained using the UCSC genome browser (<http://genome.ucsc.edu/>). Based on the FLANK10K theory of lncRNA-mRNA interaction (26), neighbors of RP11-340F14.6 labeled with the 10K region were identified, including P2X7R, P2X4R and CAMKK2 (Fig. 4A). These neighbors are likely to interact with RP11-340F14.6 through a *cis*-regulation approach. Subsequently, the expression of these neighbors was detected in RP11-340F14.6^{high} CD4⁺ T cells derived from PBMCs following transfection with RP11-340F14.6 shRNA. Of note, it was found that P2X7R expression was decreased with the loss of RP11-340F14.6 (Fig. 4B). In human clinical samples, the mRNA expression of P2X7R was increased in the JIA immune microenvironment (Fig. 4C) and its expression positively correlated with RP11-340F14.6 expression (Fig. 4D).

The phenotype for RP11-340F14.6 expression in T cell differentiation was also measured to examine whether the function of RP11-340F14.6 was mediated through interaction with P2X7R. The expression of P2X7R was knocked down using shRNA technology and the percentage of Th17 and Treg cells was determined. The overexpression of RP11-340F14.6 increased the amount of Th17 cells; however, this increase was attenuated by the loss of P2X7R expression (Fig. 5A). The percentage of Tregs was decreased by the overexpression of RP11-340F14.6, but was restored with the loss of P2X7R expression (Fig. 5B). These results indicated that the overexpression of RP11-340F14.6 may have increased the Th17/Treg ratio via a P2X7R-dependent mechanism.

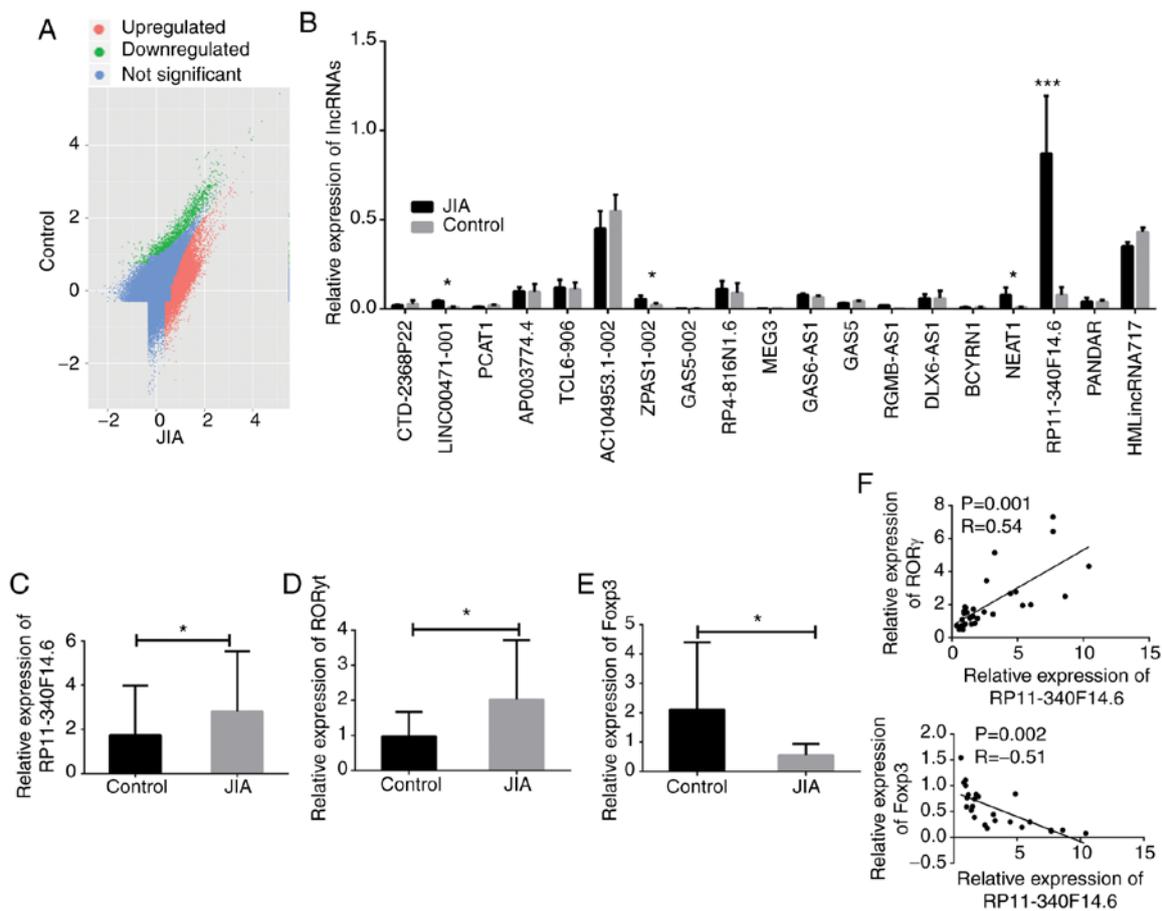


Figure 2. Aberrant expression of RP11-340F14.6 and its association with ROR γ t and Foxp3. (A) Scatter plot of the differentially expressed lncRNAs in CD4⁺ T cells of patients with JIA and healthy controls. Red dots indicate upregulated lncRNAs, while green dots indicate downregulated lncRNAs. (B) Relative expression of candidate lncRNAs in human CD4⁺ T cell samples, n=20. (C) Relative expression of RP11-340F14.6 in human CD4⁺ T cell samples of patients with JIA and paired controls, n=30. (D) Relative expression of ROR γ t in human CD4⁺ T cell samples of patients with JIA and paired controls, n=30. (E) Relative expression of Foxp3 in human CD4⁺ T cell samples of patients with JIA and paired controls, n=30. (F) Pearson's correlation analysis of the correlation between RP11-340F14.6/ROR γ t and RP11-340F14.6/Foxp3 expression. Data are presented as the means \pm SEM, * P <0.05, *** P <0.001. JIA, juvenile idiopathic arthritis; ROR γ t, retinoic acid-related orphan receptor gamma t; Foxp3, forkhead box protein 3.

The expression of P2X7R mRNA and protein was detected in cells following overexpression and/or silencing of RP11-340F14.6. The increased expression of P2X7R was observed in cells which overexpressed RP11-340F14.6, and this increase in P2X7R mRNA expression was suppressed by the knockdown of RP11-340F14.6 expression (Fig. 6A and B). CatRAPID, a bioinformatics software (http://service.tartagalialab.com/page/catrapid_group), was used to predict the potential binding fragment of P2X7R in RP11-340F14.6 (Fig. 6C). The full length of lncRNA nucleotide sequence and the full length of P2X7R amino acid peptide were used as input. The RP11-340F14.6 region between amino acids 301 to 352 was predicted to bind to P2X7R. For P2X7R, the intracellular (IC) domain and amino acids between 501 and 522 were suggested to be the most probable binding domain for RP11-340F14.6. RIP assays revealed that anti-HA (wild-type P2X7R) antibodies specifically precipitated RP11-340F14.6; however, deletions in the amino acids 501-522 of P2X7R abrogated its ability to bind to RP11-340F14.6 (Fig. 6D). These results suggested that RP11-340F14.6 interacted with P2X7R in a highly specific manner and that the predicted region (301-352) of RP11-340F14.6 is crucial for its ability to interact with P2X7R.

Discussion

JIA is a systemic autoimmune disease characterized by persistent synovial inflammation accompanied by the destruction of bone and articular cartilage. The phenotypic variability reflects the underlying fundamental biological diversity, as well as differences in PBMC gene expression patterns and serum cytokine profiles (27). lncRNAs play a critical role in regulating the differentiation and function of CD4⁺ T cells. This is evident as T cell subsets exhibit specific lncRNA expression that defines their transcriptional procedures and pedigree (28). It has been reported that lncRNAs regulate the differentiation of T helper cells by epigenetic and transcriptional reprogramming mechanisms (29). The lncRNA NeST (formally known as Tmevpgl) has been shown to promote Th1 cell differentiation by increasing WDR5 expression (30). lnc-EFGR has also been shown to enhance EGFR expression and result in the shift of Tregs and CD8⁺ T cells (23). In the present study, sample screening revealed the potential importance of RP11-340F14.6. Loss-of-function and gain-of-function assays confirmed that the expression of the closest neighbor of this lncRNA, P2X7R, was modulated by RP11-340F14.6. Furthermore, the expression

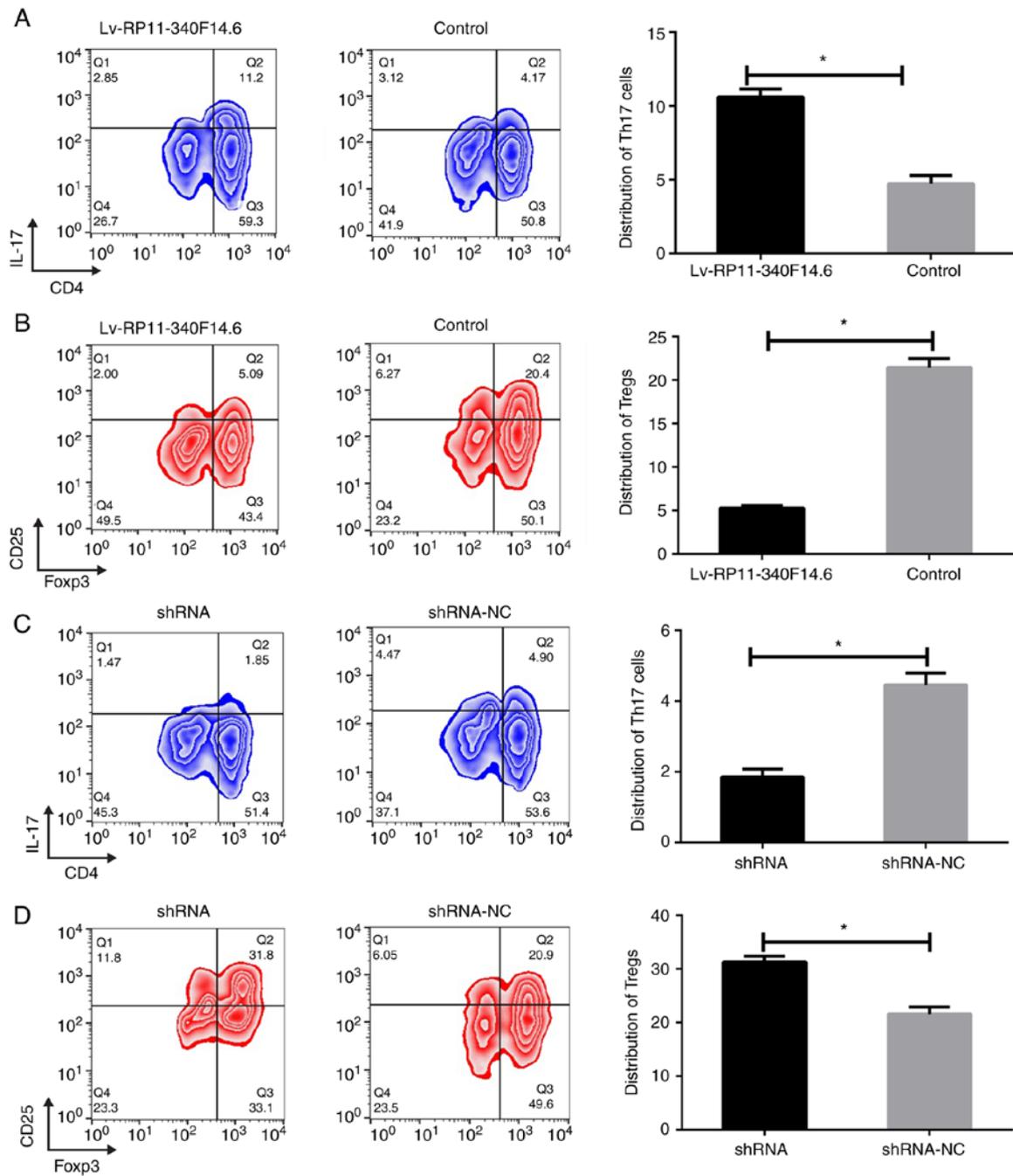


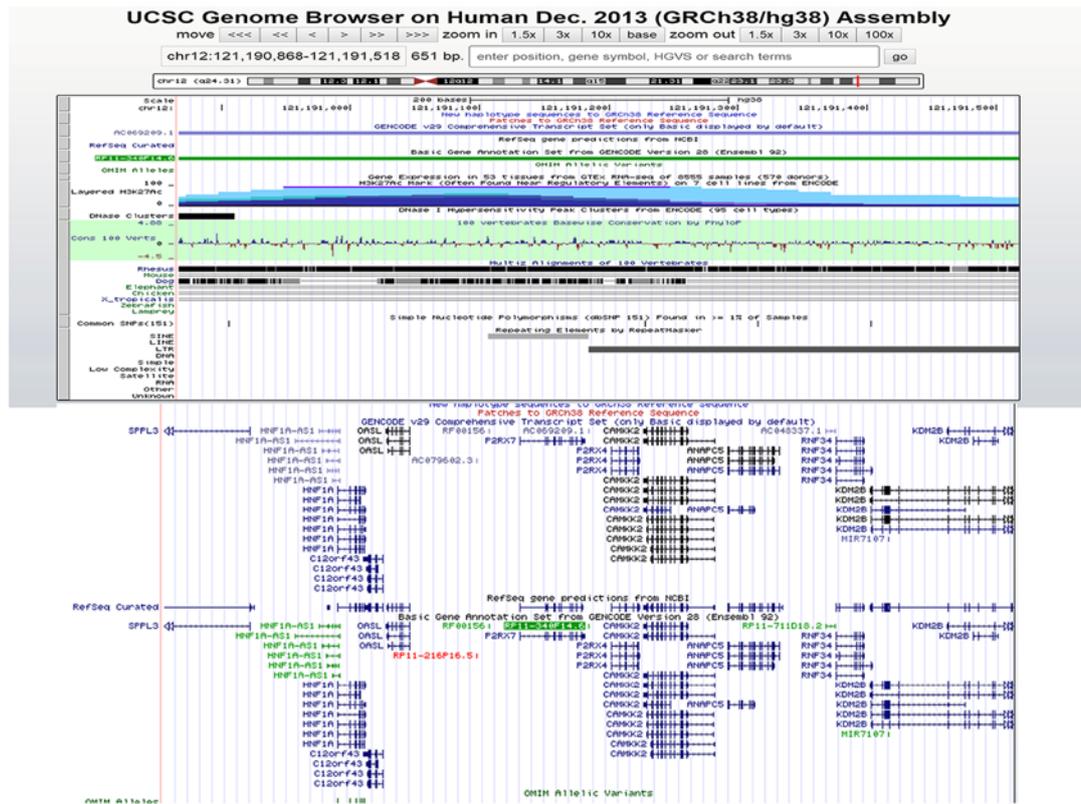
Figure 3. RP11-340F14.6 induces Th17 generation and reduces Tregs. (A) Percentage of Th17 cells in the RP11-340F14.6^{low} CD4⁺T cells transfected with RP11-340F14.6 overexpression lentivirus determined by flow cytometry. (B) Percentage of Tregs in the RP11-340F14.6^{low} CD4⁺T cells transfected with RP11-340F14.6 overexpression lentivirus determined by flow cytometry. (C) Percentage of Th17 cells in the RP11-340F14.6^{high} CD4⁺T cells transfected with RP11-340F14.6 shRNA lentivirus determined by flow cytometry. (D) Percentage of Tregs in the RP11-340F14.6^{high} CD4⁺T cells transfected with RP11-340F14.6 shRNA lentivirus determined by flow cytometry. Data are presented as the means \pm SEM, * $P < 0.05$.

of RP11-340F14.6 inhibited the differentiation of Tregs and stimulated the differentiation of Th17 cells.

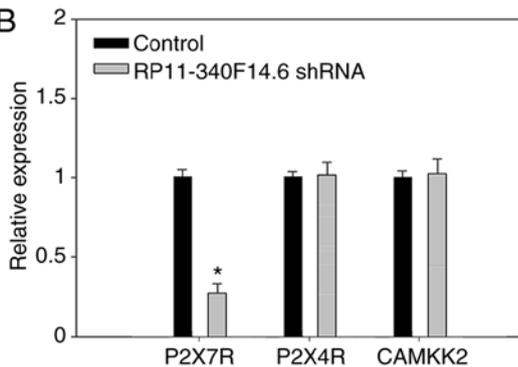
RF-positive pJIA has similar clinical manifestations and pathogenesis with adult RA. The development of RA is highly associated with Th17/Treg redistribution, specifically for an inflammatory-associated, cytokine-induced immune micro-environment. Tregs maintain immune tolerance and prevent autoimmunity by inhibiting activation and proliferation of immune effector cells (31). However, a number of questions remain unanswered as to the mechanisms through which Th17 and Tregs actively regulate JIA (32). The present study also

demonstrated that the percentage of Th17 cells was markedly increased and was accompanied by a decrease in the Treg population in patients with JIA compared to the healthy controls. The increased expression of lncRNA NEAT1 was previously found to be associated with the development of tissue inflammation in RA, and the abundance of Th17 cells in PBMCs was increased in patients with RA; consistently, NEAT1 knockdown inhibited the differentiation of Th17 cells, thus, preventing the development of RA (33). In the present study, it was found that specific expression of RP11-340F14.6 in JIA was positively associated with the ratio of Th17/Tregs.

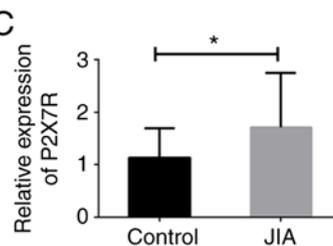
A



B



C



D

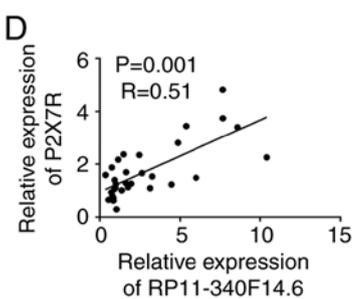


Figure 4. RP11-340F14.6 positively correlates with its neighbor, P2X7R. (A) The genome location of RP11-340F14.6 and its neighbor with the 10K region. (B) Relative expression of candidate genes in RP11-340F14.6^{high} CD4⁺ T cells transfected with RP11-340F14.6 shRNA lentivirus. (C) Relative expression P2X7R in human CD4⁺ T cell samples of patients with JIA and paired controls, n=30. (D) Pearson's correlation analysis of the correlation between RP11-340F14.6/P2X7R. Data are presented as the means \pm SEM, *P<0.05. JIA, juvenile idiopathic arthritis.

The silencing of RP11-340F14.6 significantly inhibited the differentiation of CD4⁺ T cells into Th17 cells. The silencing of RP11-340F14.6 also promoted the differentiation of JIA CD4⁺ T cells into Tregs, which proved to be helpful for the understanding of the mechanisms of JIA CD4⁺ T cell differentiation.

In the present study, RIP assays revealed a specific interaction between RP11-340F14.6 and P2X7R. This indicated that RP11-340F14.6 may induce an increase in the Th17/Treg ratio via a P2X7R-dependent mechanism. P2X7R is a distinct ligand-gated ion channel and is a member of the purinergic type 2 receptor family (34). P2X7R has been confirmed to promote Treg differentiation, and thus its expression is closely associated with Treg abundance (35). In previous studies, the authors demonstrated a novel function of P2X7R signaling in regulating CII-induced differentiation of Th17 cells (36). P2X7R can activate NLRP3, resulting in caspase-1 mediated

maturation and the release of pro-inflammatory cytokines, such as IL-1 β and IL-18 (37). In patients with RA, PBMCs can release large amounts of IL-1 β and IL-18. With ATP stimulation, high levels of P2X7R and NLRP3 inflammatory bodies can also be released (38). The P2X7R/NLRP3 pathway may play an important role in the regulation of CD4⁺ T cell differentiation. It remains to be determined whether RP11-340F14.6 promotes a shift in the Th17/Treg ratio by altering the signaling of P2X7R/NLRP3 inflammatory corpuscles.

In conclusion, the present study identified the 651 bp lncRNA RP11-340F14.6, a neighbor of P2X7R (within 10 kb). The present study aimed to investigate whether RP11-340F14.6 plays a role in stimulating Th17 differentiation and suppressing Treg distribution through a P2X7R-independent approach. A limitation to this study was the small sample size that was restricted due to time constraints. Nonetheless, the effect of the

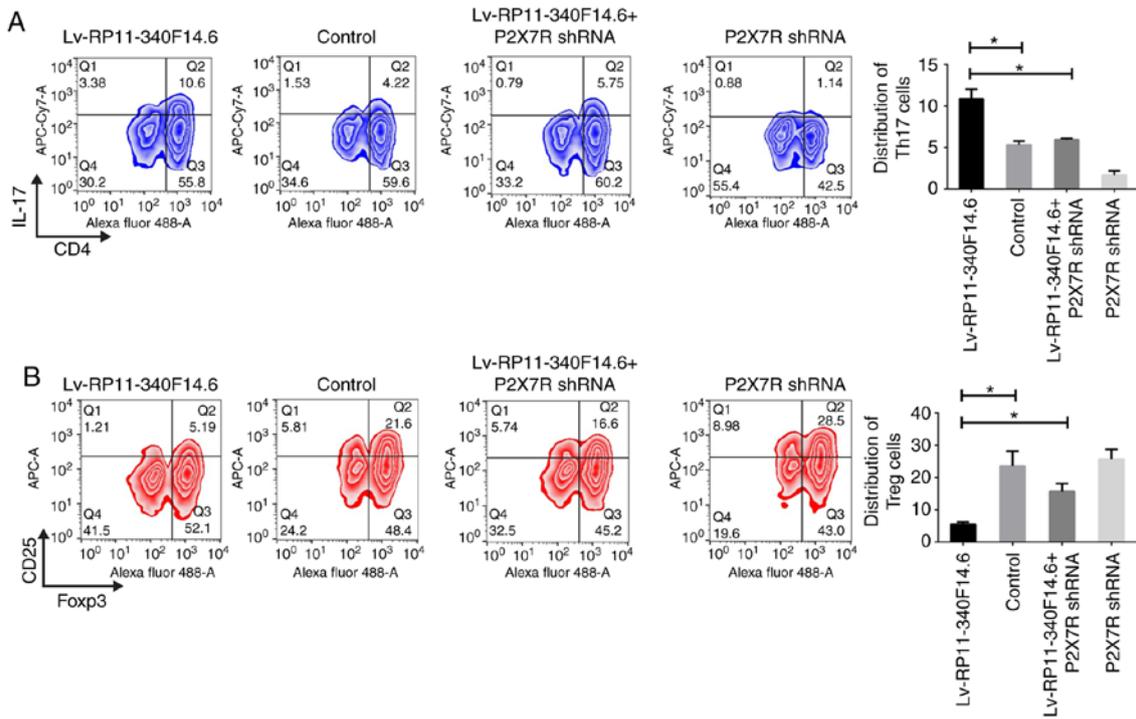


Figure 5. RP11-340F14.6 induces a shift in the Th17/Treg ratio in a P2X7R-dependent manner. (A) Percentage of Th17 cells in the RP11-340F14.6^{low} CD4⁺ T cells transfected with RP11-340F14.6 overexpression lentivirus or P2X7R shRNA determined by flow cytometry. (B) Percentage of Tregs in the RP11-340F14.6^{low} CD4⁺ T cells transfected with RP11-340F14.6 overexpression lentivirus or P2X7R shRNA determined by flow cytometry. The data are presented as the means ± SEM and were analyzed using ANOVA with Tukey's post hoc test, *P<0.05.

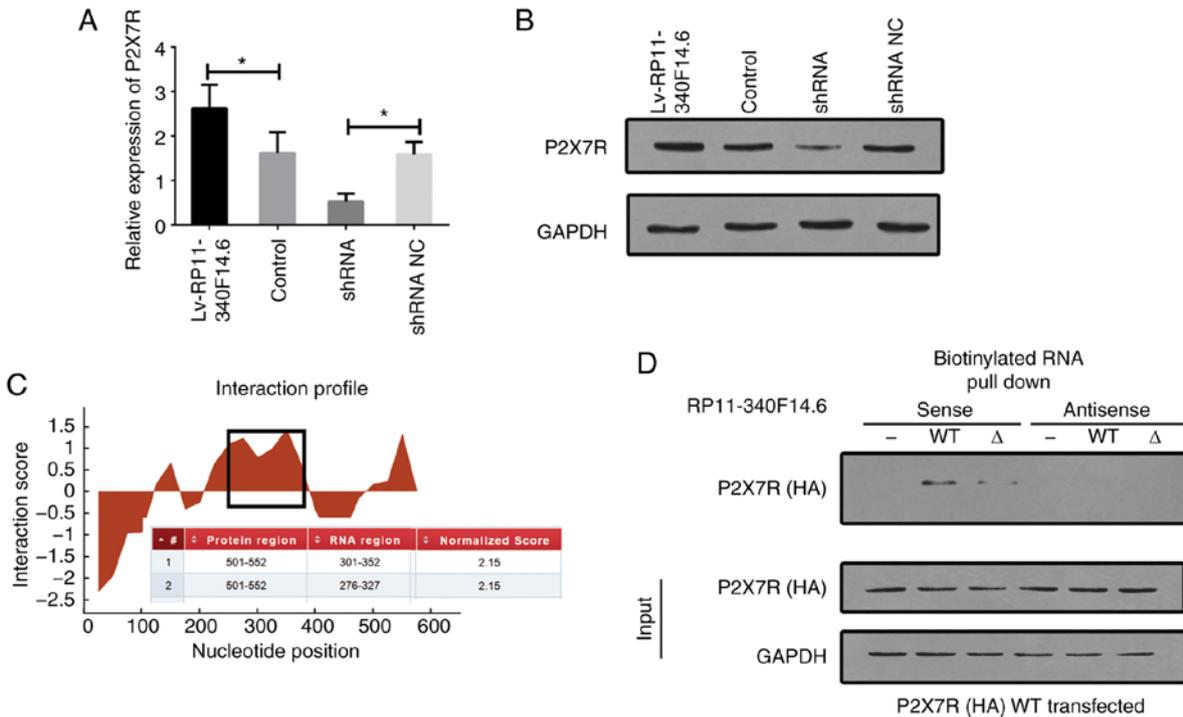


Figure 6. Cytoplasmic RP11-340F14.6 specifically binds to P2X7R. (A) relative mRNA expression of P2X7R in RP11-340F14.6^{high} CD4⁺ T cells transfected with RP11-340F14.6 shRNA lentivirus and RP11-340F14.6^{low} CD4⁺ T cells transfected with RP11-340F14.6 overexpression lentivirus. (B) Protein expression of P2X7R in cells treated with above condition. (C) Interaction between RP11-340F14.6 and P2X7R was predicted by the catRAPID method. (D) RIP assays were performed in cells transfected with P2X7R-HA (wild-type or Δ501-522) and RP11-340F14.6 (wild-type), anti-HA antibody. The precipitated RNAs were determined by RT-qPCR for RP11-340F14.6 and GAPDH. Data are presented as the means ± SEM, *P<0.05.

P2X7R signaling pathway on cell phenotype requires further investigation. At present, these findings suggest that lncRNA

RP11-340F14.6 may serve as a novel prospective intervention target for the treatment of JIA.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

NH contributed to the conception and design of the research and drafted the manuscript. ZF and LM contributed to the acquisition and analysis of the data. HM and HH contributed to the interpretation of the data. HY and XZ equally contributed to the design of the research. All authors critically revised the manuscript, agree to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed in compliance with government policies and the Helsinki Declaration. All patients or healthy controls had the consent of their legal guardians or parents who signed an informed consent form before collecting blood samples. The present study was approved by the Ethics Committee of the Children's Hospital of Nanjing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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