

The CDKN2B-AS1/ MIR497/TXNIP axis regulated macrophages

Jianzhong Xu (✉ xujianzhong2022@163.com)

First Affiliated Hospital of Zhengzhou University

Yu Li

First Affiliated Hospital of Zhengzhou University

Chenxi Gu

First Affiliated Hospital of Zhengzhou University

Guanlei Liu

First Affiliated Hospital of Zhengzhou University

Yang Yu

First Affiliated Hospital of Zhengzhou University

Research Article

Keywords: polarization, rheumatoid, macrophages, CDKN2B-AS1, miR497, TXNIP

Posted Date: July 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-701832/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Abstract

The polarization of macrophages plays a critical role in the pathophysiology of rheumatoid arthritis. The polarization states include pro-inflammatory M1 polarization and various alternative anti-inflammatory M2 polarization. Our preliminary results showed that CDKN2B-AS1/MIR497/TXNIP axis might play a role in macrophages extracted from rheumatoid arthritis patients. Therefore, we hypothesized that this axis regulated the polarization of rheumatoid macrophages. Flow cytometry was used to determine the surface polarization markers in M1 or M2 macrophages from healthy donors and rheumatoid arthritis patients. QPCR and western blotting were used to compare the expression of the CDKN2B-AS1/MIR497/TXNIP axis in these macrophages. We interfered with the expression and function of the axis from upstream to downstream in the macrophage cell line MD to test its roles in macrophage polarization. Compared to cells from healthy donors, cells from rheumatoid arthritis patients expressed a higher level of CD40 and CD80 and a lower level of CD16, CD163, CD206, and CD200R after polarization, they also expressed higher CDKN2B-AS1, lower MIR497, and higher TXNIP. In macrophages from healthy donors, there was no correlation among CDKN2B-AS1, MIR497, and TXNIP. But in macrophages patients, they showed significant correlations. The CDKN2B-AS1 knockdown, MIR497 mimics suppressed M1 polarization but promoted M2 polarization in MD cells, while MIR497 knockdown and TXNIP overexpression did the opposite. This study demonstrated that elevated CDKN2B-AS1 in macrophages promotes M1 polarization and inhibits M2 polarization of macrophage by negatively regulating MIR497, thereby upregulated the expression of TXNIP.

1. Introduction

Rheumatoid arthritis is the most common long-term inflammatory joint disease that results in joints stiffness, swelling, and pain ^[1]. As an autoimmune disease, rheumatoid arthritis results from the self-attacking of the immune system ^[2]. Although rheumatoid arthritis is not curable, clinical treatments for rheumatoid arthritis can decrease the inflammatory activities in the joints to relieve pain and inhibit joint damage ^[3]. Much as many studies have shown that the disease involves several complex interactivities between the human body and environmental factors ^[4], the exact mechanisms that drive rheumatoid arthritis remain obscure. Hence, clinical treatment of rheumatoid arthritis required further understanding of the pathogenesis of this disease.

Macrophages are one of the most critical cells in the pathophysiology of rheumatoid arthritis ^[5]. Macrophages contribute to the secretion of many pro-inflammatory cytokines and chemokines such as TNF and IL-1 β ^[6]. These pro-inflammatory factors activate many downstream cells and result in joint pain and joint damage ^[7]. On the other hand, macrophages also contribute to the recruitment of anti-inflammatory cytokines such as IL-10 ^[8]. These anti-inflammatory factors can negatively regulate autoimmune activities and protect joint tissue ^[9]. A key activity of macrophages in rheumatoid arthritis is their

plasticity, also call polarization ^[10]. The polarization of macrophages is activated by many environmental factors ^[11]. The polarized macrophages can acquire different abilities for specific functions. These different polarization states include pro-inflammatory M1 polarization and various alternative anti-inflammatory M2 polarization ^[12]. Although specific macrophage polarization surface markers in rheumatoid arthritis have not been identified ^[13, 14], several studies have reported the inflammatory regulation role of macrophages in rheumatoid arthritis ^[15].

Endogenous non-coding RNAs have been found to play critical roles in bone cells ^[16]. Micro RNAs (miRNAs) are a class of non-coding RNAs that can bind to the 3' -UTR of their regulated genes to decrease the expression of their target ^[17]. A miRNA, miR-497 (MIR497), has been reported to participate in macrophages ^[18], but its exact role has not been identified. Long non-coding RNAs (lncRNAs) are another class of non-coding RNAs that can regulate gene expression by binding to their target RNAs ^[19]. CDKN2B Antisense RNA 1 (CDKN2B-AS1) is a lncRNA that targets miR-497 ^[20], yet, whether it regulated miR-497 in macrophages is not reported. Thioredoxin-interacting protein (TXNIP) also known as thioredoxin binding protein-2 is a protein that negatively regulates the expression of Thioredoxin ^[21]. TXNIP has been identified to be a critical regulator for inflammation in macrophages ^[22-25], and its potential role in rheumatoid arthritis has previously been reported ^[26]. In addition, a study showed that TXNIP is regulated by MIR497 and CDKN2B-AS1 ^[20].

Our preliminary results showed that the CDKN2B-AS1/miR497(MIR497)/TXNIP axis might play a role in macrophages extracted from rheumatoid arthritis patients. Therefore, we hypothesized that the CDKN2B-AS1/MIR497/TXNIP axis regulated the polarization of rheumatoid macrophages. This study explored the role of the CDKN2B-AS1/MIR497/TXNIP axis in macrophages collected from healthy donors and rheumatoid arthritis patients. Then confirmed the results using CDKN2B-AS1/MIR497/TXNIP axis knockdown and overexpression experiments. Our results reveal a novel role of the CDKN2B-AS1/MIR497/TXNIP in the polarization of rheumatoid macrophages. This study sheds light on the molecular mechanisms of rheumatoid macrophage polarization and provides potential pharmacological targets for rheumatoid arthritis therapy.

2. Methods

2.1. Antibodies

APC/Cyanine7 anti-human CD40 Antibody (BP50), Brilliant Violet 421™ anti-human CD80 Antibody (B7-1), Alexa Fluor® 488 anti-human CD206 (MMR) Antibody, PE anti-human CD200R Antibody, APC/Cyanine7 anti-human CD64 antibody, and FITC anti-human CD163 Antibody were purchased from BioLegend (San Diego, CA, USA). CD16 Mouse anti-Human, V500, Clone: 3G8, BD were obtained from Fisher Scientific (Loughborough, UK). Anti-CD14 antibody [EPR21847] (ab221678), Anti-TXNIP antibody [EPR14774] (ab188865), and Anti-GAPDH antibody [6C5]-Loading Control (ab8245) were purchased from Abcam (Cambridge, UK). All the secondary antibodies were obtained from Cell Signaling Technology.

2.2. Blood samples collection

Blood samples were collected from 28 patients with rheumatoid arthritis (RA) and 36 healthy donors (HD). Patients with rheumatoid arthritis were diagnosed according to the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 rheumatoid arthritis classification criteria ^[27]. All donors were older than 18 years old. Patients with a rheumatoid arthritis disease duration of 1–3 years and a disease activity score are between 3–10 were included. The Blood samples were collected before the first treatment. Patients and healthy donors included in this study have been informed and consented to the use of the samples. The clinical information of the patients was provided in the supplementary. The study has been approved by the Ethics Committee of First Affiliated Hospital of Zhengzhou University. A positive magnetic sorting technique with anti-CD14 antibody was used to sorted CD14 + monocytes out from peripheral blood mononuclear cells. The monocytes obtained were identified by flow cytometry using a CD14-FITC antibody.

2.3. Macrophages culture

Monocytes from each donor were separately cultured. Monocytes were induced with 50 ng/ml M-CSF for 1 week for macrophage differentiation. Cells were in Iscove's Modified Dulbecco's Medium (IMDM) (ATCC® 30-2005™) supplemented with 10% Fetal Bovine Serum (FBS) (ATCC® 30-2020™). All cells were kept in a cell culture incubator at 37°C 5% CO₂. In addition, human monocyte/macrophage cell line, MD (ATCC® CRL-9850™), was purchased from ATCC used for the subsequent transfection study. MD cells were cultured and induced with the same condition.

2.4. Macrophage induction

In the macrophages not induced group (MNI), cells were not activated. In the M1 group (M1), macrophages were activated by Lipopolysaccharide (LPS, 20 ng/ml) and IFN γ (25 ng/ml) into M1 proinflammatory macrophage. In the IL10 M2 group (IL10 M2), macrophages were activated by IL-10 (50 ng/ml) into the M2 macrophage. In the IL4 M2 group (IL4 M2), macrophages were activated by IL-4 (25 ng/ml) into the M2 macrophage.

2.5. Gene knockdown and overexpression

Gene knockdown and overexpression were achieved by transfecting the cells. CDKN2B-AS1 shRNA, scrambled shRNA, miR-497 mimics, random mimics, miR-497 siRNA, scrambled siRNA, overexpression vector plasmid pcDNA3.1 cloned with human TXNIP gene, and negative overexpression vector plasmid pcDNA3.1 were designed, synthesized, or obtained commercially from the Genepharma Company (Shanghai, China). Details can be found in a previous study ^[20]. Cells were transfected using polyethylenimine (Sigma-Aldrich, St. Louis, MO, USA) according to the previous description ^[28].

2.6. Flow cytometry

We measured the surface polarization markers using flow cytometry. The methods were described previously ^[29, 30]. Briefly, after blocking, the cells were stained with corresponding antibodies, then cells

were fixed and permeabilized. Flow cytometry analysis of cells was conducted on a Miltenyi MACSQuant 10 system. FlowJo V10 software was used to analyze the results.

2.7. QPCR

The expression of CDKN2B-AS1, MIR497, TXNIP mRNA was determined using QPCR assay. The method was described previously [31]. Briefly, RNA was isolated using the RNeasy Mini kit (Qiagen, Germantown, MD, USA) following the manual. Applied Biosystems StepOnePlus instrument (Thermo, Beverly, MA, USA), PrimeScript RT Reagent kit with gDNA Eraser (Takara Bio, Japan), and PowerUp™ SYBR™ Green Master Mix (Thermo, Beverly, MA, USA) were used to do the QPCR. The following primers were used: CDKN2B-AS1 F: 5'-CTGGGACTACAGATGCACCAC-3'; CDKN2B-AS1 R: 5'-GGAGGGAGCATGTCTGTTTCT-3'; MIR497 F: 5'-CGCCAGCAGCACACTGTGG-3'; MIR497 R: 5'-GTGCAGGGTCCGAGGT-3'; TXNIP F: 5'-TGGTGGATGATGTCAATACCCCT-3'; TXNIP R: 5'-ATTGGCAAGGTAAGTGTGGC-3'.

2.8. Western Blotting

The expression of TXNIP protein was analyzed using a western blotting assay. The method was described previously [32]. Briefly, samples were lysed in lysing buffer (Pierce, Rockford, IL, USA) with protease inhibitors (Roche, Indianapolis, IN, USA). The proteins were separated in 10–12% sodium dodecyl sulfate-polyacrylamide gels followed by the transferring of the protein to nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). The membrane then was blocked in 5% skimmed milk in PBS. After the blocking, the membranes were incubated with the primary antibodies overnight at 4°C. Then, the membrane was incubated with secondary antibodies. ECL solution was used to visualize the protein on the membrane.

2.9. Statistical significance analysis

Data were present in means and standard deviations in the bar charts. A T-test or ANOVA was used to assess the significance ($p < 0.01$). Dunnett's post hoc tests were used to test the difference between groups. GraphPad Prism (version 8) was used to calculate statistics.

3. Results

3.1. Polarization of macrophages contributed to the pathogenesis of rheumatoid arthritis.

This study aimed to explore mechanisms underlying the polarization of rheumatoid macrophages. Firstly, we determined the six surface polarization markers in macrophages from healthy donors (HD) and rheumatoid arthritis patients (RA). These surface polarization markers included CD40, CD80, CD16, CD163, CD206, and CD200R. The macrophages were induced by LPS plus IFN γ , IL10, or IL4 into M1 pro-inflammatory macrophages, IL10 M2 anti-inflammatory, and IL4 M2 anti-inflammatory macrophages respectively. The surface markers of cells were analyzed by flow cytometry. Compared to cells from healthy donors, cells from rheumatoid arthritis patients expressed a higher level of CD40 and CD80

(Fig. 1AB), yet, they had a lower level of CD16, CD163, CD206, and CD200R (Fig. 1C-F). Besides, we confirmed the indication of these biomarkers for polarization states using a commercially available macrophages cell line MD (Fig. 1G).

3.2. CDKN2B-AS1/ MIR497/TXNIP axis was involved in the polarization of rheumatoid macrophages

To explore the role of the CDKN2B-AS1/MIR497/TXNIP axis in rheumatoid macrophages, we determined the expression of CDKN2B-AS1, MIR497, and TXNIP mRNA in macrophages using QPCR assay. Results showed that compared to macrophages from healthy donors, macrophages from patients expressed higher CDKN2B-AS1, lower MIR497, and higher TXNIP mRNA (Fig. 2A-C), indicating that they might play roles in the pathogenesis of rheumatoid arthritis. To analyze the association of the CDKN2B-AS1, MIR497, and TXNIP mRNA, we plotted (1) Correlation between CDKN2B-AS1 level and MIR497 level, (2) Correlation between CDKN2B-AS1 level and TXNIP mRNA level, (3) Correlation between MIR497 level and TXNIP mRNA level in monocytes from healthy donors and rheumatoid arthritis patients respectively. Results showed that in macrophages from healthy donors there was no correlation among CDKN2B-AS1, MIR497, and TXNIP mRNA. However, in macrophages from rheumatoid arthritis patients, CDKN2B-AS1 was negatively correlated to MIR497, CDKN2B-AS1 was positively correlated to TXNIP, and MIR497 was negatively correlated to TXNIP (Fig. 2D-I).

3.3. TXNIP protein expression was correlated to its mRNA level

To confirm the potential involvement of TXNIP protein in rheumatoid arthritis, we used a western blotting assay to analyze TXNIP protein expression. Results showed that compared to macrophages from healthy donors, macrophages from patients expressed a higher level of TXNIP protein (Fig. 3AB). Then we plotted the correlation of TXNIP mRNA level and TXNIP protein level in both healthy donor and patient cells. Results showed that in both healthy donor and patient cells, the TXNIP mRNA level was significantly correlated to the TXNIP protein level (Fig. 3CD). This indicated that the TXNIP mRNA level can directly affect its protein level.

3.4. CDKN2B-AS1 knockdown suppressed M1 but promoted M2 polarization in MD cells

To validate the regulation of the CDKN2B-AS1/MIR497/TXNIP axis in the polarization of macrophages, we designed knockdown and overexpression experiments of CDKN2B-AS1/MIR497/TXNIP axis in MD cells. In this study, we have confirmed that the biomarkers we tested can indicate the M1 and M2 polarization in our inducted MD cell model (Fig. 1G). Thus, to facilitate the comparison, we normalized the data by multiplying the MD cells without transfection. We started from the upstream to the downstream of the CDKN2B-AS1/MIR497/TXNIP axis. Firstly, we transfected CDKN2B-AS1 shRNA to knock down the CDKN2B-AS1 level. Results showed that CDKN2B-AS1 was successfully knocked down,

and this also resulted in an increase of MIR497 and a decrease of TXNIP mRNA and protein (Fig. 4 AB). After the induction, we found that the knockdown of CDKN2B-AS1 suppressed M1 polarization but promoted M2 (both IL10 and IL4 induced) polarization in MD cells (Fig. 4C-F). These results suggested CDKN2B-AS1 might suppress the inflammatory activities of macrophages.

3.5. MIR497 positively regulated M1 but negatively regulated M2 polarization in MD cells

Then we transfected MIR497 mimics to boost the effect of MIR497 and transfected MIR siRNA to knockdown MIR497. Results showed that MIR497 mimics were successfully detected by PCR, and this also resulted in a decrease of TXNIP mRNA and protein (Fig. 5AB). In the MIR497 knockdown experiment, the siRNA successfully knocked down the level of MIR497. Both the mimics and siRNA failed to affect its upstream target CDKN2B-AS1. After the induction, we found that the MIR497 mimics suppressed M1 polarization but facilitated M2 (both IL10 and IL4 induced) polarization in MD cells, while MIR497 siRNA remarkably promoted M1 polarization but significantly inhibited M2 (both IL10 and IL4 induced) polarization in MD cells (Fig. 5C-F). These results suggested that MIR497 is the downstream target of CDKN2B-AS1 and the upstream target of TXNIP mRNA. Additionally, MIR497 might positively regulate the inflammatory activities of macrophages.

3.6. TXNIP suppressed M1 but promoted M2 polarization in MD cells

To validate the regulation of TXNIP in the polarization of macrophages, we overexpressed TXNIP in the cells and measure their polarization. After the transfection of the overexpression vectors, the TXNIP level was significantly increased by about 6 times, but this transfection did not affect the levels of CDKN2B-AS1 and MIR497 (Fig. 6 AB). After the induction, we found that the overexpression of TXNIP significantly increased the M1 polarization but reduced M2 (both IL10 and IL4 induced) polarization in MD cells (Fig. 4C-F). These results confirmed that TXNIP might promote the inflammatory activities of macrophages.

4. Discussion

In this study, we determined the polarization of macrophages using six surface polarization markers, including CD40, CD80, CD16, CD163, CD206, and CD200R. CD40 and CD80 were considered biomarkers for M1 macrophages as they are highly expressed in M1 macrophages but not in M2 macrophages [28]. CD16 and CD163 were regarded as IL10 M2 biomarkers [29], while CD206 and CD200R were have been wildly detected as IL4 M2 biomarkers [33]. Compared to cells from healthy donors, cells from rheumatoid arthritis patients expressed a higher level of CD40 and CD80, yet, they had a lower level of CD16, CD163, CD206, and CD200R. Our study demonstrated that compared to macrophages from healthy donors, macrophages from rheumatoid arthritis patients have a higher level of M1 polarization, but had a lower

level of M2 polarization. We suggested the higher level of pro-inflammatory M1 polarization of cells is associated with the inflammatory in rheumatoid arthritis patients, on the other hand, the lower level of anti-inflammatory M2 biomarkers in rheumatoid arthritis patient cells indicated that the patient cells had a lower inflammatory.

We also explored the role of the CDKN2B-AS1/MIR497/TXNIP axis in rheumatoid macrophages. Surprisingly, in macrophages from healthy donors, there was no correlation among CDKN2B-AS1, MIR497, and TXNIP mRNA levels. These results suggested that CDKN2B-AS1/MIR497/TXNIP axis might not play role in healthy donors. However, in macrophages from rheumatoid arthritis patients, CDKN2B-AS1 was significantly negatively correlated to MIR497, CDKN2B-AS1 was significantly positively correlated to TXNIP, and MIR497 was significantly negatively correlated to TXNIP. These results indicated that CDKN2B-AS1/MIR497/TXNIP axis was activated only in rheumatoid macrophages but not in macrophages from healthy donors. We suggested that when the CDKN2B-AS1, MIR497, and TXNIP at a normal level, they are not able to significantly interacted with each other. Only when they are at an abnormal level can they regulate each other. This might also involve some unidentified feedback mechanisms that can be explored in the future.

To validate the regulation of the CDKN2B-AS1/MIR497/TXNIP axis in the polarization of macrophages, we interfered with the expression and function of the axis from upstream to downstream. The role of the CDKN2B-AS1/MIR497/TXNIP axis in macrophages was summarized in Fig. 7A. Our study demonstrated that CDKN2B-AS1 regulated TXNIP through MIR497. The complementary binding sites of the CDKN2B-AS1/MIR497/TXNIP axis were shown in Fig. 7B. Notably, because the complementary binding sites only have seven complementary base pairs, the CDKN2B-AS1/MIR497/TXNIP axis might only have relatively weak interaction, which might account for the deregulation of this axis in health donor cells. The sponging of MIR497 by CDKN2B-AS1 can inhibit the level of endogenous MIR497. Most of the miRNAs play roles in cells by their inhibition toward their target mRNA^[17]. In this study, as MIR497 can directly suppress the translation of TXNIP mRNA to TXNIP protein, the CDKN2B-AS1/MIR497/TXNIP axis regulated the level of TXNIP. Our study also revealed that TXNIP had a novel regulation role in the polarization of macrophages. TXNIP can facilitate M1 pro-inflammatory polarization but inhibited M2 anti-inflammatory polarization in macrophages, thereby positively regulated the inflammation in rheumatoid arthritis.

However, the monocytes in this study were in vitro differentiated, which were different from the macrophages obtained from patients that might be affected by the internal environment of the patient. Yet, this study is focusing on the gene expression and the roles of the CDKN2B-AS1/MIR497/TXNIP axis. We think the effect of the genotype of the cells was more critical than the internal environment that induced the cell differentiation. We presumed that the cells from patients have a genotype that results in the onset of rheumatoid arthritis and the genotype would not be affected by the internal environment of the patient. Thus, we only studied in vitro differentiated cells. In addition, the mechanism of TXNIP regulating the polarization of macrophages has not been studied in this study. One potential mechanism involves might be the ion channel regulation in macrophages. Ion channels have been found to be critical

in many cells ^[34–37], some macrophages expressed ion channels for their functions ^[38]. TXNIP has been found to be involved in some ion channel regulations ^[39]. Regarding the clinical treatment of rheumatoid arthritis, many anesthetics have been applied in therapy to relieve rheumatoid arthritis pain ^[40, 41]. These drugs usually have multiple ion channel targets ^[42, 43]. Many anesthetics can also have multiple ion channel targets ^[42] and regulate the immune system ^[44, 45] that might interfere with rheumatoid arthritis. Whether the use of these anesthetics interacts with macrophages in rheumatoid arthritis is not clear. In addition, the potential role of the CDKN2B-AS1/MIR497/TXNIP axis in other bone diseases is also interesting. Studies have found that TXNIP is closely associated with bone metabolism ^[46–48]. Rheumatoid arthritis and osteoporosis have been two of the most pervasive bone diseases ^[49, 50]. For example, many pathways have been proposed to regulated osteoporosis ^[51–54], but whether osteoporosis can be regulated by the CDKN2B-AS1/MIR497/TXNIP axis can also be explored in the future.

5. Conclusion

Generally, through studying macrophages from human blood samples and a cultured macrophage cell line, we demonstrate that elevated CDKN2B-AS1 in macrophages promotes M1 polarization and inhibits M2 polarization of macrophage by negatively regulating MIR497 for the upregulation of TXNIP. Our results revealed a novel role of the CDKN2B-AS1/MIR497/TXNIP axis in the polarization of rheumatoid macrophages and shed light on molecular mechanisms of rheumatoid macrophage polarization. This study also provided potential pharmacological targets for rheumatoid arthritis therapy.

Declarations

Conflict of Interest Statement

There is no conflict of interest.

Availability of data

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

Funding

This study was supported by the First Affiliated Hospital of Zhengzhou University.

Authorship

Yu Li had made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. Chenxi Gu, GuanLei Liu, Yang Yu, and JianZhong Xu Been had been involved in drafting the manuscript or revising it critically for important intellectual content. All authors had given final approval of the version to be published.

References

1. VERHOEVEN F, TORDI N, PRATI C, et al. Physical activity in patients with rheumatoid arthritis [J]. *Joint bone spine*, 2016, 83(3): 265–70.
2. SIOUTI E, ANDREAKOS E. The many facets of macrophages in rheumatoid arthritis [J]. *Biochemical pharmacology*, 2019, 165(152 – 69).
3. LITTLEJOHN E A, MONRAD S U. Early Diagnosis and Treatment of Rheumatoid Arthritis [J]. *Primary care*, 2018, 45(2): 237–55.
4. OKADA Y, EYRE S, SUZUKI A, et al. Genetics of rheumatoid arthritis: 2018 status [J]. *Annals of the rheumatic diseases*, 2019, 78(4): 446–53.
5. WEYAND C M, ZEISBRICH M, GORONZY J J. Metabolic signatures of T-cells and macrophages in rheumatoid arthritis [J]. *Current opinion in immunology*, 2017, 46(112 – 20).
6. TANG P M, NIKOLIC-PATERSON D J, LAN H Y. Macrophages: versatile players in renal inflammation and fibrosis [J]. *Nature reviews Nephrology*, 2019, 15(3): 144–58.
7. CROFT M, SIEGEL R M. Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases [J]. *Nature reviews Rheumatology*, 2017, 13(4): 217–33.
8. CHEN Z, BOZEC A, RAMMING A, et al. Anti-inflammatory and immune-regulatory cytokines in rheumatoid arthritis [J]. *Nature reviews Rheumatology*, 2019, 15(1): 9–17.
9. ICER M A, GEZMEN-KARADAG M. The multiple functions and mechanisms of osteopontin [J]. *Clinical biochemistry*, 2018, 59(17–24).
10. MURRAY P J. Macrophage Polarization [J]. *Annual review of physiology*, 2017, 79(541 – 66).
11. SHAPOURI-MOGHADDAM A, MOHAMMADIAN S, VAZINI H, et al. Macrophage plasticity, polarization, and function in health and disease [J]. *J Cell Physiol*, 2018, 233(9): 6425–40.
12. ORECCHIONI M, GHOSHEH Y, PRAMOD A B, et al. Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs. Alternatively Activated Macrophages [J]. *Frontiers in immunology*, 2019, 10(1084).
13. AMBARUS C A, NOORDENBOS T, DE HAIR M J, et al. Intimal lining layer macrophages but not synovial sublining macrophages display an IL-10 polarized-like phenotype in chronic synovitis [J]. *Arthritis research & therapy*, 2012, 14(2): R74.
14. BALS A, DIXEY J, SANSOM D M, et al. Differential expression of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) in rheumatoid synovial tissue [J]. *British journal of rheumatology*, 1996, 35(1): 33–7.
15. SOLER PALACIOS B, ESTRADA-CAPETILLO L, IZQUIERDO E, et al. Macrophages from the synovium of active rheumatoid arthritis exhibit an activin A-dependent pro-inflammatory profile [J]. *The Journal of pathology*, 2015, 235(3): 515–26.
16. LI X, PENG B, ZHU X, et al. Changes in related circular RNAs following ERbeta knockdown and the relationship to rBMSC osteogenesis [J]. *Biochemical and biophysical research communications*, 2017, 493(1): 100–7.

17. GEBERT L F R, MACRAE I J. Regulation of microRNA function in animals [J]. *Nature reviews Molecular cell biology*, 2019, 20(1): 21–37.
18. CUI J, REN Z, ZOU W, et al. miR-497 accelerates oxidized low-density lipoprotein-induced lipid accumulation in macrophages by repressing the expression of apelin [J]. *Cell biology international*, 2017, 41(9): 1012–9.
19. YAO R W, WANG Y, CHEN L L. Cellular functions of long noncoding RNAs [J]. *Nature cell biology*, 2019, 21(5): 542–51.
20. WANG J, ZHAO S M. LncRNA-antisense non-coding RNA in the INK4 locus promotes pyroptosis via miR-497/thioredoxin-interacting protein axis in diabetic nephropathy [J]. *Life sciences*, 2021, 264(118728).
21. ALHAWITI N M, AL MAHRI S, AZIZ M A, et al. TXNIP in Metabolic Regulation: Physiological Role and Therapeutic Outlook [J]. *Current drug targets*, 2017, 18(9): 1095–103.
22. CHOE J-Y, KIM S-K. Quercetin and ascorbic acid suppress fructose-induced NLRP3 inflammasome activation by blocking intracellular shuttling of TXNIP in human macrophage cell lines [J]. *Inflammation*, 2017, 40(3): 980–94.
23. LIU Y, LOU G, LI A, et al. AMSC-derived exosomes alleviate lipopolysaccharide/d-galactosamine-induced acute liver failure by miR-17-mediated reduction of TXNIP/NLRP3 inflammasome activation in macrophages [J]. *EBioMedicine*, 2018, 36(140 – 50).
24. LIU Z, SUN M, WANG Y, et al. Silymarin attenuated paraquat-induced cytotoxicity in macrophage by regulating Trx/TXNIP complex, inhibiting NLRP3 inflammasome activation and apoptosis [J]. *Toxicology in Vitro*, 2018, 46(265 – 72).
25. DINESH P, RASOOL M. Berberine, an isoquinoline alkaloid suppresses TXNIP mediated NLRP3 inflammasome activation in MSU crystal stimulated RAW 264.7 macrophages through the upregulation of Nrf2 transcription factor and alleviates MSU crystal induced inflammation in rats [J]. *International immunopharmacology*, 2017, 44(26–37).
26. LI X-F, SHEN W-W, SUN Y-Y, et al. MicroRNA-20a negatively regulates expression of NLRP3-inflammasome by targeting TXNIP in adjuvant-induced arthritis fibroblast-like synoviocytes [J]. *Joint bone spine*, 2016, 83(6): 695–700.
27. ALETAHA D, NEOGI T, SILMAN A J, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative [J]. *Arthritis and rheumatism*, 2010, 62(9): 2569–81.
28. LONGO P A, KAVRAN J M, KIM M-S, et al. Transient mammalian cell transfection with polyethylenimine (PEI) [J]. *Methods Enzymol*, 2013, 529(227 – 40).
29. DEGBOÉ Y, RAUWEL B, BARON M, et al. Polarization of Rheumatoid Macrophages by TNF Targeting Through an IL-10/STAT3 Mechanism [J]. *Frontiers in immunology*, 2019, 10(3).
30. LI R, XIAO C, LIU H, et al. Effects of local anesthetics on breast cancer cell viability and migration [J]. *BMC cancer*, 2018, 18(1): 666.

31. LIU H, XIONG Y, WANG H, et al. Effects of water extract from epimedium on neuropeptide signaling in an ovariectomized osteoporosis rat model [J]. *Journal of ethnopharmacology*, 2018, 221(126 – 36).
32. LIU H, XIONG Y, ZHU X, et al. Icaritin improves osteoporosis, inhibits the expression of PPARgamma, C/EBPalpha, FABP4 mRNA, N1ICD and jagged1 proteins, and increases Notch2 mRNA in ovariectomized rats [J]. *Experimental and therapeutic medicine*, 2017, 13(4): 1360–8.
33. PAGIE S, GÉRARD N, CHARREAU B. Notch signaling triggered via the ligand DLL4 impedes M2 macrophage differentiation and promotes their apoptosis [J]. *Cell communication and signaling: CCS*, 2018, 16(1): 4.
34. LIU H. Nav channels in cancers: Nonclassical roles [J]. *Global Journal of Cancer Therapy*, 2020, 6(1): 5.
35. LIU H. A prospective for the role of two-pore channels in breast cancer cells [J]. *Global Journal of Cancer Therapy*, 2020, 6(1): 001–3.
36. LIU H, DILGER J P, LIN J. The Role of Transient Receptor Potential Melastatin 7 (TRPM7) in Cell Viability: A Potential Target to Suppress Breast Cancer Cell Cycle [J]. *Cancers*, 2020, 12(1):
37. FESKE S, WULFF H, SKOLNIK E Y. Ion channels in innate and adaptive immunity [J]. *Annual review of immunology*, 2015, 33(291–353).
38. EDER C. Ion channels in microglia (brain macrophages) [J]. *Am J Physiol*, 1998, 275(2): C327-42.
39. ZHANG X, MAO Z, HUANG Y, et al. Gap junctions amplify TRPV4 activation-initiated cell injury via modification of intracellular Ca²⁺ and Ca²⁺-dependent regulation of TXNIP [J]. *Channels*, 2020, 14(1): 246–56.
40. KOHJITANI A, MIYAWAKI T, KASUYA K, et al. Anesthetic management for advanced rheumatoid arthritis patients with acquired micrognathia undergoing temporomandibular joint replacement [J]. *Journal of oral and maxillofacial surgery*, 2002, 60(5): 559–66.
41. AIRES R B, DE CARVALHO J F, DA MOTA L M H. Pre-operative anesthetic assessment of patients with rheumatoid arthritis [J]. *Revista Brasileira de Reumatologia (English Edition)*, 2014, 54(3): 213–9.
42. LIU H, DILGER J P, LIN J. Lidocaine Suppresses Viability and Migration of Human Breast Cancer Cells: TRPM7 as A Target for Some Breast Cancer Cell Lines [J]. *Cancers*, 2021, 13(2): 234.
43. LIU H, DILGER J P, LIN J. Effects of local anesthetics on cancer cells [J]. *Pharmacology & Therapeutics*, 2020, 212(107558).
44. LI R, LIU H, DILGER J P, et al. Effect of Propofol on breast Cancer cell, the immune system, and patient outcome [J]. *BMC anesthesiology*, 2018, 18(1): 77.
45. LIU H. A clinical mini-review: Clinical use of Local anesthetics in cancer surgeries [J]. *The Gazette of Medical Sciences*, 2020, 1(3): 030–4.
46. MO Y, LAI W, ZHONG Y, et al. TXNIP contributes to bone loss via promoting the mitochondrial oxidative phosphorylation during glucocorticoid-induced osteoporosis [J]. *Life sciences*, 2020, 266(118938).

47. LI Y H, TONG K L, LU J L, et al. PRMT5-TRIM21 interaction regulates the senescence of osteosarcoma cells by targeting the TXNIP/p21 axis [J]. *Aging*, 2020, 12(3): 2507–29.
48. LIANG H, GAO J, ZHANG C, et al. Nicotinamide mononucleotide alleviates Aluminum induced bone loss by inhibiting the TXNIP-NLRP3 inflammasome [J]. *Toxicology and applied pharmacology*, 2019, 362(20 – 7).
49. HAIXIA W, SHU M, LI Y, et al. Effectiveness associated with different therapies for senile osteoporosis: a network Meta-analysis [J]. *J Tradit Chin Med*, 2020, 40(1): 17–27.
50. WONG P, FULLER P J, GILLESPIE M T, et al. Bone Disease in Thalassemia: A Molecular and Clinical Overview [J]. *Endocrine reviews*, 2016, 37(4): 320–46.
51. LIU X, LIU H, XIONG Y, et al. Postmenopausal osteoporosis is associated with the regulation of SP, CGRP, VIP, and NPY [J]. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 2018, 104(742 – 50).
52. WU Z, OU L, WANG C, et al. Icaritin induces MC3T3-E1 subclone14 cell differentiation through estrogen receptor-mediated ERK1/2 and p38 signaling activation [J]. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 2017, 94(1–9).
53. CHEN G, WANG C, WANG J, et al. Antiosteoporotic effect of icariin in ovariectomized rats is mediated via the Wnt/beta-catenin pathway [J]. *Experimental and therapeutic medicine*, 2016, 12(1): 279–87.
54. WANG T, LIU Q, TJHIOE W, et al. Therapeutic Potential and Outlook of Alternative Medicine for Osteoporosis [J]. *Current drug targets*, 2017, 18(9): 1051–68.

Figures

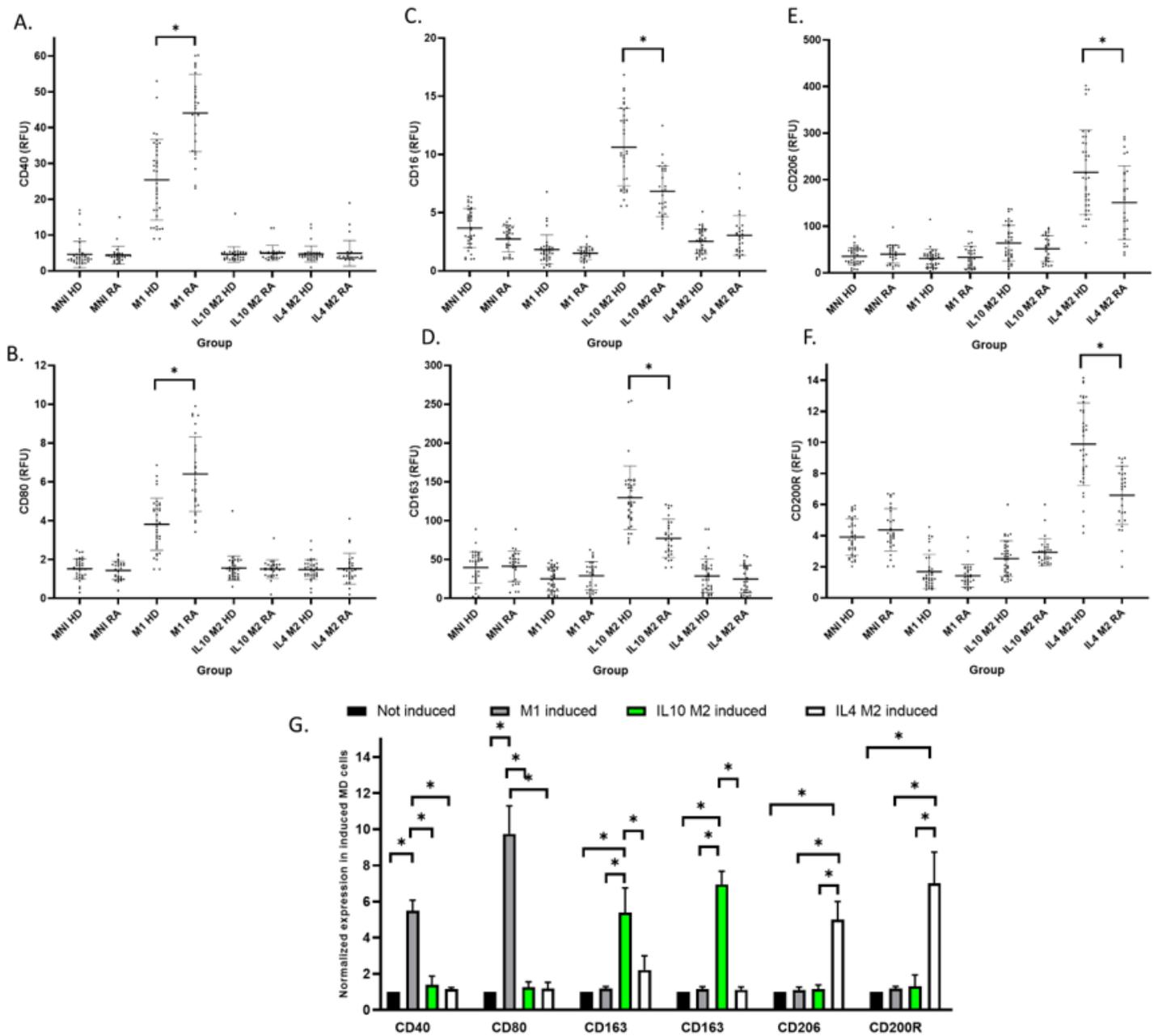


Figure 1

Surface polarization markers on induced monocytes. Blood samples were collected from 28 patients with rheumatoid arthritis and 36 healthy donors. Monocytes from each donor were separated and cultured. Surface polarization markers on cells were analyzed by flow cytometry. In the monocytes not induced group (MNI), monocytes cells were not activated. In the M1 group (M1), monocytes were activated by LPS and IFN γ into M1 proinflammatory macrophages. In the IL10 M2 group, monocytes were activated by IL-10 into the M2 macrophage. In the IL4 M2 group, monocytes were activated by IL-4 into the M2 macrophage. (AB) Surface markers for M1. (CD) Surface markers for IL10 induced M2. (EF) Surface markers for IL4 induced M2. (G) Normalized surface polarization markers on induced MD cells. (HD: healthy donor; RA: rheumatoid arthritis; MNI: monocytes not induced; * p -value < 0.01.)

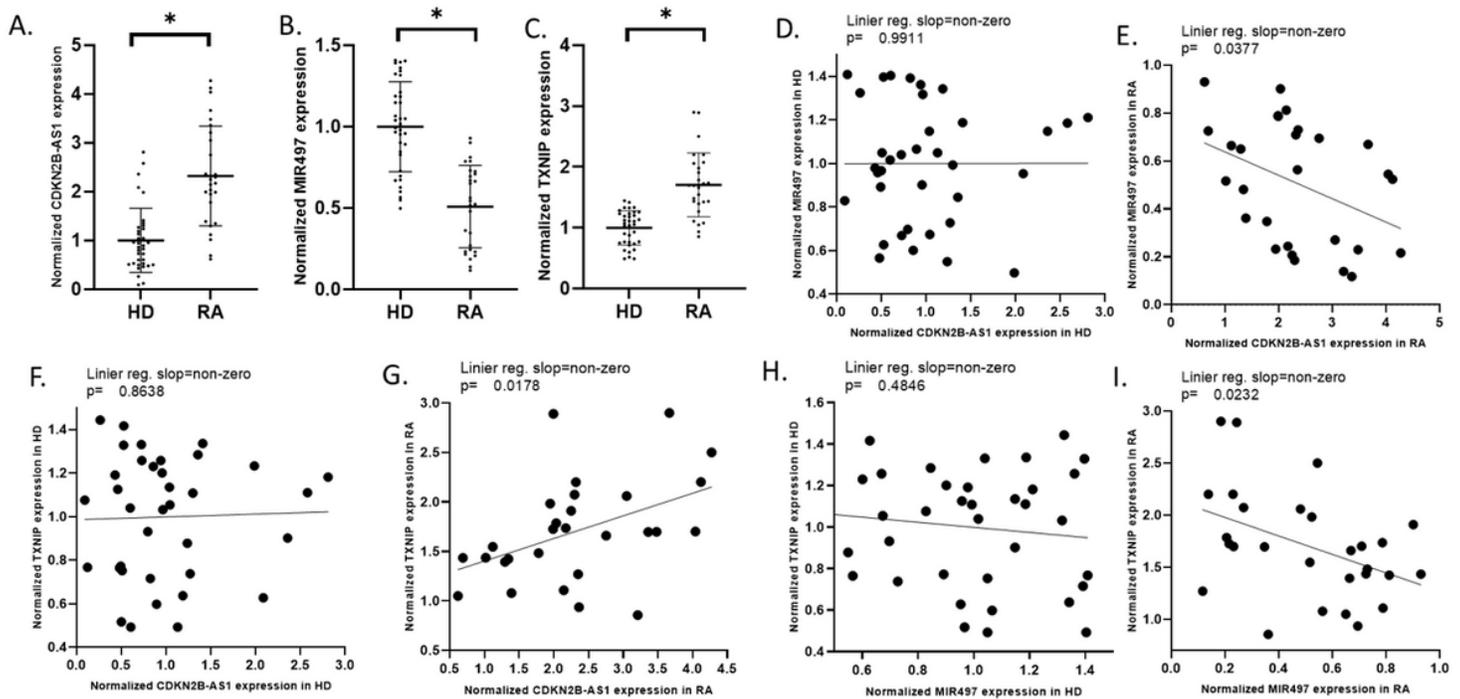


Figure 2

The expression of CDKN2B-AS1, MIR497, and TXNIP mRNA in monocytes from HD and RA patients. The RNA expression level was determined by QPCR assay. (A) CDKN2B-AS1 expression, (B) MIR497 expression. (C) TXNIP mRNA expression. (D) Correlation between CDKN2B-AS1 level and MIR497 level in HD monocytes. (E) Correlation between CDKN2B-AS1 level and MIR497 level in RA monocytes. (F) Correlation between CDKN2B-AS1 level and TXNIP mRNA level in HD monocytes. (G) Correlation between CDKN2B-AS1 level and TXNIP mRNA level in RA monocytes. (H) Correlation between MIR497 level and TXNIP mRNA level in HD monocytes. (I) Correlation between MIR497 level and TXNIP mRNA level in RA monocytes. (HD: healthy donor; RA: rheumatoid arthritis; *p-value < 0.01.)

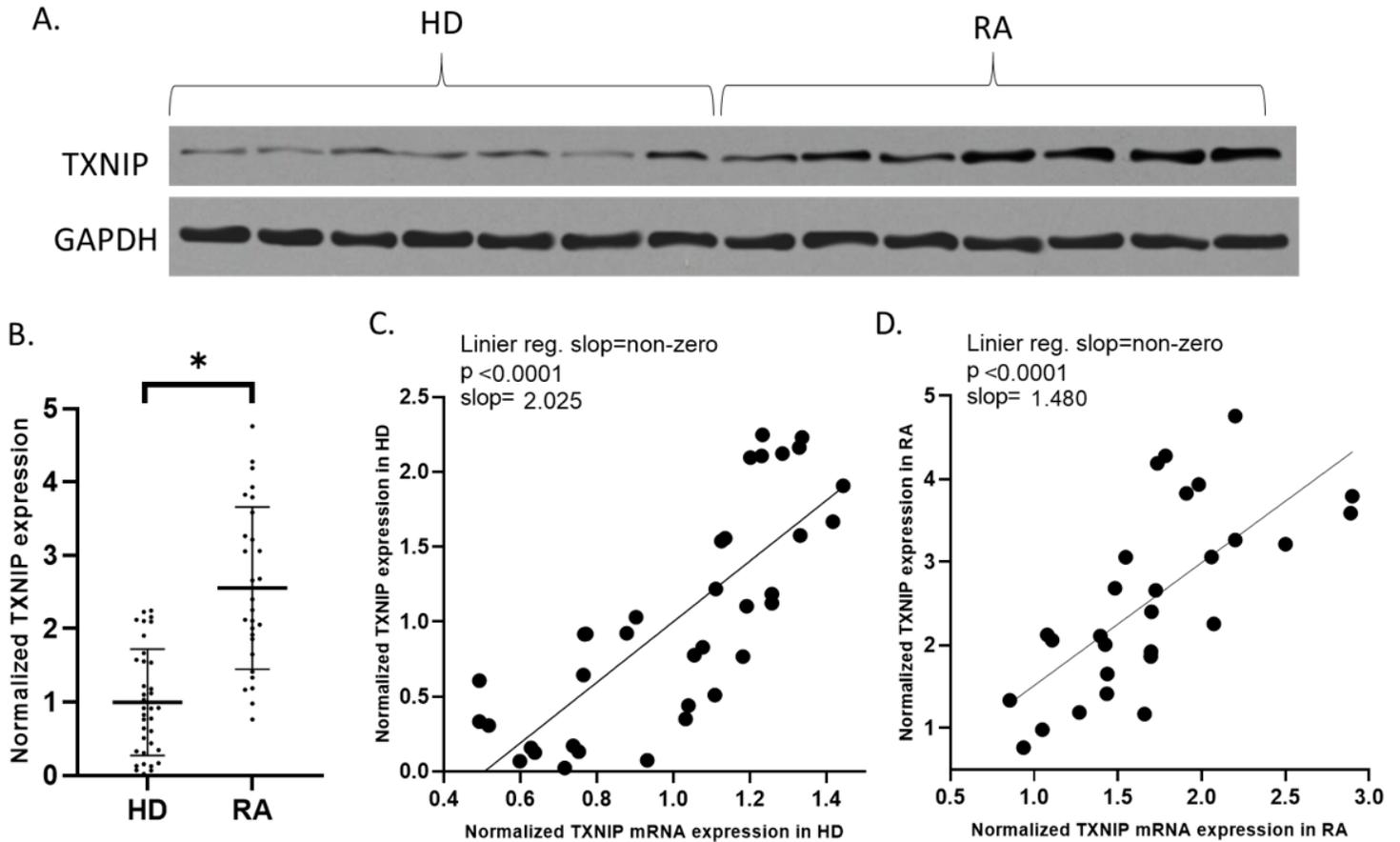


Figure 3

The expression of TXNIP in monocytes from HD and RA patients. The expression of TXNIP protein was determined by western blotting assay. (A) Representative image of western blotting assay. (B) The expression of TXNIP protein in monocytes. (C) Correlation between TXNIP mRNA level and TXNIP protein level in HD monocytes. (D) Correlation between TXNIP mRNA level and TXNIP protein level in RA monocytes. (HD: healthy donor; RA: rheumatoid arthritis; *p-value < 0.01.)

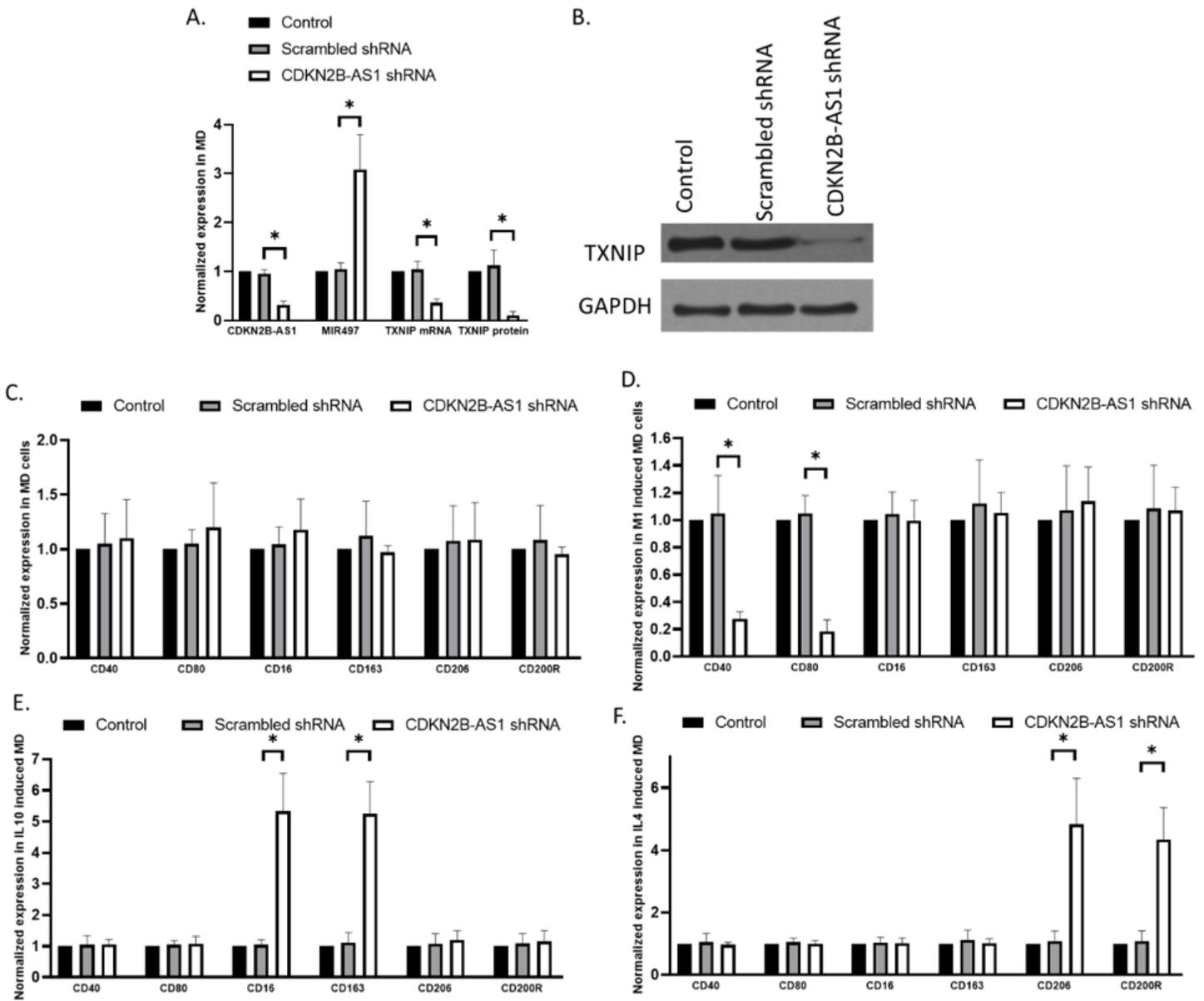


Figure 4

The effect of CDKN2B-AS1 knockdown in MD cell polarization. CDKN2B-AS1 shRNA was transfected into MD cells to achieve the knockdown. (A) Effect of CDKN2B-AS1 knockdown on CDKN2B-AS1/MIR497/TXNIP axis. (B) Representative image of western blotting assay. (C) Effect of CDKN2B-AS1 knockdown on surface polarization markers of not-induced MD cells. (D) Effect of CDKN2B-AS1 knockdown on surface polarization markers of M1 induced MD cells. (E) Effect of CDKN2B-AS1 knockdown on surface polarization markers of IL10 induced MD cells. (F) Effect of CDKN2B-AS1 knockdown on surface polarization markers of IL4 induced MD cells. (control: cells not transfected; the control was present for normalization and the difference between scrambled and knockdown shRNA was tested using a t-test, *p-value < 0.01.)

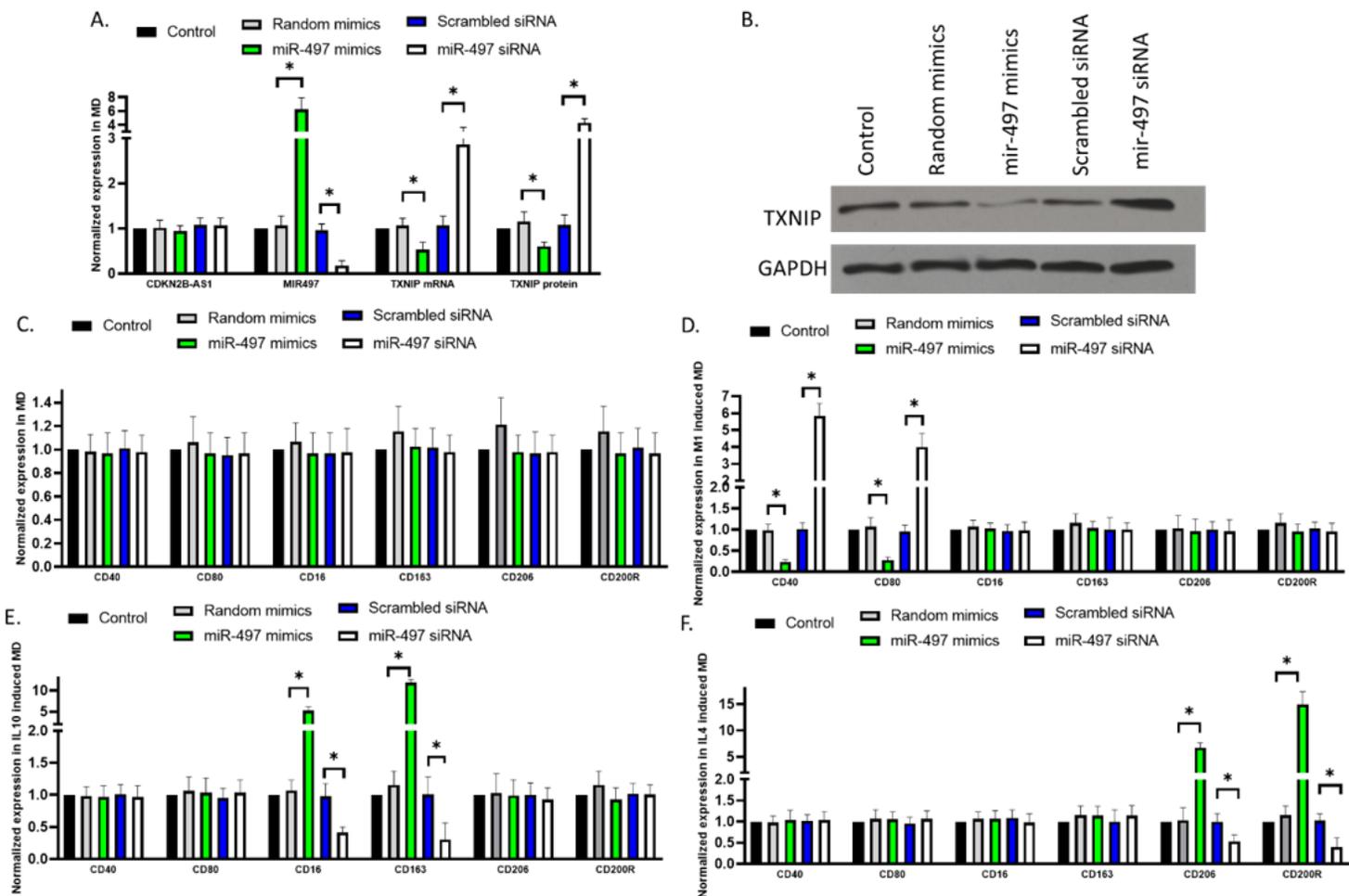


Figure 5

The effect of MIR497 in MD cell polarization. miR-497 mimics and siRNA were transfected into MD cells. (A) Effect of miR-497 mimics or siRNA on CDKN2B-AS1/MIR497/TXNIP axis. (B) Representative image of western blotting assay. (C) Effect of miR-497 mimics or siRNA on surface polarization markers of not-induced MD cells. (D) Effect of miR-497 mimics or siRNA on surface polarization markers of M1 induced MD cells. (E) Effect of miR-497 mimics or siRNA on surface polarization markers of IL10 induced MD cells. (F) Effect of miR-497 mimics or siRNA on surface polarization markers of IL4 induced MD cells. (control: cells not transfected; the control was present for normalization and the difference between scrambled and knockdown shRNA or mimic was tested using a t-test, *p-value < 0.01.)

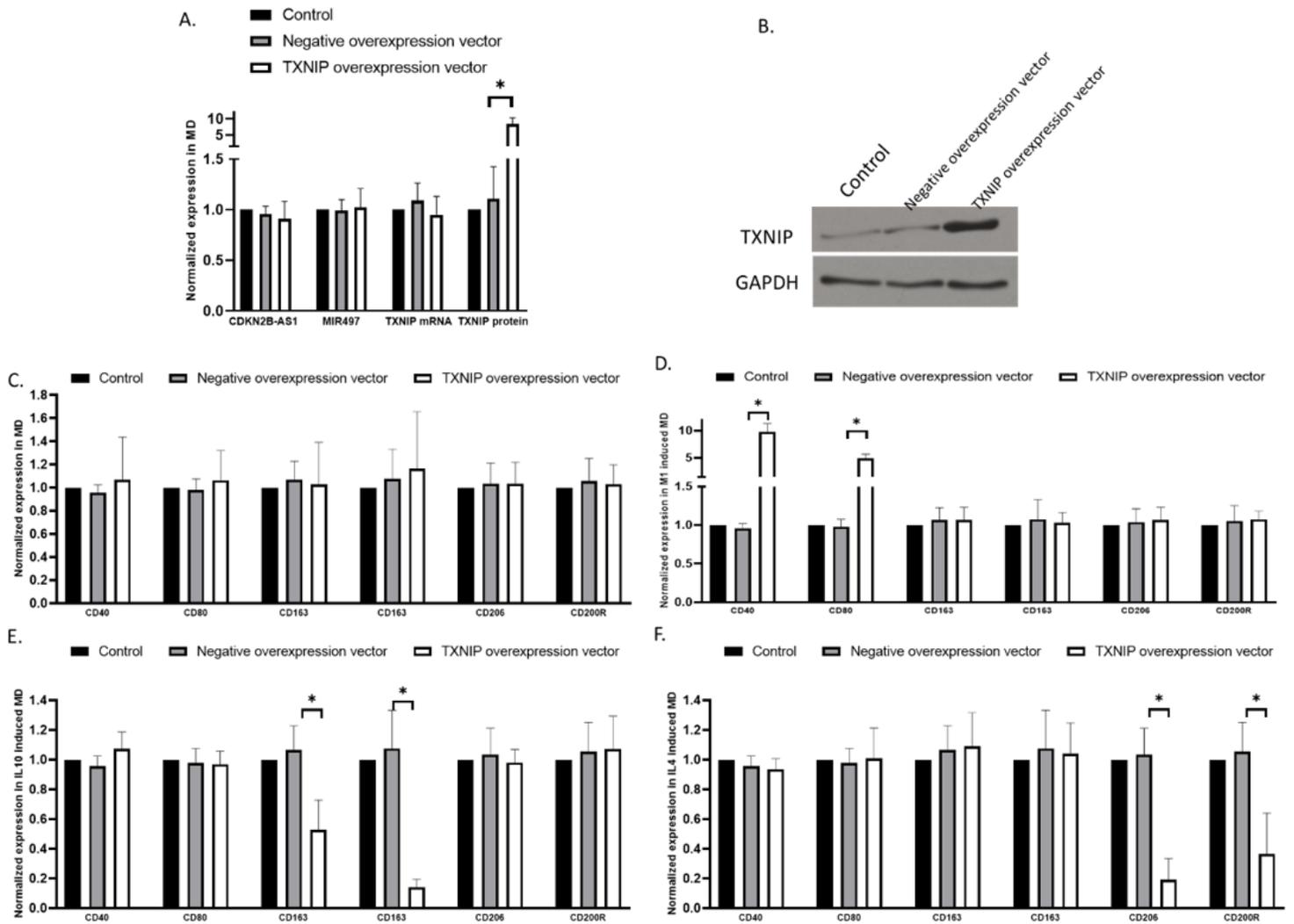
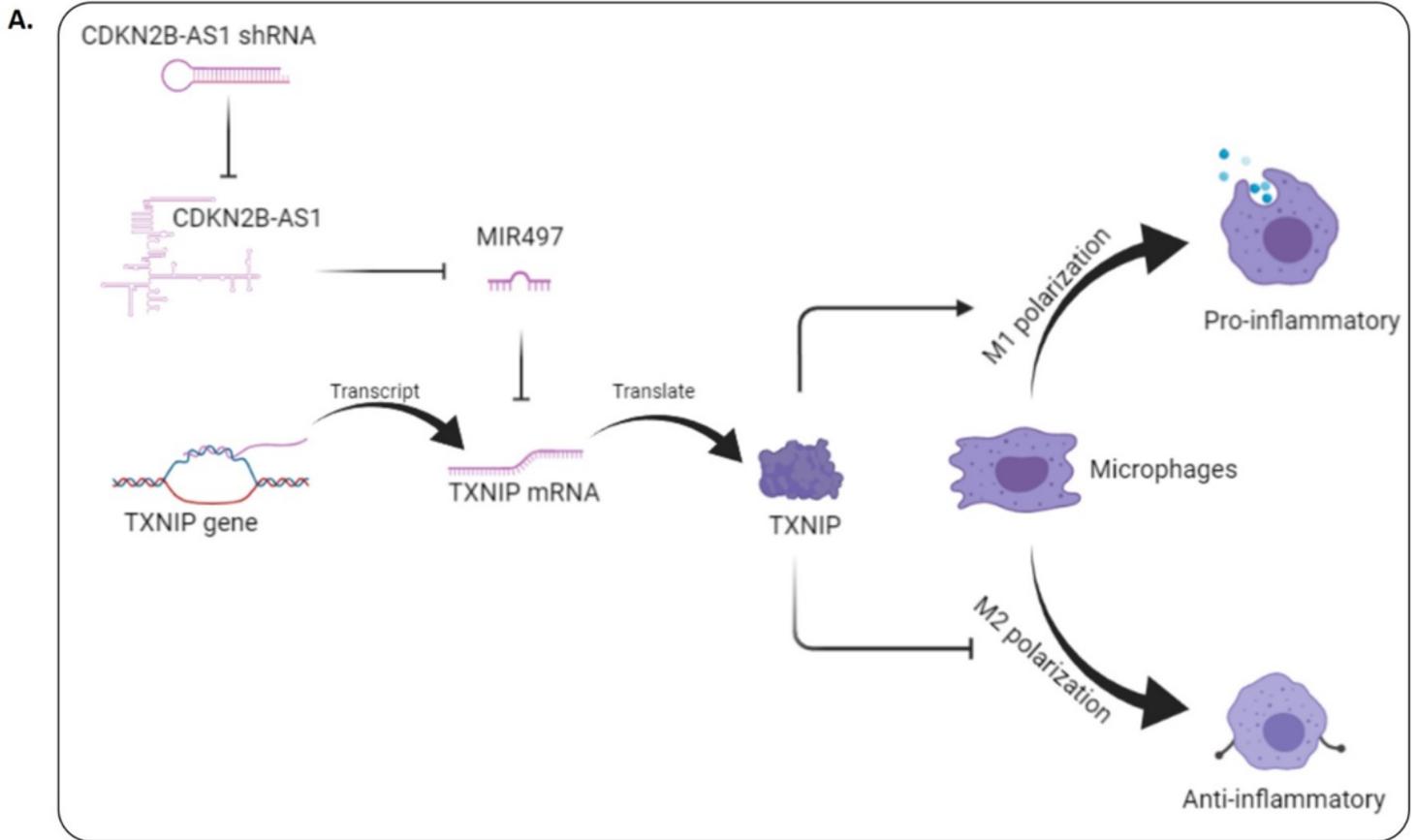


Figure 6

The effect of TXNIP overexpression in MD cell polarization. TXNIP overexpression vectors were transfected into MD cells. (A) Effect of TXNIP overexpression on CDKN2B-AS1/MIR497/TXNIP axis. (B) Representative image of western blotting assay. (C) Effect of TXNIP overexpression on surface polarization markers of not-induced MD cells. (D) Effect of TXNIP overexpression on surface polarization markers of M1 induced MD cells. (E) Effect of TXNIP overexpression on surface polarization markers of IL10 induced MD cells. (F) Effect of TXNIP overexpression on surface polarization markers of IL4 induced MD cells. (control: cells not transfected; the control was present for normalization and the difference between negative and overexpressed groups was tested using a t-test, *p-value < 0.01.)



B.

RNA	Position	Complementary binding sites
lncRNA CDKN2B-AS1	5'.....61-67.....3'	5'.....UGCUGCU.....3'
MIRNA 497	3'.....31-25.....5'	3'.....ACGACGA.....5'
TXNIP mRNA	5'.....2166-2172.....3'	5'.....UGCUGCU.....3'

Figure 7

The role of the CDKN2B-AS1/ MIR497/TXNIP axis. (A) Mode of the role of the CDKN2B-AS1/ MIR497/TXNIP axis in rheumatoid macrophages. (B) Complementary binding sites of the CDKN2B-AS1/ MIR497/TXNIP axis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [floatimage1.jpeg](#)