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miRNA-146a Inhibits LPS-induced Inflammation in THP-1 Cell Model of Kawasaki Disease

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KEYWORDS Apoptosis. Inflammation. Kawasaki Disease. Lipopolysaccharide. miRNA-146a. Proliferation

ABSTRACT This study investigated physiological effects of miRNA-146a in Kawasaki disease with THP-1 cells. An inflammatory Kawasaki disease-like environment was established in THP-1 cells with lipopolysaccharide induction, which were then transfected with miRNA-146a mimics or a control. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) analyses demonstrated that miRNA-146a significantly downregulated the expression of key pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α. After Annexin V/PI application, flow cytometric technique was used to quantify apoptosis, revealing a marked reduction in both early and late apoptotic cells following miRNA-146a overexpression. Western blot analysis further indicated increased anti-apoptotic B-cell lymphoma 2 (Bcl-2) expression, as well as decreased caspase-3 activation and BCL2-Associated X (Bax) expression. By demonstrating miRNA-146a's critical function in attenuating inflammatory responses and suppressing apoptosis, this study positions it as a promising novel therapeutic target for Kawasaki disease.

INTRODUCTION

Kawasaki disease (KD) is an acute systemic vasculitis that was initially characterised and reported by Dr. Tomisaku Kawasaki in Japan in 1967. Children are the main victims of KD, an acute vasculitis that is marked by a protracted fever, rash, corneal irritation, with a higher vulnerability of coronary artery aneurysms. KD has a multifactorial pathogenesis, which is primarily mediated by a dysregulated immune reaction, particularly the roles of inflammation and apoptosis. According to recent data, these mechanisms are essential for KD progression, leading to cardiovascular complications in affected children. The most common presentations of the clinical features of KD include elevated body temperature for more than five consecutive days, red lips, swollen and desquamated finger and toe tips, red eyes, generalised polymorphous rash, and cervical lymph node enlargement (Kuo et al. 2012; Sakurai 2019). Studies have shown that KD may be caused by an over-activated immune response following infection with a pathogen and that the over-activated immune system produces a large number of cytokines and inflammatory mediators that damage the endothelial cells of the blood vessels, resulting in vascular damage (Nakamura et al. 2019). Coronary artery abnormalities (CAAs) are the most prominent comorbidity of KD. In untreated patients, approximately 20 percent of patients have a combination of CAAs, including coronary artery dilatation, aneurysms and stenosis, with severe myocardial infarction or sudden death (Sundel 2015). Early standardised treatment with intravenous immunoglobulin (IVIG) combined with aspirin still results in CAAs in 5 percent of children with KD (Dietz et al. 2017). Therefore, it has been suggested that KD is a "coronary heart disease" in children and is associated with coronary heart disease in adults (Buda et al. 2021).

MicroRNAs (miRNAs) as post-transcriptional regulators of gene expression have been a subject of considerable interest. Research has consistently shown that the miRNA profile is profoundly dysregulated in individuals with KD. For instance, the miR-200 family, miR-429, and miR-146a may be closely associated with the pathogenesis, diagnosis, and treatment of KD (Ma et al. 2021; Wang et al. 2025; Ho et al. 2023). miRNA-146a is an NF-κB-dependent inflammation-sensitive miRNA that plays a negative feedback regulatory role in various inflammatory dis-

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eases (Ho et al. 2023; Jiang et al. 2022; Tong et al. 2021). Previous studies have demonstrated that miRNA-146a can suppress the overactivation of the NF-κB signalling pathway by targeting adaptor proteins such as TRAF6 and IRAK1, thereby attenuating inflammatory responses (Ho et al. 2023; Tong et al. 2021). In THP-1 cells, overexpression of miRNA-146a significantly reduced the production of TNF-α and IL-6, whereas its inhibition enhanced the release of these cytokines (Tong et al. 2021; Gunter et al. 2022). Furthermore, KD sufferers' peripheral blood was reported to exhibit abnormal expression of miRNA-146a, indicating its potential involvement in the immunoregulation of the disease (Cai et al. 2022). It was shown by Palomer et al. (2015) that the development of HF along with myocardial dysfunction is significantly influenced by miR-146a. miR-146a inhibited MMP-9 by affecting c-fos and reduced the c-fos/AP-1 signalling pathway to inhibit MMP-9. However, while miR-146a is known to regulate inflammatory responses in various contexts, its specific role in modulating the inflammatory response in KD, particularly in relation to LPS stimulation, requires further investigation.

LPS, a potent inducer of inflammation, mimics the pathological processes observed in KD by triggering the activation of pro-inflammatory pathways (Kansakar et al. 2022). Understanding how miR-146a interacts with LPS-induced inflammation could provide valuable insights into its potential therapeutic role in managing KD. Standard management for KD has been effective in reducing inflammation and preventing coronary artery damage, but these treatments may experience long-term complications, underscoring the need for novel therapeutic strategies. Targeting key regulatory molecules, such as miRNA-146a, could offer a new approach to enhance treatment efficacy and improve patient outcomes.

Objective

This study aimed to investigate the specific role of miRNA-146a in regulating inflammation and apoptosis within a KD THP-1 cell model. By expanding the understanding of miRNA-146a's function, the researchers hope to contribute to the development of more effective therapies for KD.

MATERIAL AND METHODS

The Huizhou Central People's Hospital Ethics Board officially acknowledged this study, [kyll20210122].

Cell Culture

The THP-1 mononuclear cell line (Institute of Cell, Chinese Academy of Sciences) was cultivated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) enriched with 10 percent foetal bovine serum (Gibco.), 1 percent penicillin/streptomycin, and 0.1 percent gentamicin in a constant temperature and humidity incubator at 37°C with 5 percent C_{o2}, . Cells were passaged at 80 percent confluence.

Cell Transfection and Grouping

To investigate the role of miRNA-146a in regulating inflammation and apoptosis, THP-1 cells were categorised into several groups, with the control group cultured under normal conditions and the LPS group stimulated by lipopolysaccharide (LPS, 0.1 μg/mL) within 24 hours to mimic inflammatory response observed in KD. miRNA-146a Mimic Group was transfected with 10 nmol/L miR-146a mimics and subsequently treated with LPS, while the Negative Control (NC) Group received transfection with 10 nmol/L mimic-NC and LPS treatment. Prior to transfection, cells were cultured in 24-well plates with the standard growth medium, excluding any double antibody. The plasmids were introduced into 150 μL of OPTI-MEM medium (31985062, Thermo Fisher Scientific, USA), along with lipo3000 (L3000150, Thermo Fisher Scientific, USA) in a 1 μL to 150 μL ratio of OPTI-MEM medium. The mixture was thoroughly combined and left at room temperature for 5 minutes. After 15 minutes of settling on an ultraclean surface, the plate was agitated and mixed. Subsequently, the plate was transferred to a serum-free medium. While the mixture was settling, the cell culture in the 24-well plate was removed, and the wells were rinsed thrice with PBS. Each well received 400 μL of OPTI-MEM medium. The plate was then placed back in the 37°C, 5 percent CO_2 incubator for continued incubation. After 15 minutes, the medium in the cell culture wells was aspirated,

and the RNA and transfection reagent mix were gently added to the 24-well plate, following the appropriate grouping. The plate was shaken and mixed thoroughly before being placed at 37°C for an additional 6 hours, using a serum-free medium. Transfection efficiency was assessed by performing a PCR assay on the cells. After transfection, various assays were conducted to evaluate inflammatory responses and apoptosis, including quantitative PCR, ELISA, Western blot analysis, CCK-8 assay, and flow cytometry. These assays collectively helped assess the effects of miRNA-146a on the inflammatory and apoptotic processes in the KD model.

The LPS-induced inflammatory response in THP-1 cells serves as a prototype for studying the pathophysiology of Kawasaki disease. The control group served as blank control. The LPS group was treated with 0.1 μg/mL LPS for 24 hours to induce the inflammatory response of THP-1 cells. LPS-induced THP-1 inflammatory cells were divided into two groups, wherein group A was transfected with 10 nmol/L mimic-NC, and group B was transfected with 10 nmol/L miR-146a-mimic.

Cells were required to meet specific inclusion criteria, that is, they must be THP-1 monocytic cells in the logarithmic growth phase and exhibit viability greater than 90 percent as assessed by trypan blue exclusion prior to any treatments. Furthermore, all experimental groups were replicated in at least three independent experiments to ensure reproducibility and statistical validity of the results.

qRT-PCR

To extract total RNA from cells, TRIzol (T9108, Takara Bio, Japan) was utilised. cDNA was synthesised from total RNA (extracted with TRIzol) using RevertAidTM First Strand cDNA Synthesis Kit (K1621, Thermo Fisher Scientific, USA). The reaction system was 10 μL, and reaction temperature and time included 95°C within three minutes (repeated one time), 95°C within ten seconds and 55°C within thirty seconds (repeated forty times). Gene expression was quantified via the $2^{-\Delta\Delta Ct}$ method using the primers listed in Table 1.

Western Blot Assay

The cells were lysed by subjecting them to RIPA buffer (P0013B, Beyotime, China) at a temperature of 0°C for 30 minutes. The resultant content included the total protein. The protein concentration was quantified using a BCA protein assay kit (ab287853, Abcam, China). The protein samples were then separated on a 10 percent SDS-PAGE and were then transferred to a PVDF membrane (Millipore, German). After blocking with 5 percent skimmed milk, the membrane was incubated overnight with the primary antibodies (TNF-α (ab183218, Abcam, China, diluted at 1:1000), IL-6 (ab233706, Abcam, China, diluted at 1:1000), IL-10 (ab189392, Abcam, China, diluted at 1:1000), TRAF6 (ab172612, Abcam, China, diluted at 1:1000) and GAPDH (ab8245, Abcam, China, diluted at 1:1000) at 4°C. After washing, the secondary antibodies (ab150077, Abcam, China, diluted at 1:10,000; HRP-conjugated goat anti-rabbit IgG H&L) were added at room temperature. To visualise the protein signals, the researchers employed a Tanon 6600 instrument (Tanon, Shanghai, China). The researchers then analysed the optical density values of proteins using Image J software.

Table 1: Primers used in qPCR

Primers	*Sequence (5→3')*	
TNF-α	Forward	CCTCTCTCTAATCAGCCCTCTG
	Reverse	GAGGACCTGGGAGTAGATGAG
IL-6	Forward	ACTCACCTCTTCAGAACGAATTG
	Reverse	CCATCTTTGGAAGGTTCAGGTTG
IL10	Forward	TCCCAGGCAACCTGCCTAAC
	Reverse	AAATCGATGACAGCGCCGTAG
miRNA-146a	mimic	UGAGAACUGAAUUCCAUGGGUU
	NC	UUGUACUACACAAAAGUACUG
GAPDH	Forward	TGCGACTTCAACAGCAACTC
	Reverse	ATGTAGGCCATGAGGTCCAC

Cell Viability Assays

Cell viability was calculated using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan). Each well containing a set of cells received 10 μL of CCK-8 solution before being placed in the incubator for continuous cultivation for 2 hours. The optical density at 450 nm (OD450) was assessed employing a microplate reader.

Flow Cytometry for Apoptosis

After a 24-hour incubation period, the cells underwent digestion and purification. 1×10^6 cells were resuspended in medium, followed by centrifugation and subsequent resuspension in PBS buffer. Afterwards, each sample received 10 μL of PI and 5 μL of annexin V-FITC (C1062S, Beyotime, China). The cells were then incubated at room temperature for 10 minutes, and the flow cytometry analysis using FACS Calibur (Becton Dickinson and Company, USA) was conducted to assess the apoptosis rate of the cells.

ELISA

Following collection, the supernatants were analysed for IL-6, IL-10, and TNF-α levels via ELISA. The procedure was performed exactly in accordance with the kit's instructions, IL-6 (PI325 Beyotime, China, minimum detectable level of 1.5 pg/ml), IL-10 (PI528, Beyotime, China, minimum detectable level of 8.8 pg/ml), and TNF-α (PT518, Beyotime, China, minimum detectable level of 14.3 pg/ml).

Luciferase Reporter Assays

The researchers constructed a luciferase reporter vector containing the 3' untranslated region (UTR) of the target gene. THP-1 cells were transfected at 70 percent to 80 percent confluence using Lipofectamine 3000, co-transfecting the luciferase vector with either miRNA-146a or a control miRNA. After 48 hours, cell lysates were collected, and a luciferase detection system was used to measure both firefly and Renilla luciferase activities,

Statistical Analysis

Data analyses and graphing were performed with Graphpad Prism 9 and complied in Adobe Illustrator 2022. All data are reported as mean ± SD. One-way ANOVA or Tukey's test was conducted for comparison, adopting a significance threshold of $p < 0.05$.

RESULTS

Efficiency of miR-146a Mimic Transfection Validated by q-PCR

qRT-PCR verified miR-146a transfection efficiency. miR-146a level was markedly increased in the transfected cells than the controls (0.00 vs. 11.52, $P<0.0001$, Fig. 1). MiR-146a expression was not different between the NC and NC+LPS groups (0.00 vs. 0.00, $P>0.05$). However, compared with the LPS group, miR-146a expression was significantly increased in the miR-146a+LPS group (0.00 vs. 26.35, $P<0.0001$).

miR-146a Inhibits LPS-induced Inflammation in THP-1 Cells

As shown in Figure 2A-C, the mRNA levels of TNF-α (0.03 vs. 6.44), IL-6 (0.00 vs. 6.93), and IL-10 (0.00 vs. 0.42) were significantly increased in THP-1 cells treated with LPS ($P<0.0001$). Furthermore, after transfection with miR-146a, the expression levels of TNF-α (6.44 vs. 4.76), IL-6 (6.93 vs. 5.81), and IL-10 (0.42 vs. 0.36) were notably lower ($P<0.05$, $P<0.001$). As shown in Figure 2D-F, ELISA assays demonstrated that the expression levels of TNF-α (10.45 vs. 13.44), IL-6 (4.72 vs. 6.74), and IL-10 (110.12 vs. 133.37) were also significantly increased in THP-1 cells treated with LPS ($P<0.001$, $P<0.0001$). Furthermore, after transfection with miR-146a, the expression levels of TNF-α (13.44 vs. 11.43), IL-6 (6.74 vs. 5.85), and IL-10 (133.37 vs. 113.98) were observed to decrease ($P<0.01$, $P<0.001$). Moreover, Western blot experiments revealed that overexpression of miR-146a inhibited the protein levels of TNF-α (0.77 vs. 0.64), IL-6 (0.45 vs. 0.36), and IL-10 (0.97 vs. 0.78) in the LPS-induced THP-1 KD cell model ($P<0.05$, Fig. 2G-J). Since ELISA detects proteins in the cell supernatant and WB detects all cellular proteins, there will be some differences in the results. These findings suggest that miR-146a can suppress LPS-induced inflammatory responses in THP-1 cells by reducing the expression of inflammatory factors TNF-α, IL-6, and IL-10.

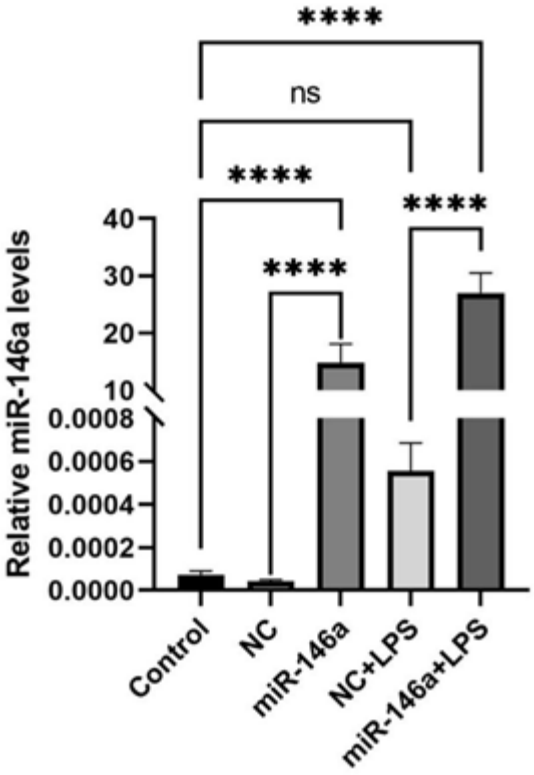

Fig. 1. The transfection efficiency of miR-146a was verified by q-PCR. *******P*<0.0001.

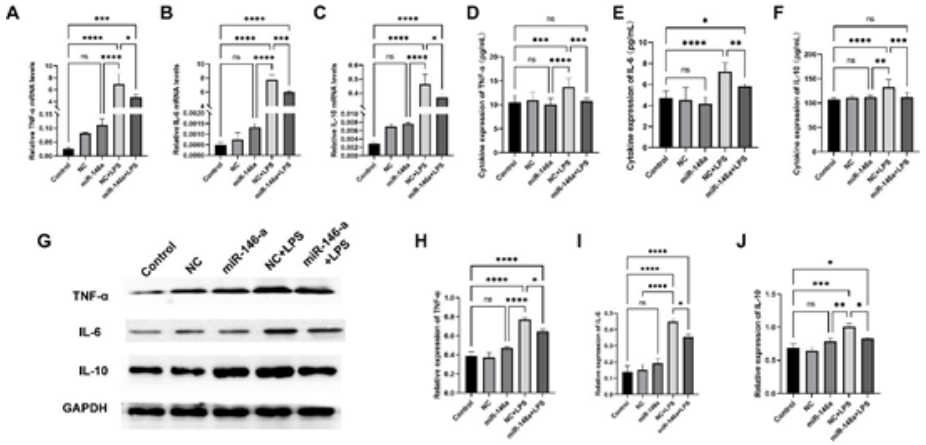

Figure 2. q-PCR for inflammatory factor expression (A-C), TNF-á (A), IL-10 (B) and IL-6 (C). ELISA for cellular supernatant inflammatory factor expression (D-F), TNF-á (D), IL-10 (E) and IL-6 (F). WB assays detected inflammatory factor expression (G-J), TNF-á (H), IL-10 (I), and IL-6 (J). ****P*<0.05, *****P*<0.01, ******P*<0.001, *******P*<0.0001.

MiR-146a Promoted LPS-induced THP-1 Cell Activity and Inhibited its Apoptosis

CCK-8 assay demonstrated that LPS treatment inhibited the survival of THP-1 cells (100% vs. 56%, $P<0.001$, Fig. 3A). Nevertheless, miR-146a+LPS group exhibited markedly higher THP-1 cell activity than NC+LPS group (56% vs. 61%, $P<0.001$, Fig. 3 A). Flow cytometry results revealed that LPS induction enhanced early apoptosis of THP-1 cells, whereas overexpression of miR-146a suppressed early apoptosis of THP-1 cells ($P<0.001$, Fig. 3 B-G). Collectively, miR-146a promotes proliferative activity of THP-1 cells and inhibits LPS-induced apoptosis.

miRNA-146a Suppresses Inflammation by Targeting TRAF6

Upon transfection with miRNA-146a, a pronounced decline in luciferase activity was observed (1.00 vs. 0.71, $P<0.05$, Fig. 4A). miRNA-146a negatively regulates TRAF6 expression by binding to its 3' untranslated region (UTR). Western blot analysis (Fig. 4B) further confirmed the downregulation of TRAF6 protein levels in the LPS+ miRNA-146a group than the LPS group (1.06 vs. 0.81, $P<0.05$).

DISCUSSION

In KD, immune cell infiltration, endothelial dysfunction, and an exaggerated inflammatory response are key drivers of vascular damage, often resulting in coronary artery abnormalities. Central to this pathogenesis are pro-inflammatory mediators, including IL-1, IL-6, and TNF-α. Inflammatory cell infiltration, endothelial edema and necrosis are seen in the blood vessels of children with KD, which in turn impede the function of the vascular endothelium, triggering excessive secretion of inflammatory mediators, pro-inflammatory cytokines, and enzymes from both endothelial cells and macrophages, which in turn induce a hyper adhesive state of platelets due to increased membrane glycoproteins. This in turn causes further platelet activation and exacerbates the inflammatory process, and atherosclerosis progression is significantly modulated by this mechanism (Burns and Matsubara

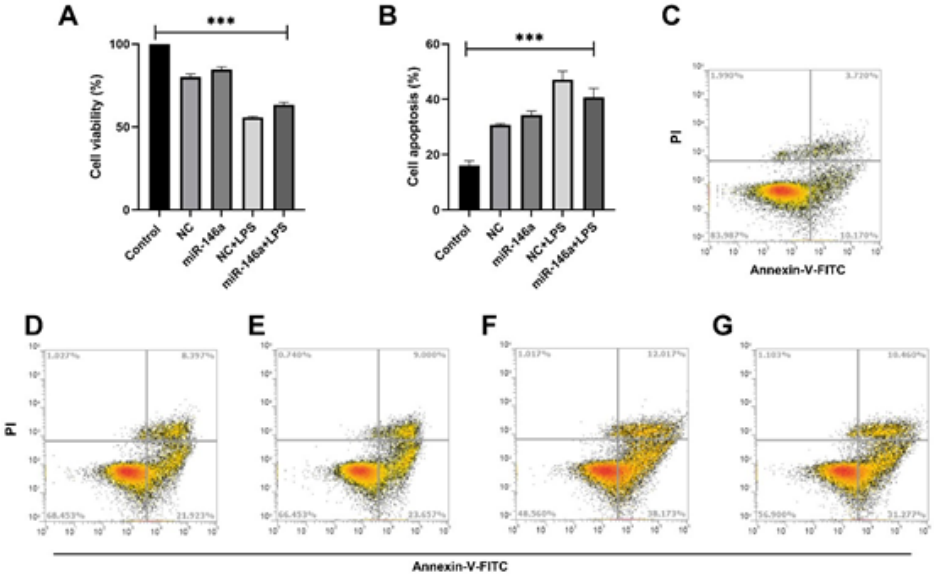

Fig. 3. Cell viability was detected by CCK-8 kit (A). The apoptosis rate was detected by flow cytometry (B). Flow cytometry results (C-G). Control (C), NC (D), miR-146a (E), NC+LPS (F) and miR-146a+LPS (G). Results were mean ± SD for three individual experiments. ***$P<0.001$.

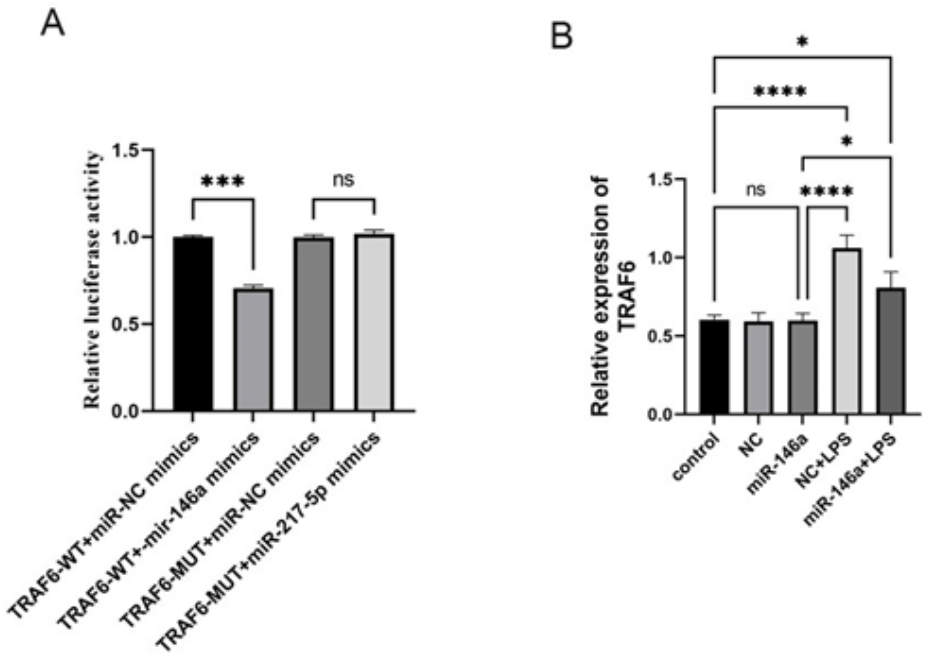

Fig. 4. miRNA-146a Suppresses Inflammation by Targeting TRAF6. (A) The luciferase reporter assay; (B) Western blot analysis.*P<0.05, ***P<0.001, ****P<0.0001.

2018). A significant characteristic of atherosclerosis is the infiltration of monocytes into the damaged arterial wall, subsequently transforming into macrophages (Amengual and Barrett 2019). In the peripheral blood during the acute phase of KD, T lymphocytes and monocytes can be aberrantly activated, and the activated cells release IL-1, IFN-γ, IL-6, TNF-α, soluble E selectin and VEGF (Hu et al. 2017; Zhou et al. 2018; Li et al. 2019; Inoue et al. 2020). Monocytes and macrophages are central in this inflammatory cascade, highlighting the need for novel therapeutic targets to mitigate inflammation in KD.

Studies have shown that serum concentrations of IL-6, IL-10, IFN-γ and TNF-á were elevated before the infusion of IVIG, while serum IL-6, IL-10 and IFN-γ levels decreased rapidly after IVIG treatment (Zhang et al. 2022). The role of cytokines released by THP1 cells in acute KD vascular endothelial injury is crucial. The THP1 cell line is extensively utilised as an in vitro model for studying the mechanisms underlying inflammatory diseases in human monocytes and macrophages (Okabe et al. 2022). Previously, the researchers selected THP-1 monocytes, which were induced by LPS to mimic the KD inflammatory response cell model, and showed that LPS stimulation increased the levels of IL-6, IL-10, and TNF-α in THP-1 cells.

The KD acute phase is marked by an imbalance of immune cells, accompanied by abnormal immune activation. Abnormally activated monocytes release large amounts of cytokines, such as TNF-α, IL-1 and IL-6, causing an excessive inflammatory response. These cytokines are thought to be produced by monocytes/macrophages (Wu et al. 2021). In this study, the transfection of miR-146a mimics was found to inhibit the secretion of those cytokines in LPS-induced THP-1 cells, thereby reducing the inflammatory response. These results indicate that miR-146a may inhibit KD. However, further investigation is required to understand its specific mechanism of action in KD.

This study confirmed that overexpression of miRNA-146a significantly suppressed the expression of TNF-α, IL-6, and IL-10 induced by LPS. In patients with systemic lupus erythematosus (SLE), deficiency of miR-146a is associated with elevated levels of inflammatory cytokines (Zhou et al. 2019). Similarly, in LPS-stimulated IPEC-J2 cells, overexpression of miR-146a-5p reversed the LPS-induced upregulation of TNF-α and IL-6 and promoted cell proliferation (Chen et al. 2022). In the present study, the inhibitory effect of miRNA-146a on IL-10 may reflect its dual role in the negative feedback regulation of inflammation. On one hand, it suppresses excessive inflammatory responses, while on the other hand, it prevents immune imbalance caused by over-secretion of anti-inflammatory factors. Furthermore, the discrepancy between the Western blot and ELISA results suggests that miRNA-146a may influence inflammatory cytokine levels through both post-transcriptional regulation (for example, mRNA stability) and secretory pathways.

Apoptosis, serving as the primary form of programmed cell demise, can maintain cellular homeostasis. However, when apoptosis occurs in an aberrant fashion, it can result in various diseases. Recent investigations have indicated that the excessive activation of the immune system and the induction of apoptosis due to oxidative stress contribute to vascular damage observed in patients diagnosed with KD (Zheng et al. 2022; Zheng et al. 2023). Overexpression of miRNA-146a markedly reduced LPS-induced apoptosis in THP-1 cells, as evidenced by decreased early apoptotic cells, inhibited caspase-3 activation, and increased Bcl-2 level. This phenomenon in this research aligns with the role of miRNA-146a in other disease models. In oxygen-glucose deprivation/reperfusion-induced HT-22 cell injury, miR-146a-5p alleviated apoptosis by suppressing the TRAF6/NF-κB pathway (Deng et al. 2025). Similarly, in Cutibacterium acnes biofilm-induced inflammation in keratinocytes, deficiency of miR-146a led to activation of the IRAK1/TRAF6 axis, thereby promoting apoptosis (Zhou et al. 2023). The present study further extends these findings by demonstrating that miRNA-146a exerts a protective effect against apoptosis in KD-related immune cells, potentially through regulation of the mitochondrial apoptotic pathway via Bcl-2 family proteins.

Using luciferase reporter assays and Western blot analysis, this study confirmed that miRNA-146a directly targets the 3'UTR of TRAF6 and inhibits its expression. TRAF6 is a key adaptor protein in the NF-κB pathway, and its downregulation blocks nuclear translocation and transcriptional activity of NF-κB (Deng et al. 2025; Lv et al. 2017). This mechanism has been validated in multiple disease models. For instance, in intervertebral disc degeneration (IDD), overexpression of miR-146a alleviated inflammation by inhibiting the TRAF6/NF-κB pathway (Lv et al. 2017). In a neuropathic pain model, miR-146a-5p mitigated pain by suppressing IRAK1/TRAF6 signalling (Wang et al. 2018). The novelty of this study lies in clarifying the central role of the miRNA-146a/TRAF6 axis in a KD immune cell model, providing mechanistic support for the observed association between miRNA-146a polymorphisms and coronary artery lesions (CAL) in KD patients (Zha et al. 2019; Fu et al. 2022). For example, the rs2910164 allele may indirectly regulate TRAF6-mediated vascular inflammatory responses by affecting the expression level of miRNA-146a.

Despite these promising results, the study has some limitations. The use of the THP-1 cell line, although widely used as a model for KD inflammation, may not fully represent the in vivo immune environment of KD patients. Future research should validate these findings using primary monocytes or tissues from KD patients to establish greater clinical relevance. Additionally, while the researchers explored miRNA-146a's effects on key inflammatory and apoptotic markers, further mechanistic studies such as luciferase reporter assays and pathway analysis are needed to confirm the direct molecular targets of miRNA-146a in KD. Finally, in vivo studies using KD animal models should be conducted to evaluate the long-term impact of miRNA-146a on coronary artery health and to further investigate its therapeutic applicability.

CONCLUSION

In conclusion, overexpression of miR-146a effectively suppresses the LPS-induced inflammatory response and apoptosis in THP-1 cells. These results indicate the considerable therapeutic potential of miR-146a in the treatment of

KD. Subsequent studies ought to concentrate on its prospects for clinical translation and its broader involvement in multi-organ injury linked to KD.

DECLARATIONS

DATA AVAILABILITY

The simulation data supporting the conclusions of this article can be obtained from the corresponding author upon reasonable request.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

None.

AUTHOR CONTRIBUTIONS

Xin Zheng carried out the study concept and design, and edited the manuscript. Shanghong Tang and Hairan Ma carried out the experiment and contributed to sample preparation.

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