Extracellular vesicles containing miR-146a attenuate experimental colitis by targeting TRAF6 and IRAK1

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ABSTRACT

Accumulating evidence indicates that microRNA-146a (miR-146a), a well-known anti-inflammatory miRNA, acts as a negative feedback regulator of the innate immune response, but its role in modulation of inflammatory bowel disease (IBD) remains unclear and the issue related to the stability of exogenous miR-146a in blood is up in the air. In this study, extracellular vesicles (EVs) from cultured medium of bone-marrow mesenchymal stem cells (BMSCs) transfected with recombinant lentiviruses can serve as a stable delivery system and overexpress miR-146a, which significantly inhibited TNF receptor–associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) expression in TNBS-induced colitis of rats. Moreover, the increased phosphorylation levels of NF-κB p65 and IκBα were down-regulated by the administration of EVs containing miR-146a. Coupled with the associated influence of over-expressed miR-146a on phosphorylated proteins above, the production of inflammation factors such as tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6) and Interleukin-1β is apparently suppressed by this non-coding RNA. Collectively, these data elucidated that EVs containing miR-146a ameliorates experimental colitis caused 2,4,6-trinitrobenzenesulfonic acid (TNBS) by targeting TRAF6 and IRAK1.

1. Introduction

Ulcerative colitis (UC), characterized by bloody diarrhea, abdominal pain, tenesmus, is a chronic and idiopathic inflammatory bowel disease (IBD), which affects the rectum and colon in an uninterrupted pattern [1]. In UC, confluent inflammation involving a pathological response in both the innate and adaptive immune systems is limited to the mucosa or superficial submucosa [2]. The pathogenesis of UC remains to be answered, however, growing evidence indicates that the disease results from an inappropriate immune response to gut microbiota in genetically susceptible hosts exposed to environmental risk factors [3]. Current medical and surgical managements usually could not keep patients in remission for a long term.

MicroRNAs (miRNAs), highly conserved endogenous 8–25-nt long non-coding RNAs, can form the RNA-induced silencing complex with the Argonaute family and then interact with partially complementary sequences located primarily in the 3′ untranslated region of their target messenger RNAs(mRNAs), eliciting either inhibition of translation or degradation of the target mRNA [4–6]. miRNAs are involved in diverse biological processes - including cell proliferation, differentiation,

apoptosis, cancer, autoimmunity, and inflammation [7–9]. To date, > 2000 miRNAs have been found in the human genome [10].

MiR-146a is a member of the miR-146 miRNA family, comprising two evolutionary conserved copies: miR-146a and miR-146b. In people, these loci are located at chromosomes 5 and 10 respectively, and they differ in their mature sequence only by 2 nt at the 3′end. It has been affirmed that miR-146a acts as a negative feedback regulator of the innate immune response by targeting two adapter proteins, TRAF6 and IRAK1 [11]. In vivo, the stability of exogenous miRNAs should be considered in that miRNAs could be readily degraded due to the RNases in plasma [12]. Consequently, it is a central question how miRNAs in circulating blood are protected from degradation.

Mesenchymal stem (or stromal) cells (MSCs), nonhematopoietic adult stem cells, which can be isolated from bone marrow (BM), umbilical cord, placental or adipose tissue, are multipotent cells with the ability to differentiate into several cell types, thus serving as a potential solution for tissue repair and wound healing [13]. In addition to their direct role in tissue regeneration, MSCs have potent anti-inflammatory and/or immunosuppressive properties [14]. Extensive research and clinical trials suggest that MSCs transplantation can be used as

regenerative agents in many diseases including IBD [15]. However, MSC transplantation presents some limitations, such as immune-mediated rejection and malignancy transformation [16]. What's more, recent studies show that MSCs achieve a therapeutic effect in vivo via paracrine action [17]. And the paracrine functions of MSCs could, at least in part, be mediated by extracellular vesicles (EVs). Compared to their parent cells, EVs may have a superior safety profile and can be safely stored without losing function [18].

Extracellular vesicles (EVs), mainly including exosomes, microvesicles and apoptotic bodies, which are all naturally occurring cell-derived vesicles that are enclosed by a lipid bilayer, ranging from 30 nm to 2000 nm in diameter depending on their origins. EVs can be involved in a diverse range of biological processes, consisting of stem cell maintenance [19], tissue repair [20], immune surveillance [21], blood coagulation [22]. One of the way by which EVs exert their biological effects is delivering effectors including small and large non-coding regulatory RNAs (such as miRNAs), mRNAs, transcription factors, oncogenes, and infectious particles into recipient cells [23–25]. Thanks to the horizontal transfer of genetic information, these vesicles also have potential as drug delivery vehicles [26]. Studies demonstrated that miR-200b could be packaged into EVs and functioned as an endogenous form of miR-200b in recipient cells [27]. This study sheds new light on how the EVs can be served as a new delivery system with high transport efficiency and stability.

Based on these findings, we hypothesized that BMSCs transfected with lentivirus could overexpress miR-146a and miR-146a is able to be overexpressed and packaged into BMSC-EVs, and miR-146a-containing EVs attenuate experimental colitis by targeting of TRAF6 and IRAK1 expression.

2. Materials and methods

2.1. Isolation and identification of BMSCs by flow cytometry

BMSCs were obtained and cultured from healthy male rats 3 weeks of age as described previously [28]. The passage 2 of BMSCs (P2) was identified by flow cytometric analysis with the following fluorescent antibodies: anti-rat CD29, CD90, CD11b, and CD45 (BioLegend, San Diego, CA, USA). Labeled cells were analyzed by a FACSCalibur flow cytometer with the use of FlowJo software (FACSCalibur flow cytometer; Becton-Dickinson, Franklin Lakes, NJ, USA). The adipogenic and osteogenic differentiation potential of the BMSCs was investigated as described before with the use of rat BMSC adipogenic/osteogenic induction medium (Cyagen, Guangzhou, China) [29].

2.2. Recombinant lentivirus construction

The rat miRNA-146a gene fragments were acquired through a chemical synthesis method, completed by Shanghai Generay Biotech Co., Ltd. The gene sequences were synthesized as follows:
AGAGGGAGTGCCTCTGGATTTATCAGAGATTTCTCTTGTCTTTACA GGGCTGGCAGGATCTGACCTGTGAGGAAGGACCAGGCCTTACCTGGA GAGTCTGTGTGTATCCTCAGCTCTGAGAACTGAATTCCATGGGTTATA GCAATGTCAGACCTGTGAAGTTCAGTTCTTTAGCTGGGATAGCTCTAT CGTCATGGACCTGAGGAACACCGTCTAGGACAACCTCTGAAGGCTTTC TTATTCTGCCAAGGAACTTGAAGAGTGGAGAGAGTGGGGTGAAGGAT GGGACTGGTGG. The lentiviral vector Ubi-MCS-SV40-puromycin (named GV342, Genechem, Shanghai, China) was digested by the restriction enzyme *Age*I/*Eco*RI. Then the miRNA-146a gene fragments were ligated into the lentiviral vector mentioned above. The primers (5′-CCAACTTTGTGCCAACCGGAGAGGGAGTGCCTCTGGATTTATC-3′ and 5′-CACACATTCCACAGGAATTCCACCAGTCCCATCCTTCACCC-3′) located in the vector were used in PCR to identify positive transformants. Positive clones were chosen for sequencing. Recombinant lentiviruses, which coexpressed anti-puromycin gene and miRNA-146a sequence, were produced by 293 T cells following the cotransfection of

Ubi-MCS-SV40-puromycin- miRNA146a and the packaging plasmids pHelper1.0 and pHelper2.0 (Genechem). The virus titer was detected through a drug screening method.

2.3. In vitro transduction and culture expansion

When P2 BMSCs reached 30%–40% confluence, transfection was performed at a multiplicity of infection of 10 in the presence of 10 mg/mL polybrene (Genechem) according to the manufacturer's instructions. Ad-miRNA-146a-BMSCs were genetically engineered with recombinant lentivirus expressing both the miRNA-146a and anti-puromycin gene. Ad-null-BMSCs were manipulated with lentivirus only expressing the anti-puromycin genes that were used as a negative control. Successfully transfected cells were selected with puromycin at a final concentration of 2.5 mg/mL (Sigma-Aldrich, St. Louis, MO, USA) for 7 days. All these cells were used for the EVs harvest.

2.4. Isolation and characterization of EVs

Only < 6 passages of BMSCs were used to produce BMSC-EVs. BMSCs were thoroughly washed with phosphate-buffered saline (PBS) for three times and then the cells were starved by subjecting them to basal media without serum for 48 h and the supernatants were collected. EVs were isolated from cultured medium by differential centrifugation with some modifications and characterization as previously described [30]. Briefly, the cultured supernatants of BMSCs were centrifugated at 1000g for 10 min, 2000g for 30 min to remove cells and debris. Cell-free supernatants were centrifuged (Beckman Coulter OptimaL-100K ultracentrifuge, Fullerton, CA, USA) again at 120,000g for 70 min at 4 °C, washed in PBS and submitted to a second ultracentrifugation in the same conditions. The protein content of EVs was quantified by BCA assay (Beyotime Biotechnology, Shanghai, China). Then EVs were observed under 100kv transmission electron microscope (TEM) (HITACHIH-7000FA, Tokyo, Japan) to identify its morphology [31]. Surface epitope protein expression (CD9 and TSG101) in EVs were analyzed by Western blot.

2.5. Experimental animals

Male Sprague-Dawley rats weighed 160–180 g were purchase from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed under specific pathogen-free conditions at constant humidity and temperature (22–24 °C), with 12/12 h darkness-light cycles and free access to food and water. This study was conducted strictly according to the Animal Research Institute Committee guidelines of HUST, and approved by the Institutional Animal Care and Use Committee of HUST.

2.6. Induction of experimental colitis and treatment

Male Sprague-Dawley rats were assigned randomly to four groups: the normal group, the TNBS group, the NC group, and the miR-146a group (the TNBS group n = 11, the other groups, n = 10). Rat model of colitis was induced with 2,4,6-trinitrobenzenesulfonic acid (TNBS) enema as described previously [32]. In TNBS group and the treatment groups (the NC group and the miR-146a group), animals were treated with TNBS dissolved in ethanol through the anus of rats. In the normal group, equal volume of PBS was used instead of TNBS to clyster. On the third day after the enema, the animals in the miR-146a group received EVs overexpressing miR-146a suspended in 1 mL PBS via tail vein injection at a dose of 100 μg per rat, while rats in the NC group received EVs without enforced miR-146a expression. The other two groups (the normal group and the TNBS group) were injected with 1 mL PBS instead. On the seventh day after tail vein injection, animals were anesthetized, and colons were dissected and analyzed for subsequent studies. To identify whether these EVs could reach the colon in vivo after administration, two other rats received lipophilic red fluorescence

Table 1
Primer sequences used for polymerase chain reaction.

Gene name		Primer sequences (5′ to 3′)
U6	Forward	CTCGCTTCGGCAGCACATA
	Reverse	CGAATTTGCGTGTCATCCT
miR-146a-5p	Forward	GGCTGAGAACTGAATTCCA
	Reverse	CAGTGCAGGGTCCGAGGTAT
TRAF6	Forward	AAGCTGTCCTCTGGCAAATATC
	Reverse	CATGTGCAACTGGGTGTTCTC
IRAK1	Forward	CTGGGTTATGTGCCGTTTCTAC
	Reverse	GTGAGGATGTGAACGAGGTCAG
TNF-α	Forward	CACCACGCTCTTCTGTCTACTG
	Reverse	GCTACGGGCTTGTCACTCG
Il-6	Forward	TGGAGTTCCGTTTCTACCTGG
	Reverse	GGTCCTTAGCCACTCCTTCTGT
IL-1β	Forward	GTGGCAGCTACCTATGTCTTGC
	Reverse	CCACTTGTTGGCTTATGTTCTGT
IL-10	Forward	GCAGGACTTTAAGGGTTACTTGG
	Reverse	ATCATTCTTCACCTGCTCCACT

dye PKH-26 (Sigma-Aldrich, St. Louis, MO, USA) labeled EVs after TNBS clyster. The PKH-26 staining process was accomplished according to the manufacturer's manual before intravenous injection. The laser scanning confocal microscope (Olympus-FV1000, Tokyo, Japan) was used to observe the frozen section of colon.

2.7. Assessment of colonic inflammation and damage

During the treatments, body weights, stool consistency, and stool occult blood were observed daily to score the disease activity index (DAI) as described previously [33]. Colons were dissected and processed for histological analysis [34].

2.8. miRNA and mRNA qRT-PCR

The expression of IL-10, IL-6, IL-1β, TNF-α, TRAF6 and IRAK1 mRNAs and miR-146a was quantified by qRT-PCR as described previously [35]. All mRNA and miRNA primers are shown in Table 1.

2.9. Western blot

Western blot analyses were performed as described previously [33]. Anti-TRAF6 (1:2000, Abcam, Cambridge, UK), Anti-IRAK1 (1:500, Abcam), Anti-pIκα (Ser32/36) (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-NF-κBp65 (1:2000, Cell Signaling Technology), anti- pNF-κBp65 (Ser536) (1:1000, Abcam), anti-TNF-α (1:500, Abcam), anti-IL-6 (1:500, Abcam) antibodies were used as primary antibodies, respectively. Anti-GAPDH antibody (1:10,000, Abcam) was used as a loading control.

2.10. Immunohistochemistry and immunofluorescent staining

Anti-TNF-α (1:100, Abcam), anti- IL-6 (1:50, Abcam) antibodies were used as primary antibodies for immunohistochemistry, respectively. The localization of PKH26 labeled EVs in the intestine and TNF-α (Sigma-Aldrich, USA) treated HT-29 cell line (human colon cancer epithelial cell line) (ATCC, Rockville, MD) were observed by a laser confocal scanning microscope (Olympus-FV1000, Tokyo, Japan).

2.11. Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-10, IL-1β, IL-6 in colon homogenate supernatants were measured using ELISA kits (NeoBioscience, Shenzhen, China) employing the quantitative sandwich enzyme linked immunoassay technique, according to the manufacturers' instructions.
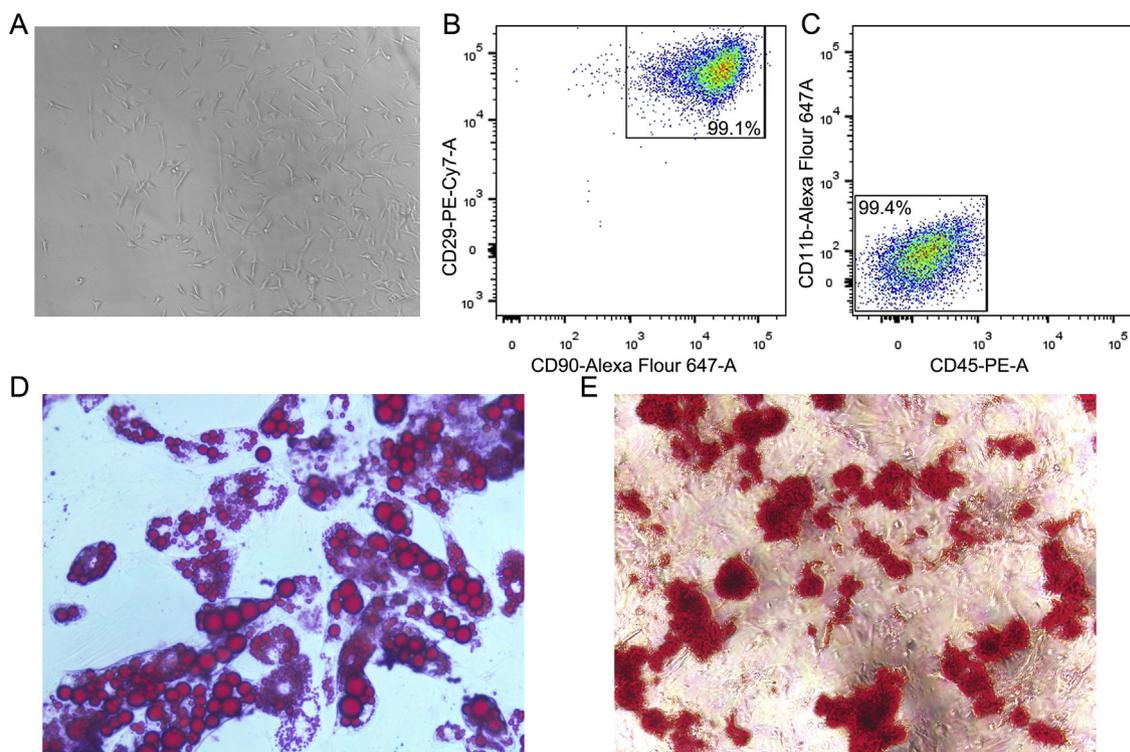

Fig. 1. Identification of rat BMSCs. (A) Representative micrographs of light microscopes obtained from rat BMSCs of the P0 passage. Original magnification, ×100. (B and C) Flow cytometric characterization of BMSCs at passage 2. (D) Adipogenic differentiation of BMSCs stained with Oil Red O. Original magnification, ×400. (E) Osteogenic differentiation of BMSCs stained with Alizarin red. Original magnification, ×100.

2.12. Statistical analyses

Data are presented as mean ± standard deviation (SD) and were analyzed by SPSS 19.0 (IBM, Chicago, IL, USA) software. The statistical significances between two groups were analyzed using unpaired two tailed Student *t*-test; more than two groups were compared using One-way ANOVA with Bonferroni post hoc test. An effect was considered significant when P ≤ 0.05. All experiments were conducted at least 3 separate times.

3. Results

3.1. Characterization of BMSCs and BMSC-EVs

The BMSCs cultured as plastic-adherent cells showed a flattened and spindle-shaped morphology and distributed as swirling in vitro (Fig. 1A). The expression of cell surface markers on BMSCs was analyzed by flow cytometry. As illustrated in Fig. 1B and C, the cells strongly expressed CD29 and CD90 (99.1%), which are bone marrow progenitor cell markers and weakly expressed CD45 and CD11b (99.4%), which are two specific cell surface markers of hematopoietic cells. To authenticate the pluripotential capacity of the cultured cells, the cells were exposed to the induction medium. BMSCs differentiated into both adipocytes (Fig. 1D) and osteoblasts (Fig. 1E) three to four weeks after induction. These data demonstrate the morphology, purity and differentiation potential of the BMSCs. Purified EVs were visualized by TEM (Fig. 2A). Images revealed that pellets obtained following ultracentrifugation showed a round and oval shaped, membrane-bound structures and that the diameter of majority EVs were < 1 μm. The results of Western blot showed that markers of EVs, including TSG101 and CD9, were expressed in EVs from BMSCs of rats (Fig. 2B).

3.2. Localization of EVs in vivo and cellular internalization of EVs in vitro

PKH26-labeled BMSC-EVs (100 μg per rat) were intravenously injected, and 12 h later rats were culling to remove colons to make frozen section. In confocal microscopy images, PKH26-labeled EVs (red) were detectable in the cross-sections of TNBS induced colitis tissue (Fig. 3A). Furthermore, the interactions with and uptake of EVs by TNF-α treated HT-29 can be observed after HT-29 were incubated with PKH26-labeled EVs for indicated time, 5 h (Fig. 3B) and 14 h (Fig. 3C), respectively.

3.3. Expression of miR-146a was up-regulated in BMSCs, BMSC-EVs and colons

Lentiviral-based microRNA system was applied to establish a stable cell line with miR-146a overexpression. The expression of miR-146a has increased 155.24-fold than negative control cells (nc group) (P < 0.0001) (Fig. 4A). We also found that the level of miR-146a in miR-146a-EVs increased 3.16-fold than the null-EVs (P < 0.0001) (Fig. 4B). As expected, the administration of miR-146a-EVs was effective to improve the expression of miR-146a (1.65-fold and 1.34-fold, respectively) in inflamed colon tissue compared with the TNBS group

and the NC group (Fig. 4C), which was also verified by qRT-PCR analysis.

3.4. Effect of EVs administration on disease severity of rats with TNBS induced colitis

To estimate the severity of symptoms, such as body weights, stool consistency, and stool occult blood, the DAI score have been recorded daily. The DAI score in the miR-146a group was decreased significantly compared to the TNBS group (Fig. 5A). The progression and severity of the damage of colon can be reflected by the shortening of the colon. The intra-rectal instillation of TNBS distinctly reduced colon length (Fig. 5B and C) when compared to the normal group (P < 0.01). However, the administration of 100 μg BMSC-EVs containing miR-146a significantly alleviate this change (P < 0.01). Histological analysis showed miR-146a mitigated mucosal damage, characterized by colonic inflammatory cell infiltration and ulceration, and the miR-146a group had a lower histological score (Fig. 5D and E). Taken together, these data demonstrate that miR-146a can suppress intestinal mucosal inflammatory response in TNBS-treated rats.

3.5. MiR-146a negatively regulates TRAF6 and IRAK1 in TNBS-induced colitis of rats

It has been well documented that TRAF6 and IRAK1, which are important components in TLR/IL-R mediated NF-κB activation pathways, are bona fide targets of miR-146a [11]. So we then examined the effect of miR-146a on the expression of TRAF6 and IRAK1 in the presence and absence of TNBS-induced colitis. Fig. 6A and B shows that the protein levels of TRAF6 and IRAK1 in the miR-146a group were significantly decreased by 87.15% and 72.88%, respectively, compared with the TNBS group. Administration of null-EVs did not obviously alter the levels of TRAF6 and IRAK1 in vivo. Those results above were further confirmed by qRT-PCR (Fig. 6C).

3.6. Increased miR-146a expression inhibits TNBS-induced inflammatory cytokine production in rats by suppressing NF-κB activation pathway

TRAF6 and IRAK1, two adapter molecules, often work together in a linear cascade, even small changes in their expression levels could have profound effects on downstream NF-κB and MAPK (mitogen-activated protein kinase) signaling. To further investigate the role of miR-146a as a key negative regulator of the TLR-mediated NF-κB activation pathway and inflammation/autoimmunity [11,36], we examined the expression levels of many inflammatory factors.

In comparison with normal group, TNBS treatment apparently increased TNF-α (4.23-fold), IL-6 (3.21-fold), and IL-1β (2.85-fold) at the mRNA level in colonic tissues, while the expression of TNF-α, IL-6 and IL-1β in miR146a group were 0.48-fold, 0.59-fold and 0.64-fold compared to TNBS group, respectively, indicating that miR-146a can mitigate inflammation in TNBS-induced colitis (Fig. 7A, B and C). The similar tendency was also observed in the protein level of TNF-α (Fig. 7D, E and M), IL-6 (Fig. 7D, F, G and N) and IL-1β (Fig. 7H). As to the anti-
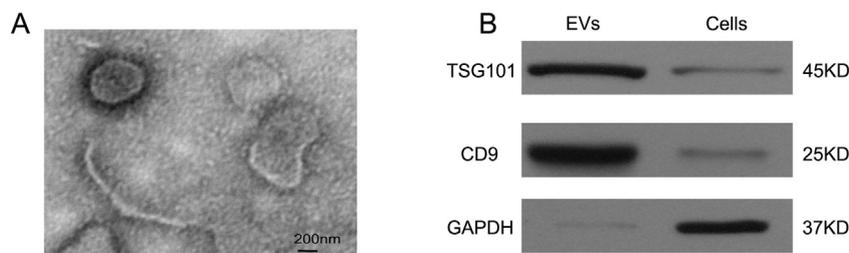

Fig. 2. The characterizations of BMSC-EVs. (A) A representative transmission electron microscopic image of EVs derived from BMSCs. Scale bar, 200 nm. (B) Western blot assay for TSG101 and CD9 expression in EVs.
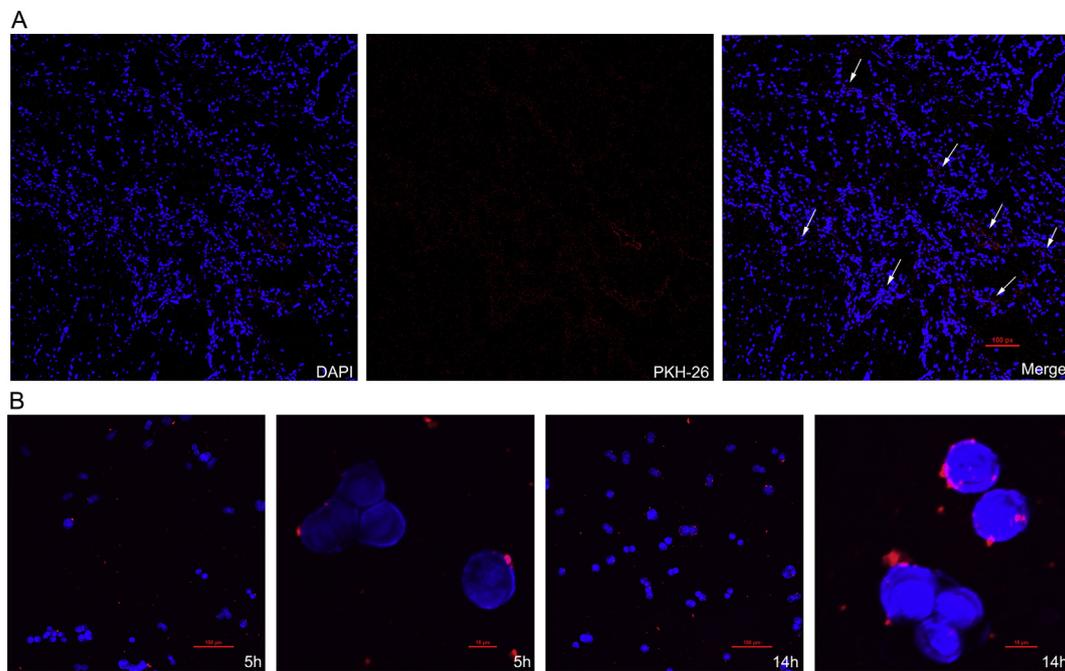
Fig. 3. Confocal microscopy analysis of PKH26-labeled EVs localization. (A) Representative micrograph of inflammatory colon with the treatment of PKH26-labeled EVs (red) at the dose of 100 μg. (B and C) Internalization of EVs by HT-29 cell line. Significant colocalization of PKH26 labeled-EVs (red) (10 μg/mL) with the HT-29 cell (blue) at different time points was observed. Cells incubated with PKH26-labeled EVs for 14 h (C) showed significantly higher uptake compared with that of 5 h (B). In merge images, cell nuclei were stained with DAPI (blue). Scale bar, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inflammatory cytokines IL-10 (Fig. 7I and J), qRT-PCR and Elisa analysis showed a significant raise in mRNA and protein levels in groups receiving EVs treatment compared with the TNBS group. Furthermore, we observed that overexpression of miR-146a in the miR-146a group also suppressed TNBS-induced IκBα phosphorylation (Fig. 7D and L) and NF-κBp65 subunit phosphorylation (Fig. 7D and K), indicating that increased miR-146a levels attenuates TNBS-induced NF-κB activity. Overall, our findings implicate that miR-146a plays a key role as a molecular brake on TNBS-induced inflammation in colitis of rats via preventing NF-κB activation.

4. Discussion

The precise cause of inflammatory bowel disease is unknown; however, genetically susceptible individuals seem to have a dysregulated mucosal immune response to commensal gut flora, which results in bowel inflammation [3]. Treatment for ulcerative colitis now consists

mainly of mesalazine, corticosteroids, immunosuppressive drugs, and monoclonal antibodies to TNF-α. Nevertheless, it seems that things are not going well and existing data highlight the need for research into prevention of inflammatory bowel disease and development of novel treatment strategies. Previous studies have indicated that miR-146a may function as negative regulators that help to fine-tune the immune response [36]. However, studies concerning the relationship between miR-146a and IBD are limited, and the role of miR-146a in the immunomodulatory effects of IBD is unclear. Natalie Stickel and his colleagues confirmed the anti-inflammatory role for miR-146a in acute GVHD [37]. Our results are consistent with these early studies [38] and show that overexpression of miR-146a by gene engineering technique significantly ameliorates the symptoms of UC, including reducing the score DAI and histological score and colon length, and suppressing the excessive inflammatory response in TNBS-induced colitis model. We demonstrated that the mechanisms by which miR-146a protects the colon tissues from TNBS-induced damage involve inhibition of
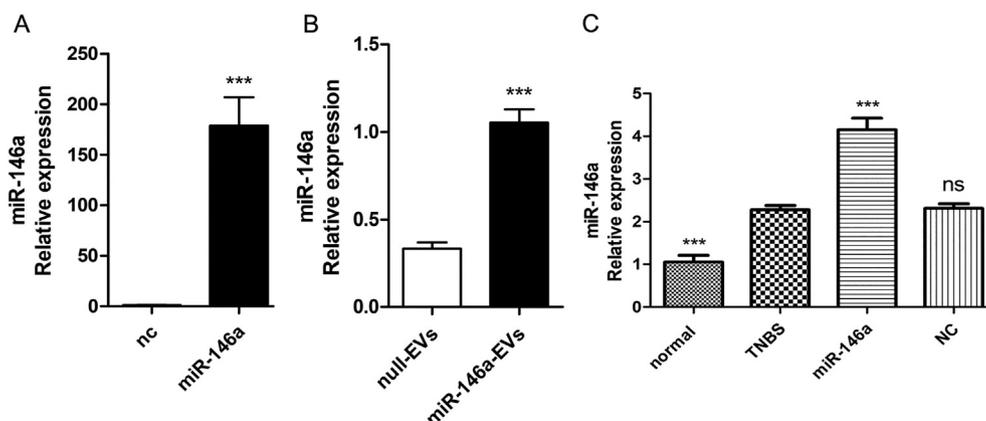

Fig. 4. miR-146a expression in BMSCs, BMSC-EVs and colons. Real-time PCR analysis of miR-146a expression in BMSCs (panel A) and EVs (panel B) derived from BMSCs, as well as in colon tissue (panel C). The expression level of the miRNA was normalized to U6. ***P < 0.0001 vs. nc group or null-EVs group or TNBS group.
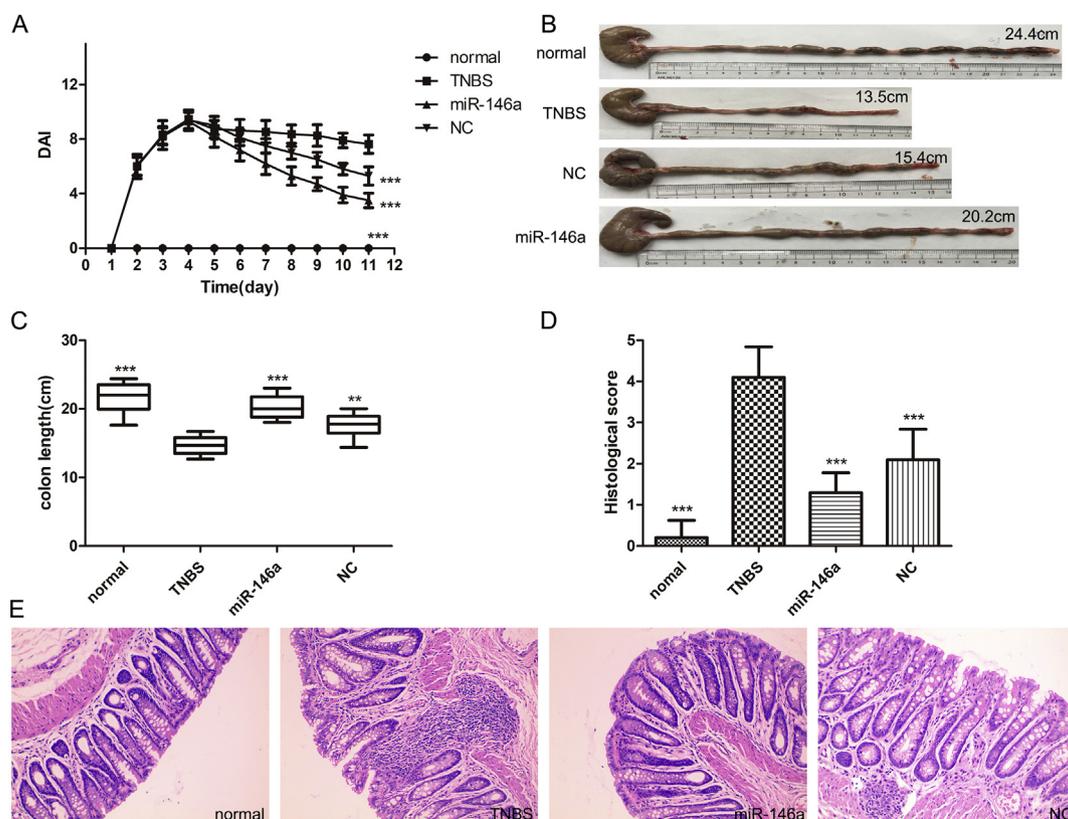
Fig. 5. miR-146a protects against TNBS-induced damage of colon in rats. (A) DAI of rats were presented. (B) Colons gross appearances were showed. (C) Colonic length of rats. (D) Colonic histological scores. (E) Hematoxylin and eosin (HE) analysis of colon specimens. Original magnification, ×200. ***P < 0.0001, **P < 0.01 vs. TNBS group.

inflammatory responses via inhibition of NF-κB binding activity by targeting IRAK1 and TRAF6.

The innate immune system is currently seen as the probable initiator of events which culminate in the development of IBD with Toll-like receptors (TLRs) known to be involved in this disease process [39]. Many regulators of TLRs have been described, and dysregulation of these may also be important in the pathogenesis of IBD [40]. TLR4 expression is substantially increased in lamina propria cells of patients with ulcerative colitis [41]. Activation of TLRs triggers innate and adaptive immune responses and predominately activates NF-κB [42] which is an important transcription factor directly regulating inflammatory cytokine production and adhesion molecule expression in
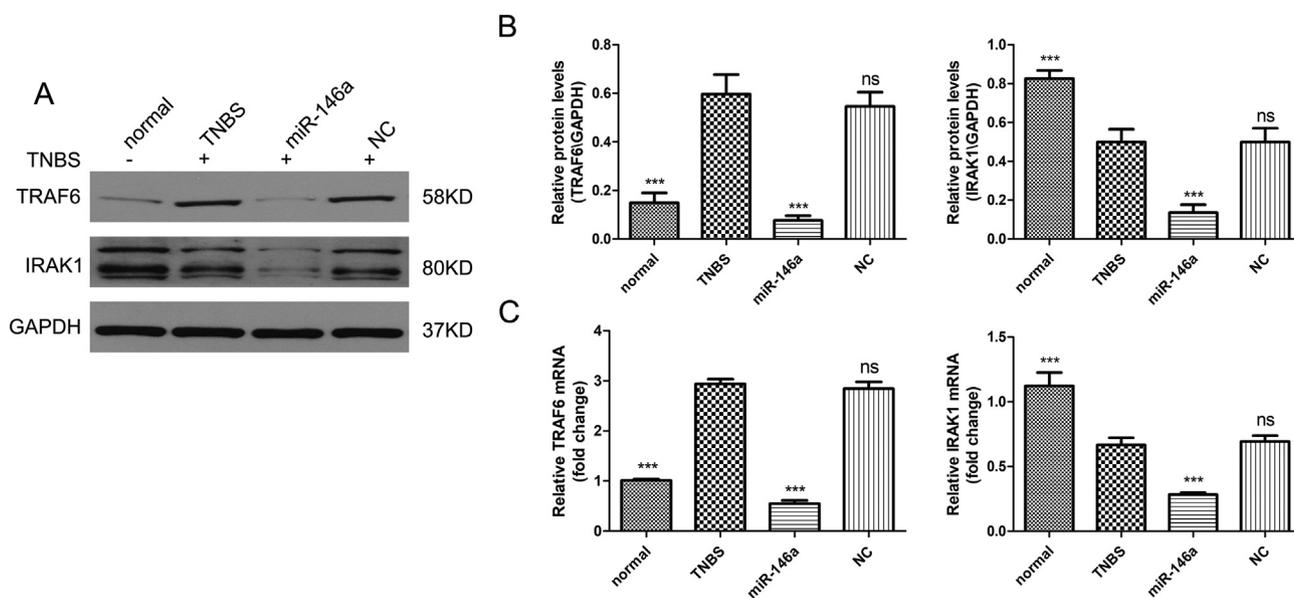

Fig. 6. Administration of miR146a-EVs dampens TRAF6 and IRAK1 expression. (A) Western blot analysis of TRAF6 and IRAK1 protein expression. (B) Grey value histogram of western blot detection. GAPDH was used as a control. ***P < 0.0001 vs. TNBS group. ns, no significance. (C) Expression of TRAF6 and IRAK1 genes was analyzed by qRT-PCR and normalized by rat GAPDH expression.
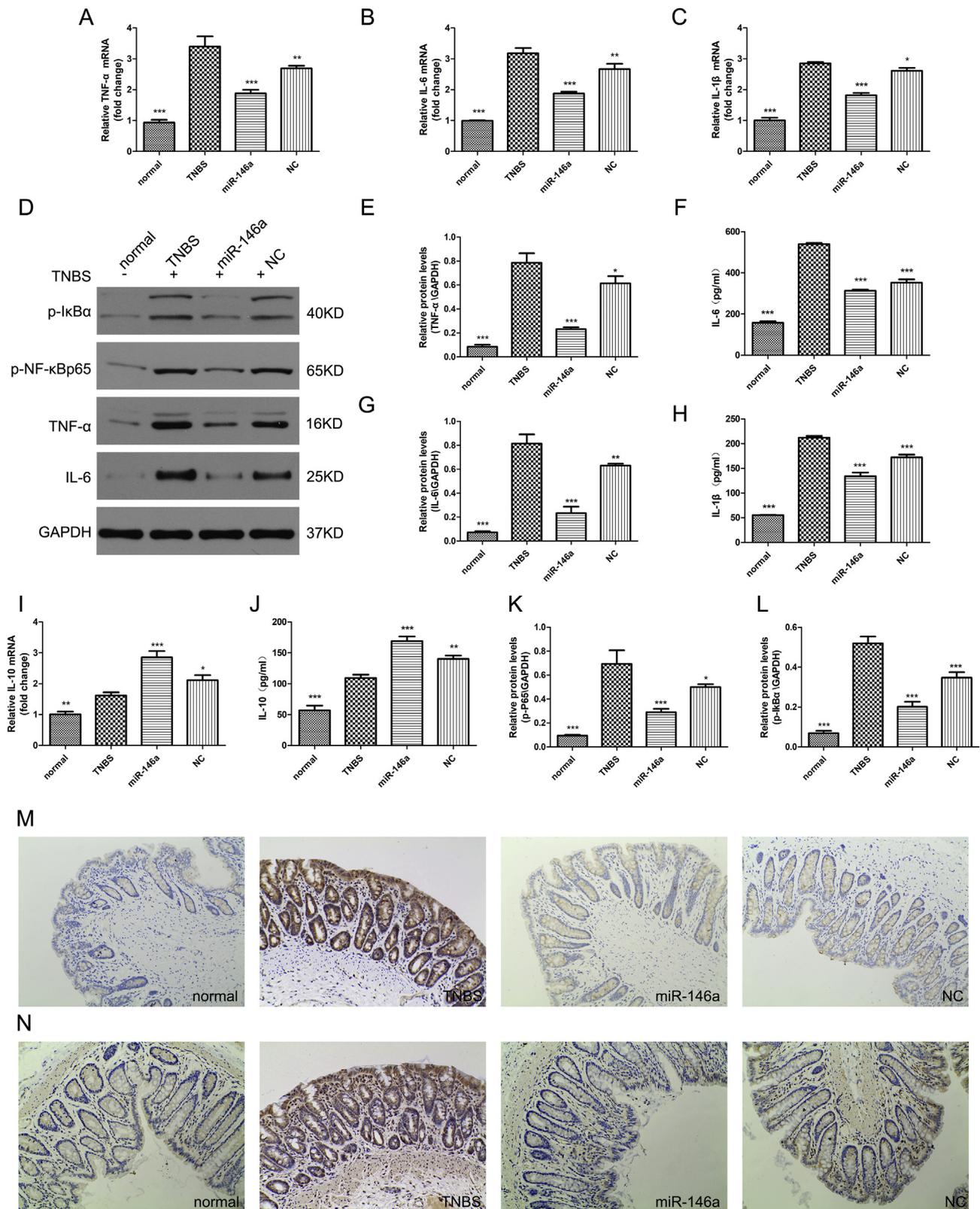
Fig. 7. MiR-146a suppresses TNBS-induced inflammatory cytokine production in colon tissue of rat through suppressing NF-κB activation pathway. Increased expression of miR-146a attenuates TNBS-induced TNF-α (A, D, E and M), IL-6 (B, D, F, G and N), and IL-1β (C, H) production, decreases phosphorylation of NF-κBp65 subunit (D and K) and IκBα (D and L) and increases the expression of IL-10 at the mRNA (I) and protein (J) level in colon tissue.

UC [43]. Therefore, suppression of NF-κB activation may be an important approach for the attenuation of chronic intestinal inflammation in IBD.

MicroRNA-146a has been demonstrated to serve as a negative regulator of the NF-κB activation pathway [36,44]. Further research revealed that NF-κB plays a critical role in induction of miR-146a transcription by LPS, TNF-α, and IL-1β through promoter analysis of miR-146a gene [36]. Similarly, we have also observed that the level of

miR-146a was markedly increased in the colon following TNBS-induced rat model. The data is consistent with the report by Gao et al. [45] that activation of NF-κB regulates miR-146a expression. Based on results mentioned above, we hypothesize that increased expression of miR-146a will reduce the pro-inflammatory phenotype and attenuate lesion in IBD. But how miR-146a can be transferred appropriately? To address the problem that exogenous miRNAs might be degraded by various RNAases in vivo, we take membrane-derived EVs into account. It has been reported that miRNAs can be packaged into EVs and then be delivered to recipient cells [26]. In this study, we found that the expression level of miR-146a in miR146a-EVs group was evidently up-regulated compared to that in the miR146a-EVs group, implying that miR-146a can be selectively packaged into these extracellular nanovesicles, which is in line with the findings of other researchers [46].

To investigate whether increased expression of miR-146a will attenuate colon damage in TNBS-induced colitis, we transfected BMSCs with lentivirus expressing miR-146a, isolated EVs from these BMSCs with genetic modification, and then delivered miR146a-EVs into the focus through tail vein injection two days after induction of IBD by TNBS. We observed that increased colonic expression of miR-146a significantly alleviates pathological manifestations of acute inflammatory disorders in UC model. Data of qRT-PCR also shows that the colon efficiently took up extracellular vesicles containing miR-146a when the miR146a-EVs was transported through the caudal vein. This indicates that miR-146a may target the TLR-mediated NF-κB pathway, resulting in reduced production of inflammatory cytokine in the colon tissues. Our in vitro data demonstrated that increased expression of miR-146a in colon suppresses the expression of IRAK1 and TRAF6, prevents TNBS-induced activation of NF-κB as well as phosphorylation of IκBα and NF-κB P65 subunit and attenuates increases in the levels of TNFα and IL-6. Though increased activation of NF-κB has been implicated in the pathogenesis of several inflammatory diseases including IBD [47], accumulating evidence has suggested that NF-κB signaling plays a particularly important role for the maintenance of physiological immune homeostasis in epithelial tissues which include the intestine [48]. In the current study, we only examined the effects of one-time systemic administration in acute model, the long term impact of increased expression of miR-146a on NF-κB signaling pathway related epithelial homeostasis remain elusive and need further study.

Boldin et al. have confirmed TRAF6 and IRAK1 genes as bona fide miR-146a targets by using a combination of gain and loss of function approaches [11]. TRAF6, which is a downstream molecule of IRAK1 and can be associated with phosphorylated IRAK1, plays a crucial role in the induction of inflammatory responses via activation of IKK, leading to NF-κB activation [42,49]. TRAF6 can activate NF-κB and MAPK signaling pathways when stimulators of TLR/IL-1R arise [42,49]. TRAF6 also contains a RING domain that confers E3 ligase activity [50] which induces TRAF6 autoubiquitination via catalyzation of lysine-63 (K63) polyubiquitination. K63 polyubiquitination activates IKK and MAPKs, as well as in RIG-I signal transduction at multiple steps [50]. Hence, suppression of TRAF6 will significantly down-regulate inflammatory responses mediated by NF-κB and MAPK signaling pathways. In the present study, notable reductions in the levels of TRAF6 and IRAK1 in colons were observed, which may be a critical mechanism by which extracellular vesicles containing miR-146a keep inflammation in check in TNBS administration.

In conclusion, our study demonstrated that miR-146a plays a protective role in TNBS-induced colitis. The mechanism involves suppression of activation of the NF-κB pathway in inflamed colons in rats with TNBS-induced colitis. Therefore, increased miR-146a expression may be a potential approach for prevention and/or treatment of IBD.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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