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Original Article

Inflammatory response of macrophages cultured with *Helicobacter pylori* strains was regulated by miR-155

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Abstract: MicroRNA-155 plays an important role in the inflammatory response macrophages, while present studies identified that miR-155 was up-expressed in gastritis induced by *Helicobacter pylori*. We found that miR-155 was over expressed in macrophages infected with *H. pylori in vivo* or *in vitro*. Subsequently, inflammatory cytokines IL-23, IL-10, TNF-α and IL-8 were increased significantly, and the expression of CD80, CD86 or COX2, NOS2. Were enhanced in *H. pylori* infection macrophages by regulated with miR-155mimics. Furthermore, the apoptosis of macrophages induced by *H. pylori* was increased obviously due to the over-expression of miR-155. Therefore, these observations indicated that miR-155 may act as a inflammatory promoter in *H. pylori* infected macrophages. These findings contribute us to understand the functions of miR-155 in gastritis induced by *H. pylori* furtherly.

Keywords: Macrophage, miR-155, *Helicobacter pylori*

Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium that could inhabit in the stomach and cause gastritis, peptic ulcer, and gastric cancer [1]. In acute or chronic *H. Pylori* infection, inflammation is thought to be a major determinant of both peptic ulceration and gastric malignancy [2], but the regulatory mechanisms of *H. pylori*-induced inflammation are still not well understood.

Gastric epithelial cells constitute the first line of defense against *H. pylori*, and they produce interleukin-8 (IL-8), which promotes the recruitment of poly nuclear cells [3]. Immune cells in gastric tissures including macrophages, dendritic cells (DC) and mucosa infiltrating lymphocytes take part in the innate and adaptative immune responses to *H. pylori*. Host defense against pathogens requires the induction of appropriate innate immune responses, as excessive or inappropriate activation of the immune system can be deleterious to the organism. Therefore, various immune regulators, including microRNAs (miRNA), also take part in the immune responses [4].

MicroRNAs (miRNAs) are non-protein coding 20~22 nucleotide small RNAs that induce translational repression or degradation of their mRNA targets [5, 6]. MiRNA received considerable attention because of their implication in maintaining homeostasis in fundamental biological processes in non-pathological states, and their deregulation in pathological states [7]. A growing body of evidence suggests bacterial and viral infection of mammalian and plant cells can modulate miRNA expression [8]. Changes in miRNA expression in response to bacterial infection have been reported, including *H. pylori* infection in the gastric mucosa, in gastric epithelial cells and in immune cells [9-12].

Recent reports show that during *Helicobacter pylori* infection, miR-155 play an negative regulatory role in the inflammatory response and highlighted the regulatory role of miR-155 in *H. pylori* infection and associated diseases [13]. Meanwhile, miR-155, have recently emerged as crucial regulators of innate immunity and inflammatory responses, and is regulated by Toll-like receptor (TLR) ligands in monocyte-derived cells and has been shown to be induced

in macrophages during *H. pylori* infection [14]. Macrophages cells are key players *in vivo* in terms of inflammation and T or B cell lymphoma initiation. To date, however, there few studies of the regulation of miRNAs by *H. pylori* in macrophage cells.

Here, we investigated the function of miR-155 in the inflammatory response of *H. pylori* infection macrophages, and its role in the activation or apotosis of macrophages infected with *H. pylori*.

Materials and methods

Peripheral blood mononuclear cells collection

Blood samples were collected from 30 *H. pylori* infection patients and 30 healthy individuals from the Cancer Institute of ChongQing in 2014. Peripheral whole blood (5 mL) was collected in EDTA-anticoagulated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque™ Plus (Amersham Pharmacia Biotech, Shanghai, China) according to the manufacturer's instructions. After Ficoll density gradient centrifugation, the upper layer was removed carefully, and the PBMCs were collected and washed twice with PBS by low-speed centrifugation (200× *g*, 10 min). PBMCs were frozen at -80°C within 4 h of blood collection. All procedures were approved by the ethics committee of the Cancer Institute of Chongqing.

Macrophage cultures

The human acute monocytic leukemia cell line THP-1 was purchased from the Center for Type Culture Collection of Wuhan University. Cells were cultured in wells or flasks at 37°C under 5% CO_2, in RPMI 1640-GlutaMAX™ (HyClone Laboratories, GE Healthcare Lifesciences, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (HyClone), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. Differentiation of THP1 cells into macrophage-like cells was induced by stimulation with 0.1 mmol/l phorbol 12-myristate 13-acetate, PMA (Sigma, St Louis, MO, USA) for 24 h.

H. pylori culture

The wild-type H. pylori strain 26695 obtained from ATCC were cultivated for 48 h at 37°C under microaerobic conditions (5% O_2) on selective agar consisting of 21.5 g of Wilkins Chalgren agar, 50 ml of human blood, 10 μg/ml of vancomycin, 10 μg/ml of cefsulodin, 5 μg/ml of trimethoprim, and 10 μg/ml of amphotericin B.

siRNA transfection

THP-1 were cultured in a 12-well plate for 24 h, the cells were washed with PBS and 500 μL OpitMEM (Naomi Biotech, WuXi, China) was added to each well of the 12-well plate. Then, 20 pmol miR-155 mimics, miR-155 inhibitors, negative siRNA, or a blank control were added to 50 μL OpitMEM and combined with 50 μL OpitMEM containing 5 μL IR that was heat shocked for 10 s at 37°C. The mixed solutions were then added to the cells. After 6 h, the medium was replaced and the cells were cultured for 24 h. The transfected cells were observed by fluorescence microscopy. Differentiation of these cells into macrophage-like cells was induced with 0.1 mmol/l Phorbol 12-myristate 13-acetate, PMA (Sigma, St Louis, MO, USA) for 24 h.

Co-cultures of macrophages and H. pylori

Immature macrophages were washed once in PBS and plated onto 24-well plastic plates at a density of 5×10^5 cells per well in 1 ml of RPMI-1640 growth medium. *H. pylori* were recovered from the agar plates using a swab and resuspended in RPMI-1640 growth medium at an optical density of 0.6 at 600 nm, which corresponds to 3×10^7 CFU/ml. The bacteria were added to macrophages at the indicated multiplicity of infection (MOI) 10:1 and the co-cultures were further incubated at 37°C in a 5% CO_2 atmosphere for 24 h.

Quantitative RT-PCR of miR-155

Levels of miR-155 expression were analyzed by quantitative RT-PCR performed with the Eppendorf PCR system (Eppendorf, Germany). Total RNA was isolated from PBMCs or macrophages treated with LPS or *H. pylori* using TRIzol reagent and quantified by a photometer. cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

For RT-PCR, 1 μL cDNA, 0.5 μL forward primer and 0.5 μL reverse primer, 2 μL 2.5 mM dNTPs, 0.5 μL DNA polymerase (10 U/μL) (Takara, Da lian, China), 4 μL 5× buffer, and 11.5 μL dd H_2O

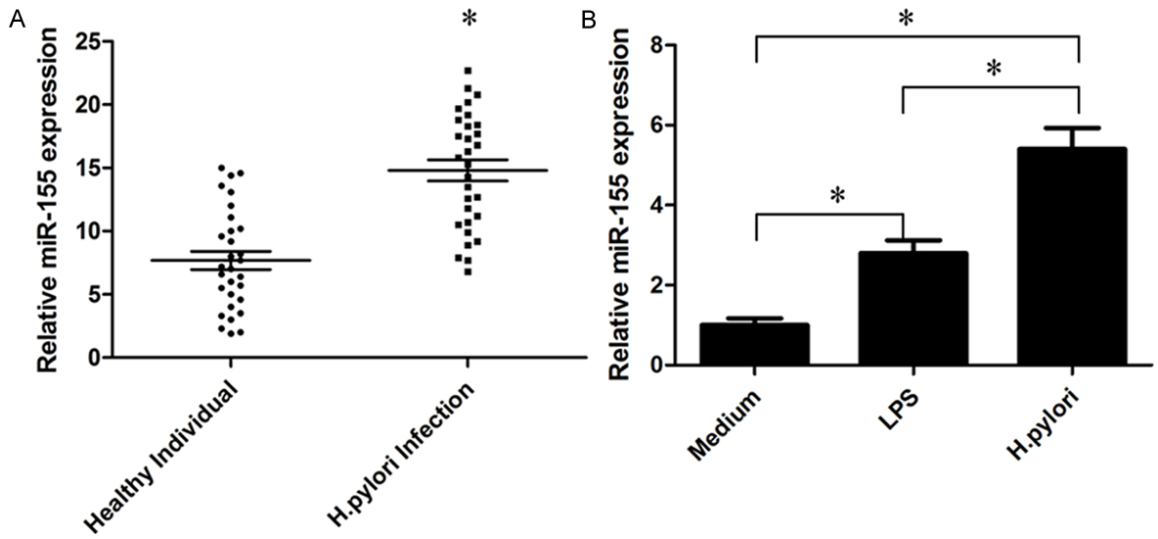

Figure 1. Analysis of the expression of miR-155 in PBMCs from *H. pylori* infection patients or *H. pylori* infection macrophage. A: The expression levels of miR-155 in 30 cases of PBMCs of *H. pylori* infection patients were detected by quantitative PCR. B: Quantitative PCR results of miR-155 expressed in *H. pylori* infection macrophage; U6 was used as the control (mean ± SEM, three independent experiments). *$P<0.05$.

were reacted for 35 cycles at 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. A U6 small nuclear RNA was used as endogenous control for data normalization, and relative expression was calculated using the comparative threshold cycle (Ct) method. The primers were: 5'CGCTGCTACCTGAGAGTAGACCAGATGCAGCG ACCCCT3' (reverse trans-scription), R 5'TTAATGCTAATCGTGATAG3', and F 5'ACCTGAGAGTAGACCAGA 3' for miR-155; R 5'GGAACGCTTCACGAATTTG3' and F 5'ATTGGAACGATACAGAGAAGATT3' for U6.

The expression levels of miR-155 were normalized to those of U6 miRNA. Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ formula: $\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{sample}$, where ΔCt is the difference in the cycling threshold between the gene of interest and the housekeeping gene U6, ΔCt_{sample} is the Ct value for miR-155 normalized to U6, and $\Delta Ct_{reference}$ is the Ct value corresponding to control samples normalized to U6. Mean Ct values were calculated from the duplicates.

Cytokine quantifications by ELISA

Macrophages cultured in six well plates were infected with *H. pylori* (MOI of 10; circles) for 24 h. IL-23, IL-10, TNF-α, IL-8 in above cell culture supernatants were analyzed by ELISA kits, according to the instructions of Boster Biotechnology Company (WuHan, China), and cytokine concentrations were calculated by referring to standard curves.

Flow cytometry analysis

Macrophages transfected with siRNA cultured in six well plates were treated for 24 h with *H. pylori* (MOI of 10; circles), then were harvested and washed twice with PBS containing 0.2% BSA. Cells were then stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies (BD, USA) to CD80, CD86, or the appropriate isotype controls. Macrophages were washed and fixed in 10% (vol/vol) formaldehyde-PBS. Finally, cell sorting and analysis were performed on a flow cytometry (Beckman MOFLO XDP, USA). Median fluorescence intensities (MFIs) and the percentages of positively expressing cells were determined after subtraction of the values for the isotype controls.

Western blotting analysis

For Western blotting, equal concentration of proteins from cell lysates, as quantitated by the Micro BCA Protein Assay (Merck Millipore, USA) were loaded on 10% SDS-PAGE gels, electrophoresed, and transferred onto polyvinylidene difluoride membrane (Milipore, Bedford, MA). The membranes were probed for COX2 (R&D, 1:600 dilution), NOS2 (R&D, 1/400 dilution). Immune-detected protein bands were quantified with ImageJ and statistically analyzed by ANOVA software.

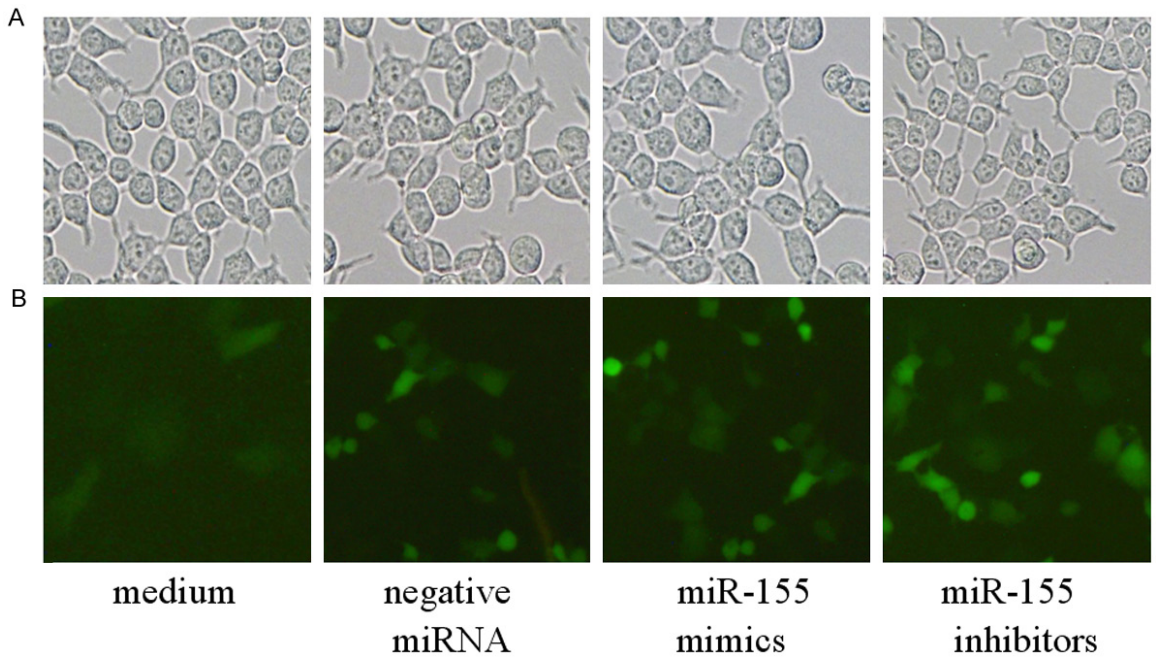

Figure 2. Immunofluorescence microscopy of macrophages cells transfected with siRNAs (×40). A: Untransfected macrophages cells; B: Macrophages cells transfected with siRNAs. Experiments were repeated 3 times.

Apoptosis and necrosis

Macrophages interferenced with siRNA were infected with *H. pylori* (MOI of 10; circles) for 24 h. Cell apoptosis was quantitatively determined by flow cytometry using an annexin V-FITC/PI apoptosis detection kit (BD, USA). Following the treatment, cells were harvested by trypsination, washed with PBS, and incubated with annexin V-FITC and PI at 25°C for 10 min in the dark. The stained cells were analyzed by an FACS Calibur flow cytometer and CellQuest analysis software (Beckman MOFLO XDP, USA).

Statistical analysis

All data were expressed as mean ± SEM. Values were analyzed by SPSS16.0 software for Windows, and the statistical significance of difference among the values was evaluated by one-way analysis of variance. P value <0.05 was defined as significant.

Results

The expression of miR-155 in H. pylori infection patients or macrophages

To investigate the role of miR-155 in *H. pylori* infection cells, the expression levels of miR-155 were first measured in 30 cases of PBMCs from *H. pylori* infection individual by quantitative PCR (**Figure 1A**), then the levels of miR-155 expression in macrophages stimulated with LPS or *H. pylori*, miR-155 levels were detected by quantitative PCR (**Figure 1B**). Above results show that miR-155 expression levels were significantly higher in *H. pylori* infection patients than healthy individual, and were also overexpressed in macrophages stimulated with LPS or *H. pylori*.

Macrophages transfected with siRNAs

Macrophages cells were transfected with siRNAs including miR-155 mimics, miR-155 inhibitors, or negative control siRNA for 24 h by ImagenFect RNAi kit (Naomi Biotech, Wuxi, China). Then transfection macrophages cells were observed under a fluorescence microscope (Olympus, Osaka, Japan) to assess the transfection efficiency. Most macrophages cells emitted green fluorescence, indicating that the siRNAs were transfected into macrophages cells successfully (**Figure 2**).

Analysis of proinflammatory cytokines secreted from macrophaes

Macrophages are able to produce a variety of cytokines such as IL-6, IL-8, IFN-γ, TNF-α and so on to resist microbial invasion. Macrophages

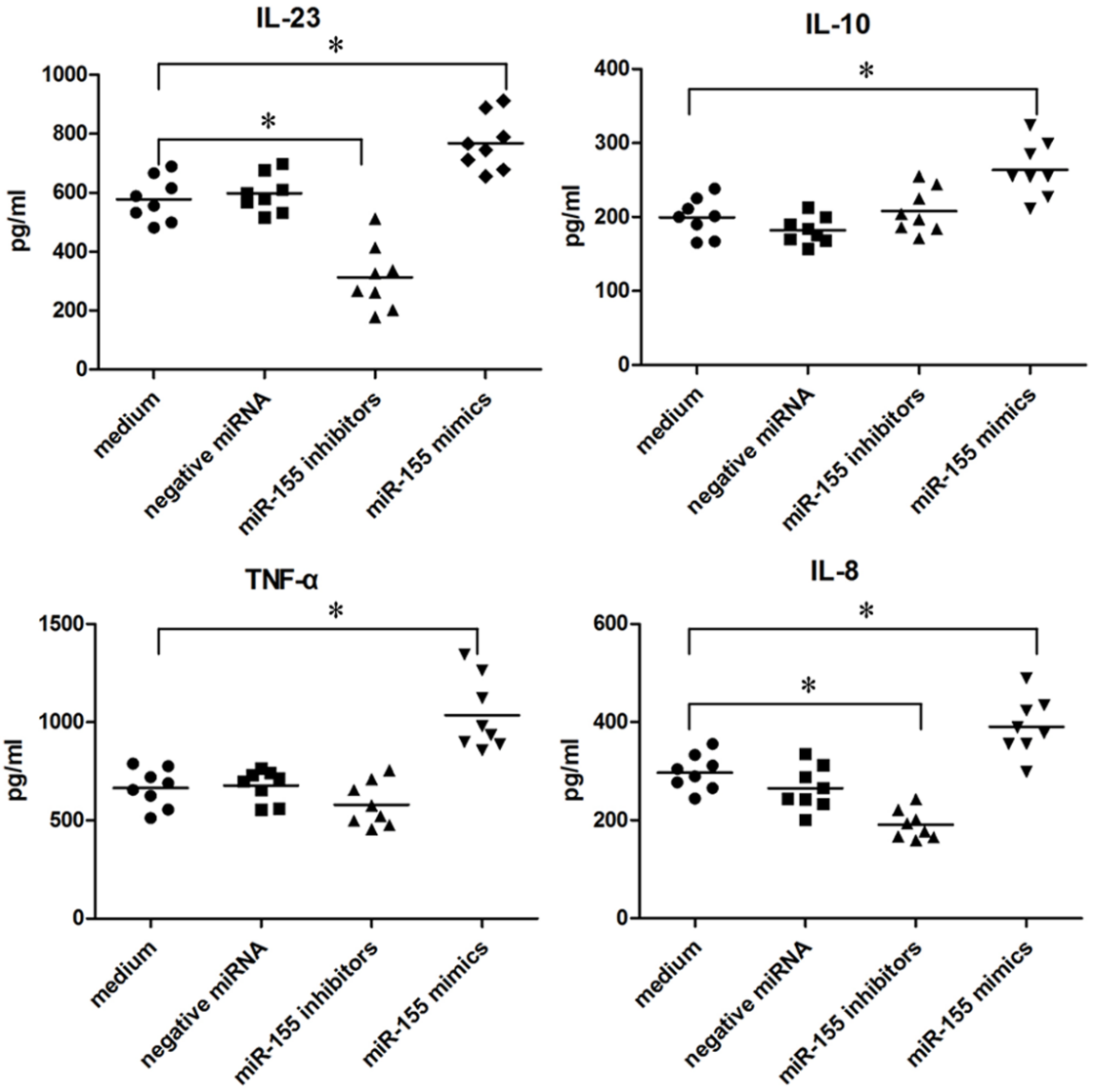

Figure 3. Cytokines released from *H. pylori* infection macrophages transfected with siRNA. A total of 10^6 macrophages interferenced with miR-155mimics, miR-155inhibitors or negative miRNA, were infected with *H. pylori* (MOI of 10; circles) for 24 h. Concentrations of cytokines in the supernatants were determined by ELISA, and each symbol per condition represents the data obtained with cells from one donor. Horizontal lines show the median values of 8 experiments. *P<0.05 (Friedman test and Dunn's multiple comparison test).

transfected with miR-155 mimics, miR-155 inhibitors, or negative control siRNA were infected with *H. pylori*, then IL-23, IL-10, TNF-α and IL-8 in the supernatant were detected by ELISA method (**Figure 3**). Notably, IL-23, IL-10, TNF-α and IL-8 were all increased significantly in *H. pylori* infection macrophages interferenced by miR-155 mimics, but IL-23, IL-8 were decreased in *H. pylori* infection macrophages treated with miR-155 inhibitors. Therefore, above results indicated that an inflammatory response of *H. pylori* infection macrophages could be regulated by miR-155.

Expression of CD80 and CD86 in siRNA interferenced macrophages infected with H. pylori (flow cytometry)

In order to confirm the enhancement of CD80/86 proteins expression by H. pylori infection in macrophages interferenced by miR-155 mimics, miR-155 inhibitors and negative miRNA, we quantified CD80/86 levels by flow cytometry. Macrophages were interferenced by miR-155 mimics, miR-155 inhibitors and negative miRNA for 24 h, and infected with H. pylori, then subjected to flow cytometry for the detec-

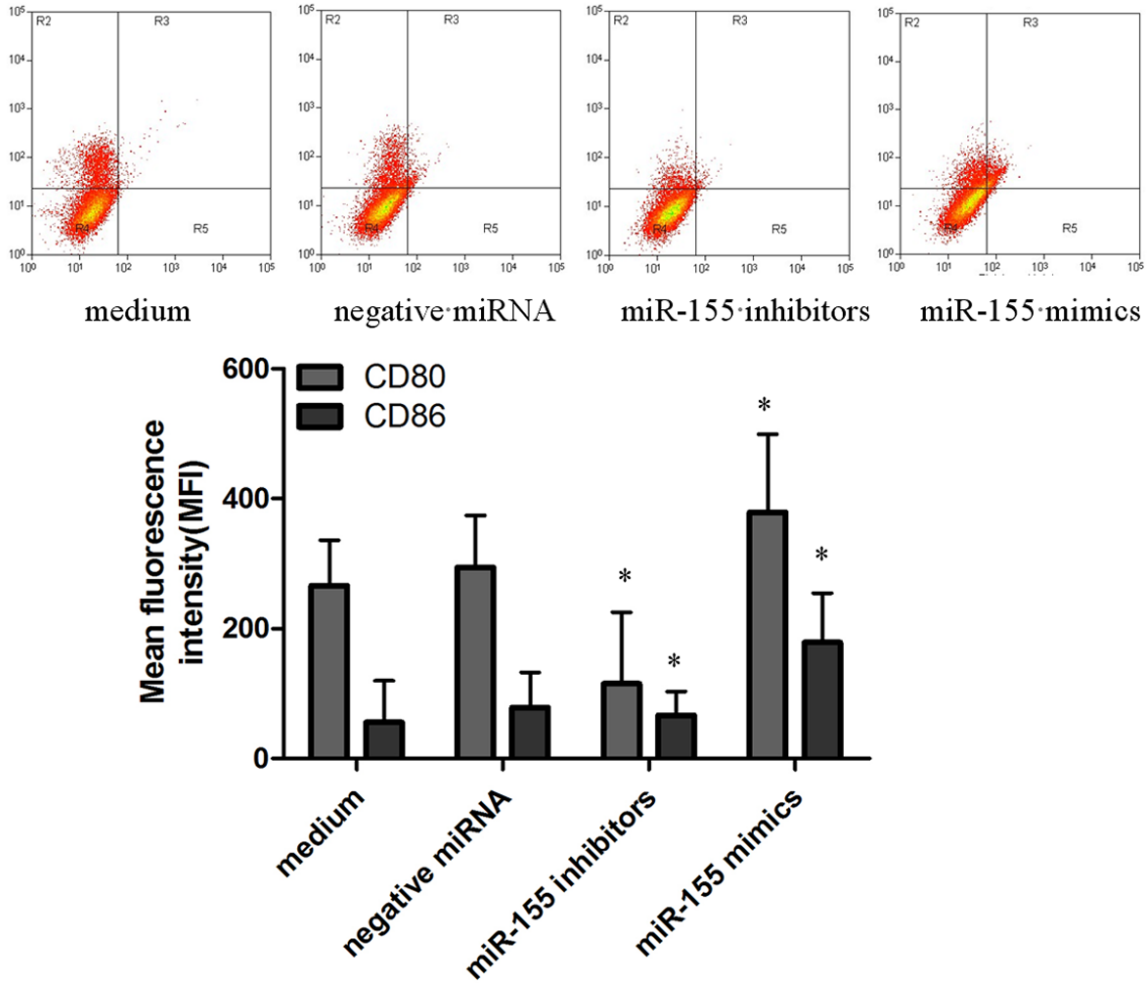

Figure 4. Analysis of surface CD80/CD86 by flow cytometry in siRNA interferenced macrophages infected with *H. pylori*. Macrophages interferenced with miR-155mimic, miR-155 inhibitors, then 10^6 cells were analyzed on FACS and obtained fluorescence intensities of CD80/CD86. Bar graphs show the mean fluorescence intensities (MFI) of CD80/CD86 (mean ± SE, 3 independent experiments), and statistical analysis was by SPSS 16.0, with asterisks indicating the pairs of values compared for which significant differences were observed (*$P \leq 0.05$).

tion of CD80 or CD86. The results show that CD80 levels increase in miR-155 mimics transfected macrophages, but decreased in miR-155 inhibitors transfected macrophages. Meanwhile, the expression of CD86 was similar with macrophages the expression of CD80 (**Figure 4**).

Proteins expressed in macrophages

COX2, NOS2 in siRNA interferenced macrophages infected with H. pylori were analyzed by Western blotting (**Figure 5**), and the gray of bands was scanned by Image J software, GAPDH as standard calibration. As a result, COX2, NOS2 proteins were all decreased in miR-155 inhibitors interference macrophages, but up-expressed in miR-155 mimics interference macrophages.

Apoptosis of macrophages

Macrophages interferenced by miR-155 mimics, miR-155 inhibitors and negative miRNA were incubated with H. pylori for 24 h, then were analyzed the asymmetry and permeability of the cell membrane with annexin- VFITC and PI staining by flow cytometry (**Figure 6**). As shown in results, the apoptosis or necrosis of macrophages interferenced by miR-155 mimics were decreased significantly compared with macrophages treated with miR-155 inhibitors, negative miRNA or medium.

Discussion

Recently, miR-155 has been indicated to play a key role in the regulation of normal immunity or inflammation response [15, 16]. Expression of

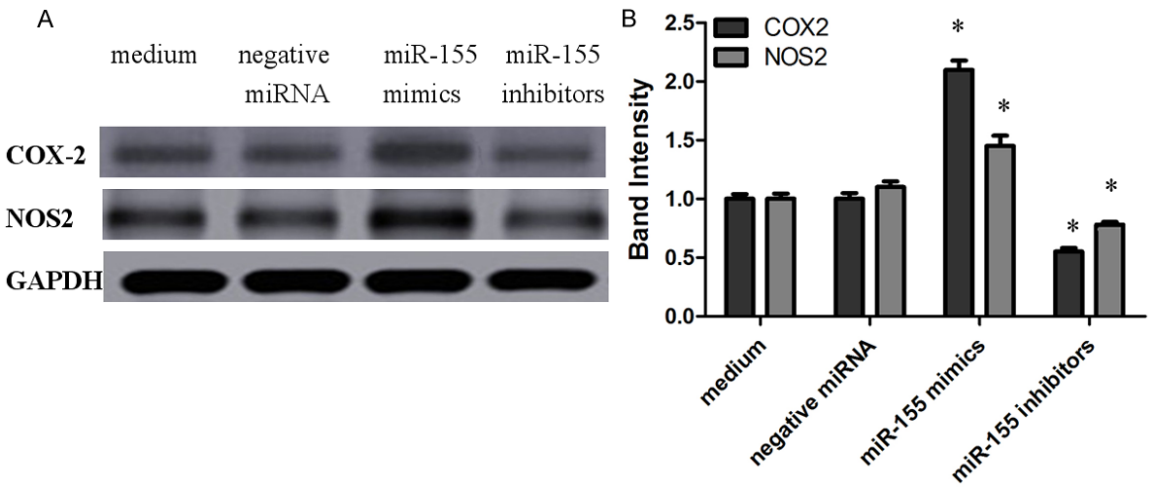

Figure 5. Analysis of the expression of COX2 and NOS2 proteins detected by Western blotting. COX2 and NOS2 proteins in macrophages interferenced with miR-155 mimics, miR-155 inhibitors and negative miRNA were infected with H. pylori (MOI of 10; circles), then were analyzed by Western blotting, and controls included macrophages incubated with medium alone (mean ± SEM, 3 independent experiments). Statistical analysis was by SPSS 16.0, with brackets indicating the pairs of values compared for which significant differences were observed (*P<0.045).

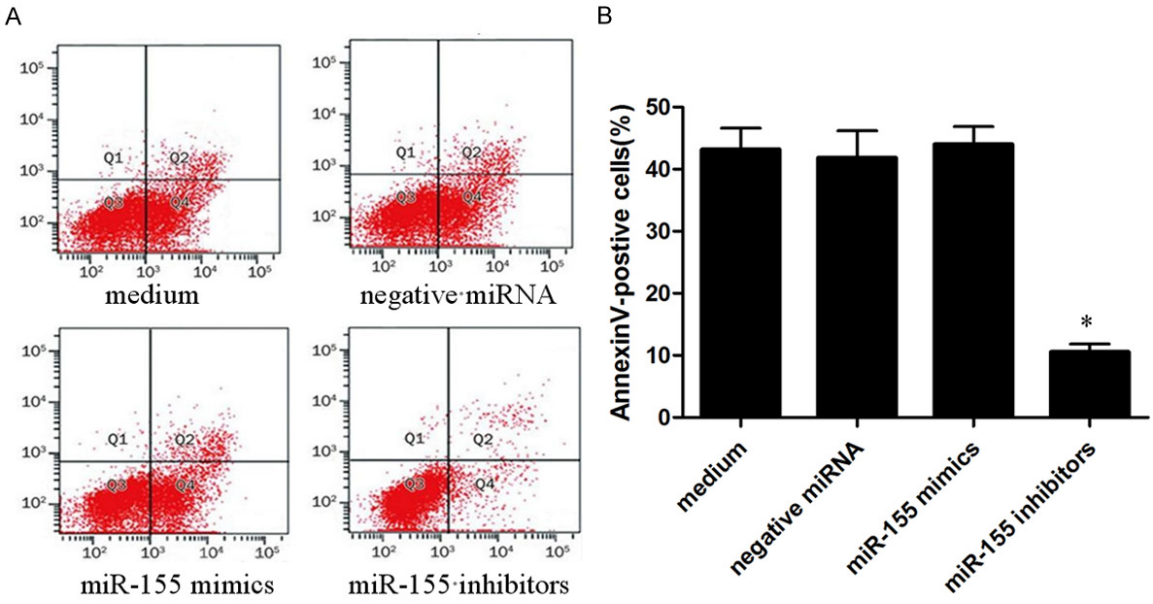

Figure 6. The apoptosis of siRNA interference macrophages infected with H. pylori was detected by flow cytometry. A: Results of flow cytometry, which macrophages interferenced by miR-155 mimics, miR-155 inhibitors and negative miRNA were infected with H. pylori (MOI of 10; circles) respectively for 24 h; B: Quantified assay of the apoptosis and necrosis by SPSS 16.0, with asterisks indicating the pairs of values compared for which significant differences were observed was presented as mean ± SEM (n = 3 represented for three time experiments, *P<0.05).

miR-155 in monocytes or macrophages is strongly induced by a broad range of stimuli including bacterial lipopolysaccharide (LPS), IFN-β polyriboinosinic-polyribocytidylic acid (poly IC) or tumor necrosis factor-a (TNF-α) [17-19]. In addition, the up-regulated miR-155 expression has been found in synovial fibroblasts and tissue from rheumatoid arthritis patients [20]. Therefore, the mechanism by which miR-155 modulate the immune response to *H. pylori* is needed to be further investigated. The expression miR-155 in PBMCs of *H. pylori* infection patients or in macrophages infected with *H. pylori* was enhanced remarkerly *in vivo*

and *in vitro*. Meanwhile, macrophages were transfected with miR-155 mimics, miR-155 inhibitors, or negative siRNA successfully to study functions of miR-155 in *H. pylori* infection macrophages.

Macrophages as myeloid antigen-presenting cells (APCs), are present in the *H. pylori*-infected mucosa and are likely involved in both the induction and maintenance of *H. pylori* specific immune responses and inflammatory reactions and have a high capacity to kill *H. Pylori* [21]. Macrophages could be activated by *H. pylori*, which the production of IL-6, IL-10 and IL-23 and the expression of CD80, CD86 were increased in macrophages infected with *H. pylori* [22]. In our studies, IL-23, IL-10, TNF-α and IL-8 increased in obviously in miR-155 mimics transfection macrophages infected with *H. pylori*, but IL-23 and IL-8 decreased in miR-155 inhibitors interference macrophages infected with *H. pylori*. The expression of CD80 and CD86 was enhanced in miR-155 mimics transfection macrophages infected with *H. pylori*, but the results was opposite in miR-155 inhibitors interference macrophages infected with *H. pylori*. All above results indicate that miR-155 could promote the inflammatory response through cytokine secretion and the expression of cell surface signaling molecules to kill or inhibit *H. pylori*.

COX-2 involved in the early inflammation in cells, but has anti-inflammatory function in chronic inflammatory stage. Animal experiments show that, in COX-2 gene knockout mouse using indomethacin could induce the gastrointestinal ulcer or peritonitis and enhanced the susceptibility of colitis. Meanwhile COX-2 could enhance the adoptive cell protective effect of gastric tissue, and involve in the regulation of gastric epithelial cell proliferation and enhance the stress resistance of mucosal injury. Proinflammatory proteins such as COX2, IL-6, IL-8, are markers of chronic inflammation in the tumor microenvironment, and these proinflammatory mediators directly correlate with inducible nitric oxide synthase (NOS2), which is an emerging biomarker of aggressive tumors that predicts poor survival in patients with elevated tumor NOS2 expression [23-27]. The over expression of miR-155 induced the expression of COX2, NOS2 proteins in *H. pylori* infection macrophages, while the expression of COX2, NOS2 proteins was down expressed in miR-155 inhibition macrophages infected with *H. pylori*.

Gram-negative bacteria may act as pro- as well as antiapoptotic factors for macrophages. *H. pylori* encodes both pro- and antiapoptotic effector molecules, such as CagA and VacA [28]. Primary monocyte derived cells seemed to be resistant to *H.pylori*-induced apoptosis [29]. The antiapoptotic potential of miR-155 has been described for several cell types, including B lymphocytes [30], and in pancreatic tumors by targeting *TP53INP1* [31] and *JARID2* [32]. Moreover, miR-155 has been reported to promote resistance to specific chemotherapeutics in breast cancer cells by targeting *FOXO3A* [33], and recent reports suggested proapoptotic potential of miR-155 through targeting *SKI2* in human melanoma cells [34] or *Kpc1* in murine DCs [35]. The low expression of miR-155 would be induce apotosis of macrophages infected with *H. pylori*, but the over expression of miR-155 has no any effect on macrophages infected with *H. pylori*.

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Disclosure of conflict of interest

None.

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