Molecular Immunology 77 (2016) 205–212

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

MiR-146a modulates macrophage polarization in systemic juvenile idiopathic arthritis by targeting INHBA

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ARTICLE INFO

Article history:
Received 27 June 2016
Received in revised form 2 August 2016
Accepted 7 August 2016

Keywords:
Systemic juvenile idiopathic arthritis
MiRNA-146a
Macrophage polarization
INHBA

ABSTRACT

Monocytes from patients with systemic juvenile idiopathic arthritis (SJIA) have both features of classical activated M1 and alternatively activated M2 macrophages. An increasing number of studies have indicated that microRNAs (miRNAs) are critical regulators of monocyte polarization. Here, we focused on miR-146a expression in SJIA and investigated the function of miR-146a in monocyte polarization. We found that miR-146a expression was highly up-regulated in SJIA monocytes and correlated with the systemic features. miR-146a was expressed at a higher level in monocytes polarized with M2 conditions than those polarized with M1 conditions. miR-146a overexpression significantly decreased the production of M1 phenotype markers such as IL-6, IL-12, TNF-α, CD86 and iNOS in M1 macrophages, but increased the production of M2 marker genes such as Arg1, CCL17, CCL22 and CD206 in M2 macrophages. Conversely, knockdown of miR-146a promoted M1 macrophage polarization but diminished M2 macrophage polarization. We subsequently demonstrated that miR-146a targeted the 3′-untranslated region (UTR) of INHBA to inhibit its expression. Additionally, INHBA overexpression rescued the reduced IL-6, IL-12, and TNF-α levels induced by miR-146a overexpression in M1 macrophages, and rescued the increased Arg1, CCL17, and CCL22 levels induced by miR-146a overexpression in M2 macrophages. Similarly, the effects of miR-146a inhibition in monocyte polarization were all partly reversed by INHBA inhibition. Taken together, the data suggest that miR-146a serves as a molecular regulator in monocyte polarization and might play an important role in monocytes from patients with SJIA.

1. Introduction

Systemic juvenile idiopathic arthritis (SJIA) is a childhood-onset disease characterized by a combination of chronic arthritis and systemic features, including remitting fever, typical skin rash, adenopathy and serositis (Petty et al., 2004). Although new treatments have been identified, many children still suffer long term disability and high mortality (Ramanan and Grom, 2005), at least partly due to the high risk of developing a fatal complication called macrophage activation syndrome (MAS) (Ravelli et al., 2012). The pathogenetic mechanisms of SJIA are not well understood, and the contribution of innate immunity has attracted more attention. SJIA has features consistent with an auto-inflammatory disorder, characterized by dysfunction of innate immune effector cells including neutrophils, monocytes and macrophages (Mellins et al., 2011). Children with SJIA demonstrate increased levels of circulating monocytes with upregulated expression of monocyte differentiation genes (Fall et al., 2007; Macaubas et al., 2010; Ogilvie et al., 2007), as well as high levels of monocyte-derived cytokines (Ling et al., 2010), suggesting that monocyte activation plays an important role in SJIA pathophysiology.

The monocyte/macrophage lineage differentiates into specific activation phenotypes, depending on the environmental stimuli. The population activated by LPS and INFγ is referred to classically activated M1 macrophages with a "pro-inflammatory" cytokine profile, whereas activated by IL-4 or IL-13 results in alternatively activated M2 macrophages with an "anti-inflammatory" cytokine repertoire. Numerous molecular mechanisms are involved in the regulation of macrophage polarization (Zhou et al., 2014). In this regard, microRNAs (miRNAs) have been found to be important modulators (Graff et al., 2012). MiRNAs are a class of small endogenous 19–24 nt long non-coding RNAs. Mature miRNAs bind to the

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http://dx.doi.org/10.1016/j.molimm.2016.08.007
0161-5890/

3′-UTR of target mRNAs to degrade the mRNA or inhibit the post-transcriptional processing of target mRNA. They can function at different levels to modulate physiological and pathological processes such as cell division, metabolism and immunity (Mendell and Olson, 2012). Several studies have examined miRNA expression profiles during macrophage polarization and found marked differences associated with distinct polarization states (Jiménez et al., 2014; Zhang et al., 2013). One recent study found that miR-146a, which is highly expressed under alternative activating conditions, might play a pivotal role in suppressing M1 macrophage polarization whilst promoting M2 activation (Huang et al., 2016).

Monocytes in SJIA patients appear to have a mixed polarization phenotype, with features of both classically and alternatively activated populations (Ogilvie et al., 2007); however the molecular mechanisms that regulate monocyte polarization in SJIA are unknown. The purpose of the present study was to determine the expression of miR-146a in freshly isolated $CD14^+$ monocytes from children with SJIA, and to investigate the effects of miR-146a in monocyte polarization. We hypothesized that monocytes from children with SJIA would have altered expression of miR-146a and that the differentially expressed miR- 146a may be involved in the regulation of monocyte polarization in particular.

2. Materials and methods

2.1. Patients

Patients with SJIA were enrolled from Affiliated Children' Hospital of Xi'an Jiaotong University. This study was approved by the Clinical Research Ethics Committee of Affiliated Children' Hospital of Xi'an Jiaotong University, and informed consent was obtained from all patients and/or their legal guardians. SJIA was diagnosed based on the International League of Associations for Rheumatology diagnostic criteria (Petty et al., 2004). Patients were considered to have clinically inactive disease (CID) based on the Wallace criteria (Wallace et al., 2011). Comprehensive clinical information was collected during routine clinic visits (Supplementary Table S1). Control age-matched patients were enrolled, after consent, from healthy children undergoing routine phlebotomy at Affiliated Children' Hospital of Xi'an Jiaotong University. Patients who were acutely ill, with a prior history of autoimmune or hematologic diseases, or taking anticonvulsant or immunosuppressive medications, were excluded.

2.2. Isolation of human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated using Ficoll-Hypaque (Pharmacia, NJ, USA) density-gradient centrifugation according to standard procedures. Monocytes were purified from PBMCs by magnetic cell sorting using $CD14^+$ Microbeads (Miltenyi Biotec). $CD14^+$ monocytes were cultured at 0.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 g/ml streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere with 5% CO_2. Total RNA was extracted immediately following cell separation using TRIzol reagent (Invitrogen, Carlsbad, USA) to evaluate miR-146a expression.

2.3. Cell culture and monocyte polarization

Human THP-1 cell line (American Type Culture Collection) was cultured in RPMI 1640 medium supplemented with 10% FCS in 5% CO_2 at 37 °C with saturated humidity. Prior to polarization THP-1 cells were differentiated overnight with the addition of 50 ng/ml PMA. Primary $CD14^+$ monocytes were isolated as described above and maintained in RPMI 1640 supplemented with 10% FCS. THP-1 cells or primary $CD14^+$ monocytes were left untreated in RPMI 1640 with 10% FCS or polarized as follows: M1 (20 ng/ml IFNγ (R&D Systems, Minneapolis, MN), in combination with 10 ng/ml LPS (Sigma Aldrich)), M2 (20 ng/ml IL-4 (R&D Systems)). Cells were exposed to each polarizing conditions for the times indicated in each experiment.

2.4. Transfection of miRNAs

THP-1 monocytes were differentiated with PMA as described above and then transfected with miR-146a mimic or miR-146a inhibitor using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Negative control mimic or inhibitor (Shanghai GenePharma Co., Shanghai, China) was transfected to serve as matched controls. The cells were transfected with mimics, inhibitor or negative control at a final concentration of 10 nM. Efficiency of miR- 146a overexpression or inhibition was determined using qRT-PCR 24 h post-transfection as described below.

2.5. Flow cytometry assay

Flow cytometry was used for M1 and M2 surface marker analysis. Single-cell suspensions were washed in PBS with 2% FCS and adjusted to a concentration of $1–5 \times 10^6$ cells/ml. Cells were stained with FITC-conjugated antibody against CD80, CD86, CD163, or CD206 (BD Biosciences, San Jose, CA) or the appropriate isotype control, respectively. All antibodies were used at 5 μg/ml. The cells were incubated with the antibodies for 30 min at 4 °C and washed with PBS. The samples were analyzed using FACSCalibur (BD Biosciences, CA, USA) and the results were analyzed using FlowJo software (Tree star Inc, Ashland, OR).

2.6. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent according to the manufacturer's instructions, and the RNA concentration was determined with an ND-1000 spectrophotometer (NanoDrop). cDNA was synthesized by the PrimeScript RT Reagent Kit (TaKaRa) from total RNA. The analysis of miR-146a was performed using TaqMan microRNA assays (Applied Biosystems), and RNU48 was used as an endogenous control, as it has been reported to have stable expression levels during monocyte polarization (Graff et al., 2012). SYBR Premix Ex Taq kit (Takara, Dalian, China) was used to quantify the expression of mRNA transcripts, and β-actin was used as an endogenous control. qRT-PCR was performed using a 7300 Real-time PCR machine (Applied Biosystems). The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blot

Cells were lysed with lysis buffer and mini protease inhibitor. After centrifugation at 12 000 rpm for 20 min, the concentrations of supernatant proteins were quantified using a BCA Protein Assay Kit (Thermo scientific, IL, USA). Forty micrograms of total protein were electrophoresed in an SDS-PAGE gel, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) and blocked with 5% skimmed milk powder for 1 h. The PVDF membranes were incubated with primary antibodies against CXCL9 (ab9720, Abcam), CXCL10 (ab47045, Abcam), iNOS (ab3523, Abcam), Arg1 (ab91279, Abcam), CCL17 (ab182793, Abcam), CCL22 (ab9847, Abcam), INHBA (ab97705, Abcam) or GAPDH (sc-32233, Santa Cruz) overnight in 4 °C and washed with TBST for 10 min three times. Then the PVDF membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature and washed four times for 10 min each. Finally, the immunoblot was visualized using Odyssey