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Dynamic expression of microRNAs in M2b polarized macrophages associated with systemic lupus erythematosus

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ABSTRACT

Macrophage polarization contributes to the initiation and perpetuation of systemic lupus erythematosus (SLE). Our previous study demonstrated that M2b polarized macrophages induced by activated lymphocyte-derived DNA (ALD-DNA) have a crucial function in the initiation and progress of SLE disease. Accumulated data suggest that microRNAs (miRNAs) serve as critical regulators to control macrophage polarization. To investigate miRNA regulation during macrophage M2b polarization of SLE, miRNA microarrays of murine bone marrow derived macrophages (BMDMs) were performed following stimulation with ALD-DNA for 6 and 36 h. Over 11% of the 1111 analyzed miRNAs appeared differentially expressed during ALD-DNA triggered macrophage M2b polarization. Cluster analysis revealed certain patterns in miRNA expression that are closely linked to ALD-DNA induced macrophage M2b polarization. Analysis of the network structure showed that the predicted functions of the differentially regulated miRNAs at 6 h are significantly associated with inflammatory response and disease. Differentially regulated miRNAs identified at 36 h were determined to be significantly related to cell proliferation by biological network analysis. In this study, dynamic miRNA expression patterns and network analysis are described for the first time during ALD-DNA induced macrophage M2b polarization. The data not only provide a better understanding of miRNA-mediated macrophage polarization but also demonstrate the future therapeutic potential of targeting miRNAs in SLE patients.

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1. Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory and autoimmune disease characterized by the production of autoantibodies against cell nucleus components associated with diverse clinical manifestations affecting various organs and systems (Herrmann et al., 2000; Rahman and Isenberg, 2008). The different immune cells and inflammatory mediators involved in both innate and adaptive immunity participate in the initiation and progression of SLE (O'Neill and Schrieber, 2005). Multiple studies have demonstrated the key functions of T and B cells in the pathophysiology and therapy of SLE (Peng, 2009; Shlomchik et al., 2001). In addition to auto-reactive T and B lymphocytes, macrophages and other myeloid cells also have important functions in SLE. Dysregulations in macrophage phenotype and function have been documented in several patients with SLE and contribute to the initiation and perpetuation of the systemic autoimmune response and tissue damage (Katsiari et al., 2010; Paulson, 2007). However, the molecular and cellular mechanisms underlying the pathogenesis of SLE have yet to be completely explored.

In our previous study, we demonstrated that administration of activated lymphocyte-derived DNA (ALD-DNA) to syngeneic female BALB/c mice could induce a lupus-like autoimmune disease that closely resembles human SLE as manifested by high levels of anti-double-stranded DNA antibodies, glomerulonephritis, and proteinuria (Qiao et al., 2005; Wen et al., 2007). This finding implies that ALD-DNA can function as an auto-antigen that elicits autoimmune responses that can eventually lead to SLE pathogenesis (Walport, 2000). Our previous study also demonstrated that a substantial degree of macrophage infiltration and aberrant M2b polarization occurs in the kidneys of ALD-DNA induced lupus mice, which indicates the significance of macrophages in the mediation of SLE disease progression (Zhang et al., 2010, 2011). Through diverse external stimulations, macrophages are able to adopt a spectrum of divergent phenotypes (M1, M2a, M2b, and M2c), as shown in Table 1 (Mantovani et al., 2004; Mosser and Edwards, 2008). In general, the M2b macrophage state is stimulated by immune complexes and agonists of TLRs or IL-1 receptors; ALD-DNA can also induce macrophage M2b polarization in vivo and in vitro (Zhang et al., 2010). However, the mechanism of ALD-DNA-induced macrophage M2b polarization has yet to be elucidated.

Abbreviations: SLE, systemic lupus erythematosus; ALD-DNA, activated lymphocyte-derived DNA; UnALD-DNA, unactivated lymphocyte-derived DNA; BMDMs, murine bone marrow derived macrophages; miRNAs, microRNAs; PBMCs, peripheral blood mononuclear cells; FACS, fluorescence-activated cell sorting; MR, mannose receptor; qPCR, quantitative PCR; IPA, Ingenuity Pathways Analysis; GO, gene ontology; DAVID, Database for Annotation, Visualization, and Integrated Discovery; BP, biological process.

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Table 1
Characteristics and functional properties of polarized macrophages.

	M1	M2a	M2b	M2c
Products	IL-12^{high}, IL-10^{low}, TNF-α, IL-1β, IL-6	IL-10, IL-1ra	IL-10^{high}, IL-12^{low}, TNF-α, IL-1β, IL-6	IL-10, TGF-β
Markers	MHC II, CD80, CD86, MR	MHC II, MR	MHC II, CD80, CD86	MR
Arginine metabolism	iNOS	Arg1	iNOS	Agr1

MicroRNAs (miRNAs) are a class of small (~22 nucleotides) non-coding RNAs that were initially discovered in *Caenorhabditis elegans* and are ubiquitously expressed in a variety of species, including viruses, worms, flies, plants, and animals (Bartel, 2004; Lee et al., 1993). Modulation of gene expression by miRNAs occurs at the post-transcriptional level. Even small changes in miRNA levels may have a significant impact on the expression levels of target genes (He and Hannon, 2004). Emerging data show that miRNAs participate in generally all biological processes, including cell proliferation, differentiation, and metabolism. Moreover, miRNA control is believed to be a critical regulatory factor in the mammalian immune system (Kim et al., 2009). miRNA dysregulation is associated with the pathogenesis of multiple inflammatory and autoimmune diseases. For instance, a miRNA profile has revealed a SLE-specific miRNA signature upon comparison with control individuals in peripheral blood mononuclear cells (PBMCs), T cells, B cells, body fluids, and renal tissues from patients with SLE (19–26). However, miRNA regulation during macrophage M2b polarization in SLE pathogenesis remains incompletely understood.

The aim of the present study is to analyze dynamic and global miRNA expression patterns at different time points (0, 6, and 36 h) occurring during stimulation of ALD-DNA, a characteristic inducer of macrophage M2b polarization. A unique miRNA pattern in polarized bone marrow-derived macrophages (BMDMs) and dynamic regulation of miRNAs during macrophage M2b polarization are presented. Data obtained provide a valuable resource that can further enhance the current understanding of miRNA-mediated macrophage polarization as well as SLE pathogenesis.

2. Materials and methods

2.1. Animals

Female BALB/c mice between 6 and 8 weeks old were purchased from the experimental animal center of the Chinese Academy of Sciences (Shanghai, People's Republic of China). The mice were housed in a specific pathogen-free room under controlled temperature and humidity. All animal experiments were approved by the University of Soochow Animal Care and Use Committee.

2.2. Isolation and culture of murine bone marrow macrophage

Bone marrow cells were isolated from uninfected and normal mice at 8–12 weeks old. Single cell suspensions were prepared by passing samples through a cell strainer and adjusting the concentration to 2×10^6 cells/mL. Cells were induced for differentiation to macrophages with RPMI 1640 medium containing 10% FBS and 20% L929 culture supernatant for 6 d. Mature macrophage formation was assessed using fluorescence-activated cell sorting (FACS) analysis of F4/80 surface antigen expression. The purity of F4/80$^+$ cells was >90%.

2.3. DNA extraction and purification

ALD-DNA and unactivated lymphocyte-derived DNA (UnALD-DNA) were prepared using murine splenocytes generated from the surgical resected spleens of 6–8-week-old female BALB/c mice and cultured with or without Con A (Sigma-Aldrich) in vitro. To generate ALD-DNA, splenocytes were seeded at 2×10^6 cells/mL in a cell culture flask and cultured with Con A (5 μg/mL) for 6 d to induce apoptosis. Apoptotic cells were stained with FITC-labeled Annexin V (BD Biosciences) and propidium iodide (PI; Sigma-Aldrich) and then sorted using a FACSAria instrument (BD Biosciences). Genomic DNA from the syngeneic apoptotic splenocytes was treated with S1 nuclease (TaKaRa) and proteinase K (Sigma-Aldrich) and then purified using DNeasy blood and tissue kits (Qiagen) according to the manufacturer's instructions. UnALD-DNA was prepared from unactivated (resting) splenocytes and extracted using the same methods.

2.4. FACS analysis

To assay the phenotype of differentiated macrophages, flow cytometry analysis was performed. BMDMs were stained with the following monoclonal antibodies according to the manufacturer's instructions: APC-labeled anti-F4/80, PE-labeled anti-MHC class II, PE-labeled anti-CD80, PE-labeled anti-CD86, and PE-labeled anti-mannose receptor (MR) (eBioscience Inc., USA).

2.5. Cytokine analysis

The cell culture supernatant was collected and stored at −80 °C until the assay. The protein levels of TNF-α, IL-1β, IL-6, IL-10, IL-12, and MCP-1 in the cell culture supernatants were quantified using ELISA (eBioscience) according to the manufacturer's instructions.

2.6. Time-series experiments

To investigate the dynamic regulation of miRNAs, time-course experiments were performed. BMDMs were either stimulated with ALD-DNA or UnALD-DNA (final concentration of 50 μg/mL) for the indicated time or left untreated (control). Cells were collected at the end of each time period and frozen at −80 °C until analysis.

2.7. RNA extraction

Total RNA was extracted using an RNAiso Plus kit (Takara, Japan) according to the manufacturer's protocol. The RNA concentration was assessed using a NanoDrop spectrophotometer at a 260 nm/280 nm absorbance ratio of 2.0.

2.8. Real-time quantitative PCR of mRNA

The cDNA was synthesized from RNA using a PrimeScript™ first-strand cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's instructions. cDNA was subsequently subjected to real-time quantitative PCR (qPCR) using an ABI Prism 7500 real-time PCR system (Applied Biosystems, USA) and SYBR® Pre-mix Ex Taq™ II (TaKaRa) according to the manufacturer's instructions. All reactions were performed in triplicate. qPCR samples were normalized to the housekeeping gene GAPDH using the $2^{-\Delta\Delta Ct}$ method. Differences between groups were determined by Student's *t*-test, and statistical significance levels were set as ***$p < 0.001$, **p: 0.001–0.01, and *p: 0.01–0.05. Supplemental S1 shows the primer sequences used in this study.

2.9. Real-time quantitative PCR of miRNA

Small RNAs were transcribed into cDNA via miRNA-specific reverse transcription reactions using stem-loop primers specific to each

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