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HMGB1 silencing in macrophages prevented their functional skewing and ameliorated EAM development: Nuclear HMGB1 may be a checkpoint molecule of macrophage reprogramming

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ABSTRACT

High-mobility group box 1 (HMGB1), an important inflammatory factor, plays significant roles in CD4$^+$T cell differentiation, cancer and autoimmune disease development. Our previous data have demonstrated that HMGB1 contributes to macrophage reprogramming and is involved in experimental autoimmune myocarditis (EAM) development. In contrast to the well-explored function of HMGB1, little is known about the nuclear function. Whether HMGB1 can serve as an architectural factor and control functional skewing of macrophages remains unclear. Therefore, the present work was performed to address the above speculation. The adenovirus-mediated shRNA (Ad-shRNA) was employed to knock down HMGB1 in RAW264.7 and monocytes/macrophages of EAM mice. Our data showed that *in vitro* HMGB1 silencing limited functional skewing of macrophages and down-regulated inflammatory factors secretion, which can't be reversed by the exogenous HMGB1. In M1 polarization system, the phosphorylations of NF-κB, p38 and Erk1/2 were inhibited following HMGB1 silencing. *In vivo*, HMGB1 silencing could effectively ameliorate EAM development. Our data suggest that HMGB1 may be a checkpoint nuclear factor of macrophage reprogramming. Our findings also provide an exciting therapeutic method for inflammatory disorders.

1. Introduction

Monocytes/macrophages play key roles in innate and adaptive immunity and are crucial mediators of inflammatory diseases. They can differentiate into the proinflammatory M1 phenotype or the anti-inflammatory M2 phenotype depending on their environment and cytokine exposure [1, 2]. The M1 macrophage is typically activated by interferon-γ (IFN-γ) and/or lipopolysaccharide (LPS); releases different proinflammatory cytokines, such as necrosis factor-α (TNF-α), interleukin (IL)-6, and nitric oxide synthase; and up-regulates MHCII, and CD86/80 expression [3, 4]. Conversely, M2 macrophage usually secretes anti-inflammatory cytokines, such as IL-10 [5]. Macrophage polarization is determined by environmental condition or cytokine exposure; however transcription and/or architecture factors are also involved in functional skewing of macrophages, similar to NF-κB [6, 7].

High-mobility group box 1 (HMGB1) is an abundant non-histone nuclear protein and plays a significant role in the DNA architecture and transcription of eukaryotic cells [8, 9]. HMGB1 can release/secrete to the extracellular environment and serves as an essential damage-associated molecular patterns (DAMPs) to activate proinflammatory signaling pathways by binding with certain pattern recognition receptors (PRRs) [10, 11]. Our previous data have suggested that HMGB1 promoted Th17 cell expansion and facilitated macrophage reprogramming toward the M1-like phenotype in experimental autoimmune myocarditis (EAM) [12, 13]. As an architectural factor, HMGB1 contributes to DNA bending and organization of various regulatory protein complexes, thus regulating transcriptional activation; furthermore, HMGB1 can also directly bind to sequence-specific binding proteins, for example, NF-κB/Rel family [14–16]. It remains unclear whether nuclear HMGB1 can also control macrophage reprogramming. Therefore, in the present work, we hypothesized that nuclear HMGB1 may be a checkpoint of functional skewing of macrophages.

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2. Materials and methods

2.1. Reagents

Phospho-antibodies (p-Abs) against Erk1/2, p38, NF-κB (p50) and corresponding total antibodies, iNOS, β-actin and HMGB1 were obtained from Abcam (Shanghai, China). U0126, SB203580 and SN50 (inhibitors of Erk1/2, p38 and NF-κB, respectively) were purchased from univ-bio (Shanghai, China); recombinant HMGB1 (rHMGB1) was obtained from HMGbiotech, with a purity of > 95% and free from lipopolysaccharide (LPS); fluorochrome conjugated mAbs against F4/80, MHCII and CD86 were obtained from (Becton Dickinson Biosciences, Shanghai, China); IL-1β, IL-6 and TNF-α ELISA kits were obtained from MultiSciences (Lianke) BiotechCo., Ltd. (Hangzhou, China); LPS (*Escherichia coli* serotype 055:B5) was purchased from Sigma-Aldrich (Shanghai, China). M-CSF and Angiotensin II (ANG II) were obtained from R&D Systems (Minneapolis, MN, USA). Fetal calf serum (FBS) was purchased from Gibco, Life Technologies (Shanghai, China).

2.2. Cells and animals

BALB/c mice were maintained in the Animal Center of Jiangsu University in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). The experimental protocols were approved by the Committee for Ethical Affairs of Jiangsu University (Zhenjiang, China). Bone marrow-derived macrophages (BMDMs) were isolated from BALB/c mice and cultured in DMEM with 50 ng/mL M-CSF for 3–4 days [17]. RAW264.7 cells were cultured with RPMI 1640 containing 10% FBS.

2.3. Induction of myocarditis

BALB/c mice were inoculated with 150 μg of MyHC-$\alpha_{614–629}$ (Ac-SLKLMATLFSTYASAD-OH), that was emulsified at a 1:1 ratio in PBS/CFA on day 0 and 7 [18]. After 21 days, the mice were anesthetized with pentobarbital sodium (30 mg/g body weight, i.p.), sacrificed by cervical dislocation, and rapidly removed the corresponding excision.

2.4. Histopathology

Hearts were fixed in 10% formalin, paraffin embedded and stained with hematoxylin and eosin (HE). Severity scores of myocarditis were graded in a double blind manner by two independent investigators according to the Dallas criteria, based on the presence of inflammatory cells and cardiac myocytes necrosis [19]. The grades were as follows: 0, no inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes; 2, larger foci of > 100 inflammatory cells; 3, involving > 10% per cross-section; and 4, involving > 30% per cross-section [20].

2.5. AAV vector and adenoviral vector package

The AAV and adenoviral (Ad) vector with green fluorescent protein (GFP) and targeting HMGB1 were constructed by Hanbio Biotechnology Co., Ltd. (Shanghai, China). The sequence targeting on HMGB1 was GAAGATGATGATGATGAATAA. After packaging, AAV and Ad viral vectors were produced in HEK293T cells using a three-plasmid co-transfection technique and then purified. The titers of the virus were 1.0×10^{11} viral genome/mL, as detected using quantitative PCR (qPCR).

2.6. Immunofluorescence

After deparaffinization, rehydration, and antigen unmasking, samples were immersed in blocking buffer for 60 min; then PE-conjugated anti-F40/80 and FITC-conjugated anti-CD86 antibodies were added overnight at 4 °C. After washing, DAPI was added for 10 min. Sections were viewed with a fluorescence microscope (Olympus, Japan).

Table 1
The primers used in the present research.

Genes	Sequence	Size (bp)
IL-6	5′-TGGAGTACCATAGCTACCTGG-3′ 5′-AAAAAGTGCCGCTACCCTGA-3′	188
TNF-α	5′-GAAAGAAGCCGTGGGTTGGA-3′ 5′-ATCCCATGCCTAACTGCCCT-3′	268
IL-1β	5′-CCCTGCAGTGGTTCGAGG-3′ 5′-GACAGCCCAGGTCAAAGGTT-3′	208
GAPDH	5′-ACGGCAAATTCAACGCACAG-3′ 5′-AGACTCCACGACATACTCAGCAC-3′	215

2.7. RT-qPCR analysis

TNF-α, IL-6, IL-1β levels were assessed by RT-qPCR. Briefly, total RNA was isolated from BMDM or RAW264.7 cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. After cDNA synthesis, qPCR was performed with iQ SYBR Green Supermix using a Real-Time PCR System (Bio-Rad, Life Science, Shanghai, China) with GADPH used as an internal control. The primers were listed in Table 1. Quantification of gene expression was calculated relative to GADPH.

2.8. Western blot analysis

Proteins were extracted from RAW264.7 cells or BMDMs and electrophoresed on 12% SDS-PAGE gels, and then they were transferred onto PVDF membranes (PerkinElmer, USA). Membranes were blocked with 5% (w/v) non-fat dry milk/1% (v/v) Tween 20 in PBS for 1 h at room temperature and incubated overnight with primary antibodies against HMGB1, iNOS, p-p38, p38, p-Erk1/2, Erk1/2, p-p50, p50 and β-actin. After washing, HRP labeled secondary antibodies were added for 1 h at 37 °C. Detection was performed with electrochemiluminescence (ECL) and relevant blots were quantified by densitometry using the accompanying computerized image analysis program (Amercontrol Biosciences, USA).

2.9. Cytokine assay

Mouse serum and cell culturing supernatants were collected and stored at −80 °C until use. IL-6, IL-1β and TNF-α were measured using ELISA kits according to manufacturer's instructions.

2.10. Flow cytometry analysis

Macrophages were stained with PE-conjugated anti-MHC II and FITC-conjugated anti-CD86 antibodies. After washing with PBS, stained cells were re-suspended and analyzed. All samples were analyzed by FACS Calibur (BD Biosciences, USA).

2.11. Statistical analysis

All statistical analysis was performed using Graphpad Prism 5 software. Data were shown as the mean ± standard deviation (SD). Comparisons between groups were conducted using the paired *t*-test or one-way ANOVA with a Bonferroni correction; $p < .05$ was considered statistically significant.