Cytokine 108 (2018) 89–95

Contents lists available at ScienceDirect

Cytokine

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

Intracellular osteopontin negatively regulates toll-like receptor 4-mediated inflammatory response via regulating GSK3β and 4EBP1 phosphorylation

Haiou Yang[a,1], Xingchen Ye[b,1], Xiaoqing Zhang[c], Xiaoliang Li[c], Qihua Fu[c,*], Zhenhua Tang[a,*]

[a] Department of Laboratory Medicine, The International Peace Maternity & Child Health Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200030, PR China
[b] Department of Laboratory Medicine, Huzhou Central Hospital, Huzhou, Zhejiang 303000, PR China
[c] Department of Laboratory Medicine, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, PR China

ARTICLE INFO

Keywords:
Osteopontin
Toll-like receptors
Inflammation
Negative regulation
Immune response

ABSTRACT

Toll-like receptors (TLRs) play an important role in host defense against invading pathogens. By initiating a signal transduction cascade, TLRs lead to the release of pro-inflammatory cytokines. However, the inappropriate activation of TLR signaling could result in inflammatory disorders or autoimmune diseases. Osteopontin (OPN) has been reported to be an inflammatory cytokine participating in cell-mediated immunity. However, the role of OPN in TLR-mediated immune responses is poorly understood. In the present study, OPN-deficient (OPN-/-) macrophages exhibited significantly higher levels of pro-inflammatory cytokines after stimulation with lipopolysaccharide (LPS). Our study also demonstrated that the intracellular OPN (iOPN) isoform acted as a negative regulator to inhibit LPS-induced inflammatory responses. Compared to WT macrophages, OPN-/- macrophages had lower Akt phosphorylation levels and higher GSK3β phosphorylation levels, which were downregulated by p-Akt. Moreover, as a down-stream target of Akt, 4EBP1 was hypo-phosphorylated in OPN-/- macrophages compared to 4EBP1 in WT macrophages. These findings reveal that iOPN can regulate GSK3β and 4EBP1 phosphorylation to inhibit TLR4-mediated inflammatory responses.

1. Introduction

Toll-like receptors (TLRs) play a pivotal role in host defense against invading microbial pathogens by recognizing pathogen-associated molecular patterns (PAMPs) [1–4]. In humans, the TLR family currently comprises at least 10 functional members that differ in their subcellular distributions and microbial agonists. For instance, TLR4 is expressed on the cell surface and primarily recognizes LPS, whereas TLR3 is localized to the endoplasmic reticulum and detects viral dsRNA [5–11]. After recognizing their specific agonists, TLRs initiate a signal transduction cascade through recruiting the cytoplasmic adapter proteins MyD88 and TRIF; this action results in the activation of transcription factors, such as NF-κB, and eventually leads to the release of type I interferon and pro-inflammatory cytokines, such as IL-6, IL-12 and TNF-α [6,7]. Although TLR-mediated signaling pathways are important for the host defense system to clear pathogens, the inappropriate activation of TLR signaling may result in inflammatory disorders, such as septic shock and autoimmune diseases. Therefore, it is important to elucidate the mechanism underlying the fine regulation of TLR-mediated inflammatory responses [12]. In addition to PAMPs, several positive regulators, including Peli1, SHP-1, CD14 and MHC class II molecules, have been reported. Moreover, negative regulators, such as NLRC3, epidermal growth factor receptors, TRIM35 and CD11b, have also been successfully identified [13–19]. However, additional regulators of TLR signaling need to be discovered.

Osteopontin (OPN) is expressed by different types of immune cells, including T cells, macrophages and dendritic cells [20,21], and can be detected in most tissues and body fluids. As a potential immuno-modulatory factor, OPN is considered a pro-inflammatory cytokine that is involved in regulating cell adhesion, migration and survival [21–24] and participates in cell-mediated immunity and granulomatous inflammation [25,26]. Nevertheless, previous studies also revealed that OPN acts as an anti-inflammatory molecule in various pathological settings. Da salva, A.P. et al. reported that OPN deficiency exacerbates tissue destruction and reduces the repair process in an acute colitis model [27,28]. Furthermore, macrophages derived from aging mice with higher OPN expression levels than young mice were less responsive to LPS stimulation [29]. Our previous work also demonstrated that OPN-deficient mice exhibited more hepatic granuloma formation and released higher levels of pro-inflammatory cytokines than WT mice

* Corresponding authors.
E-mail addresses: qihuafu@126.com (Q. Fu), 15900897010@163.com (Z. Tang).
[1] These authors contributed equally to this work and should be considered co-first authors.

https://doi.org/10.1016/j.cyto.2018.03.013
Received 31 August 2017; Received in revised form 12 February 2018; Accepted 12 March 2018

in a *Propionibacterium acnes*-induced hepatic granuloma mouse model [30]. These results indicated that OPN may play diverse roles in different inflammatory diseases. However, the role of OPN in the innate immune response, especially in the regulation of TLR-mediated inflammatory cytokine release, is poorly understood.

In the present study, the role of OPN in regulating TLR-mediated inflammatory responses was investigated. We found that compared with peritoneal macrophages derived from WT mice, peritoneal macrophages from OPN-/- mice exhibited higher protein and mRNA expression levels of pro-inflammatory cytokines after LPS stimulation. WT peritoneal macrophages transiently transfected with the intracellular OPN (iOPN) construct showed significantly decreased IL-6 mRNA expression levels.These results indicate that iOPN negatively regulated TLR4-mediated inflammatory responses. Furthermore, aphospho-specific protein microarrayand western blot analyses were performed to reveal the underlying mechanisms. Our results indicate that iOPN could act as a negative regulator of TLR4-mediated signaling pathways. Our research also provides novel insights into the mechanism underlying fine-tuned TLR-mediated inflammatory responses.

2. Materials and methods

2.1. Mice

OPN-/- mice were purchased from Jackson Laboratory (http://www.jax.org/). The genotype of the mice was confirmed by RT-PCR as suggested by the Jackson Laboratory. Male WT C57BL/6 mice (6–8 weeks old) were purchased from the Shanghai Experimental Animal Center of the Chinese Academic of Sciences (Shanghai, China). All animals were housed in a pathogen-free environment and maintained in accordance with the guidelines of the Animal Committee of Shanghai Children's Medical Center.

2.2. Reagents

Lipopolysaccharide (LPS) from Escherichia coli O111:B4 was purchased from Sigma (Sigma-Aldrich, St Louis, MO). Enzyme-linked immune sorbent assay (ELISA) kits for TNF-α, IL-6 and IL-12 were purchased from Dakewei (Dakewei, Beijing, China). Antibodies against Src (Tyr416), AKT (Ser473), GSK3β (Ser9), and 4EBP1 (Thr37/46) and HRP-linked secondary antibody were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA, USA). HRP-linked anti-GAPDH antibody was purchased from Kangchen (Kangchen Biotech, Shanghai, China).

2.3. Cell culture

Peritoneal macrophages were prepared and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco). Briefly, WT and OPN-/- mice were anaesthetized with 1% pentobarbital sodium and then injected intraperitoneally with 5 ml of pre-cooled phosphate-buffered saline. After a gentle massage of the peritoneum, the cells were collected from the lavage fluid and cultured in DMEM with 10% FBS.

2.4. LPS stimulation and ELISAs

For the ELISAs, peritoneal macrophages derived from WT or OPN-/- mice were plated in 24-well plates (1×10^5 cells per well) overnight. Titrations of 1 μg/ml LPS were added for different times. The concentrations of TNF-α, IL-6 and IL-12 in the supernatants from triplicate wells were measured with the ELISA kits according to the manufacturer's instructions.

2.5. Real-time PCR analysis

A SYBR RT-PCR kit (Takara, Dalian, China) was used for quantitative real-time PCR analyses as described. Briefly, after stimulation with 1 μg/ml LPS for different times, total mRNA was extracted from cultured peritoneal macrophages derived from WT or OPN-/- mice or from WT macrophages transfected with pEGFP-N1-iOPN.cDNA was reverse transcribed from RNA using a reverse transcription system (Takara, Dalian, China). Real-time PCR was performed using a Rotor-Gene Q system (Qiagen, Hilden, Germany). Primers were synthesized by Sangong, Inc., Shanghai, China. The data were normalized to GAPDH expression.

2.6. Phospho-specific protein microarray analysis

A phospho-specific protein microarray was obtained from Full Moon Biosystems, Inc. (PEX100). This antibody microarray included 1318 highly specific and well-characterized antibodies for reference proteins and for 582 phosphorylation sites in 452 proteins that participate in many signaling pathways; each antibody had six replicates. The paired antibodies for the same (but unphosphorylated) target sites were also included in the microarray to determine the relative level of phosphorylation. Briefly, cell lysates were obtained from peritoneal macrophages derived from WT or OPN-/- mice and stimulated with 1 μg/ml LPS for different times. A total of 100 μg of cell lysate in 50 μl of reaction mixture was labeled with 1.43 μl of biotin in 10 μg/ml N,N-dimethyformamide. The resulting biotin-labeled proteins were diluted 1:20 in a coupling solution before application to the array for conjugation. The PEX100 microarray was first treated with a blocking solution for 30 min at room temperature. Then, the microarray was rinsed with Milli-Q reagent grade water for 3 min and incubated with the biotin-labeled cell lysates at 4 °C overnight. After three rinses with 1 × wash solution for 10 min each time, the conjugated proteins were detected using Cy3-streptavidin. For each antibody, we computed the phosphorylation ratio as follows:

$$\text{Phosphorylation ratio} = \frac{\text{phospho - stimulated}}{\text{unphospho - stimulated}} / \frac{\text{phospho - control}}{\text{unphospho - control}}$$

2.7. Plasmid construction

The plasmid encoding mouse iOPN with codons 1–15 deleted was constructed in two steps. First, mouse OPN was constructed by PCR-based amplification of the cDNA from mouse peritoneal macrophages. Then, the product was sub-cloned into the pEGFP-N1 eukaryotic expression vector and confirmed by DNA sequencing. The specific OPN PCR primers with restriction sites (XhoI and BamHI) (Takara, Dalian, China) were as follows: 5′-CTC GAG ATG AGG CTG CAG TTC TCC TGG-3′ and 5′-GGA TCC TTA GTT GAC CTC AGA AGA-3′. Second, the expression plasmid for iOPN was generated by PCR-mediated mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, The Netherlands) according to the manufacturer's instructions. The iOPN plasmid DNA clones were sequenced entirely in their sense and antisense directions to exclude any random base exchanges.

2.8. Transfection

WT-derived peritoneal macrophages were cultured in 24-well plates at 1×10^5 cells per well. After culture for 1 day, the cells were transiently transfected with OPN-specific siRNA or the pEGFP-N1-iOPN plasmid using Lipofectamine 3000 (LifeTechnologies, Inc.) according to the manufacturer's instructions. OPN-specific small interfering RNA (siRNA) (5′-GGA UGA CUU UAA GCA AGA A-3′) was used to suppress endogenous OPN expression, and a nonsense sequence (5′-UUC UCC GAA CGU GUC ACG U-3′) was used as the control siRNA.

2.9. Western blot analysis

Macrophages were lysed with RIPA cell-lysis buffer (Cell Signaling