International Immunopharmacology 23 (2014) 288–293

Contents lists available at ScienceDirect

International Immunopharmacology

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

Role of TREM-1 in response to *Aspergillus fumigatus* infection in corneal epithelial cells

Li-ting Hu, Zhao-dong Du, Gui-qiu Zhao *, Nan Jiang, Jing Lin, Qian Wang, Qiang Xu, Lin Cong, Sheng Qiu

Department of Ophthalmology, Affiliated Hospital of Qingdao University, Qingdao, China

ARTICLE INFO

Article history:
Received 21 April 2014
Received in revised form 6 August 2014
Accepted 9 September 2014
Available online 19 September 2014

Keywords:
TREM-1
Fungal keratitis
Aspergillus fumigatus
TLR-4

ABSTRACT

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a cell surface receptor that is highly expressed in inflammatory lesions caused by infectious agents such as bacteria and fungi and amplifies immune responses. The aim of the current study was to investigate TREM-1 expression in corneal epithelial cells infected by *Aspergillus fumigatus* (*A. fumigatus*) and evaluate its role. In this study, infection with *A. fumigatus* upregulated TREM-1 expression in corneal epithelial cells both in vitro and in vivo. Furthermore, treatment with the antagonistic peptide of TREM-1 decreased the levels of inflammatory cytokines that were enhanced by the fungal infection. We speculated that cross-talk occurs between TREM-1 and Toll-like receptor-4 (TLR-4) in cornea fungal infection. Inhibitors of TLR-4 and myeloid differentiation factor 88 (MyD88) could partially inhibit the upregulation of TREM-1 induced by *A. fumigatus* respectively. In addition, TLR-4 blockade enhanced the inhibitory effect of the antagonistic peptide of TREM-1 on *A. fumigatus*-induced inflammation. These findings suggest that TREM-1 plays critical roles in fungal infection, and targeting it may represent a novel therapeutic strategy for patients with fungal keratitis.

1. Introduction

Fungal keratitis (FK) is an opportunistic infection of the cornea that is caused by pathogenic fungi and has a high blindness rate. The incidence of FK has been increasing in recent years due to eye injury, long-term antibiotic or corticosteroid use, and decreased body immunity [1]. There are two main pathogenic fungi, *Fusarium* and *Aspergillus* [2]. Due to the lack of effective antifungal agents, FK has become the most pressing infectious keratitis problem. After being invaded by a fungus, the corneal epithelium identifies fungus and toxins, initiates immune responses, and secretes cytokines to mediate inflammatory cells to the site of the infection. A large number of cells, cytokines, and chemokines are involved in the removal of fungus and the injury and repair of the cornea [3–5]. Neutrophils kill and clear fungus by respiratory burst, phagocytosis, and secreting inflammatory cytokines. Meanwhile, neutrophils continue to generate elastase, collagenase, and myeloperoxidase (MPO) to break down the cornea. As such, corneal inflammation is a double-edged sword. Appropriate inflammation will stimulate effective host defense responses without obvious tissue damage, while excessive inflammation will seriously destroy visual function [6]. As the first barrier of the cornea against pathogenic microorganisms, corneal epithelial cells play an irreplaceable role in the innate immune response [7].

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a cell surface receptor of the immunoglobulin superfamily that is constitutively expressed by monocytes and polymorphonuclear leucocytes (PMNs) [8]. Bacterial or fungal infections can cause upregulation of membrane-bound TREM-1, rendering it a useful early inflammatory biomarker for systemic infection [9]. Although the endogenous TREM-1 ligand remains unknown, it is activated upon bacterial recognition by host cells, triggering a number of intracellular signaling events that result in amplified Toll-like receptor-initiated responses [8,10,11]. The activation of Toll-like receptor-4 (TLR-4) initiates the transmembrane signaling cascade and triggers intracellular signaling molecules, including myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase 1 and 4 (IRAK1 and IRAK4), and tumor necrosis factor receptor-associated factor 6 (TRAF6), and results in the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [12,13]. On the other hand, TREM-1 blockade protects mice against lipopolysaccharide (LPS)-induced septic shock and microbial sepsis caused by live *Escherichia coli* [8]. This blockade reduces the TREM-1-mediated inflammatory response but still allows sufficient control of the bacterial infection by downregulating the production of pro-inflammatory cytokines as well as the total number of infiltrating PMNs and macrophages [11].

It was reported that TREM-1 expression is increased during pulmonary fungal infection, suggesting that this receptor might be involved in anti-fungal immune responses [14]. TREM-1 amplifies the inflammation induced by both bacteria and fungi; thus, it represents a potential therapeutic target. Since nothing is known about the role of TREM-1

* Corresponding author at: Department of Ophthalmology, Affiliated Hospital of Qingdao University, 16th Jiangsu Road, Qingdao City, Shandong Province, 266003, China.
E-mail address: guiqiuzhao@126.com (G. Zhao).

http://dx.doi.org/10.1016/j.intimp.2014.09.011
1567-5769/

in FK, this study investigated the expression and role of TREM-1 in *Aspergillus fumigatus* (*A. fumigatus*) keratitis.

2. Materials and methods

2.1. Materials and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin/0.03% ethylenediaminetetraacetic acid solution, and Ham F-12 were purchased from Gibco (San Diego, CA, USA). RNAiso Plus and reverse transcription–polymerase chain reaction (RT-PCR) kits and SYBR Premix Ex Taq™ (Tli RNaseH Plus) were purchased from TaKaRa (Dalian, China). Polyclonal goat anti-human TREM-1 and polyclonal goat anti-human TLR-4 were obtained from R&D Systems (Minneapolis, MN, USA). Monoclonal rabbit anti-human MyD88 was purchased from Cell Signaling Technology (Boston, MA, USA). CLI095 was obtained from the InvivoGen (San Diego, CA, USA). ST2825 was obtained from ApexBio Technology LLC (Boston, MA, USA). Bicinchoninic acid assay and ECL Western Blotting Detection Reagent were purchased from Beyotime (Shanghai, China). Phenylmethylsulfonyl fluoride (PMSF) and cell lysis buffer (RIPA) were purchased from Solarbio (Beijing, China).

2.2. Preparation of A. fumigatus

The standard *A. fumigatus* strain was purchased from China General Microbiological Culture Collection Center and grown in Sabouraud medium at 28 °C for 5–7 days. The fungal conidia were collected and inactivated in 6 h by treatment with 75% ethanol. After washed three times in sterile phosphate-buffered saline, solution was adjusted to a concentration of 5×10^7/mL with DMEM.

2.3. Cell culture

The telomerase-immortalized human corneal epithelial cells (THCEs) were kindly provided by XiaMen Ophthalmic Center and cultured in DMEM with 10% FBS, 0.075% epidermal growth factor, 0.075% insulin, and 1% SPA sulfate at 37 °C in a humidified atmosphere containing 5% CO_2. Confluent corneal epithelial cultures were switched to serum-free DMEM and treated with *A. fumigatus* spores (5×10^7/mL) in 12- or 6-well plates.

2.4. Examination of clinical specimens

To determine whether TREM-1 mediates corneal inflammation after fungal infection, patients with fungal keratitis at the Department of Ophthalmology (The Affiliated Hospital of Qingdao University) from September 2012 to October 2013 were included. The patients' diagnoses were clinically confirmed by fungal culture, staining of corneal scrapings, or confocal microscopy. A total of 25 first-visit patients were divided into three groups according to keratomycosis severity, which was scored visually with the aid of a slit lamp: mild group (8 cases, 8 eyes), corneal ulcer diameter ≤ 2 mm, invasion depth of corneal opacity invasion depth ≤ 1/3 of the corneal stroma, no hypopyon; moderate group (10 cases, 10 eyes), of corneal ulcer diameter = 2–4 mm, corneal opacity invasion depth = 1/3–2/3 of the corneal stroma, no hypopyon; and severe group (7 cases, 7 eyes), corneal ulcer diameter ≥ 4 mm, corneal opacity invasion depth ≥ 2/3 of the corneal stroma, with hypopyon. Corneal epithelial scrapings were collected, and the mRNA was analyzed using real-time PCR.

Controls were normal corneal tissues remaining after corneal transplantation. For the use of these clinical materials for research purposes, the patient's consent and approval from the Institutional Research Ethics Committee were obtained.

2.5. Quantitative RT-PCR

The corneal epithelial scrapings or cultured cells were harvested and saved at −80 °C. The total RNA of the isolated cells was extracted using RNAiso plus reagent (TaKaRa) and rapidly quantified using spectrophotometry. Complementary DNA was generated by reverse transcription of 2 μg of total RNA and then used in the following quantitative PCR reactions with SYBR Green using specific primers: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and a final stage of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The oligonucleotide primers used were as follows: β-actin TAACACCCAGCACAATGAA and CTAAGTCATA GTCCGCCTAGAAGCA; TREM-1 GTTGGAGCTGGTGCACAGGA and TTCT GGCTGCTGGCAAACTTC; IL-1β GCTGATGGCCCTAAACAGATGAA and TCCATGGCCACAACAACTGAC; TNF-αTGCTTGTTCCTCAGCCTCTT and CAGAGGGCTGATTAGAGAGAGGT; IL-6 AAGCCAGAGCTGTGCAGATGAG TA and TGTCCTGCAGCCACTGGTTC; and IL-8 TTTCAGAGACAGCAGAGC ACACAA and CACACAGAGCTGCAGAAATCAGG. The gene expression levels were quantified by RT-PCR using the housekeeping gene β-actin as an internal control. Quantification was performed using the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated at least three separate times.

2.6. Western blot analysis

Cell culture proteins were extracted via RIPA lysis buffer plus 1 mM PMSF at 4 °C for 40 min. The lysate was centrifuged every 10 min, followed by centrifugation at 14,000 rpm for 15 min at 4 °C.

Total protein was quantified via bicinchoninic acid assay, denatured with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer at 95 °C for 5 min. Proteins (60 μg/well) were separated by 12% SDS–PAGE in Tris/glycine/SDS buffer and electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking by Western blocking buffer for 2 h, the membranes were incubated with goat anti-TREM-1, rabbit anti-β-actin, and goat anti-TLR-4 at 4 °C overnight and then incubated with secondary antibody for 1.5 h. All blots were detected with BeyoECL Plus (Beyotime, Shanghai, China). Band intensity was measured by Quantity One Software (Bio-Rad, CA, USA).

2.7. Statistical analysis

All of the results are shown as mean ± SD. Data analysis was performed by one-way analysis of variance, and further pairwise comparisons were made using the least significant differences test using SPSS 17.0 software (SPSS, Chicago, IL, USA). Statistical significance was set at the $p < 0.05$ level.

3. Results

3.1. TREM-1 expression in human corneal epithelium with fungal infection

To determine whether TREM-1 mediates the pathogenesis of fungal keratitis, TREM-1 expression was tested in 25 cases of fungal keratitis versus 5 normal human corneas. PCR data (Fig. 1) showed that, compared with controls, TREM-1 was significantly enhanced in the human corneal epithelium with fungal infection. Moreover, the expression levels of TREM-1 increased with increasing keratomycosis severity.

3.2. Enhanced TREM-1 expression in THCEs by A. fumigatus

To determine the effect of *A. fumigatus* infection on TREM-1 expression in THCEs, we treated the cells with *A. fumigatus* spores (5×10^7/mL) in 12-well plates. As shown in Fig. 2A, although no difference was seen at 2 h, there was an upregulation of TREM-1 expression at 4 h and 8 h. However, there was no further increase in TREM-1 mRNA expression at 16 h. Western blot analysis also showed an obviously elevated TREM-1 level in *A. fumigatus*-treated cells (Fig. 2B, C).