Fish & Shellfish Immunology 43 (2015) 357–363

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Mannose receptor mediated phagocytosis of bacteria in macrophages of blunt snout bream (*Megalobrama amblycephala*) in a Ca^{2+}-dependent manner

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ARTICLE INFO

Article history:
Received 8 October 2014
Received in revised form
30 December 2014
Accepted 2 January 2015
Available online 9 January 2015

Keywords:
Megalobrama amblycephala
Mannose receptor
Macrophage
Phagocytosis
Bacteria

ABSTRACT

Mannose receptor (MR) is an important pattern-recognition receptor in macrophages and plays a critical role in immune responses. It is has been reported that mammalian macrophages are able to engulf a wide range of microorganisms mediated by Ca^{2+}-dependent MR binding to terminal mannose residues which are frequently found on the pathogen surfaces. However, little is known about the MR-mediated phagocytosis in macrophages of fish. In this report, the distributions of MR in the macrophage and head kidney tissue from blunt snout bream were examined using MaMR specific antibody generated in our lab. Mannan and MaMR specific antibody inhibition experiments results collectively showed that MR was involved in the GFP-expressed *E. coli* engulfed in the macrophages, resulting in respiratory burst, nitric oxide production as well as inflammatory cytokines secretion, and the MaMR-mediated phagocytosis was Ca^{2+}-dependent. These results will shed a new light on the immune functions of teleost MRs.
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1. Introduction

Mannose receptor (MR), an important pattern-recognition receptor (PRR) in macrophages and dendritic cells, playing a critical role in both innate and acquired immune responses [1]. MR is able to bind and mediate the phagocytosis of a wide range of microorganisms, including viruses, bacteria, yeasts and parasites, such as HIV [2], *Mycobacterium tuberculosis* [3], *Leishmania donovani* [4], *Schistosoma mansoni* [5], *Pneumocystis carinii* [6] and *Saccharomyces cerevisiae* [7]. MR is a typical type I transmembrane receptor, it contains an N-terminal cysteine-rich domain (CR), a single fibronectin type II domain (FNII), eight tandemly arranged C-type lectin-like domains (CTLDs), a single transmembrane domain and a short C-terminal cytoplasmic domain [8–10]. MR CTLDs display Ca^{2+}-dependent binding to the terminal mannose, fucose and N-acetylglucosamine residues that are frequently found on the pathogen surfaces and mediate diverse immune responses [5]. It has been demonstrated that Ca^{2+} was essential to the activity of MR in the mammalian macrophages [11]. Although the structure and function of mammalian MR have been well characterized, little is known in fish and very few papers about receptor-mediated phagocytosis are reported. The capture of antigens by MR or glucan receptor was reported in the leukocytes of gilthead seabream (*Sparus aurata*) [7,12]. In zebrafish, it has been reported that EDTA was the specific inhibitor of MR binding which could efficiently inhibit the uptake of heat-killed FITC-*Edwardsiella ictaluri* [13]. Given that macrophages express high levels of MR [14] and the abundance of mannose residues on the surface of *E. coli* [7], studies on the interactions between *E. coli* and MR could offer insights into the mechanism of phagocytosis by fish macrophages.

In our previous report, we have cloned and characterized the first two teleost MRs from grass carp (*Ctenopharyngodon idella*) and blunt snout bream (*Megalobrama amblycephala*), the two principal fish cultured in China [15,16]. Subsequently, we have successfully isolated macrophages from blunt snout bream, and further showed

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http://dx.doi.org/10.1016/j.fsi.2015.01.002
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Table 1
The primers used for qRT-PCR assay. The sequences of the primers and the accession numbers of the target genes deposited in the NCBI gene bank are shown.

Primer names	Sequence (5′ → 3′)	GenBank	Application
β-actin F (forward)	ACCCACACCGTGCCCATCTA	ADV57164.1	qRT-PCR
β-actin R (reverse)	CGGACAATTTCTCTTTCGGCTG		qRT-PCR
IL-1β (forward)	GTGCCAGGTGCCAAGTAGC	KF245425.1	qRT-PCR
IL-1β (reverse)	AAGCCCAAGATATGCAGGAGT		qRT-PCR
TNF-α (forward)	CCGCTGCTGTCTGCTTCA	HQ696609.1	qRT-PCR
TNF-α (reverse)	GCCTGGTCCTGGTTCACTCT		qRT-PCR

that oligochitosan and LPS of bacteria stimulated phagocytic activity in the macrophages and it was associated with respiratory burst coupled with nitric oxide production [17]. Amino acid sequences alignment analysis revealed that the MR of blunt snout bream (MaMR) contains typical CTLDs which possess two conserved site of carbohydrate recognition domain (CRD), EPN (Glu^{725}-Pro^{726}-Asn^{727}) and WND (Trp^{749}-Asn^{750}-Asp^{751}) [15]. The EPN motif dictates specificity of mannose containing ligands [10]. In addition, there were two conserved Ca^{2+} binding sites (Asn^{728}-Asn^{731}- Glu^{737}-Asn^{753} and Glu^{725}-Asn^{727}-Asn^{750}-Asp^{751}) which characterized the calcium-dependent agglutination activity of MR, however it was lack of direct evidence to approve it [15]. In this report, taking the advantage of generated antibody against the CTLD4-8 domains of MaMR (MaMR-CTLD4-8 specific antibody) in our lab, the distributions of MaMR in the macrophage and head kidney tissue from blunt snout bream were observed. In addition, MR was involved in the GFP-expressed *E. coli* engulfment in the macrophages, resulting in respiratory burst, nitric oxide production as well as inflammatory cytokines secretion, and the MaMR-mediated phagocytosis was Ca^{2+}-dependent.

2. Materials and methods

2.1. Immunohistochemistry assay for MR distribution in head kidney of blunt snout bream

Immunohistochemistry (IHC) was performed to observe the distribution of MR in the head kidney tissue from blunt snout bream, since we have reported that the mRNA of MR was highly expressed in the head kidney [15]. Fresh head kidney tissue was fixed with 4% paraformaldehyde, prior to the tissue was embedded and sectioned using standard method. The sections were incubated with 100% methanol containing 3% H_2O_2 at room temperature for 10 min to block endogenous peroxidase activity. After washing with PBS, the slides were incubated in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate) at 95–100 °C for 10 min to unmask the antigenic epitope. Subsequently, the sections were incubated with blocking buffer (10% bovine serum albumin in PBS) at room temperature for 1 h. Thereafter, the sections were incubated with MaMR-CTLD4-8 specific antibody at the dilution of 1:1000 at room temperature for 1 h. After washing with PBS, the slides were incubated with goat anti-rabbit IgG-HRP for 30 min and then revealed in DAB substrate solution (Guge Biology, China).

2.2. Subcellular localization of MR in macrophages from blunt snout bream

Macrophages were isolated according to the previous protocol established in our lab [17] and seeded onto cover slips in 24-well plates. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 2 min. 10% normal goat serum (Guge Biology, China) as blocking solution was added to minimize non-specific fluorescence and then MaMR-CTLD4-8 specific antibody (diluted 1:500 in blocking solution) was incubated at 28 °C for 2 h, followed by incubation with FITC conjugated goat anti-rabbit IgG (Invitrogen) for 30 min. The nuclei were stained with DAPI (4′, 6-Diamidino-2-phenylindole, dihydrochloride) for 5 min. The slides were imaged by a fluorescence microscope (Axio imager A2).

2.3. Phagocytosis of GFP-expressed E. coli in macrophages

Macrophages (2.5×10^6 cells) were pre-incubated with medium containing mannan (Sigma), MaMR-CTLD4-8 specific antibody or negative serum at 28 °C for 30 min, respectively. Mannan was known to be the specific inhibitor of MR binding [2]. Phagocytosis was evaluated by incubation with green fluorescent protein expressed *E. coli* strain MC1061 containing plasmid pFPV25.1 (GFP-expressed *E. coli*, a kind gift from Professor Jinxing Wang, School of Life Sciences, Shandong University, China). Cells were incubated with heat-inactivated GFP-expressed *E. coli* at multiplicity of infection (MOI) of 10 at 28 °C for 2 h as described previously [17]. Then cells were washed four times with pre-cooling PBS to remove the non-binding *E. coli*. Thereafter, the phagocytic activity of macrophages was measured in two different ways. Cells were either fixed with 4% paraformaldehyde, stained with fluorescent dyes DAPI and DiI (Beyotime, China, for cell membrane staining), for imaging by a fluorescence microscope, or lysed in cell lysis buffer (Beyotime, China), for fluorescence intensity measurement in the fluorescence microplate at 509 nm.

To address whether the GFP-expressed *E. coli* binding was Ca^{2+}-dependent, five groups of macrophages were pre-incubated with PBS in the absence or the presence of 2.0 mM EDTA for 30 min at 28 °C as follow: group A (cells were incubated with PBS alone and was used as control), group B (cells were incubated with 2.5 mM Ca^{2+}), group C (cells were incubated with 2.0 mM EDTA), group D (cells were incubated with 2.5 mM Ca^{2+} and 2.0 mM EDTA), group E (cells were incubated with 2.5 mM Mg^{2+} and was used as Ca^{2+}-dependent control). After 30 min treatment, these solutions were discarded and the cells were incubated with GFP-expressed *E. coli* resuspended in PBS for 2 h at 28 °C. After washing and lysis, the fluorescence intensity in the lysates was measured in the fluorescence microplate at 509 nm.

2.4. Respiratory burst activity and nitric oxide production

Nitro Blue Tetrazolium (NBT) reduction assay and Griess assay were performed to determine the production of superoxide anion and NO based on the methods of Kemenade et al. (1994) and Nakhro et al. (2013) respectively [18,19]. Cells were pre-incubated with MaMR-CTLD4-8 specific antibody, negative serum or L-15 medium alone for 2 h. Subsequently, cells were washed and incubated with complete medium with heat-inactivated GFP-expressed *E. coli* except for the blank control group. For superoxide anion assay, cells were incubated with GFP-expressed *E. coli* together with NBT for 70 min, supernatant was removed and then cells were fixed with 100% methanol. Cells were washed twice with 70% methanol and air-dried. The formazan was dissolved by adding 120 μl of 2 M KOH to each well, followed by 140 μl of dimethylsulphoxide (DMSO). After mixing, the absorbance was measured at 620 nm. For NO assay, the supernatant was detected after 6 h incubation. 100 μl of supernatant was added into another 96-well plate, then 50 μl of 1% sulphanilamide in 2.5% phosphoric acid was added to each sample, after 10 min incubation at 37 °C, 50 μl of 0.1% N-naphthylethylenediamine in 2.5% phosphoric acid was added to incubate for 10 min, finally absorbance was measured at 540 nm. The molar concentration (μM) of nitrite in the sample was estimated according to the NO standard curve.

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