Molecular Immunology 103 (2018) 182–190

Contents lists available at ScienceDirect

Molecular Immunology

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

Interaction of Group B *Streptococcus* sialylated capsular polysaccharides with host Siglec-like molecules dampens the inflammatory response in tilapia

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ARTICLE INFO

Keywords:
Oreochromis niloticus
Group B *Streptococcus* (GBS)
Sialylated capsular polysaccharides
Siglec-like
Anti-inflammatory cytokine
Proinflammatory cytokines

ABSTRACT

Group B *Streptococcus* (GBS, *S. agalactiae*) infection in tilapia (*Oreochromis niloticus*) causes widespread death of this species and is a significant issue for the aquaculture industry. The major virulence factor for GBS is its sialylated capsular polysaccharides (CPs). These CPs interact with sialic acid-binding immunoglobulin-like lectins (Siglecs) on the host immune cells to regulate the downstream inflammatory response and evade detection. Previously, we cloned multiple Siglec-like molecules from an *O. niloticus* cDNA library, all of which were shown to interact with the sialylated CPs of GBS. In the present study, we investigated the effects of GBS infection on the expression of pro- and anti-inflammatory cytokines in *O. niloticus* as well as *On*Siglec-like-transfected macrophage cells. Eukaryotic expression vectors containing full-length *On*Siglec-1-like, -4b-like, -14-like were constructed and used to transfect RAW264 macrophages *in vitro* as well as live tilapia *in vivo* prior to GBS infection. The expression of the anti-inflammatory cytokine interleukin *(IL)-10* and the pro-inflammatory cytokines tumor necrosis factor *(TNF)-α*, *IL-6,* and interferon *(INF)-β* were then analyzed by qPCR. Our results indicate that as infection progressed, *IL-10* expression was significantly upregulated, while that of *TNF-α* and *IL-6* were significantly downregulated in the *On*Siglec-like-transfected cells. *INF-β* expression was also downregulated in cells transfected with *On*Siglec-1-like and -4b-like, but was not significantly effected in *On*Siglec-14-like-transfected cells. Notably, the magnitude of these cytokine expression changes was greatly decreased when a Δ*neuA* GBS mutant was used to infect the *On*Siglec-1-like-transfected cells. In GBS-infected tilapia, *IL-10* expression was significantly upregulated in all tissues, whereas *INF-β* expression in the spleen, kidney, and gills was significantly downregulated at 12 hpi. While the expression of *TNF-α* was slightly upregulated, this change was not significant. In GBS Δ*neuA* mutant-infected *O. niloticus*, *IL-10* expression in all of the tissues was significantly lower than that observed for the wild-type GBS group, while *TNF-α* expression was higher in the mutant infected group. There was no significant difference in *INF-β* expression between the two groups. Taken together, sialylated CPs on GBS appear to interact with host *On*Siglec-like molecules to transmit negative regulatory signals *via* enhanced anti-inflammatory cytokine IL-10 production and reduced pro-inflammatory cytokine production, ultimately leading to dampening of the host immune response. The results of this study further elucidate the molecular mechanism underlying GBS infection in tilapia and also provide candidate drug target molecules.

1. Introduction

Sialic acids (Ne5Acs) on the cell surface play an important role in many biological processes, such as cell adhesion, cell proliferation, and regulation of the immune response (Mandal et al., 2015). Sialic acid-binding immunoglobulin-like lectins (Siglecs) are cell membrane receptors that are capable of recognizing specific cell surface sialic acids. Siglecs are predominantly expressed by immune cells, and the majority of them (Siglecs 2–12) transmit negative regulatory signals and downregulate the host immune responses *via* activation of one or multiple cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). These ITIMs recruit src homology 2 domain containing

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https://doi.org/10.1016/j.molimm.2018.09.014
Received 14 May 2018; Received in revised form 20 September 2018; Accepted 21 September 2018
0161-5890/

phosphatase 1 (SHP-1), SHP-2, or suppressor of cytokine signaling 3 (SOCS3) *etc.*, which subsequently suppress tyrosine kinase-dependent signaling required for immune activation and downstream cytokine signaling (Kitzig et al., 2002). Interestingly, Siglec function has been mimicked in some foreign species to evade the host immune system.

Group B *streptococcus* (GBS) is a Gram-positive bacterium that can colonize in various hosts. GBS can cause neonatal septicemia and meningitis in humans and can also infect live stock (*e.g.*, pigs, cattle, and sheep) as well as fish (Atul Kumar et al., 2006; Zhang et al., 2017). GBS can biosynthesize sialic acid as well as internalize it from the external environment (Raetz and Whitfield, 2002). The bacterium then integrates the sialic acid into the termini of its own glycoconjugates through biosynthesis and modification using related genes, thus forming sialylated capsular polysaccharides (sialylated CPs) (Carlin et al., 2009). CPs are the major virulence factor of GBS and its terminal $Neu5Ac_{\alpha2\text{-}3}Gal_{\beta1\text{-}4}GlcNAc$ unit is very similar to sialylated polysaccharides found at the termini of glycoproteins on the surface of immune cells (Pillai et al., 2012). By molecularly mimicking the host sialylated glycans, CPs can then bind the host Siglecs to activate the cytoplasmic ITIMs, transmit negative regulatory signals, and destroy the homeostasis of the host’s inflammatory response, allowing the bacteria to escape the host's immune defense and cause systemic infection (Fittipaldi et al., 2012; Maisey et al., 2008; Pangburn et al., 2009; Weiman et al., 2010). GBS has been show to evade the host immune response in a variety of species.

Nile tilapia (*Oreochromis niloticus*) has been recommended for consumption by the United Nations Food and Agriculture Organization (FAO) in many countries and has become an important farmed fish species in China (Globefish, Tilapia-January 2015). However, recent GBS outbreaks in this species have caused huge economic losses to the tilapia aquaculture industry and no effective preventive or control measures have been established. Notably, in a recent study, we cloned multiple Siglec-like molecules from a GBS-infected *O. niloticus* cDNA library (designated *On*Siglec-like) and demonstrated that these receptors interact with GBS using eukaryotic transfection and flow cytometry techniques (Dong et al., 2016). Erythrocyte adhesion assays and ELISAs were then used to further confirm that GBS binds these *On*Siglec-like molecules *via* its sialylated CPs. While this previous study indicates that GBS does indeed mimic Siglec signaling to avoid the host’s immune system, very little is known about the molecular mechanisms of this interaction in fish, particularly the effects of infection on downstream signaling cascades.

In the present study, we investigated the effects of GBS infection on the expression of pro- and anti-inflammatory cytokines in *O. niloticus* as well as *On*Siglec-like molecule-transfected macrophage cells. To our knowledge, this is the first time the downstream inflammatory molecules have been investigated in the GBS CP-Siglec signaling cascade in this particular species. The results of this study further elucidate the molecular mechanism underlying GBS infection in tilapia and provide candidate drug targets for the prevention and control of streptococcal infection in tilapia aquaculture production.

2. Materials and methods

2.1. *O. niloticus* and GBS strains

A total of 100 fish with an average weight of 50 ± 5 g were obtained from the Gaoyao Aquaculture Germplasm Conservation Station, PRFRI, Chinese Academy of Fishery Sciences, Guangdong Province. The fish were maintained in an indoor aquarium at 29–31 °C with a continuous oxygen supply for 15 days prior to the experiment.

The GBS strain WC1535, with the molecular serotypes Ia and ST7, was isolated and maintained in our laboratory (Lu et al., 2010; Ye et al., 2011; Zhang et al., 2015). The temperature sensitive suicide vectors pSET1s and pSET4s used for GBS gene knockout were kindly provided by Professor Takamatsu at the Bacterial and Parasitic Diseases Research Division, National Institute of Animal Health (NARO), Japan (Takamatsu et al., 2001a,b) (see Supplemental Table S1).

The mouse macrophage cell line RAW264 is widely used in the study of cellular inflammation (Ramana et al., 2006). Endogenous Siglec expression (Siglec-3/-E/-F/-G/-H) in RAW264 cells is extremely low (< 0.28) (Ando et al., 2008), and therefore RAW264 cells can be used to study the effect of exogenous *On*Siglec-like gene and protein expression on immune cells.

2.2. Macrophage transfection

Eukaryotic expression vectors (pcDNA3.1(+)) containing one of three *On*Siglec-like proteins (similar to Siglec-1, -4b, and -14) were constructed. RAW264 cells were transiently transfected with the individual vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Monoclonal stable transformants were selected in the presence of G418. After confirming the transfection level by qPCR, cultured GBS was added to the *On*Siglec-like-transfected RAW264 cells at a cells:GBS ratio of 1:100. The cell mixture was then centrifuged at 1200 rpm for 5 min, incubated at 37 °C with 5% CO_2 for 15 min, and then washed to remove unbound GBS. Transfection of each *On*Siglec-like expression vector was performed in triplicate along with the control groups (untransfected RAW264 cells and uninfected *On*Siglec-1-like-transfected RAW264 cells). A density of 4×10^4 cells were obtained at 0, 6, and 24 h post infection (hpi) from each group for analysis.

2.3. Time-series analysis of cytokine expression after GBS infection of macrophage

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A nanodrop spectrophotometer was used to determine RNA concentration and purity (OD_{260}/OD_{280} = 1.8–2.0), and RNA integrity was assessed by agarose gel electrophoresis. The isolated RNA was then stored at −70 °C until use. The RNA was reverse transcribed into cDNA using a Reverse Transcription Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Briefly, a 20 μL reaction, containing 1 mg of total RNA, 1 μL of DNase I buffer (10×), 1 μL of DNase I, 0.5 μL of RNA inhibitor, and RNase Free H_2O, was mixed and incubated at 37 °C for 30 min. Then, 1 μL of ethylenediaminetetraacetic acid (EDTA) and 1 μL of oligo dT was added into the mixture and incubated at 65 °C for 10 min. After adding 4 μL of Reaction buffer, 0.5 μL of RNase Inhibitor, 2 μL of Deoxynucleotide Mix, and 0.5 μL of Transcriptor Reverse Transcriptase, the mixture was incubated at 25 °C for 10 min, 55 °C for 30 min and 85 °C for 5 min. The resulting cDNA was stored at −20 °C.

Specific primers (see Supplemental Table S2) were used to quantify gene expression of various inflammatory cytokines using qPCR. The PCR products were sequenced to confirm amplification specificity. The *GAPDH* gene was selected as the internal reference gene (Ando et al., 2008). Each 20 μL qPCR mixture contained: 10 μL of SYBR Green Realtime PCR Master Mix (Life Technologies, USA), 0.5 μL of the respective primer sets, 5 μL of cDNA (diluted 10-fold), and ddH_2O to a total volume of 20 μL. Each reaction was run in triplicate, and reactions without cDNA were used as the negative controls. Fluorescence quantitative analysis was performed using an ABI PRISM 7300 Fluorescence Quantitative PCR system (Life Technologies, USA) with the following conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 31 s, and 72 °C for 30 s. After amplification, the PCR products were heated to 60 °C to generate the melting curve for verification of amplification specificity. The data was analyzed with ABI7300 SDS V2.0 software. The software automatically set the baseline, threshold, and threshold cycle (Ct) values, and the results were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The relative expression of each gene is reported as the mean ± standard deviation (SD) of the triplicate experiments. Duncan’s significance tests were used to compare the differences of the means. $P < 0.05$ was considered statistically