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Pathogen-associated gene expression profiles in rainbow trout macrophages

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Abstract

Pathogens can be distinctively recognized by the cells of the immune system through interactions between the Pathogen-Associated Molecular Patterns (PAMPs) that they produce and the innate immune receptors of leukocytes. The present paper reports on the PAMP-modulated expression of a group of genes expressed in trout macrophages. The genes were identified in subtracted libraries from lipopolysaccharide (LPS)-stimulated macrophages and their expression was analyzed using quantitative real time PCR following stimulation of the cells with *E. coli* LPS, poly (I:C) and zymosan; representing Gram-negative bacteria, viruses and fungi, respectively. Genes (SPINT1L, DDIT4L, STEAP4, and TNFAIP3), the expression of which was induced by LPS and zymosan, were not significantly up-regulated by poly(I:C) and the opposite was found for transcripts (HMGB1 and PSMB9) up-regulated by poly(I:C). Overall, the differences in gene expression were greater at a later stage of macrophage activation (24 h) at a time when stimulation with poly(I:C) resulted in substantially different responses as compared to LPS and, to a lesser extent, zymosan.

Keywords: Rainbow trout; Macrophage; Pathogen-associated molecular patterns; Lipopolysaccharide; Zymosan; Poly(I:C); Gene expression

1. Introduction

Pathogens are "betrayed" to the innate immune system by substances (usually called pathogen-associated molecular patterns, PAMPs) that are vital for their homeostasis and are found only within certain microorganismal taxa but not in their multicellular hosts (Janeway, 1989). These include lipopolysaccharide (LPS), peptidoglycan, double-stranded RNA (dsRNA) and beta-glucans which are exclusively produced by Gram-negative and Gram-positive bacteria, viruses and fungi, respectively.

Innate immunity is able to detect PAMPs via a restricted number of genotypically encoded receptors (Janeway and Medzhitov, 2002) including toll-like receptors (TLRs) and intracellular molecules such as members of the nucleotide-binding oligomerization domain (NOD)-like receptors and the DexD/H box RNA helicases (Werts et al., 2006). The activation of these molecules, widely referred to as pattern recognition receptors (PRRs), triggers more or less overlapping intracellular signaling cascades and, eventually, a set of transcription factors including NFkB, AP-1 and IRF-1/3/5 and 7 (Kawai and Akira, 2005). Since the PRRs exhibit specificity towards concrete PAMPs, recognition of certain pathogens may elicit pathogen-specific reactions early during an infection. For example, activation of TLR2 (in heterodimers with TLR1 or TLR6) by lipoproteins or zymosan (Ozinsky et al., 2000; Takeuchi et al., 2001) and TLR5 by flagellin (Hayashi et al., 2001), will preferentially activate MAPK and NFkB signaling. As a result these bacterial PAMPs will induce the expression of antibacterial peptides, proinflammatory cytokines and enzymes involved in the production of mediators of inflammation. On the other hand, activation of TLR3, retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (Mda5) by double

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stranded RNA (or its analog poly(I:C)), results in IRF3/7-driven induction of type I interferon (IFN) which further activates STAT-1-dependent antiviral responses (Werts et al., 2006).

Although a number of PRRs have already been identified in non-mammalian vertebrates including birds and different fish species (Oshiumi et al., 2003; Jault et al., 2004; Iqbal et al., 2005), very few of them have been characterized functionally. These include chicken TLR7 and TLR2 homologs (Fukui et al., 2001; Philbin et al., 2005) that have been shown to recognize loxoribine and lipopeptides respectively; trout TLR5 which signals the presence of flagellin (Tsujita et al., 2004); and zebrafish TLR3 which reportedly is involved in the activation of NFkB (Phelan et al., 2005). In addition, the mRNA expression of specific fish TLRs is dynamically regulated in response to infections with virulent bacteria; for example, catfish TLR 3 and 5 following challenge with *Edwardsiella ictaluri* (Bilodeau and Waldbieser, 2005; Bilodeau et al., 2006).

It has been perceived that the PRRs, and the superfamily of the TLRs in particular, are both structurally and functionally well-conserved in vertebrates (Roach et al., 2005; Purcell et al., 2006). Notwithstanding, it appears that the response to certain PAMPs may be fundamentally different between vertebrate classes. Based exclusively on gene expression and in silico analyses, we have recently hypothesized that the TLR4-mediated recognition of endotoxin may be absent in fish and other non-mammalian vertebrates (Iliev et al., 2005b).

In the current work we have sought to further evaluate the capacity of primary rainbow trout mononuclear phagocytes (referred to as trout macrophages throughout the paper) to recognize and respond to different PAMPs with specific changes in gene expression. Trout macrophages respond to various PAMPs including poly(I:C) and zymosan (Iliev et al., 2005a,b). In addition, despite the possibility that TLR4-mediated endotoxin recognition may be absent in fish, trout macrophages do mount a robust cytokine response to high levels of bacterial LPS that could result from stimulation via an alternative pathway and/or involve other components in the LPS preparation (Iliev et al., 2005b). The genes analyzed here and listed in Table 1, were identified in subtracted cDNA libraries of LPS-stimulated vs. non-stimulated cells and preliminary analysis suggested that they may be regulated upon macrophage activation. Their expression was analyzed in trout macrophages stimulated with LPS, poly(I:C) and zymosan for 6 and 24 h.

Generally, the transcripts (SPINT1L, DDIT4L, STEAP4, and TNFAIP3) up-regulated by LPS were also inducible by zymosan but not poly(I:C). Instead, poly(I:C) distinctively enhanced the expression of PSMB9 and HMGB1. The differences in the gene expression levels were much more pronounced after prolonged stimulation (24 h) suggesting that autocrine immune mediators may have contributed to the specificity of the gene expression profiles at the late stages of macrophage activation.

2. Materials and methods

2.1. Animals

Mature rainbow trout (*Oncorhynchus mykiss*) were purchased from Rushing Waters Fisheries (Palmyra, WI). The fish were held under natural photoperiods in tanks with flow-through water maintained at ~10 °C and were fed on commercial pellets.

2.2. Cell culture and stimulation

Rainbow trout macrophages were isolated, cultured and stimulated according to methods that have been previously described (MacKenzie et al., 2003; Goetz et al., 2004). The cells were plated either on 100 mm cell culture dishes at 1.5×10^7 cells/dish or on 60 mm cell culture dishes at 5×10^6 cells/dish and were kept at 15 °C and 5% CO_2. Both types of dishes (Becton Dickinson) were coated with poly D-lysine. The cells were washed approximately 24 h after initial plating and supplied with fresh medium. The adherent cells were incubated for another 4 days before stimulation.

LPS from *E. coli* (serotype O26:B6, cat.# L-8274, 2.2% protein) and zymosan A from *Saccharomyces cerevisiae* (cat.# Z-4250), were purchased from Sigma-Aldrich. Polyinosine-polycytidylic acid (poly (I:C)) was purchased from InvivoGen (cat. code tlrl-pic).

To obtain RNA for the generation of subtracted libraries, cells cultured in 100 mm dishes were either left untreated or were stimulated with 10 μg/mL of LPS for 24 h prior to RNA extraction.

For real time SYBR Green QPCR analysis of gene expression, three experiments were conducted using cells from 3 fish. For each fish, separate experiments were conducted consisting of 8 treatments: a stimulant-free control, 10 μg/mL

Table 1
List of genes whose expression was analyzed using comparative SYBR green QPCR

Accession #	Best annotated hit (blastx)	Hit Accession #	% identity	Hit coverage	*E* value
DQ403259	PSMB9A (low molecular mass polypeptide complex subunit 2) [*Oncorhynchus mykiss*]	AAD28715	100%	27%	2.00E-26
DQ403260	ID2B (inhibitor of DNA binding/differentiation 2B) [*Oncorhynchus mykiss*]	AAX46288	100%	42%	4.00E-24
DQ400409	CSRP1 (Cysteine and glycine-rich protein 1) [*Danio rerio*]	AAH65956	78%	100%	3.00E-94
DQ399551	DUSP6 Dual specificity phosphatase 6) [*Danio rerio*]	AAH60937	93%	35%	5.00E-58
DQ403261	HMG-1 (high-mobility-group-1) [*Oncorhynchus mykiss*]	AAA58771	99%	70%	9.00E-80
DQ400411	PSTPIP2 (Proline-serine-threonine phosphatase-interacting protein 2) [*Mus musculus*]	AAH57654	61%	38%	9.00E-37
DQ400410	DDIT4L (DNA-damage-inducible transcript 4-like) [*Mus musculus*]	NP_084419	39%	100%	6.00E-18
DQ400414	TNFAIP3 (tumor necrosis factor, alpha-induced protein 3) [*Homo sapiens*]	CAH72937	68%	18%	2.00E-47
DQ400412	SPINT1L (Serine protease inhibitor, Kunitz type 1-like) [*Danio rerio*]	AAH53239	63%	20%	2.00E-32
DQ400413	STEAP4 (Tnfa-induced adipose-related protein) [*Mus musculus*]	AAH06651	55%	62%	4.00E-65