Fish & Shellfish Immunology 42 (2015) 473–482

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Production of inflammatory mediators and extracellular traps by carp macrophages and neutrophils in response to lipopolysaccharide and/or interferon-γ2

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ARTICLE INFO

Article history:
Received 23 June 2014
Received in revised form
13 November 2014
Accepted 14 November 2014
Available online 29 November 2014

Keywords:
Carp
Monocytes/macrophages
Neutrophilic granulocytes
Interferon gamma2
Extracellular traps

ABSTRACT

Neutrophilic granulocytes and macrophages are crucial for the innate immune response against infections. They migrate into the focus of inflammation, where they efficiently bind, engulf and kill bacteria by proteolytic enzymes, antimicrobial peptides, reactive oxygen (ROS) and nitrogen (RNS) species. Moreover, activated neutrophils and macrophages can form extracellular traps (ETs). Fish neutrophils and macrophages are morphologically, histochemically, and functionally similar to their mammalian counterparts, but their significance for regulation of inflammatory responses and pathogen killing needs further elucidation. We compared the activity of head kidney monocytes/macrophages and neutrophilic granulocytes of common carp and established that upon lipopolysaccharide stimulation, not only neutrophils, but also carp monocytes/macrophages release extracellular DNA and are capable to form macrophage extracellular traps (METs). To clarify whether many specific LPS functions reported for piscine phagocytes might be due to impurities in the commonly used LPS preparations we studied expression of inflammatory mediators, release of DNA, ROS and RNS in cells stimulated with LPS or its highly purified form (pLPS). Also IFN-γ2 stimulation and its synergism with LPS/pLPS in stimulating expression of pro-inflammatory mediators was studied. Results substantiate that a classical stimulation of TLR4 by LPS may indeed be absent in carp as most of the classically reported LPS effects are abolished or diminished when pLPS is used. Interestingly, we also observed a potent IL-10 expression in neutrophilic granulocytes upon LPS stimulation, which, apart from their pro-inflammatory function, clearly indicates a role in restrictive control of the inflammatory reaction.

1. Introduction

Neutrophilic granulocytes and macrophages are crucial for the innate immune response against bacterial, viral and fungal infections [1–3]. In response to pathogens and/or inflammatory mediators phagocytes migrate into the focus of inflammation, where they efficiently bind, engulf and kill bacteria by proteolytic enzymes, antimicrobial peptides, reactive oxygen (ROS) and nitrogen (RNS) species [4,5]. Moreover, activated neutrophils [6–11] and macrophages [12–17] can release extracellular traps (ETs) that consist of DNA, histones and protein components of cytoplasmic granules specific for a certain cell population, e.g. myeloperoxidase (MPO), neutrophil elastase (NE) and antimicrobial peptides like cathepsin G and lactoferrin for neutrophils [6] and cathelicidin-related antimicrobial peptide (CRAMP) for macrophages [12]. ETs are responsible for trapping and extracellular killing of bacteria, fungi and parasites [e.g. Refs. [6,18–23]]. Both cell populations are also actively involved in regulation of the inflammatory response through synthesis and release of pro- and anti-inflammatory mediators including cytokines and chemokines [24,25].

Fish neutrophils and macrophages are morphologically, histochemically, and functionally similar to their mammalian counterparts [26], but their differential significance for regulation of the inflammatory response as well as for pathogen killing has not yet been determined in detail. Both cell types display phagocytosis,

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http://dx.doi.org/10.1016/j.fsi.2014.11.019
1050-4648/

have an active respiratory burst to produce oxygen radicals/reactive oxygen species (ROS) and synthesize cytokines/chemokines [27,28]. Moreover, a potent bactericidal activity is found within both the macrophage and the neutrophil phagosome [29]. Recently it was shown by us and others that fish neutrophils, upon stimulation with PMA or with pathogen-associated molecular patterns (PAMPs) released Neutrophil Extracellular Traps (NETs) containing DNA and neutrophil elastase [30–32], that may be functional in bactericidal activity. Furthermore we provided evidence for ROS-dependent and independent mechanisms of NET formation in carp [30].

To detect PAMPs or damage-associated molecular patterns (DAMPs), phagocytes use pattern recognition receptors (PRRs) from which the Toll-like receptors (TLRs) are most studied. In mammals particular TLRs recognize a specific group of infectious agents [33,34]. Until now, 20 TLRs have been identified in teleost species [35–38]. The key structures of the TLRs and the crucial factors involved in the signaling cascade have high similarity to their mammalian counterparts although fish TLRs also show distinct features [39] and some of the piscine TLRs appear to have different ligand repertoires and a different functional role.

In mammals, lipolysaccharide (LPS) binding to TLR4 is one of the best understood PAMP–PRR interactions [40–42]. LPS is the main component of the external layer of the outer membrane of gram-negative bacteria that contributes to its structural integrity and protects the membrane from chemical attack [43,44]. It is a potent PAMP that stimulates various cell types, including granulocytes and macrophages, to secrete pro-inflammatory cytokines and numerous inflammatory mediators for an early innate and subsequent adaptive antibacterial responses [41,45]. TLR4 and its associated molecules (CD14, LY96 (MD-2) and TICAM2 (TRAM)) are required for a TLR4-mediated response to LPS [40]. Also in fish, LPS stimulates phagocytes to produce pro- and anti-inflammatory cytokines/chemokines and NO. However, in fish LPS may act in other ways to mediate pro-inflammatory cytokine expression [46], as indicated by their high resistance to the toxic effect of LPS [47]. In fish LPS induces an immune response at concentrations 1000-fold higher than those commonly found in mammals (μg/ml vs. ng/ml) [48]. Moreover, most fish species are devoid of a TLR receptor with a sequence similar to mammalian TLR4 [49], whereas in zebrafish and carp, which posses TLR4, it cannot recognize LPS and has no capability to induce downstream signaling [50], probably due to the absence of the accessory molecules [39,51]. This raises questions about the specific receptors involved in a classical LPS stimulation. Moreover the extensive literature in mammals that reports LPS induced effects may be significantly biased by the fact that in the majority of cases the LPS used contains a considerable contamination with e.g. peptidoglycans that may cause a significant stimulation via TLR2 receptors [52,53]. The low LPS sensitivity in teleosts, combined with the lack of TLR4 or the accessory molecules, suggests that many of the reported effects of LPS as well as the potent synergistic effect with IFN-γ may have been evoked by contaminating factors.

This study extends our specific knowledge on selective activation and function of carp macrophages and granulocytes with regard to expression of pro- and anti-inflammatory mediators, extracellular trap formation, ROS production and NO or lysozyme release. To clarify the issue of the LPS effects we have stimulated purified macrophage- and granulocyte fractions with commonly used *E. coli* LPS or with its highly purified form, alone or in combination with recombinant carp IFN-γ2 (rc IFN-γ2). Indeed a differential stimulation could be observed and significant differences were noted with the use of both LPS preparations indicating major effects of the contaminating factors in the LPS preparations via multiple TLR receptors.

2. Materials and methods

2.1. Animals

Common carp (*Cyprinus carpio* L.) (50–60 g b.w.), from the Department of Ichthyobiology and Aquaculture, Polish Academy of Science, Golysz, Poland were reared at 20 °C in recirculating tap water [54]. Animals were anaesthetized with tricaine methane sulphonate (TMS; Sigma–Aldrich, St.Louis, MO; 0.2 g/l) buffered with $NaHCO_3$ (POCH, Gliwice, Poland; 0.4 g/l) and bled through puncture of the caudal vein using a heparinised syringe. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the appropriate committee.

2.2. Cell isolation and in vitro culture

Head kidney cell suspensions were obtained by passing the tissue through a 50 μm nylon mesh with carp RPMI (cRPMI), (RPMI 1640, Invitrogen, Carlsbad, CA, adjusted to carp osmolarity of 270 mOsm/kg with distilled water) containing 10 IU/ml heparin (Leo Pharmaceutical Products Ltd., Weesp, the Netherlands) and washed once. This cell suspension was layered on a discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient (1.020, 1.070, and 1.083 g cm^{-3}) to retrieve enriched populations of monocytes/macrophages (1.070 g cm^{-3}) and neutrophilic granulocytes (1.083 g cm^{-3}) [29] and centrifuged for 30 min at 800 × g with the brake disengaged. Cells were collected, washed, and seeded at 5×10^6 cells/ml in cell culture plates at 27 °C, 5% CO_2 in complete $cRPMI^{++}$ (cRPMI supplemented with 0.5% (v/v) pooled carp serum, 1% L-glutamine (Merck, Whitehouse Station, NJ), 200 nM β-mercaptoethanol (Bio-Rad, Hercules, CA), 1% (v/v) penicillin G (Sigma–Aldrich, St. Louis, MO), and 1% (v/v) streptomycin sulphate (Sigma–Aldrich, St. Louis, MO). Cells were stimulated either with lipopolysaccharide (LPS, 50 μg/ml, *Escherichia coli* serotype O55: B5, Sigma–Aldrich, St. Louis, MO, L2880, purified by phenol extraction, impurities: <3% of protein), highly purified LPS (pLPS, 50 μg/ml, *E. coli* serotype O55: B5, Sigma–Aldrich, St. Louis, MO, L4524, purified by ion exchange chromatography, impurities: <1% of protein and <1% of RNA), recombinant carp interferon gamma2 (rcIFN-γ2, 10, 100 ng/ml) [55], the combination of LPS (50 μg/ml) and rcIFN-γ2 (10, 100 ng/ml) or with culture medium (control). Experiments were repeated for 3–5 independent fish. Additionally, release of nitric oxide was tested in culture medium of cells that were stimulated with different concentrations of LPS and pLPS (0.1–100 μg/ml).

2.3. Cell activity

2.3.1. Respiratory burst

The respiratory burst was measured with nitroblue tetrazolium (NBT) [56]. Neutrophilic granulocytes and monocytes/macrophages were stimulated 90 min in the presence of NBT (1 mg/ml, Sigma Aldrich, St. Louis, MO). Following incubation, the monolayers were rinsed and the reaction was stopped with methanol. The plates were washed 3 times in 70% ethanol, dried and 120 ml of 2 M potassium hydroxide and 140 ml of dimethyl sulphoxide were added to each well. The O.D. was recorded in an ELISA reader (ASYS Hitech GmbH) at 690 nm with a reference at 414 nm.

2.3.2. Formation of extracellular traps

The release of DNA from monocytes/macrophages and granulocytes in response to stimuli was determined as described