Molecular Immunology 48 (2011) 846–859

Contents lists available at ScienceDirect

Molecular Immunology

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

TLR agonists extend the functional lifespan of professional phagocytic granulocytes in the bony fish gilthead seabream and direct precursor differentiation towards the production of granulocytes

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ARTICLE INFO

Article history:
Received 18 November 2010
Received in revised form 9 December 2010
Accepted 11 December 2010
Available online 7 January 2011

Keywords:
Apoptosis
Cell signaling
Evolution
Fish
Neutrophil
Pathogen-associated molecular pattern

ABSTRACT

Neutrophils are major cells participants in innate host responses. They are short-lived leukocytes, although microbial products activate intracellular signaling cascades that prolong their survival by inhibiting constitutive apoptosis. To gain insight into the phylogeny of this important cell type, we examined the ability of toll-like receptor agonists to extend the lifespan of gilthead seabream (*Sparus aurata* L.) acidophilic granulocytes, which are the functional equivalent of mammalian neutrophils. The results obtained demonstrated that apoptosis was also the default state of seabream acidophilic granulocytes and that toll-like receptor agonists were able to dramatically extend their functional lifespan (up to 10 days) by inhibiting apoptosis and inducing a long lasting activation of phagocytic and respiratory burst activities, together with the expression of genes coding for several proinflammatory molecules. This process was independent on contaminating cells and interleukin-1β production. In addition, the results showed that p38 mitogen-activated protein kinase, but not nuclear factor κB, c-Jun terminal kinase or phosphatidylinositol 3-kinase, was involved in the inhibition of acidophilic granulocyte apoptosis following toll-like receptor engagement. Finally, stimulation of head kidney hematopoietic precursor cells with toll-like receptor agonists promoted their terminal differentiation to acidophilic granulocytes. These results demonstrated that the extension of neutrophil lifespan by microbial products is conserved in lower vertebrates although the magnitude of the response is much higher in fish.

1. Introduction

Neutrophils play an important role in innate host responses. They are short-lived leukocytes that show constitutive apoptosis, which is the mechanism involved in maintaining a normal level of circulating neutrophils and ensuring the rapid resolution of inflammation. Previous studies have shown that, during inflammation, the lifespan of neutrophils is extended by cytokines, growth factors and the activated endothelium (Colotta et al., 1992; Lee et al., 1993; Ginis and Faller, 1997; Watson et al., 1998; Coxon et al., 1999; Klein et al., 2000; Baumann et al., 2003; Cowburn et al., 2002; Wang et al., 2003; François et al., 2005; Marshall et al., 2007), which contributes to the persistence of inflammation. Microbial products activate intracellular signaling cascades, which prolong neutrophil survival by inhibiting constitutive neutrophil apoptosis (Colotta et al., 1992; Lee et al., 1993; Ginis and Faller, 1997; Watson et al., 1998; François et al., 2005; Marshall et al., 2007) Therefore, neutrophils have the capacity to regulate their survival by persisting in an activated state in the face on an acute challenge, but to regress and die once the challenge has been overcome. While microbial products inhibit apoptosis, and so permit neutrophils to successfully eradicate viable microorganisms, the final steps of this process, the phagocytosis of the organism, accelerate the programmed cell death of the neutrophil and the resolution of inflammation (Zhang et al., 2003).

Abbreviations: AGs, acidophilic granulocytes; CFSE, carboxyfluorescein acetate succinimidyl ester; CM, conditioned media; CSFR, colony-stimulating factor receptor; DHR, dihydrorhodamine 1,2,3; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal regulated kinase; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; GMPs, granulocyte/macrophage progenitors; HK, head kidney; HSCs, hematopoietic stem cells; Ig, immunoglobulin; IL-1β, interleukin-1β; JNK, c-Jun terminal kinase; LPS, lipopolysaccharide; MACS, magnetic-activated cell sorting; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; NF-κB, nuclear factor κB; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PAMPs, pathogen-associated molecular patterns; PRR, pattern recognition receptor; ROS, reactive oxygen species; SSC, side scatter; TCR, T cell receptor; TLR, toll-like receptors; TNFα, tumor necrosis factor α; *Va*DNA, genomic DNA from *Vibrio anguillarum*.

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doi:10.1016/j.molimm.2010.12.010

TLRs are the major cell surface pattern recognition receptors (PRRs) in the recognition of microbial components and the induction of immune responses (Akira and Takeda, 2004). Neutrophils express the full repertoire of toll-like receptors (TLRs), with the exception of TLR3 (Hayashi et al., 2003). Several intracellular signaling pathways have been proposed as being involved in the inhibition of neutrophil apoptosis by inflammatory mediators, including the nuclear factor κB (NF-κB), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPKs), and cAMP/protein kinase A signaling cascades (Frasch et al., 1998; Klein et al., 2000; Akgul et al., 2001; Webb et al., 2000; Cowburn et al., 2002, 2004; François et al., 2005). Nevertheless, the involvement of TLR activation in the survival of neutrophil is controversial. For example, it has been shown that monocyte contamination of neutrophil preparations may be important in the apparently direct response to lipopolysaccharide (LPS) or tumor necrosis factor α (TNFα) (Prince et al., 2004; Walmsley et al., 2004). Prolonged neutrophil survival was seen to be dependent on monocyte-released neutrophil survival factors. Furthermore, the antiapoptotic action of IL-1β, thought to be involved in neutrophil survival in response to LPS, is also mediated by peripheral blood mononuclear cell (PBMC) contaminants (Prince et al., 2004).

In fish, definitive hematopoiesis occurs in the head kidney, which is the larval and adult location for the production of all hematopoietic cell types and comparable to the bone marrow of mammals (Sepulcre et al., 2002). In the bony fish gilthead seabream (*Sparus aurata* L.), acidophilic granulocytes (AGs) are, at least numerically, the major cell type participating in innate host responses and the head kidney is the central immune organ that provides a source for AGs (Sepulcre et al., 2002; Chaves-Pozo et al., 2005). Similar physiological responses have been documented for mammalian neutrophils and seabream AGs: (i) they are the most abundant circulating granulocyte (Sepulcre et al., 2002); (ii) they show strong phagocytic and reactive oxygen species (ROS) production capabilities (Sepulcre et al., 2002, 2007); (iii) they produce cytokines in response to several immunological stimuli (Sepulcre et al., 2007; Chaves-Pozo et al., 2004); and (iv) they express a broad range of TLRs but seem to lack a functional TLR3, at least in resting conditions (Sepulcre et al., 2007). Therefore, AGs might be considered as functionally equivalent to mammalian neutrophils.

The aim of the current study was to gain further insight into gilthead seabream AG biology and to examine the ability of TLR agonist to extend their lifespan. The results demonstrated that apoptosis is the default state of seabream AGs, while TLR agonists are able to dramatically extend their functional lifespan by inhibiting apoptosis and inducing the long lasting expression of genes coding for several proinflammatory molecules. This process is independent of contaminating cells and IL-1β production. In addition, the results showed that p38 MAPK, but not NF-κB, c-Jun terminal kinase (JNK) or PI3K, is involved in the inhibition of AG apoptosis upon TLR engagement. Finally, the stimulation of head kidney hematopoietic precursor cells with TLR agonists promoted their terminal differentiation to AGs. These results demonstrated that the extension of the neutrophil lifespan by inflammatory mediators is conserved in lower vertebrates, highlighting the relevance of this process in neutrophil biology.

2. Materials and methods

2.1. Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*S. aurata*, Actinopterygii, Sparidae) were maintained at the Oceanographic Centre of Murcia (Spain) in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Trouvit, Burgos, Spain). Fish were fasted for 24 h before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (approval no. #333/2008) for the use of laboratory animals.

2.2. Isolation of phagocytes

AGs were obtained by magnetic-activated cell sorting (MACS) as described earlier (Roca et al., 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a mAb specific to gilthead seabream acidophilic granulocytes (G7) (Sepulcre et al., 2002), washed twice with phosphate buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma–Aldrich) and 5% fetal calf serum (FCS, Invitrogen) and then incubated with 100–200 μl per 10^8 cells of micro-magnetic-bead-conjugated anti-mouse immunoglobulin G (IgG) antibody (Miltenyi Biotec). After washing, $G7^+$ (AG) and AG-depleted cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al., 2006).

2.3. Cell culture and treatments

Head kidney leukocytes and isolated AGs were stimulated at 23 °C with 50 μg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (*Va*DNA), 100 ng/ml flagellin (Invivogen), 25 μg/ml poly I:C (Invivogen) or 10 μg/ml gelatin (Sigma–Aldrich) in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl] supplemented with 5% FCS and 100 I.U./ml penicillin and 100 μg/ml streptomycin (Biochrom). These concentrations of PAMPs and gelatin have been found to be optimal for *in vitro* activation of seabream AGs (Sepulcre et al., 2007; Castillo-Briceño et al., 2009).

In some experiments, cells were pretreated for 1 h with a rabbit monospecific antibody to gilthead seabream IL-1β (17 μg/ml) (López-Castejón et al., 2007), recombinant soluble type II IL-1 receptor from gilthead seabream produced in HEK-293 cells (1/10 dilution) (López-Castejón et al., 2007), the NF-κB inhibitors Bay 11-7085 (1 μM, Sigma–Aldrich) and NAI (6-amino-4-(4-phenoxyphenylethylamino) quinazoline, 10–100 nm, Calbiochem), the pan-caspase inhibitor Z-VAD-FMK (50 μM, Calbiochem), the p38 MAPK inhibitor SB220025 (1 and 10 μM, Sigma), the JNK inhibitor SP600125 (3 and 30 μM Sigma–Aldrich) or the PI3K inhibitor wortmannin (2 and 10 μM, Santa Cruz Biotechnology).

Conditioned media (CM) from control and 100 ng/ml flagellin-stimulated AG-depleted cell fractions were collected after overnight incubation, clarified with a 0.45 μm filter, and immediately added to AGs at a final dilution of 1/2.

2.4. Cell viability

Aliquots of cell suspensions were diluted in 200 μl PBS containing 40 μg/ml propidium iodide (PI). The number of red fluorescent cells (dead cells) from triplicate samples was analyzed by using flow cytometry (BD Biosciences).

2.5. Immunofluorescence staining and flow cytometry analysis

Aliquots of 10^5 cells were washed in PBS containing 2% FCS and 0.05% sodium azide (FACS buffer) and incubated for 20 min on ice with G7 (1/1,000) (Sepulcre et al., 2002) or commercial anti-IgM (1/100, Aquatic Diagnostic Ltd., Stirling, UK) mAbs. After washing,