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Research Article

The expression profile of *TLR9* mRNA and CpG ODNs immunostimulatory actions in the teleost gilthead seabream points to a major role of lymphocytes

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Abstract. The potential effects of synthetic unmethylated oligodeoxynucleotides (ODN) containing CpG motifs, mimicking bacterial DNA, has never been evaluated on the immune response in the teleost fish gilthead seabream (*Sparus aurata*), the most important fish species in Mediterranean aquaculture. First, binding and competition studies have demonstrated that binding is saturated and promiscuous, suggesting the participation of several receptors. Moreover, leucocyte cytotoxic (NCC) activity, production of ROIs (reactive oxygen intermediates), and expression of immune-relevant genes was greatly primed by ODNs. Focusing on the mechanism, the *TLR9* gene is widely distributed in seabream tissues and differently regulated *in vitro* by several stimuli. Moreover, and for the first time in fish, *TLR9* mRNA has been detected in lymphocytes as the main cell-source. To conclude, ODNs containing GACGTT, GTCGTT (optimal for mouse and human, respectively) or AACGTT motifs are the most potent inducers of seabream immunity, whilst the involvement of TLR9 is under debate.

Keywords. CpG ODN, TLR9, NCC, innate immune system, gilthead seabream.

Introduction

Bacterial DNA, in sharp contrast to eukaryotic DNA, is a pathogen-associated molecular pattern (PAMP) that strongly primes the immune system [1, 2]. This difference resides in the presence of unmethylated cytosine-phosphodiester-guanosine (CpG) motifs within the bacterial genomic DNA, which appear in lower numbers and are methylated in eukaryotic cells [2, 3]. Thus, bacterial DNA or agonist synthetic oligodeoxynucleotides (ODNs) containing CpG motifs can directly or indirectly induce the production of reactive oxygen intermediaries (ROIs), proliferation, increase antigen presentation by major histocompatibility complex (MHC) class I and II and up-regulate the expression at the gene or protein level of co-stimulatory molecules (B7–1, B7–2, CD80, CD83, etc.), immunoglobulins (IgG, IgM), Fc receptors and a wide range of cytokines (interleukin (IL)-1, IL-6, IL-10, IL-12, IL-15, interferon (IFN)α, IFNγ or tumor necrosis factor (TNF)α) [1, 2, 4, 5] by dendritic cells, macrophages, natural killer (NK) cells, B and T

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lymphocytes, monocytes, granulocytes or even mast cells. Moreover, ODNs may act as strong adjuvants, increasing the B- and T cell response to antigens, and increasing protection from pathogenic bacteria, protozoa and viruses in mammalian species such as human, mouse, sheep, chicken or monkey [5–7]. The biological activity of bacterial DNA or synthetic ODNs starts by binding to a pattern recognition receptor (PRR) defined within the Toll-like receptor (TLR) family, TLR9, since *TLR9*-deficient mice showed responsiveness to CpG motifs [8]. TLR9 receptor is not only present on the cell-surface or in endosomal compartments of B lymphocytes and dendritic cells but also in primed fibroblasts, monocytes, macrophages, granulocytes and NK cells [4, 5, 9–12]. However, the presence and activation of Fc and scavenger receptors might also be involved in ODN and bacterial DNA recognition and leucocyte activation in cooperation with TLR9 [13, 14]. Although the mechanisms leading to the activation of the immune system are fairly well known, more effort is now focusing on the potential use of ODNs as vaccine adjuvants in humans and veterinary-relevant species, including studies dealing with how to improve their lifetime and immunostimulatory activity. Aquaculture has become an important industry in the supply of fish of sufficient quality and quantity for human consumption to replace wild-fishing. Intensive culture of fish, however, has led to health and disease problems that still need to be overcome. In this way, the use of immunostimulants as effective preventive tools for fish farming success has demonstrated many benefits [15]. Among the proposed substances, ODNs containing unmethylated GpG motifs have been demonstrated to produce good immunostimulatory effects *in vitro* and *in vivo* in teleost fish [16, 17]. Thus, ODN treatment increased leucocyte proliferation, ROI production, chemotaxis, non-specific cytotoxic cell (NCC), bactericidal, antiviral and IFN-like activities, as well as the expression of several immune-relevant genes and increased the disease resistance to some pathogens in several important fish-farmed species such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), chinook salmon (*Oncorhynchus tshawytscha*), catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idellus*), goldfish (*Carassius auratus*), Japanese seabass (*Lateolabrax japonicus*) or flounder (*Paralichthys olivaceus*). Unfortunately, very little is known about the mechanisms involved in fish leucocyte recognition and activation by ODNs. First, fish orthologs of the *TLR9* gene have been found in some fish species (zebrafish *Danio rerio*, fugu *Takifugu rubripes*, pufferfish *Tetraodon nigroviridis*, flounder *Paralichthys olivaceus* or gilthead seabream *Sparus aurata*) but very little is known about fish TLR9 regulation and function [18–20]. More importantly, the best immunostimulatory CpG motifs for the human (GTCGTT) and mouse (GACGTT) might not work for fish, since they tend to show species-specificity [2, 4, 5, 16, 17]. Strikingly, non-CpG motifs (GTGCTT and AAGCTT) or methylated CpGs may be as immunostimulatory as those considered canonically so in fish [21, 22]. Thus, further studies are needed to deepen our knowledge of the best immunostimulatory ODNs for each teleost fish species and to elucidate the involvement of the TLR9 receptor in the activation pathway.

Although the immunostimulatory potential of ODNs has been evaluated in some fish-farmed species, no information exists on the gilthead seabream despite the fact that this is the most economically important marine farmed fish species in the Mediterranean area. In this sense, we have investigated the *in vitro* effects of several ODNs containing different CpG sequences and numbers on the main innate cellular immune parameters and gene expression of immune-relevant genes of seabream leucocytes. Moreover, in an attempt to shed some light on the mechanism at play in fish we also studied the binding of ODNs to seabream leucocytes and the regulation of the *TLR9* gene.

Materials and methods

Animals. Twenty specimens (50–100 g body weight) of the sexually immature male seawater teleost gilthead seabream were obtained from CULMAREX S.A. (Murcia, Spain). Animals were kept in 450–500 l running seawater (28‰ salinity) aquaria at 20 ± 2 °C and with a 12 : 12 h light/dark photoperiod. They were fed daily with 2 g of a commercial pellet diet (Skretting, Spain) per fish. Animals were acclimated for 15 days prior to the experiments. The Bioethical Committee of the University of Murcia approved the studies carried out herein.

Leucocyte isolation and treatment with synthetic unmethylated CpG ODNs. Fish were anaesthetized (2-phenoxyethanol at 0.1 % v/v) and bled, and leucocytes of the head-kidney (HK), the main haemopoietic organ of fish, were isolated in sRPMI (RPMI-1640 culture medium (Gibco) supplemented by 0.35 % sodium chloride, 10 % foetal calf serum (FCS; Gibco), 100 i.u./ml penicillin (Flow) and 100 μg/ml streptomycin (Flow)) in sterile conditions, as described elsewhere [23]. HK leucocytes (HKLs) were adjusted to 10^7 cells/ml in sRPMI. Cell viability, assayed by the

Table 1. Phosphorothioate-backbone synthesized oligodeoxynucleotides used in this study.

Name	ODN sequence (5′→3′)	CpG motifs	Length
1668	TCCATGA***CG***TTCCTGATGCT	1	20
1826	TCCATGA***CG***TTCCTGA***CG***TT	2	20
D	AC***CG***ATAA***CG***TTGCCAA***CG***TTGGT	3	24
1670	AC***CG***ATAA***CG***TTGC***CG***GTGA***CG***	4	22
2006	T***CG***T***CG***TTTTGT***CG***TTTTGT***CG***TT	4	24
1668 m	TCCATGA***CmG***TTCCTGATGCT	1*	20

* Methylated ODN used as control. CpG motifs are in both italics and bold.

trypan blue exclusion test, was always higher than 95 %.

Synthetic unmethylated CpG ODNs (Table 1) were purchased from Eurogentec (Spain). They were synthesized with phosphorothioate (PS) backbone and resuspended in sterile phosphate buffer (PBS) at 1 mM. HK leucocytes were incubated in flat-bottomed 96-well microtiter plates (Nunc) with different ODNs and concentrations (0, 0.2, 5, 10 or 20 μM). Samples consisting of mixed ODNs were also included. Control samples consisted of leucocytes incubated with culture medium alone. As a positive control, phenol-extracted *Vibrio anguillarum* R-82 (Va) genomic DNA (50 μg/ml) was used. Negative controls were incubated with methylated CpG ODN 1668 (1668 m), calf thymus genomic DNA (Sigma) (50 μg/ml) or DNase I-treated bacterial DNA. Samples were incubated for 24 h at 22 °C in an atmosphere with 85 % relative humidity and 5 % CO_2.

ODNs binding to leucocytes and competition. We performed a series of experiments to evaluate the involvement of one or several membrane receptors involved in the binding and internalization of the ODNs by seabream leucocytes. For ODN binding assays, fluorescein isothiocyanate (FITC)-labeled CpG ODNs 1668 and D (Sigma) (serial dilutions from 0 to 160 μM) were incubated with 10^5 HKLs for 90 min at 4 °C. After two washes, HKLs were analyzed in a flow cytometer (Becton Dickinson) and the percentage of R1 and R2 HKL populations positive for each FITC-labeled ODN determined. For cold competition assays, 10^5 HKLs were incubated with 5-fold excess (125 or 300 μM) of different ODNs or DNAs for 90 min at 4 °C. After washing, HKLs were incubated with 25 or 60 μM of FITC-ODN 1668 or FITC-ODN D, respectively, as above. Samples were then analyzed by flow cytometry and the percentage of binding inhibition determined as the decrease in total binding.

Cytotoxic or NCC activity. The NCC activity of gilthead seabream (which is mediated by a heterogeneous population consisting of lymphocytes, acidophilic granulocytes and macrophages – in order of importance – and are functionally equivalent to mammalian NK cells) was evaluated using a flow cytometry technique based on double-fluorescent labelling [23]. Briefly, tumor target cells from the L-1210 line (mouse lymphoma, ATCC CCL-219) in exponential growth were labelled with 10 μg/ml of 3,3′dioctadecyloxacarbocyanine perchlorate (DiO, Sigma) for 1 h in darkness. After labelling, free DiO was removed by washing three times in PBS and cell-staining uniformity was examined by flow cytometry. HKLs (effectors) incubated with ODNs or DNAs were mixed with DiO-labelled L-1210 cells (targets) (effector:target ratio of 50:1). The samples were centrifuged (400 g, 1 min, 22 °C) and incubated at 22 °C for 3 h. Cytotoxic samples incubated for zero h (control) were used to determine initial target viability. After incubation, 30 μl of propidium iodide (400 μg/ml, Sigma) were added and all samples were analysed in a flow cytometer set to accept the positive FL1 region, which corresponds to DiO-labelled target cells ($FL1^+FL2^-$). The percentage of dead or non-viable target cells showing green and red fluorescence ($FL1^+FL2^+$) was related to the cytotoxic activity of gilthead seabream leucocytes. Cytotoxic activity, a parameter describing the percentage of non-viable target cells, was calculated by the formula:

$$\text{Cytotoxic activity (\%)} = 100 \times (\%_{\text{sample}} - \%_{\text{control}})/(100 - \%_{\text{control}}).$$

ROI production. The ROI production or respiratory burst activity of gilthead seabream HK leucocytes was studied by a chemiluminescence method [24]. Briefly, 100 μl of Hank′s balanced salt solution (HBSS) containing 1 μg/ml phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) were added to the samples. The plate was shaken and immediately read in a plate reader for 1 h at 2 min intervals. The kinetics of the reactions were analysed and the maximum slope of each curve calculated. Backgrounds of lumines-

Table 2. Gene and primers used for real-time PCR experiments.

Gene name	Acc. number	Primer sequence (5′→3′)
18S	AY587263	Fw CGAAAGCATTTGCCAAGAAT Rev AGTTGGCACCGTTTATGGTC
IL-1β	AJ277166	Fw GGGCTGAACAACAGCACTCTC Rev TTAACACTCTCCACCCTCCA
TNFα	AJ413189	Fw TCGTTCAGAGTCTCCTGCAG Rev TCGCGCTACTCAGAGTCCATG
COX-2	AM296029	Fw GAGTACTGGAAGCCGAGCAC Rev GATATCACTGCCGCCTGAGT
TLR9	AY751798	Fw GGAGGAGAGGGACTGGATTC Rev GATCACACCGTTCACTGTCTC
TLR5	AM296028	Fw CCTGTCTGCAACTGTCAGGA Rev TGTGGATCTGGTTCAAGCTG
Hep	EF625900	Fw GCCATCGTGCTCACCTTTAT Rev CTGTTGCCATACCCCATCTT
MHCIIα	DQ019401	Fw CTGGACCAAGAACGGAAAGA Rev CATCCCAGATCCTGGTCAGT
IRF-1	AY962255	Fw ACAAACGGACCAAAGCAAAG Rev GAGTGTCGCTGTCCTCTTCC
Mx	AF491302	Fw AAGAGGAGGACGAGGAGGAG Rev TTCAGGTGCAGCATCAACTC
IgM_H	AM493677	Fw CAGCCTCGAGAAGTGGAAAC Rev GAGGTTGACCAGGTTGGTGT
NCCRP-1	AY651258	Fw ACTTCCTGCACCGACTCAAG Rev TAGGAGCTGGTTTTGGTTGG
CSF-1R	AM050293	Fw ACGTCTGGTCCTATGGCATC Rev AGTCTGGTTGGGACATCTGG

cence were calculated using reactant solutions containing luminol but not PMA.

Effect of ODN on leucocyte gene expression studied by real-time PCR. HKLs were incubated with 10 μM of the different ODNs or with 50 μg/ml of genomic DNAs for 24 h. Samples were washed twice at 4 °C and pooled for RNA isolation. Total RNA was isolated with TRIzol reagent (Invitrogen) following the manufacture's instructions. For this, 1 μg of total RNA was treated with DNAse I to remove any genomic DNA contamination, and the first strand of cDNA was synthesized by reverse transcription using the ThermoScript™ RNAse H- Reverse Transcriptase (Invitrogen) with an oligo-dT_{12-18} primer (Invitrogen) and finally treated with RNAse H (Invitrogen). Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 amplification cycles (15 s at 95 °C and 1 min at 60 °C) and a dissociation cycle (15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C). For each mRNA, gene expression was corrected by the expression of the ribosomal RNA 18S subunit in each sample. The primers used are shown in Table 2. In all cases, each PCR was repeated twice and confirmed.

Leucocyte sorting. Different leucocyte populations were purified using plastic adherence, MACS (magnetic activated cell sorting) and FACS (fluorescence-activated cell sorting), as previously described [25–28]. Macrophages (MM) were first separated by plastic adherence after incubation for 24 h at 25 °C in culture flasks in the absence of serum. Macrophage monolayers were carefully washed four times and used to obtain the RNA. Acidophilic granulocytes (AG) were separated by means of MACS. Briefly, 2×10^8 HK leucocytes were washed in MACS buffer (PBS with 2 mM EDTA and 0.5 % BSA), incubated with a house-produced D2 mAb specific for gilthead seabream acidophilic granulocytes [27, 28] for 40 min, washed twice and resuspended in MACS buffer. Cells were then incubated with 100 μl of paramagnetically labelled goat anti-mouse IgG (Miltenyi Biotec) for 15 min and washed again. Cells were separated using a MACS LS^+/VS^+ column according to the protocol. Both the cells passing through ($D2^-$) and remaining in the column ($D2^+$; AG) were collected. Lymphocytes (Ly) were then obtained from the $D2^-$ fraction by fluorescence-activated cell sorting (FACS) in an

EPICS ALTRA flow cytometer set to sort the cells with very low SSC and FSC parameters at a ratio of 300–800 cells/s. Leucocyte fractions were resuspended in TRIzol Reagent (Invitrogen) for total RNA isolation following the manufacturer's instructions. Each cell fraction (AG, Ly and MM) was analyzed by flow cytometry and gene expression for separation efficiency and leucocyte purity. Immunoglobulin M heavy chain (IgM_H) and colony-stimulating factor-1 receptor (*CSF-1R*) were used as B lymphocyte and monocyte-macrophages cellular markers, respectively.

Analysis of *TLR9* gene expression and regulation. *TLR9* gene expression was evaluated by real-time PCR (see above) in samples from resting seabream samples, sorted (Ly, AG and MM) leucocyte populations (see above) and *in vitro* stimulated seabream HKLs. Briefly, pooled brain, skin, liver, gut, gills, HK, spleen, thymus, peripheral blood (PBLs) or peritoneal exudate (PEL) leucocytes from three specimens as well as seabream fin tumor cells (SAF-1; ECACC-00122301; maintained at exponential growth in sRPMI culture medium) were obtained and kept in TRIzol Reagent for RNA isolation as previously described [25–28].

To study the *in vitro* regulation of *TLR9* gene expression, HKLs from three different fish (10^7 per fish) were incubated with: medium alone (controls), concanavalin A (ConA; 5 μg/ml; Sigma), lipopolysaccharide (LPS; 5 μg/ml; Sigma), ConA+LPS (5 and 10 μg/ml, respectively), phytohemagglutinin (PHA; 10 μg/ml; Sigma), poly I:C (25 μg/ml; Sigma), heat-killed *Vibrio anguillarum* R-82 (five bacterial cells per leucocyte) (pathogenic for seabream), heat-killed *Saccharomyces cerevisiae* S288C (two yeast cells per leucocyte), sonicated and RNase treated SAF-1 or L-1210 tumor cells (10 leucocytes per tumor cell). The mixed leucocyte reaction (MLR) was carried out by co-incubation of leucocytes from three specimens (10^7 leucocytes from each fish) in triplicate. After 4 h of incubation, leucocytes from the replicas were washed, pooled and the RNA obtained as described [25–28].

Statistical analysis. The data from the flow cytometric assays were analysed using the statistical option of the Lysis Software Package (Becton Dickinson). The data are presented as means±SE ($n=6$) of fold change relative to control samples incubated with medium alone. One-way analysis of variance (ANOVA; $P \leq 0.05$) was used as statistical analysis.

Results

ODNs differentially bind to seabream HKL populations. Gilthead seabream HKLs are able to bind FITC-labelled synthetic ODNs in a dose-dependent manner until saturation is reached (Fig. 1). According to previous morpho-functional studies, HKLs appear as two populations when analyzed by flow cytometry (Fig. 1a) where R1 is mostly formed by acidophilic granulocytes (AG) (the main phagocytic cells and ROI producers in this fish species and considered functional equivalents to mammalian neutrophils) and R2 is mostly formed by lymphocytes (Ly) but also with a minor presence of macrophages (MM) [25–30]. Flow cytometry data revealed that, in seabream, both populations bind ODNs in different percentages and with different intensities (Fig. 1b-e). Histograms show that leucocyte binding for ODN 1668 (optimal motif for mice GACGTT) is higher than for ODN D (containing one ACCGAT and two AACGTT motifs) and that the R1 population has more affinity and binding sites than the R2 population (Fig. 1f). Thus, we determined the ODN concentration producing 50 % leucocyte binding for each population and each ODN. The AG (R1) population showed values of 15.6 and 53.4 μM for ODN 1668 and ODN D, respectively. In contrast, the R2 population showed higher values of 31.2 and 73.8 μM, respectively. These data demonstrate that AG possesses more binding sites for ODNs than the Ly+MM population. However, within the same HKL population, cells reached the same percentage with either ODN 1668 or ODN D, even though they have one or three CpG motifs, respectively.

ODNs and DNA compete for leucocyte receptors. Cold competition assays were performed to evaluate the presence of one or several ODN-receptors in seabream HKLs as well as the importance of the number of CpG motifs (Fig. 2). Overall, most synthetic ODNs partly inhibited (from 10 to 50 %) labelled-ODN binding, suggesting that different receptors exist or that the sequence or number of CpG motifs is important in the binding process. Strikingly, cold competition with the homologous ODN failed to completely block the binding of the FITC-labeled ODN. Moreover, the methylated control ODN (1668 m) inhibited the binding of FITC-labeled ODN D by almost 80 %. While the calf genomic DNA slightly inhibited ODN binding, the addition of bacterial DNA (highly unmethylated) completely blocked the ODN D binding. These data, together with the binding curves, suggest that ODN binding is promiscuous with little dependence of the CpG sequence and number, which make very likely the

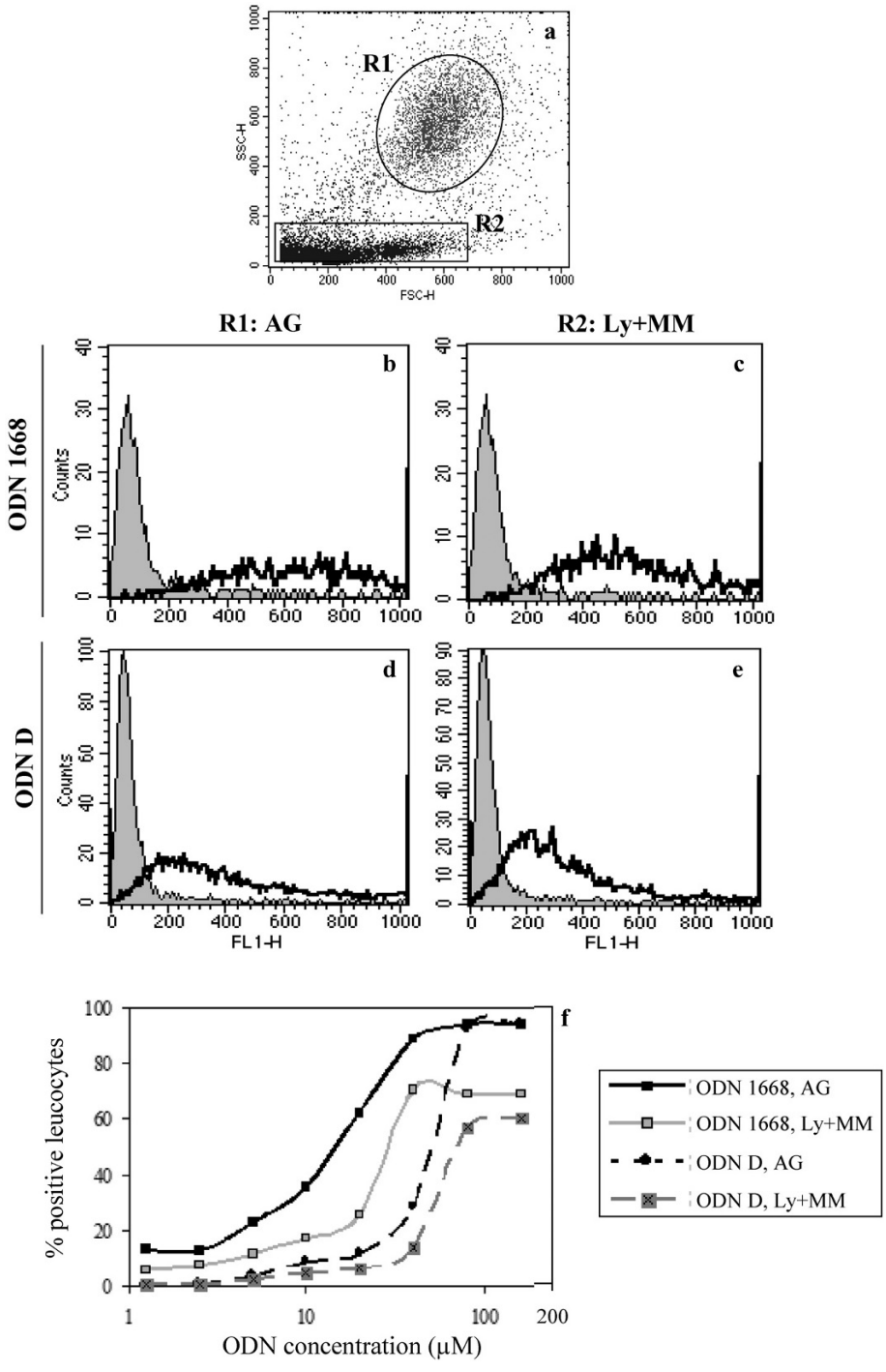

Figure 1. Synthetic ODNs bind to seabream head-kidney leucocytes. a. Representative dot-plot of seabream HKLs. b-e. Representative histograms showing ODN-binding to HKLs. Leucocytes were incubated with medium (grey) or 80 μM FITC-labeled ODNs (open) for 90 min at 4 °C. f. Percentage of HKLs positive for FITC-labeled ODNs.Experiments were independently repeated three times with similar results. AG, acidophilic granulocytes; Ly, lymphocytes; MM, macrophages.

involvement of several other receptors apart from TLR9.

ODNs greatly increase seabream innate immune responses. We next evaluated whether the main innate immunological parameters were affected by CpG ODNs in seabream HKLs. All the unmethylated ODNs and bacterial DNA were seen to strongly induce seabream immunity in a dose-dependent manner. We first found that the synthetic ODNs affect the NCC activity in a dose-dependent manner that differed from one ODN to another (Fig. 3). HKL incubation with ODNs for 24 h led to a very significant increase in NCC activity (up to 19-fold the control samples) while bacterial DNA only increased up to 3.5-fold. The best inducer was ODN 1670 (containing four different CpG motifs) followed by ODN D and ODN 1668. Negative controls consisting of methy-

lated ODN, calf DNA and digested DNA (not shown) failed to change this HKL activity. Respiratory burst activity or ROI production (Fig. 4) was also significantly increased by synthetic ODNs but not to such an extent as the NCC activity. Strikingly, ODNs 2006 (containing several copies of GTCGTT motif optimal for humans) failed to significantly increase seabream HKL production of ROIs. Moreover, ODN 1668 had the greatest effect on ROI production (up to 5.36-fold) and ODN 1826, containing two GACGTT, showed lower effects than the ODN containing only one motif (ODN 1668). As with the NCC, the mixture of ODNs failed to produce a better effect than the single ODNs. Supporting the idea that this leucocyte activation is unmethylated CpG ODN-dependent, negative controls were seen to have no effect on ROI production, whilst bacterial DNA increased production up to 17.3 ± 1.06 times compared with the leucocytes incubated with medium alone.

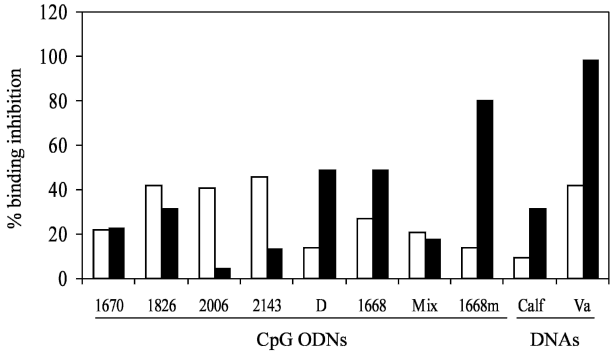

Figure 2. Binding competition of synthetic ODNs in seabream leucocytes. Head-kidney leucocytes were incubated for 90 min at 4 °C with 5-fold excess of different CpG ODNs or DNAs and further incubated with 25 or 60 μM of FITC-labeled ODN 1668 (white bars) or ODN D (black bars), respectively. Samples were analyzed by flow cytometry. Results are expressed as the leucocyte binding capacity inhibition compared to the total binding capacity of labeled-ODNs. Data are representative of three independent experiments. Va, *Vibrio anguillarum.*

HKL gene expression is up-regulated by synthetic ODNs. The expression of several genes important for the immune system was analyzed by real-time PCR. Thus, we analyzed the effects of HKL treatment with synthetic ODNs and DNAs for 24 h on the expression of genes (Fig. 5): related to inflammation (interleukin-1β (*IL-1β*), tumor necrosis factor-α (*TNFα*) and cyclooxygenase-2 (*COX-2*)), Toll-like receptors (*TLR9* and *LR5*), antimicrobial peptide (hepcidin; *hep*), major histocompatibility receptor class II (*MHCIIα*), interferon or Th1 response (*Mx* and interferon regulatory factor; *IRF-1*) as well as potential leucocyte markers (IgM_H for B lymphocytes, *NCCRP-1* for NCCs and *CSF-1R* for macrophages). All the genes were up-regulated in seabream HKLs by the ODNs, although the degree of up-regulation depended on the ODN used, whilst the use of the methylated CpG ODN, calf DNA or digested DNA

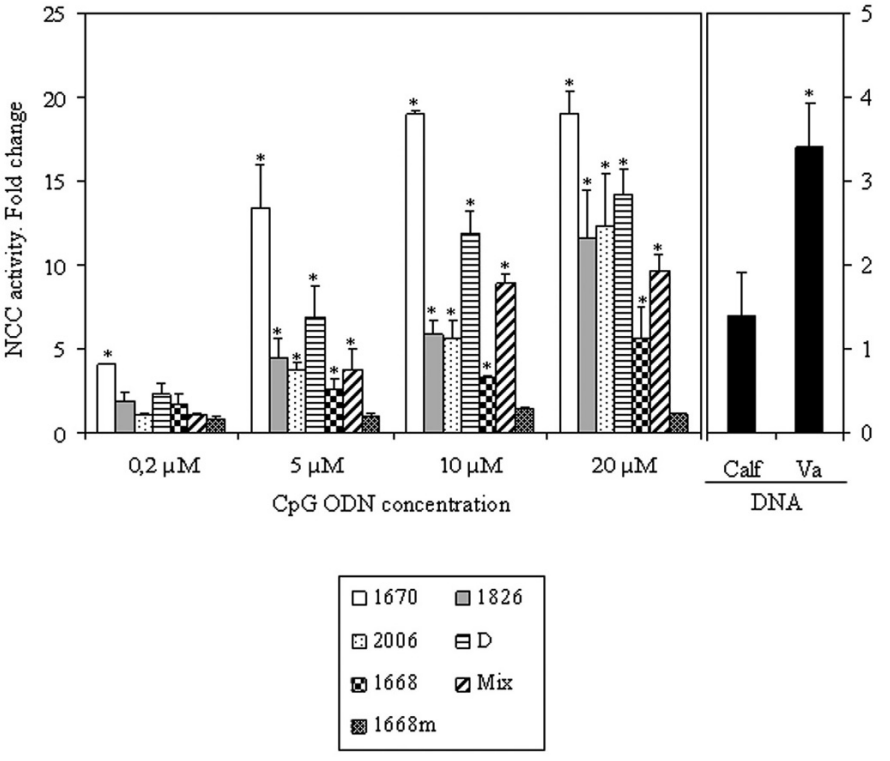

Figure 3. ODNs greatly prime seabream NCC (non-specific cytotoxic cell) activity. NCC activity of gilthead seabream head-kidney leucocytes incubated with synthetic CpG ODNs or DNAs for 24 h. Data represent mean ± SE (n = 6) fold change with respect to control samples. Asterisks denote statistically significant differences (ANOVA, $P \leq 0.05$) with respect to the control group. Va, *Vibrio anguillarum.*

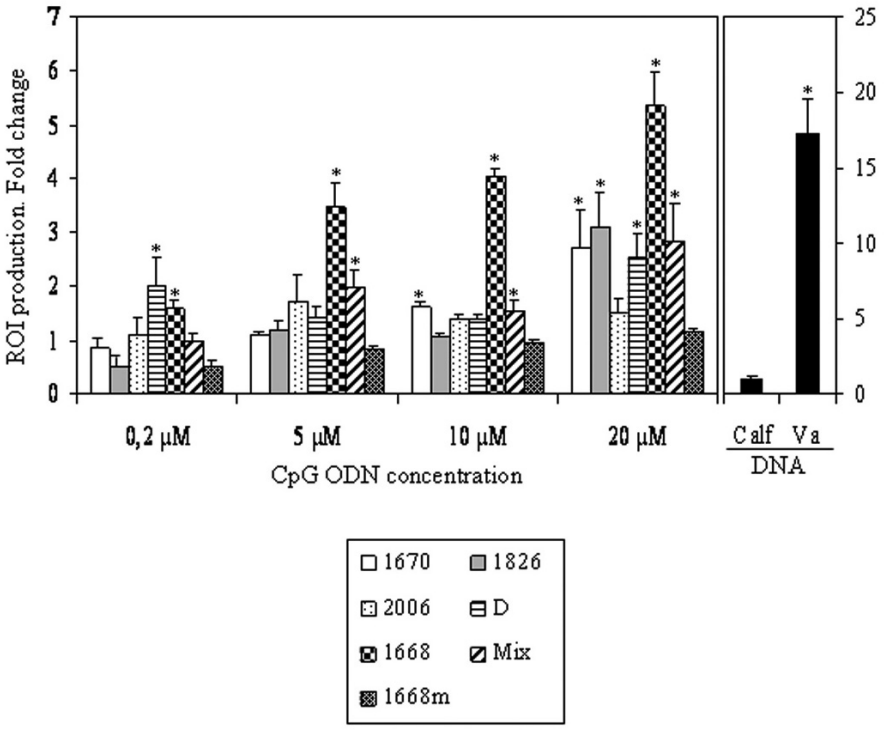

Figure 4. Seabream ROI (reactive oxygen intermediates) production is increased by ODNs. Respiratory burst activity of gilthead seabream head-kidney leucocytes incubated with synthetic CpG ODNs or DNA for 24 h. Data represent mean ± SE (n = 6) fold change with respect to control samples. Asterisks denote statistically significant differences (ANOVA, $P \leq 0.05$) with respect to the control group. Va, *Vibrio anguillarum*.

(data not shown) produced very few changes. ODN D significantly up-regulated the expression of all the assayed genes in treated HKLs and at the same time produced the greatest increase in transcript levels. One of the most surprising results is that the *TLR9* (specific for ODNs and bacterial DNA) gene was slightly up-regulated by ODN 2006 (optimal for human), ODN D and the ODN-mixture and by bacterial DNA, whilst the expression of *TLR5* (specific for flagelin) was greatly up-regulated, in sharp contrast with the observations in mammalian species [31]. These data, together with previous studies in our lab [32], suggest that, in fish, TLR9 is not exclusively involved in the ODN-activation pathway or that it is regulated at post-transcriptional level. Note that, while the ODN-combination failed to produce better innate cellular immune parameter effects than the individual ODNs, it had an additive effect on gene regulation. Among the genomic DNAs, calf DNA exerted little effect on immune gene expression while bacterial DNA significantly increased the expression of most genes except in the case of *Mx* and *CSF-1R*.

***TLR9* gene is mainly expressed in lymphocytes.** Taking in consideration binding properties, immunostimulation effects, and scarce effect on *TLR9* gene expression by synthetic ODNs, we were prompted to further investigate seabream *TLR9* gene expression and regulation by real-time PCR. In agreement with previous data [18], *TLR9* gene expression was evident in most tissues of resting seabream (Fig. 6a). Maximum transcript levels were found in PBLs and PELs, followed by gill, spleen and head-kidney. However, *TLR9* transcript showed lower expression in brain, gut, liver and thymus and was undetected in skin and SAF-1, a seabream tumor cell line. A subsequent study moved us to determine the leucocyte-types in which the *TLR9* gene is expressed in the HK. Thus, HK leucocytes purified by plastic adherence, MACS and FACS provided highly purified macrophages, acidophilic granulocytes and lymphocytes, respectively [25–28]. By means of real-time PCR we also detected the transcript in all the populations, although the highest expression was detected in the Ly fraction followed by MM and AG (Fig. 6b). These data agree with previous observations made in our lab, whereby AG and MM express the *TLR9* gene [32], but this is the first time in which *TLR9* mRNA has been found in lymphocytes and that they represent the main *TLR9* source, at least under resting conditions.

Finally, HKLs were primed for 4 h and then *TLR9* gene expression assayed (Fig. 6c). Whilst HKL incubation with the mitogens PHA and ConA up-regulated *TLR9* gene expression, treatment with LPS and ConA+LPS significantly down-regulated it. Interestingly, while leucocyte treatment with bacteria or yeast cells decreased the transcript levels, incubation with autologous cells (in MLR or tumor cells) provoked an

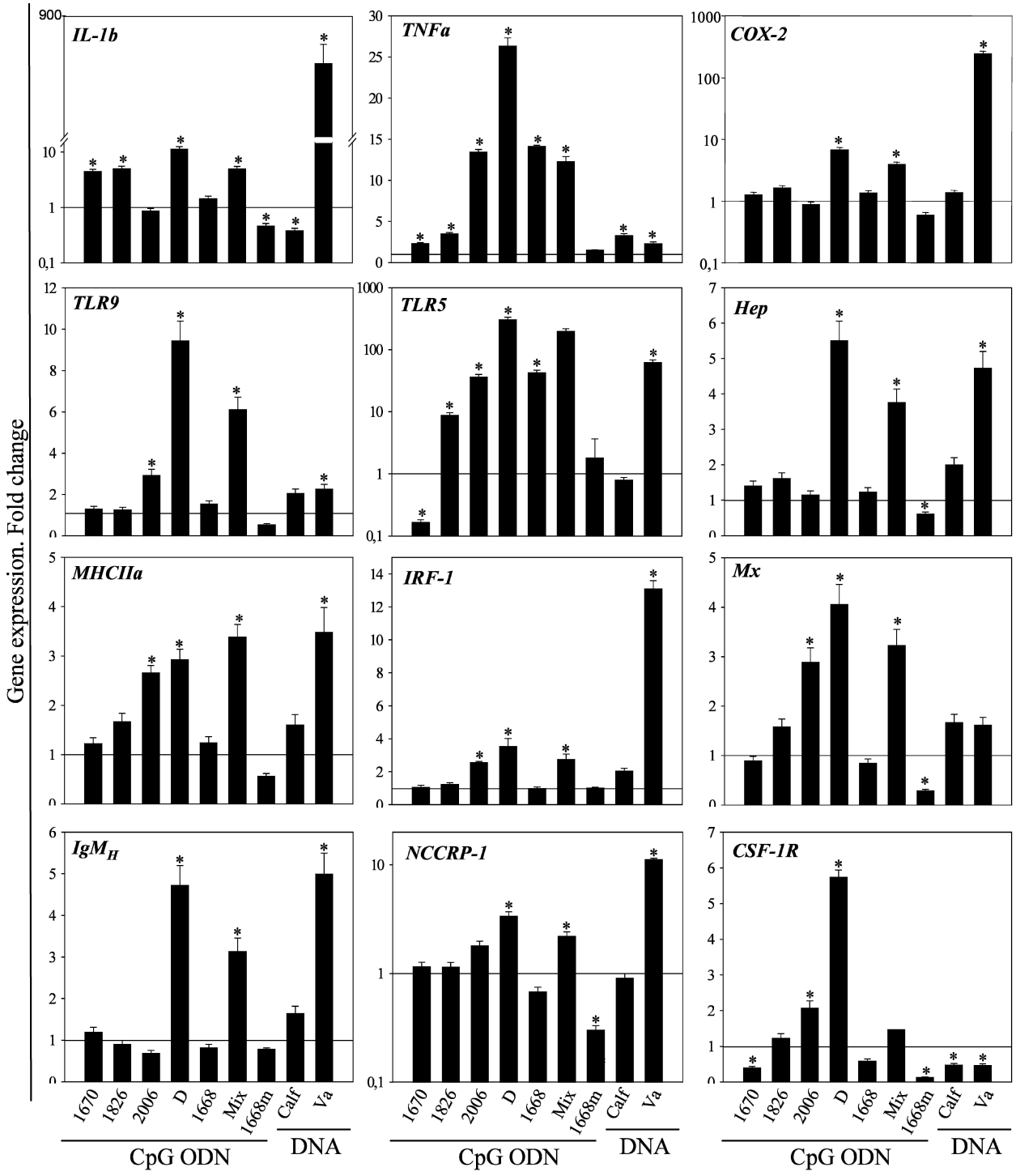

Figure 5. ODNs up-regulate leucocyte gene expression. Expression profile of immune-relevant genes after incubation of seabream head-kidney leucocytes with synthetic CpG ODNs (10 μM) or DNAs (50 μg/ml) for 24 h as determined by real-time PCR. Data represent mean ± SE of gene mRNA fold increase expression in stimulated leucocytes compared with control cells (horizontal line). Ribosomal *18S* gene was amplified as a house-keeping gene. Asterisks denote statistically significant differences (ANOVA, $P \leq 0.05$) with respect to the control group. Va, *Vibrio anguillarum.*

up-regulation of its expression. Strikingly, poly I:C or xenogeneic tumor cells failed to produce any change in the gene expression.

Discussion

Different classes of ODNs have been described according to their structure and function [2]. CpG-A

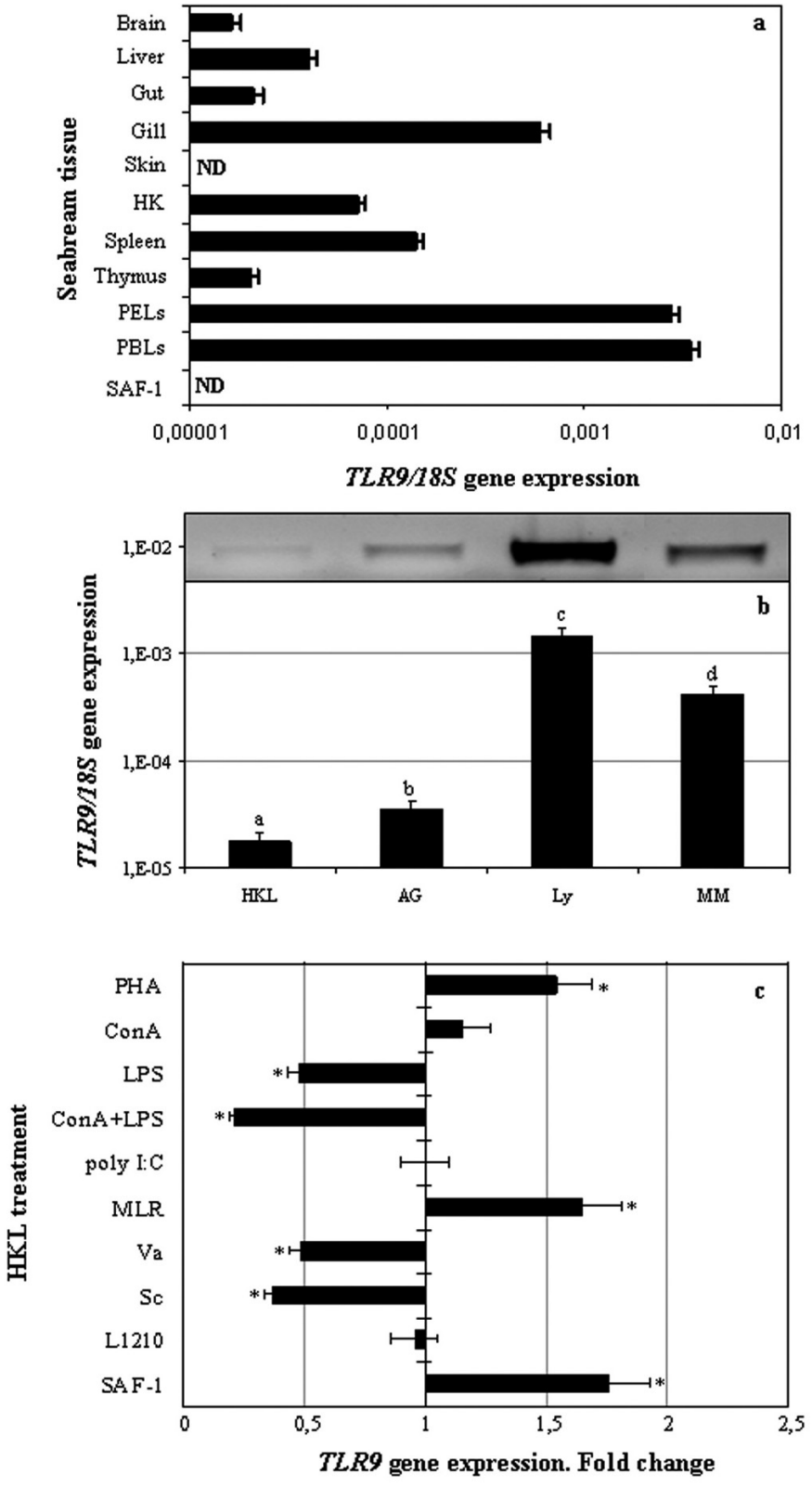

Figure 6. *TLR9* mRNA is mainly detected in lymphocytes. *TLR9* gene expression and regulation in gilthead seabream as determined by real-time PCR. *a. TLR9* transcript in different tissues from naïve seabream specimens. *b.* Gene expression in highly purified HK leucocyte populations separated by means of adherence, MACS and FACS. Products were also run in a 2% agarose gel, stained with ethidium bromide and visualized under UV light. Band corresponds to the 130-bp band of the *TLR9*-amplified sequence. Different letters stand for statistically significant differences (ANOVA, $P \leq 0.05$) and Tukey's comparison of means. *c.* Regulation of *TLR9* gene expression in HKLs treated *in vitro* for 4 h with mitogens, PAMPs and particulated antigens. In all samples, ribosomal *18S* gene was amplified as a house-keeping gene. In a and b, the relative expression of *TLR9* to the house-keeping gene (*TRL9/18S*) is presented. In c the fold change with respect to the control or untreated HKLs is shown and asterisks denote statistically significant differences (ANOVA, $P \leq 0.05$) with respect to control HKLs. ND, not detected; HK, head-kidney; PELs, peritoneal exudate leucocytes; PBLs, peripheral blood leucocytes; HKL, head-kidney leucocytes; Ly, lymphocytes; AG, acidophilic granulocytes; MM, macrophages; ConA, Concanavalin A; LPS, lipopolysaccharide; PHA, phytohemagglutinin; MLR, mixed leucocyte reaction; Va, *Vibrio anguillarum*; Sc, *Saccharomyces cerevisiae.*

ODNs (also named class D) are designed with a phosphodiester (PO) backbone and are very effective at activating dendritic cells, antigen presentation and NK cells (through the release of IFNα by dendritic cells). Modifications in the 5' and 3' ends with PS backbones containing poly G motifs produce greater immunostimulatory results and improve their lefetime. Class-B ODNs, completely designed with PS linkages that make them nuclease-resistant, have much stronger stimulatory effect on B lymphocytes but little on NK cells and the production of IFNα. Finally, C-class ODNs are also constructed on PS backbones and combine the properties and effects of both A and B-class ODNs, activating dendritic, B, and NK cells. However, the mixing of PO and PS linkages, the number and separation of CpG motifs, the species-specificity for CpG motifs, and the purpose of their therapeutic or preventive applications are important factors to be considered when designing synthetic ODNs for use in different animals.

In this study we chose different B-class ODNs to study, for the first time, their potential immunostimulatory actions and possible mechanisms used in the gilthead seabream, the economically most important marine species in Mediterranean aquaculture. Both ODNs bind to seabream leucocytes but AG presented higher binding (both rate and percentage) than lymphocytes and macrophages, suggesting that AG possesses either higher affinity for the GACGTT (ODN 1668) motif or more ODN-binding sites, but there is evidence demonstrating that ODN binds non-specifically to cell-surface proteins and not necessarily to TLR9 receptors [33]. This is confirmed by the finding that the highest *TLR9* gene source is in seabream lymphocytes. Moreover, CpG ODN and non-stimulatory ODN binds equally to mammalian leucocytes [2, 33], as occurs in catfish, where NCCs showed similar binding for ODNs containing poly G runs, CpG or GpC motifs [22]. With all this in mind, the higher level of ODN binding to seabream AG than to lymphocytes may simply be due to a greater size and not to the presence and abundance of ODN-receptors. This has been confirmed by competition-binding studies which show a decrease in binding of around 10–50 %, although this inhibition was greater when using the methylated control CpG ODN or bacterial DNA. These data, together with those for catfish NCCs [22], also support the findings that ODN binding and uptake is not CpG-sequence specific and mediated by TLR9 exclusively. For example, binding of poly G runs was partly (50 %) inhibited by homologous runs but not by CpG or bacterial DNA, whilst GpC binding was inhibited by all competitors (GpC, CpG, poly G runs, and bacterial DNA). The scarce information available on fish, only for catfish NCCs, has revealed the involvement, apart from TLR9, of scavenger receptors and other PRRs, as occurs in some mammals [22, 34–37]. Further studies are needed to ascertain the ODN binding properties and the receptor(s) involved in fish leucocytes.

To evaluate the immunostimulatory potential of synthetic ODNs for the first time in seabream, we studied their effect on NCC activity and on the production of ROIs. Seabream NCCs consist mainly of lymphocytes followed by acidophilic granulocytes and macrophages [23] and are considered evolutionary precursors of mammalian NK cell functions. HKL incubation with ODNs for 24 h led to a very significant increase in NCC activity (up to 19-fold the control samples) while bacterial DNA led only to an increase of up to 3.5-fold. In sharp contrast, only a single study has demonstrated the role of CpG motifs on catfish NCC activity, finding a 2-fold change [22], likely due to the PO backbone they used instead of the PS used in our study. Moreover, NCC activity of catfish was also activated with non-CpG motifs, especially with GpC motifs (AAGCTT and GTGCTT). These observations are in agreement with data demonstrating that bacterial DNA and synthetic ODN are useful in the prevention and treatment of tumor diseases and as adjuvants for cancer vaccines [2, 5] because of their ability to prime NK and CTL (cytotoxic T lymphocytes) lytic activity and NK-mediated IFNγ production, while CpG-treated mice depleted of NK and NKT cells failed to produce IFNγ [10]. Apart from the direct effect that synthetic ODNs may have on seabream NCCs, the additive/synergic effect of IFNs produced by ODN-stimulated leucocytes would justify such a great NCC stimulation. Unfortunately, there is no data concerning the role of IFNs in fish NCC activity. On the other hand, the ROI production (mediated by macrophages but mainly by acidophilic granulocytes) by seabream HKL was significantly enhanced by most ODNs but to a much lower degree than by bacterial DNA, in contrast to NCC activity. ROI production was also increased in common carp [38–40], grass carp [41], and olive flounder [42] after *in vivo* or *in vitro* treatment with several synthetic ODNs containing CpG motifs or even GpC motifs [42] or with plasmid containing multi-copy CpG motifs (90 in total) in goldfish and Japanese sea bass [43]. Interestingly, the ROI production increase in seabream was lower than the NCC, probably because lymphocytes are not directly involved in this phagocyte-related activity but are the main effectors in tumor-cell killing activity by NCCs. Overall, most results suggest that the CpG motif sequence (mainly flanking nucleotides) is much more important in activating fish immunity since an increase in the

number of CpG motifs is not necessarily correlated with a better immune response [6, 16, 41, 42, 44–47]. Afterwards, we investigated the effect of seabream HKL incubation with PS-backbone CpG motifs and bacterial DNA in the regulation of immune-relevant genes by real-time PCR for the first time. Only few works have found up-regulation of *IL-1β, IL-10, TNFα, COX-2, MHCIIβ, CXC* and *CC*-chemokines, *lysozyme*, *Mx* and *IFN* genes in fish treated *in vivo* or *in vitro* with ODNs containing CpG motifs by means of semi-quantitative PCR approaches [16, 38–40, 48–50]. In this work, only ODN D was able to increase the expression of all the assayed genes (*IL-1β, TNFα, COX-2; TLR9, TLR5, Hep, MHCIIα, IRF-1, Mx, IgM, NCCRP-1* and *CSF-1R*), followed by ODN 2006. These data, however, cannot be clearly correlated with leucocyte activities since ODN 1668, for example, was seen to be a strongly immunostimulatory CpG but failed to produce significant changes at gene level. Another important feature is that markers for B lymphocyte, NCCs and macrophages were also enhanced by ODNs. This fact could be due to leucocyte proliferation and may explain the increase in the synthesis of cytokines. Further work, however, is necessary in this line. It is worth noting that leucocyte incubation with the mixture of ODNs produced effects comparable to those of using single motifs, which was not the case for leucocyte functions. This means that gene expression in seabream was mainly increased by the motif GTCGTT or in the presence of several copies. Our data correlate quite well with gene expression after mammalian treatment with ODN, which up-regulates the transcript for antigen presentation, co-stimulatory molecules and cytokines (IL-1, IL-6, IL-10, IL-12, IL-15, IFNα, IFNγ or TNFα) [2, 4, 5, 9]. However, the use of heterogeneous leucocyte populations and the lack of detailed knowledge of the leucocyte types producing every cytokine, and their effects and mechanisms, hamper the elucidation of the main targets of ODNs in seabream.

Surprisingly, seabream HKL incubation with ODNs or bacterial DNA produced a significant up-regulation of *TLR9* gene expression whilst *TLR5*, specific for flagelin, was up-regulated to a greater extent. These data agree with previous results in seabream [32] and suggest that, in fish, the activation pathway using TLRs might not be as specific as in mammals [31]. This important observation led us to evaluate the presence and regulation of *TLR9* gene in an attempt to elucidate its involvement in leucocyte activation in seabream. In agreement with a previous study [18], gilthead seabream *TLR9* is widely detected in most tissues with major transcript levels in PELs and PBLs. Furthermore, we used a combination of strategies based on plastic adherence, MACS and FACS to obtain highly pure Ly, MM and AG populations [25–28]. Thus, and for the first time in fish, we found that the *TLR9* gene is mainly expressed in the lymphocytes of seabream, which contrasts with the low levels found in acidophilic granulocytes and macrophages. These data are in line with the tissue distribution of *TLR9*, since tissues with high numbers of lymphocytes (peripheral blood and spleen) and tissues with very active leucocytes (gill, but mainly peritoneal cavity) show the greatest expression levels. In mammals, the *TLR9* gene is mainly expressed in antigen-presenting cells, namely dendritic cells, and B lymphocytes [2], whilst in seabream the highest *TLR9* transcript is detected in lymphocytes, considering that the presence of dendritic cells in fish has not been demonstrated so far. This finding may somehow explain why seabream NCC activity is the most activated leucocyte function since it is mainly carried out by lymphocytes. Finally, *TLR9* gene expression of HKL is up-regulated *in vitro* in mixed leucocyte reactions and after incubation with PHA and SAF-1 tumor cells, but not with other mitogens, PAMPs and particulated antigens. In our group, moreover, incubation of purified AG or MM with several PAMPs, including bacterial DNA, showed that only MM were primed to increase *TLR9* gene expression whilst both leucocyte populations were primed to increase phagocytic activity, ROI production, and expression of *IL-1β, TNFα* or *COX-2* genes [32]. Although data are scarce in fish, *TLR9* gene expression was only studied and increased in olive flounder and zebrafish challenged with *Edwardsiella tarda* or *Mycobacterium maritinum*, respectively [19, 20] but reduced in seabream [18]. Further studies are mandatory to understand how the *TLR9* gene is regulated before correlation with TLR9-dependent actions mediated by bacterial DNA or synthetic ODNs as their agonists.

To conclude, seabream leucocytes binding to ODNs and competition studies show that the process is saturated and promiscuous and suggest the involvement of several kinds of receptors. Acting as immunostimulant, unmethylated ODN and bacterial DNA exert a very important effect on seabream HKLs NCC activity and ROI production. At the gene level, ODN treatment produced significant up-regulation of *IL-1β, TNFα, COX-2; TLR9, TLR5, Hep, MHCIIα, IRF-1, Mx, IgM, NCCRP-1* and *CSF-1R* genes differing with the ODN used. Strikingly, *TLR9* and *TLR5* gene expression patterns suggest that the TLR-pathways or regulation in fish could be different from mammals. Moreover, and for the first time in fish, *TLR9* expression has been detected in lymphocytes, showing much higher transcript levels than in acidophils and macrophages. The *TLR9* gene is also regulated by incubation with several mitogens, PAMPs, and par-

ticulated antigens. To end, from our results, we cannot unmistakably identify the best immunostimulatory CpG motif since different leucocytes show differences in binding and activation by ODNs. However, taking into consideration observations made in mammals and fish, the ODN sequence is a key factor when designing ODNs, although increasing the number of CpG motifs does not necessarily improve the immune response.

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1 Hacker, G., Redecke, V. and Hacker, H. (2002) Activation of the immune system by bacterial CpG-DNA. Immunology 105, 245–251.
2 Krieg, A.M. (2002) CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol 20, 709–760.
3 Yamamoto, S., Yamamoto, T., Shimada, S., Kuramoto, E., Yano, O., Kataoka, T. and Tokunaga, T. (1992) DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol. Immunol. 36, 983–997.
4 Ishii, K. J. and Akira, S. (2006) Innate immune recognition of, and regulation by, DNA. Trends Immunol. 27, 525–532.
5 Mutwiri, G., Pontarollo, R., Babiuk, S., Griebel, P., van Drunen Littel-van den Hurk, S., Mena, A., Tsang, C., Alcon, V., Nichani, A., Ioannou, X., Gomis, S., Townsend, H., Hecker, R., Potter, A. and Babiuk, L. A. (2003) Biological activity of immunostimulatory CpG DNA motifs in domestic animals. Vet. Immunol. Immunopathol. 91, 89–103.
6 Klinman, D. M., Currie, D., Gursel, I. and Verthelyi, D. (2004) Use of CpG oligodeoxynucleotides as immune adjuvants. Immunol. Rev. 199, 201–216.
7 Modlin, R. L. (2000) Immunology. A Toll for DNA vaccines. Nature 408, 659–660.
8 Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. Nature 408, 740–745.
9 Griebel, P. J., Brownlie, R., Manuja, A., Nichani, A., Mookherjee, N., Popowych, Y., Mutwiri, G., Hecker, R. and Babiuk, L. A. (2005) Bovine toll-like receptor 9: a comparative analysis of molecular structure, function and expression. Vet. Immunol. Immunopathol. 108, 11–16.
10 Tsujimoto, H., Ono, S., Matsumoto, A., Kawabata, T., Kinoshita, M., Majima, T., Hiraki, S., Seki, S., Moldawer, L. L. and Mochizuki, H. (2006) A critical role of CpG motifs in a murine peritonitis model by their binding to highly expressed toll-like receptor-9 on liver NKT cells. J. Hepatol. 45, 836–843.
11 Wagner, H. (1999) Bacterial CpG DNA activates immune cells to signal infectious danger. Adv. Immunol. 73, 329–368.
12 Fransson, M., Benson, M., Erjefalt, J. S., Jansson, L., Uddman, R., Bjornsson, S., Cardell, L. O. and Adner, M. (2007) Expression of Toll-like receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. Respir. Res. 8, 17.
13 Boule, M. W., Broughton, C., Mackay, F., Akira, S., Marshak-Rothstein, A. and Rifkin, I. R. (2004) Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. J. Exp. Med. 199, 1631–1640.
14 Gursel, M., Gursel, I., Mostowski, H. S. and Klinman, D. M. (2006) CXCL16 influences the nature and specificity of CpG-induced immune activation. J. Immunol. 177, 1575–1580.
15 Bricknell, I. and Dalmo, R. A. (2005) The use of immunostimulants in fish larval aquaculture. Fish Shellfish Immunol. 19, 457–472.
16 Carrington, A. C. and Secombes, C. J. (2006) A review of CpGs and their relevance to aquaculture. Vet. Immunol. Immunopathol. 112, 87–101.
17 Tassakka, A. C. and Sakai, M. (2005) Current research on the stimulatory effects of CpG oligodeoxynucleotides in fish. Aquaculture 246, 25–36.
18 Franch, R., Cardazzo, B., Antonello, J., Castagnaro, M., Patarnello, T. and Bargelloni, L. (2006) Full-length sequence and expression analysis of Toll-like receptor 9 in the gilthead seabream (*Sparus aurata* L.). Gene 378, 42–51.
19 Meijer, A. H., Gabby Krens, S. F., Medina Rodriguez, I. A., He, S., Bitter, W., Ewa Snaar-Jagalska, B. and Spaink, H. P. (2004) Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol. Immunol. 40, 773–783.
20 Takano, T., Kondo, H., Hirono, I., Endo, M., Saito-Taki, T. and Aoki, T. (2007) Molecular cloning and characterization of Toll-like receptor 9 in Japanese flounder, *Paralichthys olivaceus.* Mol. Immunol. 44, 1845–1853.
21 Jorgensen, J. B., Johansen, L. H., Steiro, K. and Johansen, A. (2003) CpG DNA induces protective antiviral immune responses in Atlantic salmon (*Salmo salar* L.). J. Virol. 77, 11471–11479.
22 Oumouna, M., Jaso-Friedmann, L. and Evans, D. L. (2002) Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: binding, specificity and identification of unique immunostimulatory motifs. Dev. Comp. Immunol. 26, 257–269.
23 Cuesta, A., Esteban, M. A. and Meseguer, J. (1999) Natural cytotoxic activity of gilthead seabream (*Sparus aurata* L.) leucocytes. Assessment by flow cytometry and microscopy. Vet. Immunol. Immunopathol. 71, 161–171.
24 Bayne, C. J. and Levy, S. (1991) Modulation of the oxidative burst in trout myeloid cells by adrenocorticotropic hormone and catecholamines: mechanisms of action. J. Leukoc. Biol. 50, 554–560.
25 Cuesta, A., Angeles Esteban, M. and Meseguer, J. (2006) Cloning, distribution and up-regulation of the teleost fish MHC class II alpha suggests a role for granulocytes as antigen-presenting cells. Mol. Immunol. 43, 1275–1285.
26 Cuesta, A., Esteban, M. A. and Meseguer, J. (2005) Molecular characterization of the nonspecific cytotoxic cell receptor (NCCRP-1) demonstrates gilthead seabream NCC heterogeneity. Dev. Comp. Immunol. 29, 637–650.
27 Cuesta, A., Meseguer, J. and Esteban, M. A. (2008) The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream. Mol. Immunol. 45, 2333–2342.
28 Meseguer, J., Esteban, M. A. and Cuesta, A. (2007) Generation and use of monoclonal antibodies as leucocyte-markers to study the immune function in teleost fish. J. Immunol. 178, B219.
29 Cuesta, A., Rodriguez, A., Salinas, I., Meseguer, J. and Esteban, M. A. (2007) Early local and systemic innate immune responses in the teleost gilthead seabream after intraperitoneal injection of whole yeast cells. Fish Shellfish Immunol. 22, 242–251.
30 Cuesta, A., Salinas, I., Rodriguez, A., Esteban, M. A. and Meseguer, J. (2006) Injection of xenogeneic cells into teleost fish elicits systemic and local cellular innate immune responses. Cell Tissue Res. 326, 93–99.
31 Werling, D. and Jungi, T. W. (2003) TOLL-like receptors linking innate and adaptive immune response. Vet. Immunol. Immunopathol. 91, 1–12.

32 Sepulcre, M. P., Lopez-Castejon, G., Meseguer, J. and Mulero, V. (2007) The activation of gilthead seabream professional phagocytes by different PAMPs underlines the behavioural diversity of the main innate immune cells of bony fish. Mol. Immunol. 44, 2009–2016.
33 Hu, Z., Sun, S. and Zhou, F. (2003) The binding of CpG-oligodeoxynucleotides to cell-surface and its immunostimulatory activity are modulated by extracellular acidic pH. Vaccine 21, 485–490.
34 Biessen, E. A., Vietsch, H., Kuiper, J., Bijsterbosch, M. K. and Berkel, T. J. (1998) Liver uptake of phosphodiester oligodeoxynucleotides is mediated by scavenger receptors. Mol. Pharmacol. 53, 262–269.
35 Kaur, H., Jaso-Friedmann, L. and Evans, D. L. (2003) Identification of a scavenger receptor homologue on nonspecific cytotoxic cells and evidence for binding to oligodeoxyguanosine. Fish Shellfish Immunol. 15, 169–181.
36 Kaur, H., Jaso-Friedmann, L., Leary, J. H., and Evans, D. L. (2004) Single-base oligodeoxyguanosine-binding proteins on nonspecific cytotoxic cells: identification of a new class of pattern-recognition receptors. Scand. J. Immunol. 60, 238–248.
37 Lipford, G. B., Bendigs, S., Heeg, K. and Wagner, H. (2000) Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG-DNA affect T-cell activation via antigen-presenting cell-derived cytokines. Immunology 101, 46–52.
38 Tassakka, A. C. and Sakai, M. (2002) CpG oligodeoxynucleotides enhance the non-specific immune responses on carp, *Cyprinus carpio.* Aquaculture 209, 1–10.
39 Tassakka, A. C. and Sakai, M. (2003) The in vitro effect of CpG ODNs on the innate immune response of common carp, *Cyprinus carpio* L. Aquaculture 220, 27–36.
40 Tassakka, A. C. and Sakai, M. (2004) Expression of immune-related genes in the common carp (*Cyprinus carpio* L.) after stimulation by CpG oligodeoxynucleotides. Aquaculture 242, 1–12.
41 Meng, Z., Shao, J. and Xiang, L. (2003) CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. Dev. Comp. Immunol. 27, 313–321.
42 Lee, C. H., Jeong, H. D., Chung, J. K., Lee, H. H. and Kim, K. H. (2003) CpG motif in synthetic ODN primes respiratory burst of olive flounder *Paralichthys olivaceus* phagocytes and enhances protection against *Edwardsiella tarda.* Dis. Aquat. Organ. 56, 43–48.
43 Chen, Y., Xiang, L. X. and Shao, J. Z. (2007) Construction of a recombinant plasmid containing multi-copy CpG motifs and its effects on the innate immune responses of aquatic animals. Fish Shellfish Immunol. 23, 589–600.
44 Carrington, A. C., Collet, B., Holland, J. W. and Secombes, C. J. (2004) CpG oligodeoxynucleotides stimulate immune cell proliferation but not specific antibody production in rainbow trout (*Oncorhynchus mykiss*). Vet. Immunol. Immunopathol. 101, 211–222.
45 Kanellos, T. S., Sylvester, I. D., Butler, V. L., Ambali, A. G., Partidos, C. D., Hamblin, A. S. and Russell, P. H. (1999) Mammalian granulocyte-macrophage colony-stimulating factor and some CpG motifs have an effect on the immunogenicity of DNA and subunit vaccines in fish. Immunology 96, 507–510.
46 Rhodes, L. D., Rathbone, C. K., Corbett, S. C., Harrell, L. W. and Strom, M. S. (2004) Efficacy of cellular vaccines and genetic adjuvants against bacterial kidney disease in chinook salmon (*Oncorhynchus tshawytscha*). Fish Shellfish Immunol. 16, 461–474.
47 Shen, K. Y., Hogstrand, C. and Davies, D. H. (2006) A newly identified CpG oligodeoxynucleotide motif that stimulates rainbow trout (*Oncorhynchus mykiss*) immune cells to produce immunomodulatory factors. Dev. Comp. Immunol. 30, 311–324.
48 Jorgensen, J. B., Zou, J., Johansen, A. and Secombes, C. J. (2001) Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1beta and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. Fish Shellfish Immunol. 11, 673–682.
49 Pedersen, G. M., Johansen, A., Olsen, R. L. and Jorgensen, J. B. (2006) Stimulation of type I IFN activity in Atlantic salmon (*Salmo salar* L.) leukocytes: synergistic effects of cationic proteins and CpG ODN. Fish Shellfish Immunol. 20, 503–518.
50 Tassakka, A. C., Savan, R., Watanuki, H. and Sakai, M. (2006) The in vitro effects of CpG oligodeoxynucleotides on the expression of cytokine genes in the common carp (*Cyprinus carpio* L.) head kidney cells. Vet. Immunol. Immunopathol. 110, 79–85.