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*J Immunol* 2010; 185:3873-3883; Prepublished online 8 September 2010;
doi: 10.4049/jimmunol.0902306
http://www.jimmunol.org/content/185/7/3873

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/09/07/jimmunol.0902306.DC1

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Prolactin-Induced Production of Reactive Oxygen Species and IL-1β in Leukocytes from the Bony Fish Gilthead Seabream Involves Jak/Stat and NF-κB Signaling Pathways

Víctor H. Olavarriá,*† María P. Sepulcre, † Jaime E. Figueroa,* and Victoriano Mulero†

Prolactin (PRL), a peptide hormone produced by the pituitary gland, was shown to play an important role in the modulation of the immune system of lower and higher vertebrates. To further investigate the effects of PRL on the activation of professional phagocytes of bony fish, we stimulated head kidney leukocytes and purified macrophages from the gilthead seabream (Sparus aurata L.) with various physiological concentrations of native salmon PRL for 2 and 16 h and analyzed the respiratory burst activity and proinflammatory cytokine expression profile. The results showed that PRL was able to induce the production of reactive oxygen species and the expression of IL-1β and TNF-α in a similar way to two major pathogen-associated molecular patterns: polyinosinic-polycytidylic acid and genomic DNA from the bacterium Vibrion anguillarum. Interestingly, when the leukocytes were stimulated with suboptimal concentrations of PRL in the presence of bacterial DNA, the expression of IL-1β was synergistically induced. More importantly, all PRL activities were blocked by neutralizing Abs to PRL, as well as by pharmacological inhibitors of the Jak/Stat and NF-κB signaling pathways. In addition, EMSA and HPLC/mass spectrometry further confirmed that Stat and NF-κB were involved in the activation of seabream leukocytes by PRL. Collectively, our data identified PRL as a key regulator of the activation of fish professional phagocytes and demonstrated a cross-talk between TLR/NF-κB and PRLR/Jak/Stat signaling pathways. In addition, to the best of our knowledge, this is the first report showing that PRL modulates the activation of phagocyte NADPH oxidase through the Jak/Stat pathway in vertebrates. The Journal of Immunology, 2010, 185: 3873–3883.

The stunning advances made in immunology and endocrinology during the past 50 y revealed that health and wellness are maintained only when these two pivotal systems interact with one another in an appropriate manner. Changes in one system, such as activation of the innate immune system, cause reciprocal changes in the neuroendocrine system (1). In fact, cells are equipped with hormones and receptors for pathogen-associated molecular patterns (PAMPs) that interpret this intricate, interactive network of signals to respond in a physiologically appropriate fashion.

It is well documented that immune processes are modulated by class I helical cytokines, a large group of signaling molecules that play key roles in a plethora of physiological processes, including host defense, immune regulation, somatic growth, reproduction, food intake and energy metabolism, the regulation of neural growth, and many more. Some members of the class I helical cytokines are growth hormone, prolactin (PRL), leptin, erythropoietin, and the “gp130 cytokines” (2, 3).

PRL is a pituitary peptide hormone that shares many properties with cytokines (i.e., homologous receptor structure, similar signal-transduction pathway, and immunomodulatory action) (4, 5). Similarly to cytokines, PRL and its cognate receptor PRLR are widely expressed in cells of the immune system (6, 7). In fish, various physiological functions have been attributed to PRL, such as synergism with the production of steroid hormones in the gonads (8), pigment dispersion in the tegumentary chromatophores (9), and reproduction (10). PRL is also known to enhance immune functions in fish as in mammals (11–13). The phagocytic activity of fish leukocytes is stimulated by administration of PRL (11, 13). PRL was also found to enhance the mitotic activity of leukocytes of the chum salmon (Onchorhynchus keta) and is necessary to maintain circulating levels of IgM in the rainbow trout (O. mykiss) (14, 15).

In mammals, PRL mediates its effect on target cells, in part through activation of Jak2 and different Stat proteins, including Stat1 and Stat5 (16). The activated Stat proteins are translocated into the nucleus and subsequently upregulate the transcription of target promoters that mediate the differentiative or mitogenic effects of PRL (17–21). However, the signaling pathway involved in the activation of fish macrophages by PRL and, in particular, its cross-talk with the TLR-induced pathway, remains to be established. This is important and seems to be feasible because many fish orthologs of mammalian TLRs have recently been identified (22–24). Interestingly, although it seems that some of these orthologs are functionally analogous, such as rainbow trout (25) and zebrafish (26) TLR5, which activates the immune system upon detection of bacterial flagellin, there still seems to be fundamental differences in the recognition, signaling pathways, and the response to certain PAMPs between fish and mammals (27).

To obtain insight into the role played by PRL in the activation of fish professional phagocytes and the signaling pathways involved in this activation, we stimulated the leukocytes from the teleost fish gilthead seabream (S. aurata L.) with different concentrations...
of native salmon PRL alone or in combination with genomic DNA from the bacterium *Vibrio anguillarum* (VαDNA) or synthetic polyniosinic–polycytidylic acid [poly(I:C)]. The results showed that PRL was able to prime the production of reactive oxygen species (ROS) triggered by PMA and to induce the expression of IL-1β at similar levels to those induced by poly(I:C) and VαDNA. Notably, PRL and VαDNA synergistically increased the mRNA levels of IL-1β. More importantly, by using pharmacological inhibitors and EMSA, it was found that Jak/Stat and NF-κB signaling pathways were involved in the activation of seabream phagocytes by PRL. Collectively, to the best of our knowledge, the results provide the first evidence in bony fish that PRL regulates the immune responses of this group of animals by cross-talk between Jak/Stat and NF-κB signaling pathways.

**Materials and Methods**

**Animals**

Healthy specimens (300 g mean weight) of the hermaphroditic protrandrous marine fish gilthead seabream (*S. aurata*) were kept at the Spanish Oceanographic Institute (Mazarro´n, Murcia) in 14-m³ running-seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) with natural temperature and photoperiod and were fed twice a day with a commercial pellet diet (Trouvit, Burgos, Spain). Fish were fasted for 24 h before sampling. All animal studies were carried out in accordance with the European Union regulations for animal experimentation.

**Cell culture and treatments**

Seabream head kidney (bone marrow equivalent in fish) leukocytes, obtained as described elsewhere (28), were maintained in RPMI 1640 culture medium (Invitrogen, Carlsbad, CA) adjusted to gilthead seabream serum osmolarity (353.33 mOsm) with 0.35% NaCl, supplemented with 5% FCS (Invitrogen), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Biochrom, Berlin, Germany). Acidophilic granulocytes were isolated by MACS using the G7 mAb (28). G7 cells were then used to isolated macrophages (adherent cells) and lymphocytes (nonadherent cells) (29). The purity of acidophilic granulocyte and macrophage fractions were ~95%, as assayed by cell morphology, flow cytometry, and the expression of specific markers (28, 29). Macrophages and total leukocytes were stimulated for 2 and 16 h at 23°C with 50 μg/ml phenol-extracted VαDNA (strain ATCC19264), 25 μg/ml poly(I:C) (InvivoGen, San Diego, CA), and/or 25–250 ng/ml PRL [purified from *Salmo salar* according to the method of Andersen et al. (30) (Supplemental Fig. 1)] in RPMI 1640 culture medium adjusted to gilthead seabream serum osmolarity (353.33 mOsm) with 0.35% NaCl supplemented with 5% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. These concentrations of poly(I:C) and VαDNA were found to be optimal for in vitro activation of seabream macrophages (31). In some experiments, leukocytes were pretreated for 1 h with several pharmacological inhibitors, including AG490 (1–100 μM, Jak2 inhibitor) (32), genistein (0.2–20 μM, tyrosine protein kinase inhibitor) (33), BAY 11-7085 (1 μM, NF-κB inhibitor) (34), or dibenziodolium chloride (DPI; 10 μM, NADPH oxidase inhibitor) (35) (all from Sigma-Aldrich, St. Louis, MO). The concentrations of these pharmacological inhibitors used were similar or 1 or 2 orders of magnitude lower than the ones reported to be specific for mammalian and/or fish cells (32–35).

**FIGURE 1.** PRL activates the respiratory burst of seabream leukocytes. Leukocytes were treated with PRL at the indicated concentrations for 2 h (A) and 16 h (B). The respiratory-burst activity by these cells triggered by PMA (1 μg/ml) was measured using a luminol-dependent chemiluminescence method. Values are normalized to unstimulated cells and represent the mean ± SE of three independent experiments. *p < 0.05 versus unstimulated cells.

C. Leukocytes were treated for 2 h with PRL (250 ng/ml) alone or in the presence of the indicated concentrations of the pharmacological inhibitors DPI, genistein, or AG490. Values are normalized to unstimulated cells and represent the mean ± SE of three independent experiments. *p < 0.05 versus cells stimulated with PRL and in the presence of the lowest concentrations of the inhibitor.
and the cells were lysed for RNA or protein extraction. Cell viability was demonstrated to be >95% for each experiment.

The specificity of the PRL effects on seabream leukocytes was confirmed by pretreatment of the cells with a rabbit polyclonal Ab produced against an internal epitope of PRL (HPERNTDSTKTK, 1:100, preincubated with PRL for 2 h).

Respiratory-burst assays

Respiratory-burst activity was measured as the luminol-dependent chemiluminescence produced by seabream head kidney leukocytes (36). This was carried out by adding 100 μM luminol and 1 μg/ml PMA (both from Sigma-Aldrich) while the chemiluminescence was recorded every 117 s for 1 h in a FLUOstar luminometer (BMG Labtechnologies). The values reported are the average of quadruple readings, expressed as the slope of the reaction curve from 117–1170 s, from which the apparatus background was subtracted.

Analysis of gene expression

Total RNA was extracted from tissues or cell pellets with TRIzol reagent (Invitrogen), following the manufacturer’s instructions, and was treated with DNase I, Amplification grade (1 U/μg RNA, Invitrogen). The SuperScript III RNase H Reverse Transcriptase (Invitrogen) was used to carry out a pretreatment of the cells with a rabbit polyclonal Ab produced against an internal epitope of PRL (HPERNTDSTKTK, 1:100, preincubated with PRL for 2 h).

Gene expression is normalized against the ribosomal protein S18 (rps18) (A5277167) content in each sample using the comparative Ct method (2^ΔΔCt). The primers used were: il1β (AJ413189): forward 5'-GGGCTGAACAACAGCACTCTC-3' and reverse 5'-TTAAAGTGCACTACACACCAACA-3' and reverse 5'-TTAAAGTGCACTACACACCAACA-3' and reverse 5'-TTAAAGTGCACTACACACCAACA-3' and reverse 5'-TTAAAGTGCACTACACACCAACA-3'.

Immunoprecipitation and Western blotting

Leukocytes were lysed in ice-cold Nonidet P-40 lysis buffer containing 1% Nonidet P-40, 25 mM Tris·HCl (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM leupeptin, and 1 mM PMSF. For immunoprecipitation studies, cell lysates were incubated with the Stat5 polyclonal Ab (1:1000) (C17; Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h. Cell lysates were then mixed with protein G-coupled Sepharose beads and rotated for 1 h at 4°C. After the beads were washed three times with ice-cold Nonidet P-40 lysis buffer, the precipitated proteins were boiled for 5 min and eluted with SDS-PAGE sample buffer.

Western blot analysis was carried out according to a standard protocol (37). Briefly, leukocytes were lysed at 4°C, and the BCA protein assay kit (Pierce, Rockford, IL) was used to determine the protein concentrations of the cell lysates. Equal amounts of protein were loaded onto polyacrylamide gels, electrophoresed, and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). After transfer, the membranes were blocked overnight in 5% dry milk and probed for 1 h with a rabbit monoclonal Ab to seabream IL-1β (1:5000) (38), anti-phosphotyrosin 4G10 plus (Millipore, Bedford, MA), or anti-PRL (1:2000) and then for 1 h with an HRP-coupled secondary Ab (1:5000) (Sigma-Aldrich). The reactive bands were visualized using an ECL-detection system (GE Healthcare, Piscataway, NJ), according to the manufacturer’s procedure. The intensity of the signals was measured with a digital-imaging system (ImageJ, National Institutes of Health, Bethesda, MD).

FIGURE 2. PRL induces the production and release of IL-1β in seabream leukocytes. Macrophages were stimulated for 2 h (A) or 16 h (B) with 250 ng/ml PRL, 25 μg/ml poly(I·C), and 50 μg/ml of VdDNA, and the mRNA levels of the proinflammatory cytokine il1β were determined by real-time RT-PCR. Gene expression is normalized against rps18 and is shown as relative to the mean of unstimulated cells. Each bar represents the mean ± SE of triplicate samples. *p < 0.05 versus unstimulated cells. C, Western blot analysis of precursor (pro) or mature (m) IL-1β in cell lysates (60 μg) and supernatants (15 μl), respectively, of total head kidney leukocytes incubated for 16 h with 250 ng/ml of PRL, 25 μg/ml of poly(I·C), and 50 μg/ml of VdDNA. The results are representative of three independent experiments.
EMS

Nuclear and cytoplasmic extracts were prepared from total head kidney leukocytes from the gilthead seabream, according to the method of Sadowski et al. (39). The double-stranded oligonucleotides used as probes in the EMSA were Stat: 5'-TGTGTAAACTGCGTTTTCCCGGAAAGTTTTGA-AGATCTGT-3'/5'-ACAGATCTTCAAAACTTTCCGGGAAAACGCAG-TTATACACA-3'; NF-κB: 5'-AGTTGAGGGGACTTTCCCAGCC-3'/5'-GGCTGGGAAAGTCCCCTCAACT-3'; and the negative control probe (CRN): 5'-TACGTACGTACCTGTATAAG-3'/5'-CTTATACAGGTACG-TACGTA-3'. Binding reactions and electrophoresis were performed as previously described (40). In the supershift assays, 0.2 μg/ml anti-Stat5 or anti-Stat1 polyclonal Abs (C17 and M22; Santa Cruz Biotechnology) were added 20 min before or immediately after adding the probe.

HPLC/mass spectrometry analysis of activated NF-κB and Stat

Aliquots (100 μl slurry containing 25 μl beads) of the NF-κB oligonucleotide probe conjugated to agarose (sc-2505 ac; Santa Cruz Biotechnology) were washed three times with 1 ml binding buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 μg/ml polydeoxyinosinic-polydeoxycytidylic acid) and then incubated overnight at 4˚C with rotation with 500 μg nuclear extracts from seabream leukocytes stimulated with PRL (250 ng/ml) or poly(I:C) (25 μg/ml). Afterward, the beads were washed three times, and the proteins were eluted, by boiling them for 90 s in 40 μl SDS-PAGE, and subjected to electrophoresis. The most intense bands were cut from the gel to determine its sequence by HPLC/mass spectrometry (MS). A similar protocol was followed using a biotin-labeled Stat5 probe (Invitrogen); however, in this case, the DNA-protein complexes were pulled down with streptavidin immobilized on agarose (Sigma-Aldrich).

The gel slices were destained, equilibrated in different buffers, and subjected to in-gel tryptic digestion using Trypsin Proteomics Grade (Sigma-Aldrich). The separation and analysis of the tryptic digests of the samples were performed with an HPLC/MS system, consisting of an Agilent 1100 Series HPLC connected to an Agilent Ion Trap XCT Plus Mass Spectrometer (both from Agilent, Palo Alto, CA) using an electrospray interface. Dry samples from in-gel trypsin digestion were resuspended in 10 μl buffer A (water/acetonitrile/formic acid, 94.9:5:0.1) and injected onto a Zorbax SB-C18 HPLC column (5 μm, 150 × 0.5 mm; Agilent), thermostatted at 40˚C, at a flow rate of 10 μl/min. After the injection, the column was washed with buffer A, and the digested peptides were eluted using a linear gradient 0–80% buffer B (water/acetonitrile/formic acid, 10:89.9:0.1) for 150 min. The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V and a scan speed of 8100 (m/z)/sec from 200–2200 m/z. The nebulizer gas pressure was set to 15 psi, whereas the drying gas was set to a flow of 5 l/min at a temperature of 350˚C. Tandem MS data were collected in an automated data-dependent mode. The five most intense ions were sequentially fragmented using helium collision-induced dissociation with an isolation width of 2 and a relative collision energy of 35%. Data processing was performed with DataAnalysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik, Billerica, MA) and Spectrum Mill MS Proteomics Workbench (Agilent) (41, 42). Briefly, MS and tandem MS data were extracted and compared with the manual NCBI-nr-based.

**FIGURE 3.** PRL and PAMPs synergistically induce IL-1β expression in seabream macrophages. Seabream macrophages were stimulated for 2 h with the indicated concentrations of PRL alone or in combination with 25 μg/ml of poly(I:C) or 50 μg/ml of VaDNA (A, B) and with or without preimmune or anti-PRL sera (C, D), and the mRNA levels of the proinflammatory cytokine il1b was determined by real-time RT-PCR. Gene expression is normalized against rps18 and is shown relative to the mean of unstimulated cells (A–C) or as the percentage of activation relative to PRL-stimulated cells without antisera (D). Each bar represents the mean ± SE of triplicate samples. The results are representative of three independent experiments. A, *p < 0.05 versus cells stimulated with PAMPs alone. C, *p < 0.05 versus cells stimulated with PRL without anti-PRL. The induction of il1b gene relative to control cells is indicated on the bars. D, *p < 0.05 versus cells stimulated with PRL alone.
Statistical analysis
All data are shown as means ± SE. Differences were evaluated using ANOVA, followed by the Student t test. Statistical significance was defined as \( p < 0.05 \).

Results
PRL activates the respiratory burst of seabream leukocytes
The homology between salmon and seabream PRL and PRLR is relatively high (83 and 74% amino acid similarity, respectively), and the PRLR motifs mediating the interaction with the PRL are very well conserved in both receptors (Supplemental Fig. 2). RT-PCR was used to analyze the expression of PRLR in total head kidney leukocytes, as well as in lymphocyte, granulocyte, and macrophage fractions, which were indeed found to constitutively express the PRLR transcript (Supplemental Fig. 2). Using luminol-dependent chemiluminescence, we analyzed the time course of ROS production in head kidney leukocytes treated with PRL (25–250 ng/ml). As shown in Fig. 1A and 1B, PRL gradually increased the amount of ROS triggered by PMA, with maximal 5- and 3.7-fold increases at 2 and 16 h when the cells were incubated with 250 and 100 ng/ml doses, respectively. PRL was unable to trigger the respiratory burst of seabream leukocytes without PMA (data not shown). To confirm the involvement of NADPH oxidase, we examined the effect of DPI, a flavin-containing enzyme inhibitor. The PRL-mediated ROS increase observed at 2 h was completely inhibited by 10 \( \mu \)M DPI (Fig. 1C). Additionally, the ROS production induced by PRL was attenuated by 10 \( \mu \)M AG490, a Jak2

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**FIGURE 4.** Blockage of Jak2 attenuates PRL-induced IL-1β mRNA expression. Cell extracts from head kidney leukocytes stimulated with 250 ng/ml PRL alone for the indicated times (A) or in the presence of the Jak2 inhibitor AG490 (10 \( \mu \)M) for 30 min (B) were immunoprecipitated with an Ab directed against Stat5 and then probed with anti-Stat5 or the anti-phosphotyrosine 4G10 plus Ab. C, Seabream macrophages were coincubated with 250 ng/ml PRL and 0–100 \( \mu \)M AG490, and the mRNA levels of the proinflammatory cytokine il1b were determined by real-time RT-PCR. Gene expression is normalized against rps18 and is shown as relative to the mean of unstimulated cells. Each bar represents the mean ± SE of triplicate samples. The results shown are representative of three independent experiments. * \( p < 0.05 \) versus cells stimulated with PRL without AG490.
PRL induces IL-1β production and release in seabream leukocytes

The above results prompted us to examine whether PRL was also able to induce the expression of two major proinflammatory cytokines secreted by activated monocytes and macrophages: IL-1β and TNF-α. Real-time RT-PCR demonstrated that PRL treatment of seabream macrophages increased IL-1β (Fig. 2A, 2B) and TNF-α (data not shown) mRNA levels within 2 h and that the level remained high for 16 h. These results were further confirmed by Western blot analysis using a specific Ab to seabream IL-1β (Fig. 2C). Interestingly, PRL was more potent than poly(I:C) in increasing IL-1β mRNA levels. Mature IL-1β protein was found to be released by leukocytes stimulated with PRL or VaDNA but not by poly(I:C) (Fig. 2C).

We next examined the cross-talk between the PRLR and TLR signaling pathways by incubating macrophages with PRL with or without poly(I:C) or VaDNA for 2 h before measuring the mRNA levels of IL-1β by real-time RT-PCR. Notably, stimulation of macrophages simultaneously with PRL and poly(I:C) resulted in an additive effect on the induction of IL-1β expression, whereas treatment of macrophages with PRL and VaDNA stimuli resulted in significantly decreased levels of the IL-1β transcript (Fig. 3A). Because the concentration of VaDNA used in this experiment resulted in a dramatic increase in IL-1β transcript levels that might mask a possible synergistic effect between PRL and VaDNA, we evaluated the effect of lower doses of PRL in combination with poly(I:C) or VaDNA (Fig. 3B). The results showed that 25 ng/ml PRL and 50 μg/ml VaDNA synergistically enhanced the levels of IL-1β mRNA, whereas the different concentrations of PRL showed additive effects with 25 μg/ml poly(I:C). However, overstimulation of macrophages with high concentrations of PRL and VaDNA resulted in a lower induction of IL-1β expression. Taken together, the above results demonstrate that PRL is able to modulate IL-1β expression in seabream macrophages and strongly suggest the existence of cross-talk between the PRLR and TLR signaling pathways.

All of the above effects of PRL on seabream macrophages were found to be specific, because the addition of neutralizing monoclonal Abs to PRL (Supplemental Fig. 1) blocked the induction of IL-1β expression by PRL, whereas it had negligible effects on the induction of this gene by poly(I:C) and VaDNA (Fig. 3C). As expected, the preimmune serum was unable to block the activity of PRL (Fig. 3D).

PRL signals through the Jak/Stat pathway

To clarify the mechanism underlying PRL-induced transcription of the IL-1β gene in seabream leukocytes, we performed Western blot analysis, real-time RT-PCR, and EMSA. As shown in Fig. 4A, a 30-min exposure to PRL increased the phosphorylation of Stat5 by 2.55-fold relative to the unstimulated control. However, PRL did not alter the expression of Stat5 protein at any time (Fig. 4A, lower panel). Notably, the Stat5 phosphorylation after a 30-min incubation with PRL was partially inhibited by AG490 (10 μM) (Fig. 4B), whereas DPI had no effect (data not shown). These data indicate that PRL promotes the phosphorylation of Stat5 via the Jak/Stat pathway. Additionally, real-time RT-PCR analysis confirmed the ability of AG490 to inhibit the PRL-induced IL-1β expression in seabream leukocytes (Fig. 4C). We then performed EMSA using the IFN-γ-activated site (GAS), which is recognized by Stat proteins, as the radiolabeled oligonucleotide probe. Fig. 5A shows that nuclear extracts from leukocytes treated for 45 min with PRL were able to shift the probe containing the GAS sequence. The specificity of PRL-promoted binding was confirmed by competitively inhibiting this reaction with a 50-fold excess of unlabeled GAS oligonucleotide (Fig. 5B). In addition, the formation of the DNA–protein complexes was abrogated by inhibiting Jak2 with AG490, as well as a cold competitor probe (5- and 50-fold molar excess), CRN (50-fold molar excess), and the Abs to Stat1 and Stat5 added 20 min before (b) or immediately after (a) adding the probe. The arrows indicate the protein–DNA complexes observed in leukocytes stimulated with PRL, whereas the circles indicate the breakdown of the protein–DNA complexes by the Stat1 and Stat5 Abs. Free, probe only.

FIGURE 5. PRL signals through the Jak/Stat pathway in seabream leukocytes. A. EMSA was performed using nuclear extracts obtained from total seabream head kidney leukocytes stimulated with 250 ng/ml PRL for the indicated times, and the oligonucleotide probe containing the GAS element. B. Three identical EMSA experiments were performed to confirm reproducibility using nuclear extracts from leukocytes preincubated with 10 μM AG490, as well as a cold competitor probe (5- and 50-fold molar excess), CRN (50-fold molar excess), and the Abs to Stat1 and Stat5 added 20 min before (b) or immediately after (a) adding the probe. The arrows indicate the protein–DNA complexes observed in leukocytes stimulated with PRL, whereas the circles indicate the breakdown of the protein–DNA complexes by the Stat1 and Stat5 Abs. Free, probe only.
Jak2-dependent phosphorylation of Stat1 and Stat5, which, in turn, translocate to the nucleus, bind to GAS-containing promoters, and initiate gene transcription.

**Blockage of NF-κB attenuates PRL-induced IL-1β gene expression**

Although it was shown that the Jak2/Stat signaling pathway might participate in the induction of IL-1β gene expression (43, 44), NF-κB is the canonical transcription factor involved in its induction (45). Therefore, we assessed the involvement of NF-κB activation in PRL-induced IL-1β gene expression in seabream macrophages pretreated for 1 h with BAY 11-7085, a pharmacological inhibitor of NF-κB activation. The dose-dependent inhibition of IL-1β mRNA levels was consistent with BAY 11-7085–mediated blockage of the NF-κB signaling pathway (Fig. 6). In fact, IL-1β mRNA expression induced by PRL was attenuated by 65% with only 1 μM of the inhibitor (Fig. 6). These results suggest that PRL-mediated IL-1β gene induction in seabream macrophages is dependent on the Jak/Stat and NF-κB signaling pathways.

To further confirm the PRL-induced activation of NF-κB in seabream macrophages, nuclear protein extracts from head kidney leukocytes treated with 250 ng/ml of PRL were assayed by EMSA with those from leukocytes coincubated with BAY 11-7085 (negative controls) or stimulated with poly(I:C) and VaDNA (positive controls) (Fig. 7A). PRL, poly(I:C), and VaDNA induced activation of NF-κB complexes, albeit with different kinetics. Following poly(I:C) treatment, NF-κB complexes formed rapidly (≤15 min), peaked at 30 min, and were still evident 45 min after stimulation (Fig. 7A). In sharp contrast, PRL and VaDNA treatments did not result in the formation of NF-κB complexes until 45 min post-stimulation (Fig. 7A). Additionally, preincubation of leukocytes with 1 μM of BAY 11-7085 completely prevented the activation of NF-κB (Fig. 7B). The specificity of the NF-κB–binding activity was demonstrated by DNA-binding–competition experiments using a 50-fold excess of unlabeled wild type and negative control (Fig. 7B). Additionally, we used two different commercial Abs raised against conserved epitopes of p65 (sc-7151) and p52 (sc-298) subunits of NF-κB but, unfortunately, they did not cross-react with seabream NF-κB, as assayed by Western blotting (data not shown).

Therefore, we again used the HPLC/MS strategy and found that several NF-κB subunits and NF-κB–interacting proteins were bound to the EMSA probe in samples of nuclear extracts from seabream leukocytes treated with PRL or poly(I:C) (Fig. 7C, Table II). These results confirm that PRL is able to activate NF-κB in seabream macrophages and that this leads to the production of IL-1β.

**Discussion**

In mammals, there is evidence that indicates the involvement of PRL in the regulation of humoral- and cell-mediated immune responses (6, 7, 46). Macrophage-mediated regulation of immune response is manifested by a variety of mechanisms involving the secretion of bioactive molecules by activated macrophages, such as ROS, TNF-α, and IL-1β (47). Macrophages can be activated by a number of agents, some of which act via signal-transduction processes involving the modulation of various second messengers, such as protein kinase C and Ca2+, and the activation of several transcription factors, such as NF-κB and Stat (48). However, little is known about the effect of PRL on the activation of macrophages.

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**Table I. Sequence and homology of the tryptic peptides identified by HPLC/MS in PRL-stimulated nuclear extracts pulled down with the Stat DNA probe**

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<td>Danio rerio</td>
<td>18478684</td>
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<tr>
<td>(R)IQQLAGNGPPEGGLDILQSWCEKLAETIWQNR(Q)</td>
<td>Stat5</td>
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**FIGURE 6.** Blockage of NF-κB attenuates PRL-induced IL-1β mRNA expression. Seabream macrophages were coincubated with 250 ng/ml of PRL and 0–100 μM of BAY 11-7085, and the mRNA levels of the proinflammatory cytokine il1β were determined by real-time RT-PCR. Gene expression is normalized against rps18 and is shown relative to the mean of unstimulated cells. Each bar represents the mean ± SE of triplicate samples. The results are representative of three independent experiments. *p < 0.05 versus cells stimulated with PRL without BAY 11-7085.
In fish, although there are a few studies describing the effects of PRL on the immune response (11, 13), it is unclear how this process occurs. To the best of our knowledge, the data presented in this study provide the first evidence that connects PRL with an immunological process: the respiratory burst and the expression of proinflammatory cytokines, both mediated by Jak/Stat and NF-κB signaling pathways. We used PRL extracted from the pituitary of *S. salar* (30) to avoid the contamination that frequently occurs with endotoxins of recombinant proteins. In addition, the pituitary of *S. salar*, which is much bigger than the pituitary of *S. aurata*, allowed us to extract a larger amount of PRL. It is important to point out the high degree of conservation between salmon and other species.

**FIGURE 7.** PRL signals through the NF-κB pathway in seabream leukocytes. A, EMSA was performed using nuclear extracts obtained from total head kidney seabream leukocytes stimulated with 250 ng/ml PRL, 25 μg/ml poly(I:C), and 50 μg/ml VaDNA, for the indicated times, and the oligonucleotide probe corresponding to the NF-κB–binding site. B, Three identical experiments were performed to confirm reproducibility using nuclear extracts from cells preincubated with 1 μM BAY 11-7085, the competitor probe (5- and 50-fold molar excess), and the CRN probe (50-fold molar excess). The arrows indicate the protein–DNA complexes observed in cells stimulated by PRL. Free, probe only. C, The proteins bound to the NF-κB probe used in EMSA were pulled down, run on SDS-PAGE, excised off, and analyzed by HPLC/MS (see Materials and Methods for details). As an example, the peptide (NH2)-LNKLSQAPLHLAVITYKQPK-(COOH) was identified, and it showed 100, 79, and 82% amino acid identity with zebrafish (AY163838.1), Atlantic salmon (BT058984.1), and chicken (NP_989744) p100 NF-κB subunit, respectively. A full list of the peptides identified and their homologies to different NF-κB subunits and NF-κB–interacting proteins of other species are shown in Table II.
seabream PRL (83% amino acid similarity) and PRLR (Supplemental Fig. 2). The quality of the purified PRL used in this study was routinely checked by Western blot and HPLC (Supplemental Fig. 1), and the doses used were 25–250 ng/ml, all within physiological ranges (49, 50). We also evaluated 500 and 1000 ng/ml of PRL but, interestingly, these supraphysiologic doses decreased ROS production and cytokine expression in a dose-dependent fashion (data not shown). More importantly, the specificity of the effects of PRL in seabream leukocytes was further confirmed by the complete neutralization of its activity by using a specific Ab directed against PRL. Finally, we are confident of the specific effects of the pharmacologic inhibitors used in this study, because the concentrations used were similar or one or two orders of magnitude lower than the ones reported to be specific for mammalian and/or fish cells (32–35).

Although, to the best of our knowledge, we observed for the first time in fish that all leukocyte populations of the gilthead seabream, namely acidophilic granulocytes, macrophages, and lymphocytes, expressed PRLR, it is likely that PRL exerted its activities through two PRLRs, as it was described in the black seabream (Acanthopus schlegelii) (51), which is a close relative of the gilthead seabream. Therefore, the identification of this second PRLR in the gilthead seabream, the analysis of its expression in different leukocytes, and the functional relevance played by each receptor in fish immune cells would be major aims of future studies.

A major source of ROS in phagocytes is NADPH oxidase (52). The NADPH oxidase of phagocytic cells is composed of the 22-kDa α-subunit (p22phox) and 91-kDa β-subunit (gp91phox) membrane components; the cytosolic components p47phox, p47phox, and p67phox; and the small m.w. G-protein Rac. We found that PRL increased NADPH oxidase activity in seabream phagocytes via the Jak/Stat pathway using pharmacologic inhibitors of Jak and EMSA. This is not surprising because IFN-γ also used this signaling pathway to prime the respiratory burst of mammalian macrophages. For example, IFN-γ stimulation of murine macrophages results in the induction of Stat1/IFN regulatory factor 1-dependent transcription of gp91phox gene (53). Similarly, we also found that PRL increases the mRNA levels of p47phox, p67phox, and gp91phox in seabream macrophages and the salmon macrophage cell line SHK-1 (V.H. Olavarria, J.E. Figueroa, and V. Mulero, manuscript in preparation). To the best of our knowledge, this is the first demonstration that PRL modulates the activation of phagocyte NADPH oxidase through the Jak/Stat pathway in vertebrates.

We also demonstrated that PRL was not only able to prime the respiratory burst of seabream phagocytes but also to elicit the expression of two major proinflammatory cytokines, IL-1β and TNF-α, in macrophages. We also found a synergistic effect of PRL with VgDNA in the induction of IL-1β gene expression, suggesting cross-talk between theTLR- and Jak/Stat signaling pathways and the complexity of macrophage activation in vivo in the course of inflammation and infection. More importantly, we confirmed, by using a specific Ab to seabream IL-1β, that PRL was able to promote the production and secretion of IL-1β at similar levels as bacterial VgDNA. The secretion mechanism of IL-1β in mammals is still a matter of debate, because this cytokine lacks a signal peptide and does not follow the classical endoplasmic reticulum–Golgi apparatus route of secretion (54). However, it is evident that following the production of proIL-1β, a second signal is required to stimulate rapid and efficient processing of IL-1β by caspase-1 and its subsequent release. Our results demonstrate that PAMPs (VgDNA) and PRL stimulation is sufficient to promote the production, processing, and release of IL-1β in seabream leukocytes (this study and data not shown), suggesting that a more sophisticated two-step mechanism for the secretion of IL-1β evolved after the divergence of fish and tetrapods ~450 million years ago.

In mammals, the functions of the PRL/PRLR complex in the immune system are mediated by receptor-associated signaling proteins. Lacking intrinsic enzymatic activity, ligand-induced dimerization of the PRL/PRLR complex serves to activate these associated signaling cascades. Some of the most proximal kinases activated during PRLR signaling are members of the Jak and Src families of protein tyrosine kinases. The Jak family includes Jak 1–3 and Tyk2 (55, 56), and they are associated with early transduction events in all members of the cytokine receptor superfamily. Receptor-associated Jak2 is activated rapidly after engagement of PRL by its receptor (57, 58), and this leads to the tyrosine phosphorylation of Stat5, resulting in Stat5 dimerization and nuclear translocation (59). To the best of our knowledge, we show for the first time that a similar mechanism operates in fish: the engagement of PRLR by PRL promotes the Jak2-dependent phosphorylation of Stat5, which, in turn, translocates to the nucleus and binds to the GAS element located in the promoter of target genes. Interestingly, disruption of DNA–protein complex formation by Stat1 and Stat5 Abs and the HPLC/MS analysis of proteins bound to the Stat DNA probe suggest that PRL activates the translocation of Stat1 and Stat5 to the nucleus as a homo- or heterodimer. However, this result needs to be confirmed with additional assays. Conservation of the Jak2/Stat5 pathway has also been confirmed in the zebrafish, where genetic ablation of Jak2 resulted in reduced erythroid cell expansion and Stat5 phosphorylation, whereas the constitutively active mutant of Jak2 and Stat5 had an opposite effect (32, 60).

Most of the genes that encode inflammatory proteins depend on NF-kB for their expression (45). In mammals, physiological concentrations of PRL are able to induce the production of several cytokines in macrophages, including IL-1β, IL-12, and IFN-γ, by mechanisms involving Ca2⁺, MAPKs, Stat3, and NF-kB (33, 61, 62). One of the most exciting findings of the current study was the demonstration that PRL-induced IL-1β expression in seabream

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**Table II. Sequence and homology of the tryptic peptides identified by HPLC/MS in PRL-stimulated nuclear extracts pulled down with the NF-κB DNA probe**

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Protein Name</th>
<th>Species</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K)LNKLSQAPLHLAVITKQPKLVEMLMK(S)</td>
<td>P100/PS2 transcription factor</td>
<td>Danio rerio</td>
<td>37725728</td>
</tr>
<tr>
<td>(K)LFMRPVIEILVRKVVRK(R)</td>
<td>Similar to NF-kb-repressing factor</td>
<td>D. rerio</td>
<td>19039936</td>
</tr>
<tr>
<td>(R)ADGDDTDDLDRGPIPLHLAAAGDVTLR(V)</td>
<td>Nuclear factor NF-kb p100 subunit</td>
<td>Salmo salar</td>
<td>223647878</td>
</tr>
<tr>
<td>(K)NGSRDRATELVK(L)</td>
<td>NF-kb repressing factor</td>
<td>Homo sapiens</td>
<td>46250431</td>
</tr>
<tr>
<td>(R)YKCGRASGIPGKSDNTTK(T)</td>
<td>p65 transcription factor</td>
<td>Siniperca chuatsi</td>
<td>158939183</td>
</tr>
<tr>
<td>(K)HEAAGAANILK(Q)</td>
<td>NF-kb repressing factor</td>
<td>Xenopus tropicalis</td>
<td>113931358</td>
</tr>
<tr>
<td>(R)QFAIVFTKPYRDQNLQKPTSVFQLKR(K)</td>
<td>NF-kB1 p105 subunit</td>
<td>S. chuatsi</td>
<td>125968525</td>
</tr>
<tr>
<td>(R)INRKYTPVYK(V)</td>
<td>Nuclear factor NF-kb p100 subunit</td>
<td>S. salar</td>
<td>223647878</td>
</tr>
<tr>
<td>(R)YKCGRASGIPGKSDNTTK(T)</td>
<td>p65 transcription factor</td>
<td>S. chuatsi</td>
<td>158939183</td>
</tr>
</tbody>
</table>
macrophages is mediated, in part, by activation of the NF-κB–signaling pathway. Because the inhibitors of kinesin (IKKs) are necessary for triggering the NF-κB signaling pathway, we evaluated this signaling pathway by using BAY 11-7085, a specific pharmacological inhibitor of IKKs. Notably, we demonstrated a 65% blockage of the PRL-induced IL-1β expression with as little as 1 μM of BAY 11-7085. Unfortunately, we were unable to evaluate IKK phosphorylation because no commercial Ab able to cross-react with fish IKKs seems to be available. However, EMSA confirmed that NF-κB was indeed activated in seabream leukocytes stimulated with PRL, as occurred with poly(I:C) and 5αDNA stimulation, although poly(I:C) was able to activate the DNA-binding activity of NF-κB faster than PRL and 5αDNA. Although we did not find any cross-reacting Ab to fish NF-κB subunits to perform a supershift, the specificity of EMSA for NF-κB was clearly demonstrated by the absence of 1 μM BAY 11-7085 to block DNA–protein complex formation, competition experiments using the cold probe and a control probe lacking the NF-κB–binding sites, and by demonstrating the presence of similar NF-κB subunits and NF-κB–interacting proteins in the DNA–protein complexes from PRL- or poly(I:C)-stimulated leukocytes’ nuclear extracts. The mechanisms involved in the activation of NF-κB by PRLR engagement and Jak activation remain to be explored, but one possibility is that Jak2 is able to directly mediate the phosphorylation of IkB, allowing the translocation of NF-κB to the nucleus and the initiation of target gene transcription. This is plausible because such a mechanism was demonstrated for the NF-κB–dependent neuroprotective effect of erythropoietin (63).

In summary, our results demonstrate the capacity of PRL to promote, through the Jak/STAT and NF-κB signaling pathways, the polarization of fish macrophages to a proinflammatory M1/classically activated phenotype characterized by the production of ROS and proinflammatory cytokines. We also confirm the cross-talk between the TLR- and PRLR signaling pathways and suggest how this cross-talk may lead to the synergistic activation of macrophage proinflammatory activities or, alternatively, to the inhibition of these activities. These results pave the way for future experiments aimed at throwing light on the exact role played by PRL in the resolution of infectious activities or, alternatively, to the inhibition of these activities.

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Disclosures

The authors have no financial conflicts of interest.

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2. Arkins, S. R., W. Jonhnson, C. Minshall, R. Dantzer, and K. W. Kelley. 2001. Interleukin-6 signaling, competition experiments using the cold probe and a control probe lacking the NF-κB–binding sites, and by demonstrating the presence of similar NF-κB subunits and NF-κB–interacting proteins in the DNA–protein complexes from PRL- or poly(I:C)-stimulated leukocytes’ nuclear extracts. The mechanisms involved in the activation of NF-κB by PRLR engagement and Jak activation remain to be explored, but one possibility is that Jak2 is able to directly mediate the phosphorylation of IkB, allowing the translocation of NF-κB to the nucleus and the initiation of target gene transcription. This is plausible because such a mechanism was demonstrated for the NF-κB–dependent neuroprotective effect of erythropoietin (63).

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Supplementary Figure 1. Purification of Atlantic salmon PRL and specificity of the anti-PRL antibody. The purification protocol involved two sequential purification steps (30): (i) Size-exclusion chromatography (Sph) and (ii) ion-exchange chromatography (CM). (A) CM of salmon PRL. The elution gradient was formed with 0.05 M NH₄Ac, pH 4.6 and 0.2 M NH₄Ac, pH 9.0. Protein elution was followed by measuring the absorption at 280 nm and 1.0 ml / 3.5 min fractions were collected. (B) Left panel: SDS-PAGE profile of PRL fractions after filtration of the acid acetone pituitary extract on Sephadex G-25 (Sph) and the purified PRL after the CM. Right panel: both chromatographic fractions were immunoblotted with anti-PRL antibody (1:2000 dilution) and visualized with 3,3’-diaminobenzidine tetrahydrochloride. (C) Purity of PRL from the CM determined by HPLC on a reversed-phase μBondapak C₁₈ column. Elution was performed by 50-56% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid and the flow rate was 1.5 ml/min.
Supplementary Figure 2. Homology of gilthead seabream and Atlantic salmon PRLR and expression of PRLR by seabream leukocytes. (A) Alignment of the predicted amino acid sequence of extracellular domain of Sparus aurata (AAG17629), Salmo salar (ABF57668), Oncorhynchus mykiss (rainbow trout, NP_001118071) and Homo sapiens (AAA60174) PRLRs. As the salmon PRLR is a partial sequence that lacks the NH2-terminal, we used the O. mykiss PRLR sequence, another Salmonidae species, which is a close relative of the salmon and, therefore, their PRLRs are almost identical. The conserved features of the extracellular domain are highlighted in yellow: two pairs of disulfide bonds (between Cys12-Cys22 and Cys51-Cys62 relative to type III fibronectin receptor) and a “WS motif” (Tpr-Ser-x-Trp-Ser). These features are responsible for the receptor-ligand interactions. The salmon and trout PRLRs show 57% identity and 74% similarity with the seabream PRLR. Identical and similar residues identified in all the proteins are indicated.
by asterisks and colons, respectively. (B) The expression of seabream PRLR was evaluated by RT-PCR. The PRLR amplification product was subjected to electrophoresis in agarose, stained with ethidium bromide and visualized under UV. 1, 100 bp DNA ladder; 2, no template; L, lymphocytes; G, granulocytes; M, macrophages.