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Research Article

The tumor necrosis factor α of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner

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Abstract. Information on the bioactivities of non-mammalian cytokines is scant due to the lack of the recombinant molecules and specific antibodies. We produced the mature predicted peptide of tumor necrosis factor α (TNFα) from the bony fish gilthead seabream (*Sparus aurata* L.) (sbTNFα), and its biological role was determined in vitro and in vivo. We first demonstrated by analytical size-exclusion chromatography that sbTNFα is an oligomeric protein but the dimer appears to predominate over the trimeric form, in contrast to mammalian TNFα. Intraperitoneal injection of native sbTNFα resulted in (i) priming of the respiratory burst of the peritoneal exudate and head-kidney (HK) leukocytes, the latter being the bone marrow equivalent in fish; (ii) rapid recruitment of phagocytic granulocytes to the injection site, and (iii) induction of granulopoiesis in the HK. Interestingly, sbTNFα was able to induce a strong proliferation of HK cells in vitro, whereas human TNFα did not. Conversely, sbTNFα was not cytotoxic for murine L929 fibroblasts.

Key words. Cytokine; TNFα; inflammation; evolution; fish.

Tumor necrosis factor α (TNFα) is a pleiotropic cytokine that plays pivotal roles in the organization and functions of the immune system. TNFα exerts its functions by interacting with two distinct receptors, TNFR1 (p60TNFR or CD120a) [1] and TNFR2 (p80TNFR or CD120b) [2]. TNFR1 is expressed in virtually all mammalian cell types, whereas TNFR2 is expressed only in cells of the immune system and in endothelial cells [3, 4]. The TNFR1 intracellular sequence contains a death domain motif, which is also found in some receptors of the TNF superfamily and which allows the recruitment of death domain-containing adapter proteins involved in cell death signaling in many cell types [5]. Unlike TNFR1, TNFR2 does not contain a death domain motif but still recruits adapter proteins to perform its intracellular signaling activities. As well as being proliferative in some cell types, engagement of TNFR2 has been found to have proapoptotic effects of varying magnitude [6]. On the other hand, the binding of TNF to TNFRs, especially to TNFR1, can also activate the NF-κB transcription factor [7] that serves as a critical regulator of inducible expression of many genes [8].

Functional analogues of mammalian TNFα and TNFR have been described in non-mammalian vertebrates, such as fish, and even in invertebrates [9]. The analogy is based mainly on the cross-reactivity of antibodies elicited against mammalian TNFα, the sensitivity of non-mammalian immune cells to the action of mammalian recombinant TNFα, and the responsiveness of mammalian immune cells to non-mammalian TNF-like factors. Thus, the presence of TNF- and TNFR-like molecules in fish was

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suggested from early studies, where human recombinant TNFα (hTNFα) was shown to enhance rainbow trout *Oncorhynchus mykiss* neutrophil migration and respiratory burst activity, effects which were inhibited by monoclonal antibodies (mAbs) against human TNFR1 [10]. However, without knowing the amino acid or gene sequences of putative non-mammalian cytokine analogues, unequivocal demonstration of a phylogenetic relationship between mammalian cytokines and their invertebrate and lower vertebrate functional analogues has not been possible.

The existence of TNF in fish has been directly confirmed by the identification of a TNFα gene orthologue in several fish species [11–14], while two TNFR-like genes have been identified in the Japanese flounder *Paralichthys olivaceus* [15]. Surprisingly, however, no work has addressed the question of whether TNFα shows conserved biological activities in fish and whether or not it displays species specificity, as seems to be indicated by the above-mentioned studies. Therefore, we produced the mature predicted peptide of TNFα from the bony fish, gilthead seabream (*Sparus aurata* L.), and determined its biological role in vitro and in vivo from its effects on the respiratory burst, mobilization and proliferation of phagocytes. Overall, our results indicate that vertebrate TNFα displays selective species specificity yet evolutionarily conserved biological activities.

Materials and methods

Animals

Healthy specimens (100 g mean weight) of the hermaphroditic protandrous marine teleost gilthead seabream (*S. aurata* L.) were obtained from Culmarex S.A. (Murcia, Spain). They were kept in 260-l running-seawater aquaria (flow rate 1500 l/h) at 20 °C with a 12-h light/dark cycle, and were fed with a commercial pellet diet (Trouvit) at a feeding rate of 15 g dry diet/kg biomass of fish/day. The studies presented in this manuscript were approved by the Bioethical Committee of the University of Murcia.

Cloning of gilthead seabream TNFα into expression vector

Gilthead seabream mature TNFα (sbTNFα) (GenEMBL accession no. AJ413189) was obtained by polymerase chain reaction (PCR) amplification using a proof-reading DNA polymerase (Ecotaq Plus; Ecogen, Barcelona, Spain). The PCR primers were FE4 (5′-AAGGATCCGCTGAAGCGCATCAGCAGC-3′) and RE5 (5′-AAGGATCCTTAAAGTGCAAACACACCAAA-3′), and *Escherichia coli* lipopolysaccharide- (LPS; Sigma, Madrid, Spain) stimulated head-kidney (HK; bone marrow equivalent) cDNA was used as template. The PCR-amplified fragment was cloned into the *Sma*I site of the pBluescript SK+ vector and then subcloned into the *Bam*HI site of the pET15b vector for bacterial expression of the His_6-tagged protein.

Overexpression and purification of sbTNFα

E. coli BL21(DE3) cells freshly transformed with the pET15b-sbTNFα construct were cultured overnight in LB-ampicillin medium. After dilution into fresh LB-ampicillin, the cultures were grown at 37 °C to A_{600} of 0.8 and induced with 1 mM isopropyl-D-thiogalactoside (IPTG; Applichem, Darmstadt, Germany) for 0.25–4 h at either 25 or 37 °C. Protein expression in whole-cell extracts was checked by centrifuging 0.1 ml of induced culture (14,000 rpm in a Microfuge), and the cell pellet was lysed by boiling in SDS-loading buffer for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [16] and Western blotting using an anti-polyhistidine mAb (Sigma). To check the solubility of the expressed protein, the cell pellet (obtained as above) was suspended in buffer A (50 mM Tris, 2 mM EDTA, 200 mM NaCl, 4 mM 2-mercaptoethanol, pH 7.5), sonicated and centrifuged, and the supernatant and pellet were separately analyzed by Western blotting.

Pelleted cells from 0.25-l cultures induced for 2 h at 25 °C were suspended in 5 ml of ice-cold extraction buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.8) containing protease inhibitors (Cocktail P8340; Sigma) and sonicated as above. Recombinant His_6-tagged sbTNFα protein from clarified supernatants was purified using TALON metal affinity resin and the accompanying purification protocol (BD Biosciences, Madrid, Spain). After purification, the His_6 tag was removed using thrombin-agarose (Recom-T; Sigma) and aliquots were stored at –80 °C in the presence of 10% glycerol.

Polyclonal anti-sbTNFα antibody

Anti-sbTNFα rabbit polyclonal antibody was obtained by standard procedures using native sbTNFα for immunization [17]. The reactivity of the antiserum to sbTNFα was checked by Western blotting (see above).

Analytical size-exclusion chromatography

Analytical high-performance liquid chromatography (HPLC) size-exclusion data were obtained at room temperature using a Superdex-200 column equilibrated with buffer A with 150 mM NaCl, which is sufficient to minimize non-specific interactions with the column matrix. Column calibration was done using vitamin B12 (1.355 kDa), cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (BSA) (66 kDa), yeast alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa) (all from Sigma). Samples (150 µl) of sbTNFα at 10–100 µM were injected at 0.4 ml/min, and the elution was tracked by absorbance at 280, 235, and 220 nm. Void (Vo) and total (Vt) bed volumes were determined using blue dextran (2000 kDa;

Sigma) and vitamin B12, respectively. Elution volumes, Ve, were assigned for sbTNFα by verifying peak identities by Coomassie-stained SDS-PAGE and Western blotting. Stokes radii, RS (in nm) for the standards were obtained from Potschka [18]. The following calibration curves were generated from the data for the standards employing SigmaPlot (Jandel Scientific, San Rafael, Calif.) with correlation coefficients ≥ 0.99 in each case: log Mr = 7.56 – 0.206 Ve. This was then used to estimate the apparent Mr for sbTNFα [19].

Treatments and cell culture

For the in vivo studies, fish were injected intraperitoneally (i.p.) with 1 ml phosphate-buffered saline (PBS) containing either 10 µg of recombinant sbTNFα or buffer alone (negative control). At 4, 16 and 40 h post-injection, HK, peripheral blood and peritoneal exudate (PE) leukocytes were obtained as described elsewhere [20] and processed for subsequent chemiluminiscence and flow cytometry analysis (see below). In parallel, groups of three fish (treated as above) were injected i.p. with 50 mg/kg body weight of 5-bromo-2′-deoxyuridine (BrdU) and, after 2 h, the HK was removed and processed for light microscopy.

For the in vitro studies, seabream HK leukocytes from non-injected fish were incubated at 25 °C for different periods of time with 0.1–100 ng/ml sbTNFα in sRPMI [RPMI-1640 culture medium (Gibco, Madrid, Spain) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl] supplemented with 5% fetal calf serum (FCS) (Gibco) and 100 IU/ml penicillin and 100 µg/ml streptomycin (Biochrom, Berlin, Germany).

Murine L929 fibrosarcoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS and penicillin/streptomycin at 37 °C.

Chemiluminiscence assay

Respiratory burst activity was measured as the luminol-dependent chemiluminescence produced by HK and PE leukocytes [21]. This was brought about by adding 100 µM luminol (Sigma) and 1 µg/ml phorbol myristate acetate (PMA; Sigma), while the chemiluminescence was recorded every 117 s for 1 h in a FLUOstart luminometer (BGM LabTech, Offenburg, Germany). The values reported are the average of octuple readings, expressed as the slope of the reaction curve from 117 to 1170 s, from which the apparatus background was subtracted.

Immunofluorescence staining and flow cytometry analysis

Aliquots of 10^5 cells were washed in PBS containing 2% FCS and 0.05% sodium azide, incubated for 20 min on ice with 100 µl of a 1/100 dilution of a mAb specific to gilthead seabream acidophilic granulocytes (G7) [20], washed, and incubated with 50 µl of a 1/100 dilution of FITC-labeled anti-mouse IgG $F(ab')_2$ fragment of goat antibody (Sigma) for 20 min on ice. Cells were then washed twice and data were collected in the form of two-parameter forward scatter (FSC) and side scatter (SSC) dot plot and green fluorescence (FL1) or red fluorescence (FL2) histograms using a FACS (BD Biosciences). The specificity of staining was checked using a mAb isotype standard.

Light microscopy and immunohistochemical staining

The HKs were fixed in Bouin-Hollande fluid solution, embedded in Paraplast Plus (Sherwood Medical, Norfolk, Nebr.) and sectioned at 5 µm. After dewaxing and rehydratation, the sections were subjected to an indirect immunocytochemical method [22] using the G7 mAb [20] or an anti-BrdU mAb (BD Biosciences), at the optimal dilution of 1:10 or 1:5, respectively. The specificity of the reactions was determined by omitting the first antiserum.

Cytotoxicity and proliferation assays

Murine L929 and seabream HK cells were seeded in 96-well microtiter plates at 10^4 and 5×10^5 cells/well, respectively. The next day, a serial dilution of sbTNFα or hTNFα (Sigma) in culture medium supplemented with FCS was added to the cells in the presence or absence of actinomycin D (5 µg/ml; Sigma). After 24 h incubation, surviving mouse L929 cells were quantified by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT; Sigma) colorimetric method [23]. Similarly, the proliferation of seabream primary HK cells was determined after 48 and 72 h of incubation by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) colorimetric method [24] and further confirmed by hemocytometer counts.

Protein determination

The protein concentrations of recombinant sbTNFα samples and cell lysates were estimated by the BCA protein assay reagent (Pierce, Rockford, Ill.) using BSA as a standard.

Statistical analysis

Data were analyzed by one- or two-way analysis of variance and an unpaired Student's t test to determine difference between groups. A quantitative study of the FC results was made using the statistical option of the Lysis Software Package (BD Biosciences).

Results

Recombinant mature sbTNFα shows an oligomeric nature

Western blot using an anti-polyhistidine mAb revealed that recombinant His_6-tagged sbTNFα was induced as

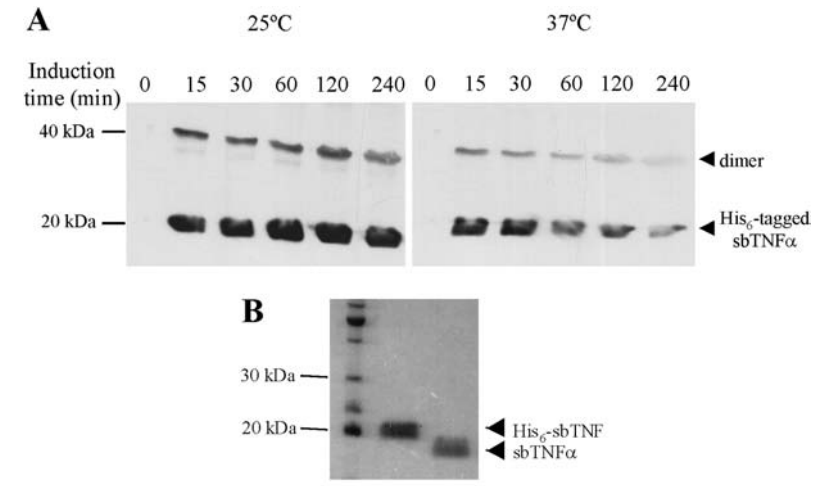

Figure 1. Production of recombinant sbTNFα. (*A*) Western blot analysis of native His_6-tagged sbTNFα in soluble fractions from *E. coli* cultures induced with 1 mM IPTG for the indicated times at either 25 or 37 °C. The filters were hybridized with anti-polyhistidine mAb. (*B*) SDS-PAGE analysis of metal affinity-purified sbTNFα before (lane 1) and after (lane 2) removing the His_6 tag using thrombin-agarose. Proteins were stained with Coomassie Brilliant Blue R-250.

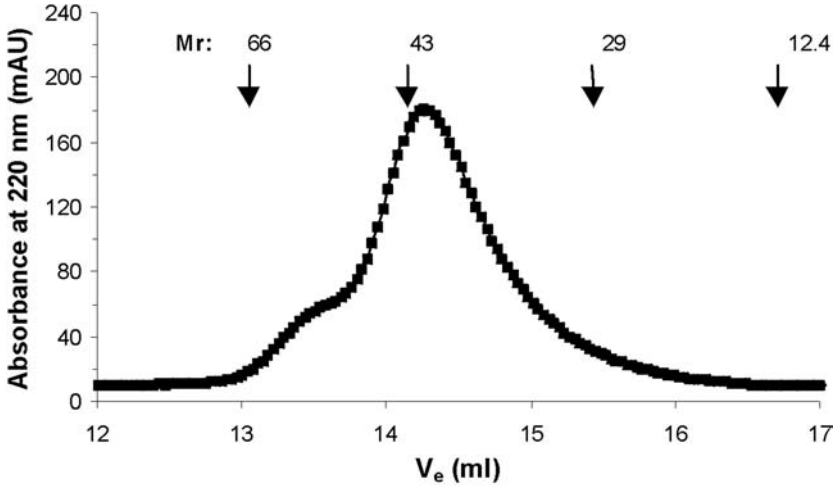

Figure 2. Oligomeric nature of sbTNFα assayed by size-exclusion chromatography. Samples of sbTNFα at 20 µM were applied to a Superdex-200 column. Protein elution was followed by measuring the absorption at 220 nm and 1-min fractions were collected. The column was calibrated with the following standards (arrows): BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

early as 15 min after the addition of IPTG at both 25 and 37 °C, although the amount of soluble protein rapidly decreased at 37 °C, probably due to the formation of inclusion bodies, while it remained soluble for up to 4 h at 25 °C (fig. 1A). Interestingly, an additional polypeptide of about 40 kDa was also detected by the anti-polyhistidine mAb, which might represent a dimeric form of the sbTNFα.

The above results, together with the trimeric nature of bioactive mammalian TNFα [25, 26], prompted us to assess the oligomeric nature of recombinant sbTNFα by analytical gel filtration HPLC [19]. Native His_6-tagged sbTNFα was affinity-purified from cultures induced at 25 °C for 2 h and the tag subsequently cleaved off by thrombin (fig. 1B). sbTNFα eluted in a main peak with a molecular mass of 41 kDa and a secondary peak with a molecular mass of 60 kDa (fig. 2), which may correspond to sbTNFα dimers and trimers, respectively. No additional peaks were detected, indicating that recombinant sbTNFα shows an oligomeric nature and that dimers predominate over trimers.

sbTNFα i.p. injection results in phagocyte activation, mobilization, and proliferation

The biological activities of recombinant sbTNFα were evaluated by injecting fish i.p. with 10 µg of the purified protein. Injection resulted in the priming of the respiratory burst activity of PE (fig. 3A) and HK (fig. 3B) cells

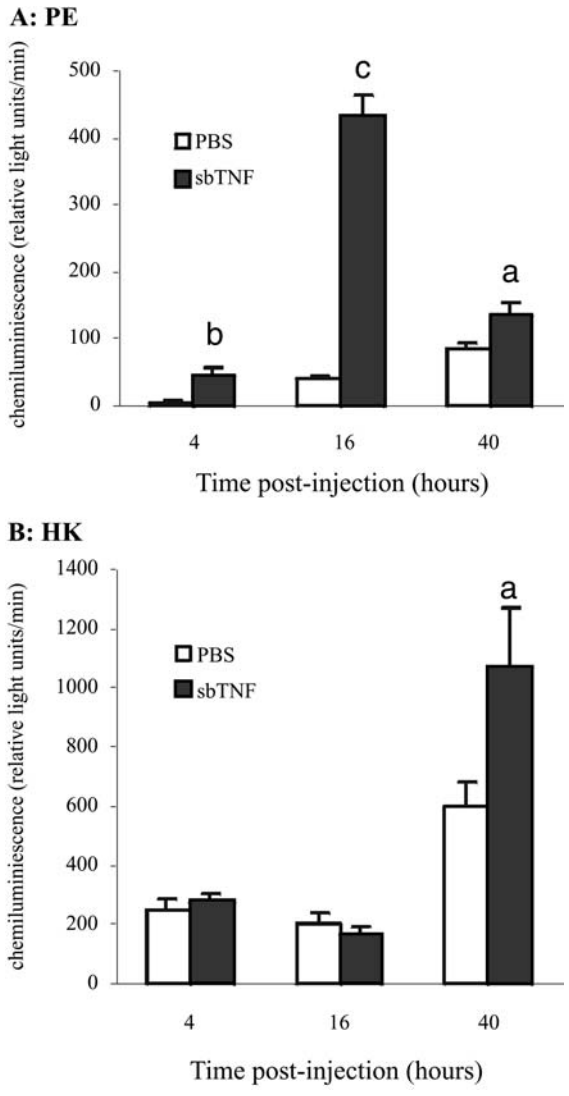

Figure 3. sbTNFα i.p. injection results in the priming of the phagocyte respiratory burst. Fish were injected i.p. with 1 ml PBS containing either buffer alone or 10 µg sbTNFα. At 4, 16, and 40 h post-injection, the respiratory burst activity of PE (*A*) and HK (*B*) cells was measured as the luminol-dependent chemiluminiscence triggered by PMA (1 µg/ml). Data are presented as mean ± SE of five fish. Versus PBS-injected fish: $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.0001$.

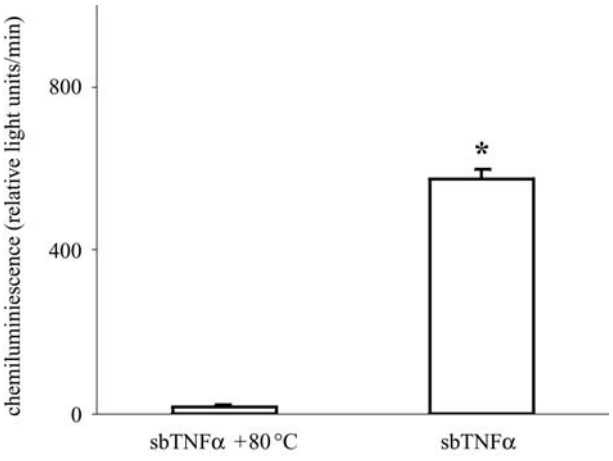

Figure 4. The bioactivity of recombinant sbTNFα samples is heat labile. Fish were injected i.p. with 1 ml PBS containing 10 µg sbTNFα that had been pre-heated or not at 80 °C for 30 min. At 16 h post-injection, the respiratory burst activity of PE cells was measured as indicated in the legend to figure 3. Data are presented as mean ± SE of three fish. Versus pre-heated sbTNFα-injected fish: $^{*}p < 0.0001$.

triggered by PMA. The maximum enhancement of PE cell respiratory burst activity was observed 16 h post-injection, although a significant priming effect was seen as early as 4 h, lasting to about 40 h post-injection. In HK cells, however, the respiratory burst was only primed 40 h post-injection but remained at basal levels at 4 and 16 h following the i.p. injection of sbTNFα. A weak, but significant ($p < 0.05$), time-dependent increase in the respiratory burst of both HK and PE cells was observed after the i.p. injection of PBS containing the protein purification buffer alone.

Although gilthead seabream leukocytes are unable to respond to concentrations of LPS below 5 µg/ml [27], we checked whether the observed priming effects on the respiratory burst of PE and HK cells after sbTNFα i.p. injection was solely due to the recombinant cytokine rather than to possible contamination with LPS from *E. coli*. For this reason and taking into account that LPS is heat resistant, we evaluated the activity of sbTNFα samples that had been pre-heated at 80 °C for 30 min. Figure 4 shows that pre-heated sbTNFα was unable to prime the respiratory burst of PE cells, confirming that sbTNFα was actually responsible for the observed in vivo priming effects.

We next evaluated the proinflammatory activity of sbTNFα by FACS using a mAb specific to gilthead seabream acidophilic granulocytes, since this cell type represents the main phagocytic cell of this species and has been considered functionally equivalent to the neutrophil of higher vertebrates [20]. Injection of fish i.p. with sbTNFα resulted in a dramatic mobilization of leukocytes 16 h post-injection (fig. 5A) concomitant with a rapid (4 h post-injection) increase in the percentage of acidophilic granulocytes (fig. 5B), while a much slower and weaker increase in both the total amount of PE cells and the percentage of acidophilic granulocytes was also observed in buffer-injected fish (fig. 5A and data not shown). These changes were matched by those occurring in the peripheral blood, where the percentage of acidophilic granulocytes significantly increased at 16 h and slightly decreased at 40 h following the injection of sbTNFα, but remained unaltered in buffer-injected fish (fig. 5B and data not shown). The amount of peripheral blood leukocytes (PBL), however, was similar in control and sbTNFα-injected groups (data not shown). Interestingly, the injection of sbTNFα also resulted in a marked increase at 16 h post-injection of the percentage of aci-

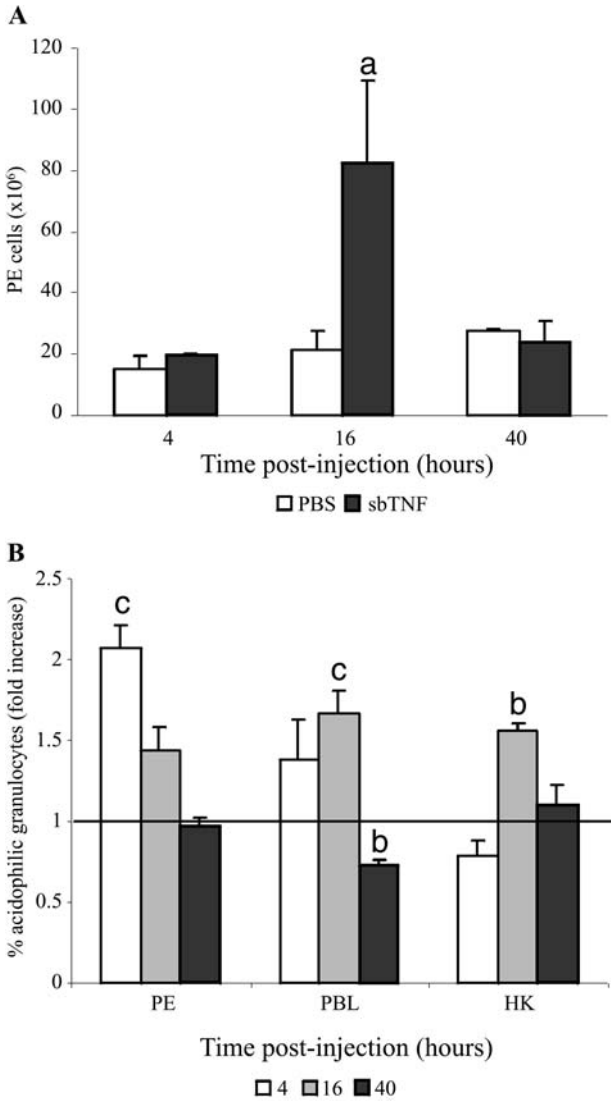

Figure 5. sbTNFα i.p. injection results in phagocyte mobilization. Fish were injected i.p. with 1 ml of PBS containing either buffer alone or 10 µg sbTNFα. At 4, 16 and 40 h post-injection, the number of PE cells was determined by hemocytometer counts (*A*) while the percentage of acidophilic granulocytes in PE, PBL and HK was evaluated by FACS after staining the cells with a mAb specific to these cells (G7) (*B*). Data are presented as mean ± SE of three fish. Horizontal line represents the control value (PBS-injected fish). Versus PBS-injected fish: $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$.

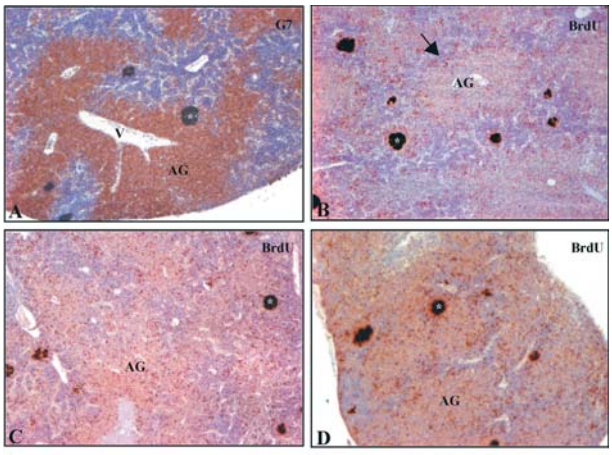

Figure 6. sbTNFα i.p. injection enhances granulopoiesis. Fish injected i.p. with 1 ml of PBS containing either buffer alone (*A*, *B*) or 10 µg sbTNFα (*C*, *D*) were pulsed for 2 h with 50 mg/kg body weight BrdU. HK sections were immunostained with the G7 mAb (*A*) or with an anti-BrdU mAb (*B*–*D*). Note that proliferating cells (arrow) were mainly restricted to the periphery of granulopoietic areas (AG) in control fish at 16 h post-injection (*B*), whereas they were abundant and distributed scattered in the HK of sbTNFα-injected fish at 16 h (*C*) and 40 h post-injection (*D*). V, blood vessel; *, melanomacrophage center. Magnification, × 10.

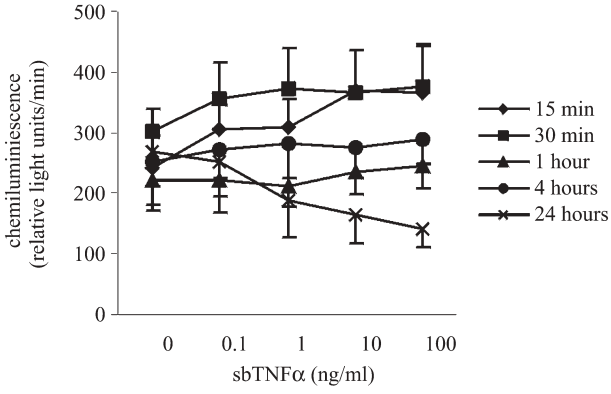

Figure 7. sbTNFα fails to prime the in vitro phagocyte respiratory burst. HK cells were incubated for the indicated times in the presence of 0–100 ng/ml sbTNFα. The respiratory burst was assayed as indicated in the legend to figure 3. Data are presented as mean ± SE of three fish.

dophilic granulocytes in the HK, the bone-marrow equivalent of fish (fig. 5B), while injection of buffer alone resulted in a slower, but significant ($p < 0.05$), increased percentage of these immune cells (data not shown).

The above results prompted us to investigate whether sbTNFα injection was able to enhance the proliferation rate of HK granulopoietic cells (fig. 6A). In fact, while proliferating cells (i.e. $BrdU^{+}$ cells) were mainly restricted to the periphery of HK granulopoietic areas in PBS-injected fish (fig. 6B), an abundant number of proliferating cells were distributed throughout the entire organ 16 h (fig. 6C) and, to some extent, 40 h (fig. 6D) following injection of sbTNFα. However, the HK from control and TNFα-injected groups showed bigger granulopoietic areas 40 h post-injection when compared to 4- and 16-h post-injected fish, confirming the increased percentage of HK acidophilic granulocytes observed by FACS at the longest post-injection time.

sbTNFα fails to prime the in vitro phagocyte respiratory burst, but acts as a growth-promoting factor

We next examined the in vitro activity of sbTNFα. Figure 7 shows that sbTNFα failed to prime the respiratory

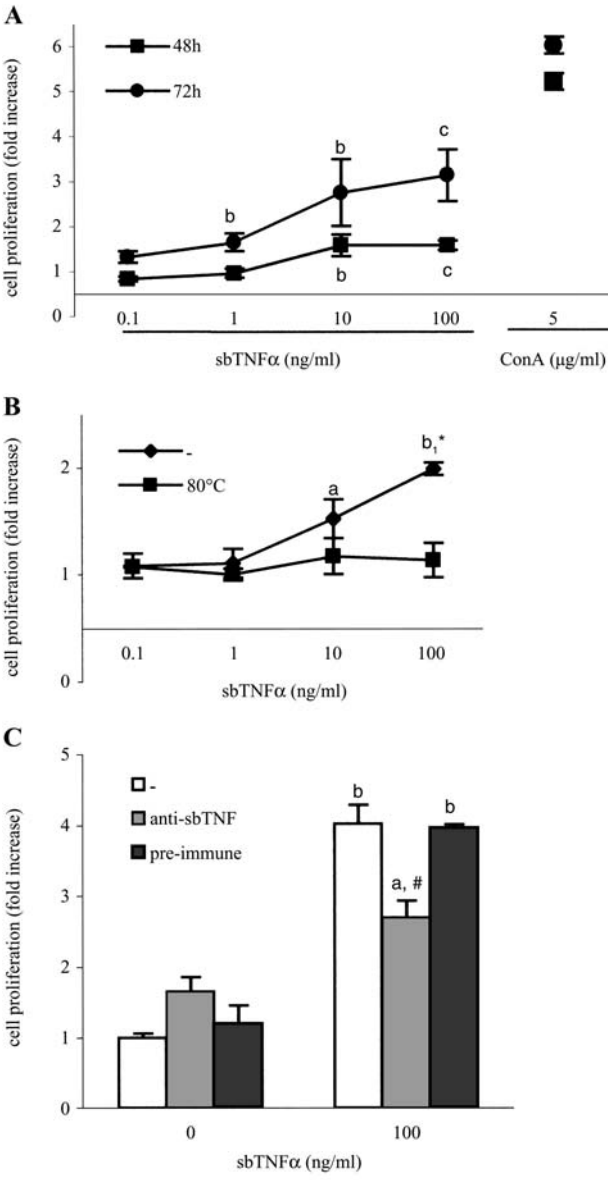

Figure 8. sbTNFα stimulates in vitro the proliferation of HK cells. Aliquots of 5×10^5 HK cells were incubated for 48 or 72 h with 0–100 ng/ml sbTNFα or 5 μg/ml ConA (*A*), or for 72 h with 0–100 ng/ml sbTNFα that had been pre-heated or not at 80 °C for 30 min (*B*), or in the absence or presence of 0.1 μl of either pre-immune or anti-sbTNFα serum (*C*). After the indicated incubation times, cell proliferation was determined as the reduction of MTT. Data are presented as mean ± SE of quadruplicate readings and are representative of three independent experiments. Versus PBS-injected fish: $^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$; versus pre-heated sbTNFα-injected fish: $^* p < 0.01$; versus pre-immune serum: $^\# p < 0.05$.

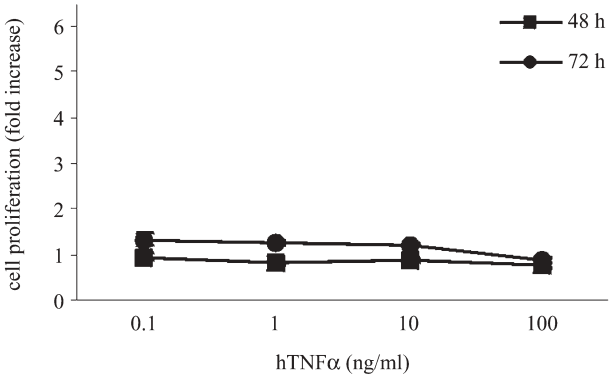

Figure 9. hTNFα was unable to stimulate the proliferation of HK cells. HK cells were incubated with 0–100 ng/ml hTNFα and cell proliferation assayed 48 and 72 h later, as indicated in the legend to figure 8. Data are presented as mean ± SE of quadruplicate readings and are representative of two independent experiments.

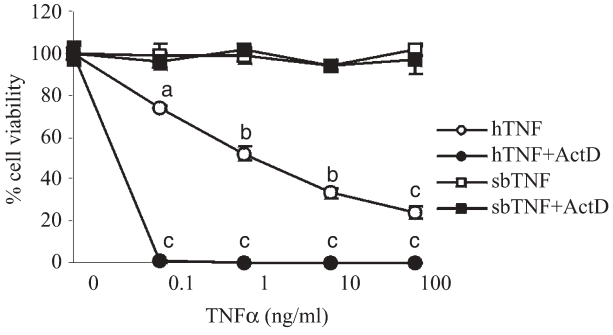

Figure 10. sbTNFα is unable to kill murine L929 cells. Aliquots of 10^4 L929 were incubated with 0–100 ng/ml of either sbTNFα or hTNFα in the presence or absence of 5 μg/ml actinomycin D (ActD). After 24 h incubation, the number of surviving cells was determined by the XTT colorimetric assay. Data are presented as mean ± SE of quadruplicate readings and are representative of two independent experiments. Versus control cells: $^a p < 0.01$, $^b p < 0.001$, $^c p < 0.0001$.

burst of HK leukocytes triggered by PMA. Similar results were obtained with PE leukocytes and using 0, 10, 100, or 1000 ng/ml PMA, 250 μg/ml concanavalin A (ConA) or 1 mg/ml opsonized zymosan to trigger the respiratory burst (data not shown). As TNFα is involved in cell death signaling in many mammalian cell types, we therefore assessed cell viability by means of the commonly used MTT colorimetric assay [24]. Of note is the fact that the addition of sbTNFα did not have a significant impact on the viability of HK cells after 24 h of incubation (data not shown), but markedly increased (in a dose-dependent manner) the proliferation of HK cells after 48 and 72 h incubation (fig. 8A). As expected, the addition of 5 μg/ml ConA induced a strong proliferation of HK cells. Notably, the growth-promoting effect of the sbTNFα was thermolabile (fig. 8B) and could be partially abrogated with neutralizing anti-sbTNFα antibodies (fig. 8C).

Mammalian and fish TNFα show selective species specificity

Earlier studies seemed to indicate that fish, and even invertebrate, cells are able to respond to recombinant hTNFα [9, 10]. We therefore studied whether seabream

HK leukocytes would respond to hTNFα and whether mouse cells would respond to sbTNFα. For the latter, we used the well-established L929 cytotoxic assay. Interestingly, hTNFα was unable to affect the proliferation of HK cells (fig. 9), in sharp contrast to the strong growth-promoting effect of sbTNFα on these cells (fig. 8A). Conversely, sbTNFα had no cytotoxic effect on L929 cells, whereas hTNFα killed these cells in both the absence and presence of actinomycin D (IC_{50} = ~1 ng/ml and <0.1 ng/ml, respectively) (fig. 10). These results suggest that sbTNFα failed to interact and/or activate either murine TNFR1 or TNFR2.

Discussion

Our current knowledge of invertebrate cytokines and, to some extent, of lower vertebrate cytokines is based mainly on immunocytochemical methods and functional assays. Cross-reactivity based on mammalian antibodies to mammalian cytokines or receptors does not unequivocally prove that the antigen in question is present in invertebrates or in lower vertebrates. In the absence of a further molecular characterization of the cross-reacting molecules, any conclusions regarding homology or common evolutionary origin remain presumptive [9]. The molecular and functional characterization of fish cytokines is now possible due to the availability of the sequences of various cytokine genes from different species as well as the full genome sequence of the fugu and zebrafish. Nevertheless, bony fish are the vertebrate group that contain the highest number of species as well as the highest phylogenetic diversity. Therefore, findings referring to other classes of vertebrate, namely mammals and birds, do not necessarily apply to all bony fish species.

In this report, we produced recombinant TNFα from the marine fish gilthead seabream, and studied its oligomeric nature as well as its biological activities in fish (seabream) and mammalian (mouse) cells. First, we demonstrated by HPLC that recombinant mature sbTNFα has an oligomeric nature, since the monomer is completely absent under native conditions, while the dimer is the main species and is accompanied by a much less abundant trimer. Although monomeric, dimeric and trimeric species of trout recombinant His_6-tagged TNFα have recently been described by means of SDS-PAGE analysis [28], the reducing conditions used during electrophoresis may induce the transition between native oligomers and denatured monomers, as occurs with mammalian TNFα after treatment with particular agents [29, 30]. In any case, the predominant dimeric nature of fish TNFα is surprising in view of the fact that bioactive mammalian TNFα is a trimer and that the monomer lacks biological activity [25, 26, 30]. Nevertheless, the demonstration that monomeric sbTNFα does not exist in native conditions suggests that oligomerization is also required for the bioactivity of fish TNFα. Whether both dimers and trimers of fish TNFα are bioactive must await further studies.

We demonstrate the in vivo proinflammatory activities of fish TNFα for the first time in a lower vertebrate. When injected i.p., sbTNFα is biologically active and able to regulate the main activities of innate immune cells at local and systemic levels. These activities include: (i) the priming of the respiratory burst of PE and HK cells; (ii) the recruitment of local, and likely HK, phagocytic cells to the site of injection, and (iii) the induction of granulopoiesis. In contrast, sbTNFα is unable to prime in vitro the respiratory burst of HK cells triggered by different agents, including PMA, ConA, and opsonized zymosan (fig. 7 and data not shown). In mammalian neutrophils and macrophages, some investigators have also noted that TNFα lacks a priming effect on PMA-triggered superoxide formation [31, 32], but others have found that TNFα primes the oxidative burst activity triggered by PMA [33, 34] or parasites [35, 36]. In the trout, a recent in vitro study showed that recombinant trout TNFα enhances leukocyte migration and phagocytosis, but the respiratory burst of leukocytes treated with trout TNFα was not evaluated [28]. Our apparently contradictory in vitro and in vivo data on the effects of sbTNFα on the phagocyte respiratory burst might be explained by taking into account that the activation of the respiratory burst and the recruitment of acidophilic granulocytes peak simultaneously in the peritoneum (16 h post-injection). Therefore, the observed in vivo effect of sbTNFα on the respiratory burst may be the result of an increased percentage of acidophilic granulocytes in this immune tissue rather than a true priming effect of resident cells. In the HK, however, the priming of the respiratory burst was observed 40 h post-injection of sbTNFα, whereas the increased percentage of acidophilic granulocytes was observed earlier (i.e., 16 h post-injection). Therefore, this will need further investigation with immunopurified acidophilic granulocytes and/or single-cell analysis of the respiratory burst by FACS.

The ability of sbTNFα to regulate cellular proliferation in vitro and in vivo was also demonstrated in this study. This is particularly important in view of the fact that a 'double-edged sword' role has been assigned to mammalian TNFα, since it either induces cellular proliferation, survival, differentiation, or apoptosis [37]. We observed no apoptotic or cytotoxic effect of sbTNFα on HK leukocytes, but rather a strong growth-promoting effect both in vitro and in vivo. Notably, while proliferation is mainly restricted to the periphery of granulopoietic areas in the HK of control fish, the injection of sbTNFα results in a marked increase in the number of proliferating cells that appear scattered throughout the hematopoietic organ. In contrast, a large number of studies have suggested that TNFα negatively regulates the growth of murine and hu-

man hematopoietic stem cell populations [38–40]. Nevertheless, in a more recent demonstration TNFα did not appear to enhance apoptosis or affect the cell cycle distribution of cultured progenitors but, rather, promoted myeloid differentiation [41]. In addition, TNFα is able to skew monocyte differentiation from macrophages to dendritic cells and this facilitates the induction of adaptive immunity [42]. Studies are in progress in our laboratory to ascertain whether similar mechanisms operate in fish, that is whether TNFα is able to regulate the differentiation of fish hematopoietic cells and/or whether it might facilitate the induction of fish adaptive immunity. This will be interesting not only from a phylogenetic point of view, but also for its potential use as vaccine adjuvant in aquaculture.

The most important finding of this study is that mammalian and fish TNFα show species specificity. Thus, hTNFα is able to kill murine L929 cells whereas sbTNFα cannot. Conversely, hTNFα is unable to affect the proliferation of seabream HK cells, while sbTNFα is a strong growth-promoting factor for such cells. In view of the results reported in this study one is tempted to speculate that the species specificity of TNFα may be related to the different oligomeric natures of hTNFα (trimeric) and sbTNFα (predominantly dimeric) and/or to the biological activities of soluble versus membrane-bound TNFα. In fact, mammalian TNFR2, unlike TNFR1, is poorly activated by soluble TNFα, and efficient activation of TNFR2 requires membrane-bound TNFα [43]. Alternatively, the homology between fish and mammalian TNFαs, which show about 35% identity and 55% similarity, may not be sufficient to allow cross-receptor engagement. This last hypothesis must be taken into account, since hTNFα binds to murine TNFR1 but not to murine TNFR2 [44], despite the high homology between mTNFα and hTNFα (78% identity and 90% similarity). Whatever the case, the molecular and functional characterization of fish TNFRs will be needed before the reasons for the species specificity of TNFα in vertebrates can be ascertained.

In conclusion, we demonstrated for the first time an in vivo physiological role for TNFα in fish. sbTNFα is mainly found as a dimer and is able to regulate the respiratory burst, the mobilization and the proliferation of phagocytes. In addition, sbTNFα is not bioactive for mammalian cells and hTNFα is inactive in fish cells. Our data point to the need for further research from a phylogenetic perspective to complete our knowledge about the complex evolution of TNFα in vertebrates.

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