Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol15-issue5-fulltext-19 *SHORT COMMUNICATION*

Immunological strategy for detecting the pro-inflammatory cytokine TNF-alpha in salmonids

Verónica Rojas[1] · Byron Morales-Lange[1] · Fanny Guzmán[1,2] · José A Gallardo[3] · Luis Mercado[1,2] ✉

1 Pontificia Universidad Católica de Valparaíso, Instituto de Biología, Laboratorio de Genética e Inmunología Molecular, Valparaíso, Chile

2 Pontificia Universidad Católica de Valparaíso, Núcleo de Biotecnología de Curauma, Valparaíso, Chile

3 Pontificia Universidad Católica de Valparaíso, Facultad de Recursos Naturales, Escuela de Ciencias del Mar, Laboratorio de Genética Aplicada, Valparaíso, Chile

✉ Corresponding author: lmercado@ucv.cl
Received August 13, 2012 / Invited Article
Published online: September 15, 2012

Abstract

Background: Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine which exerts a variety of immunological functions in vertebrates. TNF-α has been identified and cloned in a number of teleost fish species; nevertheless, the functions displayed by this cytokine in fishes remain poorly understood, given that the low sequence identity compared to their mammalian counterpart, limit fish TNF-α detection using mammalian antibodies. Then, for fish immune response characterization is fundamental the production of specific fish anti-TNF-α antibody. **Results:** We have developed a monoespecific antibody against the pro-inflammatory molecule TNF-α of salmonid fish. TNF-α epitope region was identified and characterized using bioinformatic tools. The epitope sequence was chemically synthesized using Fmoc strategy, analyzed by RP-HPLC and its molecular weight confirmed by mass spectrometry. The synthetic peptide was used to immunize mice and antibodies from ascitic fluid were purified. The resulting antibody was used for molecular and histochemical detection in gut samples from salmonid fishes treated with different food. By ELISA, we detected a differential expression of TNF-α, the western blot analysis shows recognition of the whole TNF molecule and by immunohistochemistry TNF-α positive cells were observed. **Conclusions:** We provide an immunological tool, validated through classical immunological assays, which can be a useful tool for characterizing fish TNF-α function.

Keywords: fish immunity, salmonid immunity, TNF-alpha antibody

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine produced by activated monocytes/macrophages, for example in response to pathogen-associated molecular patterns (PAMPs) exposure (Chettri et al. 2011; Roher et al. 2011). TNF-α exerts a variety of immunological functions such as regulation of cellular immune response and modulation of the expression of many other cytokines. Across all vertebrates, TNF-α plays an important role in inflammatory response to bacterial and viral infection, inducing the expression of α/β IFNs (MacKenzie et al. 2003). In fish, LPS and poly I:C induce mRNA expression of TNF-α in head kidney leucocytes and macrophages, following a similar activation kinetics observed in mammals; on the other hand, human recombinant TNF-α enhances macrophage respiratory burst activity in rainbow trout, providing indirect evidence that TNF-α

is an important macrophage-activating factor (MAF) (Roca et al. 2008; Boltaña et al. 2011); even so, the functions displayed by this cytokine in lower vertebrates remains until poorly understood.

TNF-α has been identified in all taxonomic groups of teleost fish and has been cloned in a number of fish species including Japanese flounder *Paralichthys olivaceus* (Hirono et al. 2000), rainbow trout *Oncorhynchus mykiss* (Laing et al. 2001; Zou et al. 2002), brook trout *Salvelinus fontinalis* (Bobe and Goetz, 2001), gilthead seabream *Sparus aurata* (García-Castillo et al. 2002), Atlantic salmon *Salmo salar* (Ingerslev et al. 2006; Morrisson et al. 2007), carp *Cyprinus carpio* (Savan and Sakai, 2004), channel catfish *Ictalurus punctatus* (Zou et al. 2003a), turbot *Scophthalmus maximus* (Ordás et al. 2007), mandarin fish *Siniperca chuatsi* (Xiao et al. 2007) and goldfish *Carassius auratus* L (Grayfer et al. 2008). Comparison of the gene structure and phylogenetic analysis of the amino acid sequences suggest that teleost TNFs are more closely related to mammalian TNF-α than TNF-β (Randelli et al. 2008).

In general, fishes appear to possess a repertoire of cytokines similar to that of mammals and several cytokine homologues have been cloned in fish species; nonetheless, the major trouble in identifying fish cytokines is the low sequence identity compared with their mammalian counterparts, which also limits the detection of fish cytokines using antibodies against human cytokines (Whyte, 2007). Previous works have developed antibodies to recombinant trout TNF (Zou et al. 2003b; Roher et al. 2011); nevertheless, the first one evaluates the antibody only against the recombinant protein and not against biological samples, and the second shows no bands migrating with the expected molecular weight.

Our interest was to develop an immunological tool for the detection of TNF in salmonid fish, since its key importance in fish immune response. For that, protein alignment of fish TNF available in databases were performed, epitopes were bioinformatically designed and chemically synthesized, and peptides were used to obtain a monospecific antibody against this molecule. This strategy allows generating antibodies against TNF-α based only on potential epitopes without requiring the recombinant protein. Our results show specific detection of a TNF protein in fish tissues using molecular and histological methods.

MATERIALS AND METHODS

Peptide design

The peptide was designed taking into account the homologous sequence of *Oncorhynchus mykiss* (gi:8052318). To define the best epitope we used the method described by Bethke et al. 2012. Briefly, we performed an alignment with ClustalW for conserved regions of fish TNF-α, identified hydrophilic and greater flexibility regions by Protscale of the Expasy server, then defined the best antigenic epitopes by the server of Harvard bioinformatics and finally, located the antigenic regions in a TNF trout model from the workspace of Swiss model server (Sun et al. 2009).

Peptide synthesis and purification

The candidate epitope peptide was synthesized by the solid phase multiple peptide system (Houghten, 1985) using Fmoc aminoacids (Iris and Rink resin 80.65 meq/g). Then, cleaved with TFA/TIS/H_20 (95/2.5/2.5) [Novabiochem] and 100 µg were run in a HPLC with 0-70% acetonitrile in 30 min with a flow of 1 ml/min of purified peptide. The peptide was lyophilized an analyzed by MALDI-TOF mass spectrometry.

Immunization and antibody purification

Antibodies were generated in mice as described by Bethke et al. 2012. Briefly, female CF-1 mice were injected intraperitoneally at 0, 7, 14, 21 days with 0.6 µg of the peptide diluted 1:1 in FIS and 500 µl of Freund adjuvant. At day 6 Pristane (2,6,10,14-tetramethylpentadecane) was injected to induce the tumour formation. At day 30 mice were anesthetized and drained, ascitic liquid was centrifuged and the supernatant stored at -80ºC. Antibodies were purified by immunoaffinity chromatography using the synthetic peptide bind to Cyanogen Bromide-Activated Sepharose 4B (CNBr-4B) (Narváez et al. 2010). The purified antibody, named as anti-epitope of TNF, was collected and stored at -20ºC.

To demonstrate anti-epitope recognition, antibody was first assayed by dot blot as described by Bethke et al. 2012. Briefly, nitrocellulose membranes were seeded with 2 and 1 µg of the synthetic epitope and a control dot with 0,5 µl of ascitic fluid (mouse IgG). Membranes were blocked with BSA 3% in PBS (PBSA). After washes, membranes were incubated at 37ºC for 90 min with 40 ng x μl^{-1} of the first antibody anti-epitope of TNF-α, and then, for 60 min with 1:8000 of the second commercial antibody anti-mouse IgG-HRP. Following washes, membranes were revealed with 3,3 diaminobenzidine (DAB) and hydrogen peroxide.

Antibody efficiency in peptide recognition was also evaluated by the enzyme-linked immunosorbent assay (ELISA) method (Narváez et al. 2010). Briefly, multiwell plates were incubated ON at 4ºC with serial dilutions of the synthetic peptide (from 2 ng x μl^{-1}), after blocking with PBSA they were subsequently incubated with both antibodies as described above. After both incubations plates were washed, incubated for 30 min with TMB single solution and optical densities (OD) determined by BIO-TEK Synergy HT ELISA reader at 450 nm.

***In vivo* assays**

For demonstrating TNF molecule recognition, the anti-epitope of TNF was assayed by western blotting and ELISA in gut samples obtained from rainbow trout fed with *Laminaria digitata* (Laminarin SIGMA®), and by immunohistochemistry in gill samples from Atlantic salmon infected by the ectoparasite *Caligus rogercresseyi*.

Trout (100-150 g) were obtained from Rio Blanco fish farming facility, Los Andes and maintained in the Laboratorio Experimental de Acuicultura (LEDA), Valparaíso, in tanks with dechlorinated and filtered fresh water. Fish were fed with pelleted dry food alone (controls) or supplemented with Laminarin (0.2 g/ fish kg) once a day. Salmon naturally infected with *C. rogercresseyi* were obtained from Fundación Chile, Quillaipe, Puerto Montt, Chile.

At sampling, fish were anesthetized with benzocaine (50 mg/L) and bled as completely as possible from caudal vein by excision of gill to reduce the blood volume in the tissues. Samples were removed with hypodermic scissors, homogenized in lysis buffer for western blot and ELISA analysis, or fixed in Bouin solution for immunohistochemistry.

For western blot assay, gut proteins were quantified by the BCA Protein Assay Kit and 30 ng x μl^{-1} of the protein samples were separated on a 12% polyacrylamide SDS-PAGE and transferred onto a PDVF membrane. First, the membrane was blocked with 5% PBSA for 45 min at 37ºC and then incubated with 1:100 dilution of the anti-epitope of TNF-α pre-absorbed for 2 hrs at RT with 40 µg of total protein from *O. mykiss* brain tissue. After extensive washing for 1 hr with 0,2 % of PBS-Tween, anti-IgG mouse-HRP (1:7000) was added and incubated at 37ºC for 60 min. After washing in the same condition, the membrane was revealed with chemiluminescence method.

For ELISA assay, 15 ng x μl^{-1} of the protein samples were seeded ON at RT. After blocking with 5% PBSA, plates were incubated for 90 min at 37ºC with 40 ng x μl^{-1} of the anti-epitope of TNF, and then, for 60 min with 1:20000 of commercial antibody anti-mouse IgG-HRP (Thermo Pierce). Following washes, plates were incubated for 30 min with TMB and OD determined with an ELISA reader at 450 nm. All assays were performed in triplicate. For statistical analysis, calculations of means, standard deviations and analysis of variance (ANOVA) of samples were carried out using Microsoft Excel 2007. Differences were considered significant if the *p*-value was < 0.05.

For immunohistochemistry assays, fixed gill samples were embedded in paraffin blocks, sectioned to a thickness of 5 µm and processed for immunohistochemical staining as described by Bethke et al. 2012. Briefly, sections were deparaffinized with Neoclear (Merck) and hydrated through a graded ethanol series. Then, slides were treated with hydrogen peroxide and methanol (9:1) to inactivate endogenous peroxidase.

After blocking with 5% PBSA, slides were sequentially incubated for 60 min with 1:100 dilutions in 1% PBSA of the anti-TNF and the biotinylated goat anti-mouse antibodies. Following washes, slides were incubated for 30 min with peroxidase-avidin-biotin complex (StreptABComplex/HRP DAKO). The slides

were counterstained with diluted Hansen hematoxylin solution, dehydrated through a degraded ethanol series and mounted with Neomount (Merck).

RESULTS AND DISCUSSION

Sequences of *O. mykiss* and *S. salar* TNF-α were analyzed *in silico* on basis to a semi-empirical method for prediction of antigenic determinants (Liang et al. 2009). We obtained 8 candidate epitope regions which were analyzed by flexibility and hydrophobicity, both conditions are good indicators of exposed regions in the molecule, being accessible to antibody recognition. The sequence 161-TTPLSHIIWRYSDSIGVNA-179 was defined as the most flexible (average score near 0.5) and less hydrophobic region (average score near 0) and was chosen as the candidate epitope.

To confirm surface exposition of this epitope we visualized it in the available three dimensional structure of rainbow trout TNF-α (PBD: 1TNR). Figure 1a shows that the selected epitope was highly exposed to the surface of the molecule. After chemical synthesis, the selected peptide was purified at > 95% by RP-HPLC and molecular weight was confirmed by mass spectrometry (MALDI-TOF).

Purified antibody was qualitatively tested by dot blot and quantitatively analyzed by ELISA against the synthetic peptide. Figure 1b shows positive dot blotting assay and a lineal correlation between the peptide concentration and its OD detection (r^2 = 0.995). Positive immunorecognition shows that the selected peptide was immunogenic and produced antibodies. The anti-epitope was validated in its capacity to recognize the synthetic peptide, as in Bethke et al. 2012. The specificity was demonstrated using ascitic fluids from non-immunized mice, as first antibody (not shown).

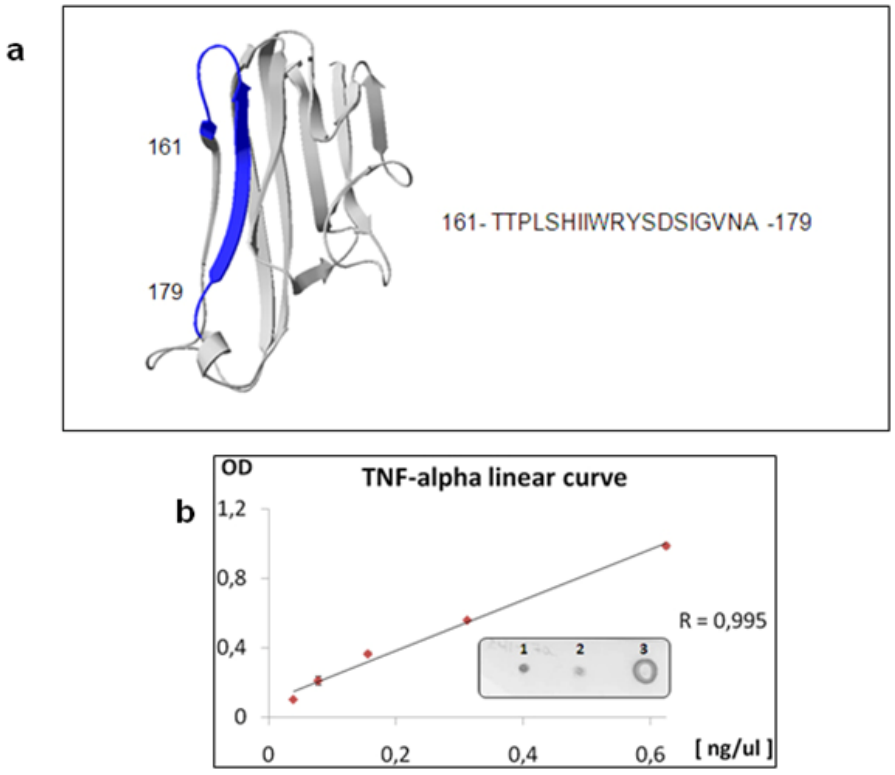

Fig. 1 (a) Epitope peptide location in the homolog model of *Onchorynchus mykiss* TNF-alpha. (b) Quantitative and qualitative immunorecognition of epitope peptide. ELISA assay shows linearity of the synthetic peptide dilution curve. Insert shows positive Dot blotting assay.

After validating the antibody, we performed an *in vivo* assay to detect TNF-α expression in gut samples from trout fed by conventional and Laminaran supplemented pellets. The versatility of the antibody was demonstrated in different assays, the western blot analysis shows a single band with the expected molecular weight (Figure 2a), but the antibody did not recognize the dimeric and trimeric forms described for the recombinant TNF (Zou et al. 2003b; Randelli et al. 2008). The anti-epitope of TNF is capable to recognize the synthetic peptide and also the whole TNF molecule in biological samples.

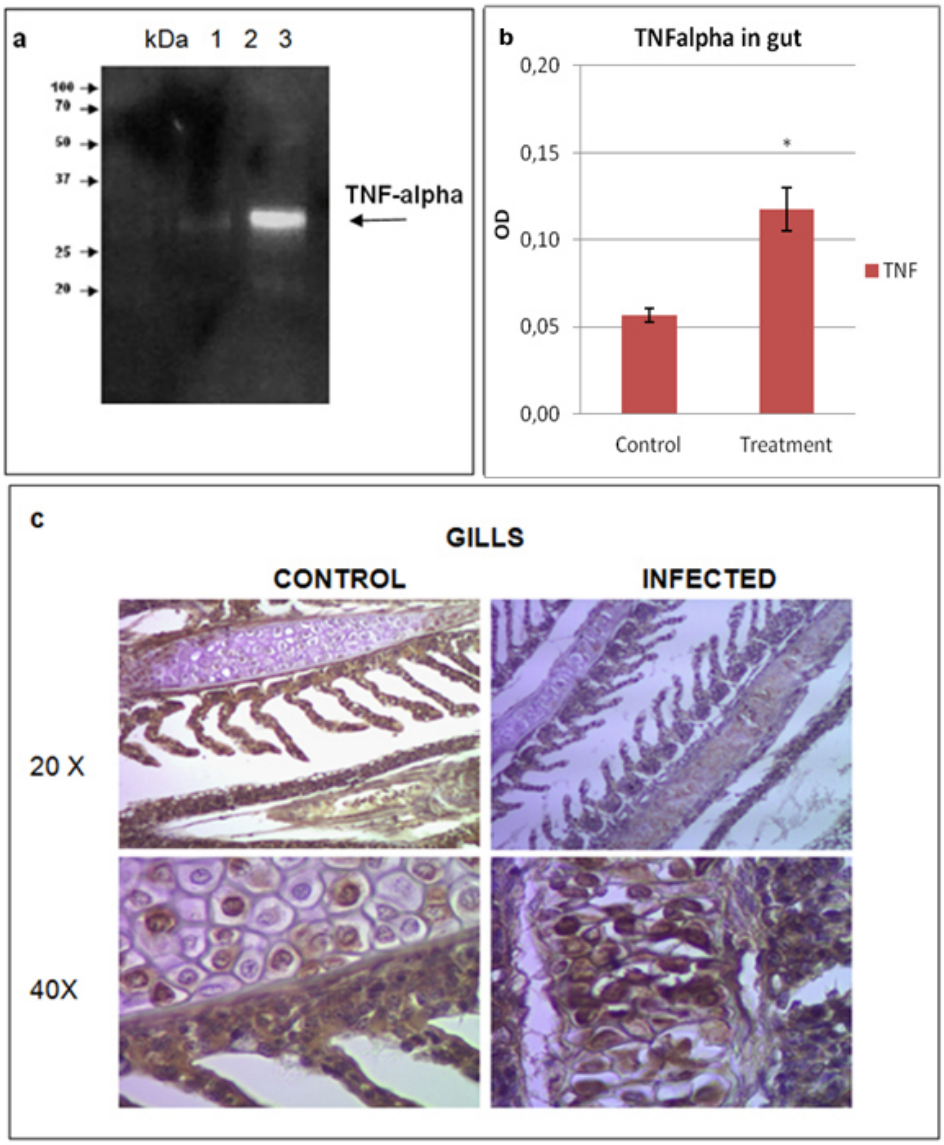

Fig. 2 (a) Western blotting, 1: Control, 2 and 3: Treatment with 10 µg and 20 µg respectively. (b) TNF-alpha detection in gut samples from control and fishes fed with laminarin (Treatment) by ELISA (*: p < 0,05). (c) Immunohistochemistry in gills tissue from controls and *Caligus* infected fishes.

By ELISA, a differentiated synthesis of TNF expression was detected in tissues from Laminaran fed fishes, with a significant increase ($p < 0.05$) at 21 days post-fed (Figure 2b). Moreover, gill histological sections show positive immunoperoxidase staining cells in *C. rogercresseyi* infected fishes (Figure 2c), allowing using the generated antibody as a very versatile and useful tool in salmonid species.

ACKNOWLEDGMENTS

We extend special thanks to Dr. Víctor Mulero, from the University of Murcia, Spain, for his background in immunohistochemistry assays.

Financial support: This work was supported by Grant 07CN13PBT-61 of InnovaCHILE-CORFO and AQUAINNOVO S.A. and ANTARES S.A.

REFERENCES

BETHKE, J.; ROJAS, V.; BERENDSEN, J.; CÁRDENAS, C.; GUZMÁN, F.; GALLARDO, J.A. and MERCADO, L. (2012). Development of a new antibody for detecting natural killer enhancing factor (NKEF)-like in infected salmonids. *Journal of Fish Diseases,* vol. 35, no. 5, p. 379-388. **[CrossRef]**

BOBE, J. and GOETZ, F.W. (2001). Molecular cloning and expression of a TNF receptor and two TNF ligands in the fish ovary. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, vol. 129, no. 2-3, p. 475-481. **[CrossRef]**

BOLTAÑA, S.; ROHER, N.; GOETZ, F.W. and MACKENZIE, S.A. (2011). PAMPs, PRRs and the genomics of gram negative bacterial recognition in fish. *Development & Comparative Immunology*, vol. 35, no. 12, p. 1195-1203. **[CrossRef]**

CHETTRI, J.; RAIDA, M.; HOLTEN-ANDERSEN, L.; KANIA, P. and BUCHMANN, K. (2011). PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*). *Development & Comparative Immunology*, vol. 35, no. 4, p. 476-482. **[CrossRef]**

GARCÍA-CASTILLO, J.; PELEGRÍN, P.; MULERO, V. and MESEGUER, J. (2002). Molecular cloning and expression analysis of tumor necrosis factor α from a marine fish reveal its constitutive expression and ubiquitous nature. *Immunogenetics*, vol. 54, no. 3, p. 200-207. **[CrossRef]**

GRAYFER, L.; WALSH, J.G. and BELOSEVIC, M. (2008). Characterization and functional analysis of goldfish (*Carassius auratus* L.) tumor necrosis factor-α. *Development & Comparative Immunology*, vol. 32, no. 5, p. 532-543. **[CrossRef]**

HIRONO, I.; NAM, B.H.T.; KUROBE, T. and AOKI, K.T. (2000). Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder *Paralychthys olivaceus*. *The Journal of Immunology*, vol. 165, no. 8, p. 4423-4427.

HOUGHTEN, R.A. (1985). General method for the rapid solid-phase synthesis of large numbers of peptides: Specificity of antigen-antibody interaction at the level of individual amino acids. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 15, p. 5131-5135. **[CrossRef]**

INGERSLEV, H.C.; CUNNINGHAM, C. and WERGELAND, H.I. (2006). Cloning and expression of TNF-α, IL-1β and COX-2 in an anadromous and landlocked strain of Atlantic salmon (*Salmo salar* L.) during the smolting period. *Fish & Shellfish Immunology*, vol. 20, no. 4, p. 450-461. **[CrossRef]**

LAING, K.J.; WANG, T.; ZOU, J.; HOLLAND, J.; HONG, S.; BOLS, N.; HIRONO, I.; AOKI, T. and SECOMBES, C.J. (2001). Cloning and expression analysis of rainbow trout *Oncorhynchus mykiss* tumour necrosis factor-α. *European Journal of Biochemistry*, vol. 268, no. 5, p. 1315-1322. **[CrossRef]**

LIANG, S.; ZHENG, D.; ZHANG, C. and ZACHARIAS, M. (2009). Prediction of antigenic epitopes on protein surfaces by consensus scoring. *BMC Bioinformatics*, vol. 10, no. 302. **[CrossRef]**

MACKENZIE, S.; PLANAS, J.V. and GOETZ, F.W. (2003). LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and *in vitro* differentiated macrophages. *Development & Comparative Immunology*, vol. 27, no. 5, p. 393-400. **[CrossRef]**

MORRISON, R.N.; ZOU, J.; SECOMBES, C.J.; SCAPIGLIATI, G.; ADAMS, M.B. and NOWAK, B.F. (2007). Molecular cloning and expression analysis of tumour necrosis factor-α in amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.). *Fish & Shellfish Immunology*, vol. 23, no. 5, p. 1015-1031. **[CrossRef]**

NARVÁEZ, E.; BERENDSEN, J.; GUZMÁN, F.; GALLARDO, J.A. and MERCADO, L. (2010). An immunological method for quantifying antibacterial activity in *Salmo salar* (Linnaeus, 1758) skin mucus. *Fish & Shellfish Immunology*, vol. 28, no. 1, p. 235-239. **[CrossRef]**

ORDÁS, M.C.; COSTA, M.M.; ROCA, F.J.; LÓPEZ-CASTEJÓN, G.; MULERO, V.; MESEGUER, J.; FIGUERAS, A. and NOVOA, B. (2007). Turbot TNF-α gene: Molecular characterization and biological activity of the recombinant protein. *Molecular Immunology*, vol. 44, no. 4, p. 389-400. **[CrossRef]**

RANDELLI, E.; BUONOCORE, F. and SCAPIGLIATI, G. (2008). Cell markers and determinants in fish immunology. *Fish & Shellfish Immunology*, vol. 25, no. 4, p. 326-340. **[CrossRef]**

ROCA, F.J.; MULERO, I.; LÓPEZ-MUÑOZ, A.; SEPULCRE, M.P.; RENSHAW, S.A.; MESEGUER, J. and MULERO, V. (2008). Evolution of the inflammatory response in vertebrates: Fish TNF-α is a powerful activator of endothelial cells but hardly activates phagocytes. *The Journal of Immunology*, vol. 181, no. 7, p. 5071-5081.

ROHER, N.; CALLOL, A.; PLANAS, J.V.; GOETZ, F.W. and MACKENZIE, S.A. (2011). Endotoxin recognition in fish results in inflammatory cytokine secretion not gene expression. *Innate Immunity*, vol. 17, no. 1, p. 16-28. **[CrossRef]**

SAVAN, R. and SAKAI, M. (2004). Presence of multiple isoforms of TNF-α in carp (*Cyprinus carpio* L.): Genomic and expression analysis. *Fish & Shellfish Immunology*, vol. 17, no. 1, p. 87-94. **[CrossRef]**

SUN, J.; WU, D.; XU, T.; WANG, X.; XU, X.; TAO, L.; LI, Y.X. and CAO, Z.W. (2009). SEPPA: A computational server for spatial epitope prediction of protein antigens. *Nucleic Acids Research*, vol. 37, no. S2, p. W612-W616. **[CrossRef]**

WHYTE, S.K. (2007). The innate immune response of finfish-A review of current knowledge. *Fish & Shellfish Immunology*, vol. 23, no. 6, p. 1127-1151. **[CrossRef]**

XIAO, J.; ZHOU, Z.-C.; CHEN, C.; HUO, W.-L.; YIN, Z.-X.; WENG, S.-P.; CHAN, S.-M.; YU, X.-Q. and HE, J.-G. (2007). Tumor necrosis factor-α gene from mandarin fish, *Siniperca chuatsi*: Molecular cloning, cytotoxicity analysis and expression profile. *Molecular Immunology*, vol. 44, no. 14, p. 3615-3622. **[CrossRef]**

ZOU, J.; WANG, T.; HIRONO, I.; AOKI, T.; INAGAWA, H.; HONDA, T.; SOMA, G.I.; OTOTAKE, M.; NAKANISHI, T.; ELLIS, A.E. and SECOMBES, C.J. (2002). Differential expression of two tumor necrosis factor genes in rainbow trout. *Oncorhynchus mykiss. Development & Comparative Immunology*, vol. 26, no. 2, p. 161-172. **[CrossRef]**

ZOU, J.; PEDDIE, S.; SCAPIGLIATI, G.; ZHANG, Y.; BOLS, N.C.; ELLIS, A.E. and SECOMBES, C.J. (2003a). Functional characterization of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. *Development & Comparative Immunology*, vol. 27, no. 9, p. 813-822. **[CrossRef]**

ZOU, J.; SECOMBES, C.J.; LONG, S.; MILLER, N.; CLEM, L.W. and CHINCHAR, V.G. (2003b). Molecular identification and expression analysis of tumor necrosis factor in channel catfish (*Ictalurus punctatus*). *Development & Comparative Immunology*, vol. 27, no. 10, p. 845-858. **[CrossRef]**

How to reference this article:

ROJAS, V.; MORALES-LANGE, B.; GUZMÁN, F.; GALLARDO, J.A. and MERCADO, L. (2012). Immunological strategy for detecting the pro-inflammatory cytokine TNF-alpha in salmonids. *Electronic Journal of Biotechnology*, vol. 15, no. 5. http://dx.doi.org/10.2225/vol15-issue5-fulltext-19