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Development of a new antibody for detecting natural killer enhancing factor (NKEF)-like protein in infected salmonids

J Bethke[1], V Rojas[1], J Berendsen[1], C Cárdenas[2], F Guzmán[2], J A Gallardo[3] and L Mercado[1,2]

1 Grupo de Marcadores Inmunológicos en Organismos Acuáticos, Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
2 Núcleo Biotecnología Curauma (NBC), Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
3 Escuela de Ciencias del Mar, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

Abstract

The main cellular responses of innate immunity are phagocytic activity and the respiratory burst, which produces a high amount of reactive oxygen species. Natural killer enhancing factor (NKEF) belongs to the peroxiredoxin family that has an antioxidant function and enhances cytotoxic cell activity. This molecule may play a key role in macrophage and cytotoxic cell communication during the innate immune response of fish against pathogens. In fish, the NKEF gene has been characterized in some species as showing an up-regulation in infected fish, suggesting a trigger effect upon NK-like cells. To detect and localize this molecule in salmonids at protein level, a monospecific polyclonal antibody was generated. A probable NKEF-like protein epitope region was identified and characterized using bioinformatic tools, and the sequence was chemically synthesized using Fmoc strategy, analysed by RP-HPLC and its molecular weight confirmed by mass spectrometry. The synthetic peptide was immunized and antibodies from ascitic fluid were obtained. The resulting antibody is a versatile tool for detecting NKEF by different immune techniques such as ELISA, Western blotting and immunohistochemistry. Analysis of NKEF-like protein is a useful method for characterizing immune properties of this molecule in fish during response to pathogens.

Keywords: ELISA, fish innate immunity, immunohistochemistry, natural killer enhancing factor, *Oncorhyncus mykiss*, *Salmo salar*.

Correspondence *L Mercado, Grupo de Marcadores Inmunológicos en Organismos Acuáticos, Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile (e-mail:lmercado@ucv.cl)*

Introduction

Natural killer enhancing factor (NKEF) was originally identified from human red blood cells, being highly expressed during erythroid differentiation, and named for its ability to enhance cytotoxicity of NK cells against tumour cells *in vitro* (Shau *et al.* 1994). NKEF belongs to a new class of peroxiredoxins (Prx) highly conserved in a wide variety of organisms, from prokaryotes to eukaryotes including mammals. Mammalian Prx are divided into five subfamilies; Prx I: NKEF-A, rat heme-binding protein 23 kDa (HBP23), mouse macrophage stress protein 23 kDa (MSP23); Prx II: human NKEF-B, human thiol-specific antioxidant protein (TSA), rat TSA, mouse TSA; Prx III: human and mouse erythroleukemic gene 5 protein (MER5); Prx IV: human antioxidant enzyme (AOE372); and Prx V: product of human open reading frame 06 (ORF06) (Shin *et al.* 2001; Dong, Li-Xin & Jian-Zhong 2007). All these proteins present a protective antioxidant activity, increasing cellular resistance to oxidative damage by hydrogen peroxide, alkyl hydroperoxide and heavy metals (Li & Waldbieser 2006). In humans, NKEF protein consists of two 22 kDa subunits, encoded by NKEF-A and NKEF-B genes, linked by disulphide bonds. They consist of 199 and 198 amino acids, respectively, and are highly

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homologous (Shau *et al.* 1994). The expression of these two genes is differently regulated, although both show similar antioxidant activity (Dong *et al.* 2007). Only the reduced form of NKEF-A enhances NK-cell cytotoxicity, while NKEF-B may mediate cellular responses to proinflammatory molecules (Sauri *et al.* 1996). In fish, the NKEF gene has been characterized in some species, such as common carp, *Cyprinus carpio* L. (Shin *et al.* 2001), rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Zhang *et al.* 2001), channel catfish *Ictalurus punctatus* (Rafinesque) (Li & Waldbieser 2006), turbot, *Scophthalmus maximus* (L.) (Chen *et al.* 2006) and pufferfish, *Tetraodon nigroviridis* Marion de Proce (Dong *et al.* 2007). They have the same genomic structure of six exons and five introns and are more similar in sequence identity and genomic structure to mammalian NKEF-A (PrxI) than to NKEF-B (Prx II) (Zhang *et al.* 2001; Dong *et al.* 2007). These researchers identified orthologous genes, and other reports have provided information about the role of NKEF during immune response in fish, revealing a differential NKEF mRNA expression in fish challenged with bacterial- (Chen *et al.* 2006, 2009), viral- or pathogen-associated molecular patterns (PAMPs) (Li & Waldbieser 2006; Dong *et al.* 2007). In fish, two types of cytotoxic cells have been described, NK cells and non-specific cytotoxic cells (NCC) that are agranular and similar to monocytes (Yoder 2004), also identified as the precursor of mammalian NK cells (Shen *et al.* 2002; Yoder 2004; Whyte 2007). NCC can destroy a variety of targets cells including tumour cells, virus-transformed cells and protozoan parasites (Yasuko *et al.* 2004). Otherwise, fish NK cells are morphologically and physiologically similar to mammalian NK cells presenting granules in their cytoplasm (Shen *et al.* 2002) and non-specific cytotoxicity to different target cells without prior induction (Shen *et al.* 2004; Yoder 2004; Utke *et al.* 2007), which distinguishes them from the NCC. The regulation of NKEF expression in virus-infected fish suggests a trigger effect upon NK cells as described in mammals (Booy *et al.* 2005; Utke *et al.* 2007). These data indicate the importance of an adequate tool for the detection and quantification of NKEF as a signaller of the immune response.

Normally, NKEF-A and NKEF-B have been analysed by RT-PCR, which gives information about the transcription of molecules that may indicate a phenotype in cells. Proteomic tools allow the characterization, quantification and identification of the regions where the molecule acts during an immunological response. Recently, a polyclonal antibody (PAb) was obtained from rabbit immunized with rNKEF-B of *Cyprinus carpio* generated in *E. coli* and used for the detection of this molecule by immunological probes in tissues (Huang *et al.* 2009). This antibody, however, recognized more than one epitope on the molecule, favouring cross-reactions. Also, it was generated to recognize NKEF-B isoform that has not been described in salmonids.

The aim of this study was to develop a monospecific polyclonal antibody for the detection of NKEF-like protein in salmonid fish. This polyclonal antibody has the ability to recognize a specific epitope providing a high detection of the molecule with low cross-reactivity. The versatility of this antibody was demonstrated in different immunological probes, using cells and tissue samples of infected salmonids.

Materials and methods

Peptide design

The peptide was designed taking into account the available NKEF homologous sequence of *O. mykiss* (Q9I886). To define the best antigenic epitope, the method of Kolaskar & Tongaonkar (1990) was used in the bioinformatics server from the immunomedicine group of the Universidad Complutense de Madrid (http://imed.med.ucm.es/Tools/antigenic.pl). Protscale (Gasteiger *et al.* 2005) by the Expasy server (Gasteiger *et al.* 2003) was used to analyse the physicochemical behaviour of the antigenic sequences and identify regions of high hydrophilicity and mean flexibility. The antigenic region in the whole molecule was located using a homology model (pdb: 1qmv) by the automatic modelling mode in the workspace of swissmodel server (Arnold *et al.* 2006). Also, ClustalW alignment (Larkin *et al.* 2007) was performed with different NKEF sequences of fish to determine conserved regions. The swissprot accession number for the different proteins was *Oncorhyncus mykiss*: Q9I886; *Salmo salar*: B5XAN1; *Paralichthys olivaceus*: Q4ZH88; *Ictalurus punctatus*: Q643S2; *Tetraodon nigroviridis*: Q4ZJF5; *Cyprinus carpio*: O93241; *Danio rerio*: A7MCD6; *Scophthalmus maximus*: Q1KLP9.

Peptide synthesis and characterization

The selected epitope was chemically synthesized by solid-phase multiple peptide system (Houghten

1985) using Fmoc-protected amino acids (Rink resin 0.65 meq g^{-1}; IRIS Biotech). The peptide was cleaved by TFA/TIS/H_20 (95%/2.5%/2.5%) (www.novabiochem.com) purified by RP-HPLC to >95% purity, lyophilized and analysed by MALDI-TOF to confirm its molecular weight. Additionally, a circular dichroism was performed; the spectrum was recorded at 20 °C using a Jasco J-810 spectropolarimeter coupled to a Peltier PFD-425S system for temperature control. Solutions of the peptide at 0.1 mg mL^{-1} in different percentages of 2,2,2-trifluoroethanol (TFE) were used (Roccatano *et al.* 2002). Spectra were collected from 195–260 nm using a quartz cell of 1 mm (parameters used were: scan speed 100 nm min^{-1}, data pitch 0.2 nm, 2 s response, 1 nm band width and 3 scans).

Antibody production

To generate the antibody, CF-1 mice obtained from the Instituto de Salud Pública de Chile were immunized at days 0, 7, 14 and 21 by intraperitoneal injection with 60 μg of the epitope and FIS (a T helper cell activator) peptide at ratio of 1:1 (Prieto *et al.* 1995) supplemented with 500 μL of Freund's complete adjuvant (Thermo). At day 6, 0.6 mL of pristane (2,6,10,14-tetramethylpentadecane) (Sigma) was injected to induce ascitic tumour formation (Narvaez *et al.* 2010). From day 30, mice were anaesthetized with a small dose of chloroform and drained with a needle 18G*1½″ (NIPRO); ascitic fluid rich in IgG was obtained and centrifuged at 10 000 ***g*** for 10 min, and supernatant was recovered and stored at −80 °C.

Antibody was purified by immunoaffinity chromatography using cyanogen bromide-activated sepharose 4B (CNBr-4b) according to the manufacturer's instructions (Sigma). Briefly, 1 mg of the synthetic epitope was coupled to CNBr-4B in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.4), and the unreacted active groups were blocked with 0.1 M Tris, pH 8. Then, the ascitic fluid diluted ten times with phosphate-buffered saline (PBS, pH 7.4) was loaded onto the column, and the IgG were eluted with 100 mM glycine (pH 2.5). The purified antibody, designated as anti-epitope of NKEF-like protein, was collected in 1 M Tris (pH 8) and stored at −20 °C.

Antibody validation was assayed by dot blot in nitrocellulose membrane 0.45 μm (Thermo) seeding 2 and 1 μg of the synthetic peptide and a control dot with 0.5 μL of ascitic fluid (mouse IgG). The membranes were blocked with BSA 3% (Boval) in PBS (PBSA) for 90 min at 37 °C; washed three times with PBS-Tween 0.05% (PBST); and incubated with 1:100 of the first antibody for 1.5 h at 37 °C. Following three washes, the second antibody anti-IgG mouse-HRP (Thermo) 1:8000 was added and incubated at 37 °C for 60 min.

Western blot was performed using *Caligus rogercresseyi*-infected and non-infected Atlantic salmon serum (12% SDS PAGE 30 μg total protein). PVDF membrane was activated in methanol for 2 min and washed in transfer buffer (20% methanol, 0.01% SDS, Tris 25 mM; glycine 192 mM). The gel sandwich was prepared in a cassette and placed in the module following the manufacturer's instructions (Mini Trans-Blot Bio-Rad). The transfer was performed at 100 volts for 90 min, PDVF membrane was washed with PBST five times and incubated with first antibody anti-epitope of NKEF-like (60 ng μL^{-1}) for 1.5 h at 37 °C, washed five times after which the second antibody at 1:8000 was added and incubated at 37 °C for 60 min. The membranes were revealed with DAB (3,3 diaminobenzidine, Sigma Aldrich) and hydrogen peroxide in PBS (10 mg; 10 μL; 10 mL). In parallel, Western blotting using anti-HSC 70 (epitope CNEVISWLDKNQTAERDE) as a housekeeping protein was performed.

Antibody efficiency in peptide recognition was also evaluated by ELISA. Briefly, multiwell plates were incubated ON at 4 °C with serial dilutions of the peptides (from 2 ng μL^{-1}), after blocking with 1% BSA for 2 h at 37 °C, they were incubated for 1.5 h at 37 °C with 40 ng μL^{-1} of the first antibody (anti-epitope of NKEF-like) and for 1.5 h with the second antibody-HRP, dilution 1:7000. After both incubations plates were washed three times with PBST in a Jencons Millenium 1000 Automatic Microplate Washer; subsequently 100 μL per well of TMB single solution (Invitrogen) was added and incubated for 30 min at RT; the reaction was stopped with 50 μL of 1 N sulphuric acid and read at 450 nm with a VERSAmax microplate reader. As a control, the peptide was incubated with ascitic fluid from a non-immunized mouse. To validate the assay, an epitope recovery test was performed. Samples were spiked with synthetic peptide at final concentrations of 0.5 and 1 ng μL^{-1}. Concentration was measured in

duplicate, and recovery percent was calculated according to Corrales, Gordon & Noga (2009).

In vitro and *in vivo* assays

The biological model for evaluating the versatility of the anti-epitope of NKEF-like protein was cultured rainbow trout and Atlantic salmon obtained from Río Blanco fish farming facilities, Los Andes, and from AQUADVISE facilities, respectively. Experimental challenges with *C. rogercresseyi* were performed as described in Torrealba, Toledo & Gallardo (2011). Briefly, fish weighing 150–200 g were infected with 100 copepodids per fish in experimental tanks. Non-infected control and infected fish were then reared in standard conditions of salinity (30–32 ppt), temperature (11–14 °C) and oxygen (8–10 mg L^{-1}). Samples were taken at 4 (early stage) and 14 days (later stage) post-infection for salmon and trout, respectively. The early stage of infection was defined as when the copepodids were settled in fins and the later stage when copepodids became mobile and settled in different body locations.

For *in vitro* assays, we used the rainbow trout spleen monocyte/macrophage cell line RTS-11 (Ganassin & Bols 1998) and head kidney primary cell culture. For the cell line, 1.5×10^6 cells per well were seeded in 6-well plates with 2 mL of L-15 medium supplemented with 2% FBS and antibiotic–antimycotic (penicillin 100 IU mL^{-1}, streptomycin 100 μg mL^{-1}, amphotericin B 2.5 μg mL^{-1}) and the cells were stabilized at 20 °C ON then stimulated for 12 h with 15 μg mL^{-1} of LPS and 10^5 mL^{-1} of ISA virus. For the detection of NKEF-like protein expression, a Western blotting was performed as described earlier, using the same housekeeping protein.

Primary cell cultures were obtained by anaesthetizing healthy rainbow trout with benzocaine (50 mg L^{-1}) and bleeding with a syringe from the caudal vein, to reduce the blood volume in the pronephros. Head kidney leucocytes were obtained according to Secombes (1990), with some modifications. Briefly, head kidneys were removed in sterile conditions and placed in ice-cold L-15 medium, passed through a 100 μm nylon net (BD Falcon Cell Strainer) using a syringe plunger and washed with L-15. Cell suspensions were carefully layered in a Percoll gradient (34%/51%) and centrifuged at 800 ***g*** for 40 min at 15 °C. The recovered cell phase was washed twice with L-15 and suspended in this medium. Cell suspensions were adjusted to $4–5 \times 10^6$ viable cells mL^{-1} in L-15 medium supplemented with 5% FBS (Gibco), 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10 U mL^{-1} heparin, seeded in 6-well plates and incubated ON at 17 °C. Cells were induced with 15 μg mL^{-1} LPS (Sigma) Zymosan as well as heat-attenuated *Vibrio ordalii* for 2 h. The induced cells were harvested and recovered by centrifugation at 400 ***g*** for 10 min, and the pellet was resuspended with lysis buffer containing Tris 20 mM, NaCl 1 M, Triton X-100 0.05%, PMSF 3 mM, EDTA 5 mM and 0.2% protease inhibitor cocktail (Sigma). A short sonication at 11 RMS was also performed for a total rupture of cells. The homogenate was centrifuged at 20 000 ***g*** for 10 min, and the supernatant was stored at −20 °C. Samples from the tissues were obtained and laid in 300 μL of lysis buffer and then frozen with liquid nitrogen and kept at −80 °C until use. Tissues were then homogenized with the same lysis buffer as cells, centrifuged at 20 000 ***g*** and the supernatant recovered. Proteins were quantified by a Thermo BCA Protein Assay Kit following the manufacturer's instructions.

For *in vivo* assays, immunohistochemistry and ELISA were performed. Fish were dissected and tissues treated in lysis buffer or fixed in Bouin's solution for up to 7 h. For immunohistochemistry, fixed tissue samples were embedded in paraffin blocks and sectioned to 5 μm; then, samples were dewaxed with Neoclear (Merck) twice for 5 min, hydrated through a descending gradient of ethanol (100%, 96%, 70%) to distilled water, each step for 3 min. Samples were treated with methanol and hydrogen peroxide (9:1) for 30 min to inactivate endogenous peroxidase; then, they were blocked with 5% PBSA in PBS for 30 min. Samples were incubated for 1 h at RT with 1:100 dilution in 1% PBSA of the first antibody anti-epitope of NKEF-like protein and then incubated in the same conditions with the second antibody, biotinylated anti-mouse IgG (Dako). Samples were incubated with peroxidase–avidin–biotin complex (StreptABComplex/HRP DAKO) for 1 h using DAB as substrate; the reaction was stopped with 50 mM Tris–HCl, pH 7.5. Counter staining was performed using diluted haematoxylin for 3 min. After washing three times with distilled water to remove excess stain, the samples were dehydrated with an ascending gradient of alcohols and mounted with Neomount (Merck).

ELISAs were performed as described earlier, using 15 ng μL^{-1} of the protein samples. As control, no protein samples were seeded.

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.

Results

According to *in silico* analysis made on the basis of a semi-empirical method for the prediction of antigenic determinants on protein antigens (Kolaskar & Tongaonkar 1990), we obtained 12 epitope candidate regions. The most flexible (average score near 0.5) and less hydrophobic region (average score near 0) was situated in the C-terminus of the molecule. The sequence 169-HGEVCPAGWKPGSDTIKPD-187 of the C-terminus region was chosen as the epitope candidate, considering the high exposure to surface as visualized in the three-dimensional structure model (template PDB: 1qmv) (Fig. 1a). The selected epitope, after chemical synthesis, had a high purity and its molecular weight was confirmed by MALDI-TOF (data not shown). Circular dichroism of the peptide confirmed that it does not acquire a secondary structure, maintaining the loop structure as in the complete molecule connecting two helical regions (Fig. 1b).

The immune recognition capacity of ascitic fluids was revealed by dot blot assay, and the antigen recognition was confirmed by indirect ELISA. The anti-epitope of NKEF-like protein purified from ascitic fluids showed a linear immune recognition capacity against the synthetic epitope (r^2 = 0.997) (Fig. 1c). The absence of natural recognition capacity of peritoneal immunoglobulin was demonstrated using ascitic fluids from non-immunized mouse as the first antibody (Fig. 1c). The antigen recovery assay displayed an optimum range of 98–108% mean recovery (Table 1), and the Western blot analysis of *Caligus* sp.-infected salmon serum showed a single band near to the NKEF molecular weight (Fig. 1d). Therefore, the anti-epitope of NKEF is able to recognize the synthetic peptide and

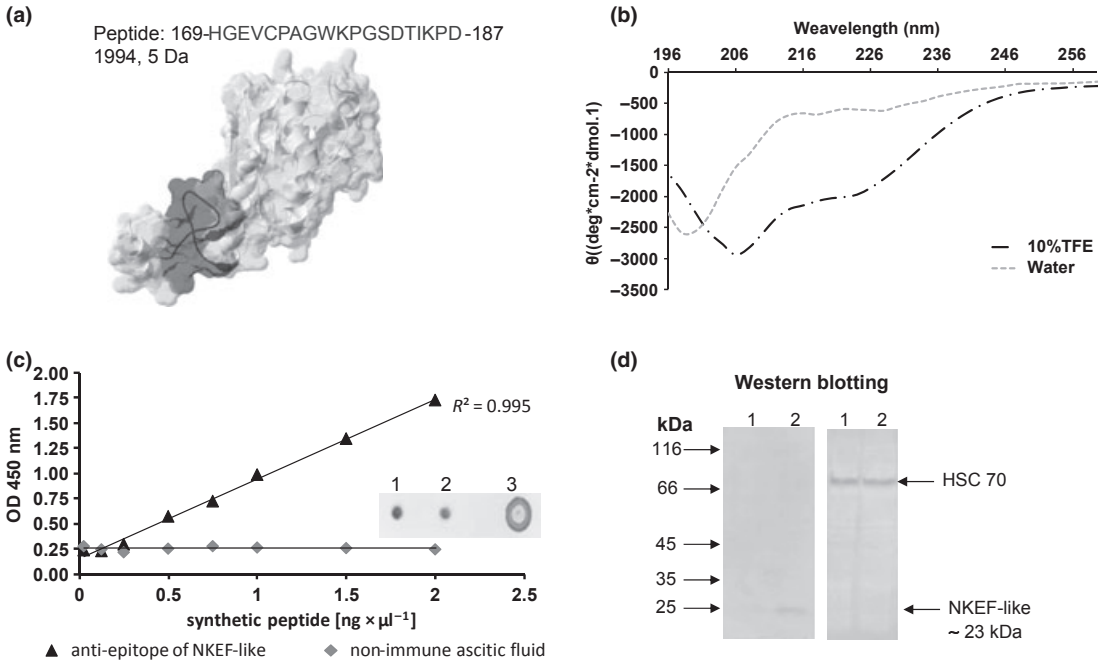

Figure 1 The selected epitope and its recognition by the antibody developed in this study. The surface projection of the epitope candidate sequence is represented in the secondary structure of natural killer enhancing factor (NKEF)-like protein (a) and the circular dichroism showing non-structured random coil (b). Dot blot shows positive recognition of the epitope peptide by ascitic fluids (1) 2 µg of synthetic peptide, (2) 1 µg of synthetic peptide and (3) control dot with 0.5 µL of ascitic fluid (insert in graph c). Linearity of ELISA shows a high correlation between peptide and Ab–peptide complex (c) using IgG purified against the epitope (▲), and no correlation was observed by the non-immune serum (◆). Western blotting of natural killer enhancing factor (NKEF)-like protein detected in serum of Atlantic salmon (d), uninfected (1) and infected (2) with *Caligus rogercresseyi*. Anti-HSC 70 is a housekeeping protein.

Table 1 Recovery of natural killer enhancing factor (NKEF)-like by ELISA from *Oncorhynchus mykiss* head kidney macrophage induced with different PAMPs supplemented with synthetic epitope

		Amount of synthetic peptide added					
		0.5 ng μL^{-1}			1.0 ng μL^{-1}		
Sample	Endogenous KKEF-like (ng $\mu L^{-1)}$	Expected	Observed	Recovery	Expected	Observed	Recovery
LPS2h	0.245 ± 0.026	0.745	0.771 ± 0.008	103	1.245	1.375 ± 0.021	110
Z2h	0.152 ± 0.003	0.652	0.726 ± 0.09	111	1.152	1.066 ± 0.002	93
Vo 2h	0.677 ± 0.124	1.177	1.051 ± 0.182	89	1.677	1.399 ± 0.103	83
C2h	0.210 ± 0.007	0.710	0.901 ± 0.05	127	1.210	1.275 ± 0.006	105
Mean				108			98

the whole NKEF molecule. The specific immune recognition of NKEF by anti-epitope of NKEF allows immunological assays to be performed.

Primary cultures of head kidney leucocytes (HKL) of trout challenged with the Gram-negative bacterium *Vibrio ordalii* showed a high induction increasing the expression of NKEF at the protein level twofold with respect to the control group, as detected by indirect ELISA (Fig. 2a). In parallel, analysis using the RTS-11 cell line, derived from trout, demonstrated the increased expression of NKEF-like protein in cells stimulated with LPS, meanwhile the infection with ISA virus produced a lower detection of this molecule (Fig. 2b). In the control group, the housekeeping protein showed the same level of detection.

Immunohistochemistry of head kidney and spleen tissues from rainbow trout infected with *Caligus rogercresseyi* showed brown positive immunoperoxidase staining cells (Fig. 3a) that were clearly differentiated from black melanomacrophages. No positive staining cells were observed in both non-infected and infected samples incubated only with the secondary antibody. Immunohistochemistry results were in agreement with indirect ELISA where a significant increase ($P < 0.05$) in NKEF-like expression was detected (Fig. 3b). On the other hand, Atlantic salmon infected with *Caligus* sp., during the first stage of infection at 4–7 days p.i, showed the gill and skin tissues with positive peroxidase-stained cells (Fig. 4); no positive cells were observed in control samples.

Discussion

The *in vitro* and *in vivo* functional biology of immune activities in teleosts is still in its infancy (Randelli, Buonocore & Scapigliati 2008); therefore, the use of antibodies for characterizing communication between immune cells is of growing importance for the understanding of the immune response in fish. To obtain an antibody against NKEF, a molecule that could play an essential role in the immune response, the sequence of rtNKEF available was analysed. According to *in silico* analysis, the most flexible (average score near 0.5) and less hydrophobic region (average score near 0) was situated in the C-terminus of the molecule. Both conditions are good indicators of a possible surface-exposed domain, and thus accessible to

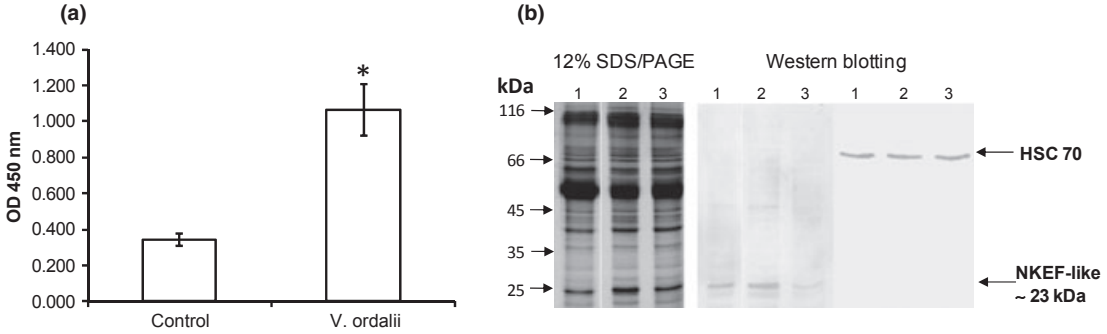

Figure 2 *In vitro* analysis of natural killer enhancing factor (NKEF) protein expression. (a) Indirect ELISA of induced HKL primary cell culture. (b) SDS PAGE and Western blotting of induced RTS-11 cell line, (1) control, (2) LPS, (3) ISAV. HSC 70 was used as housekeeping protein (*$P < 0.05$).

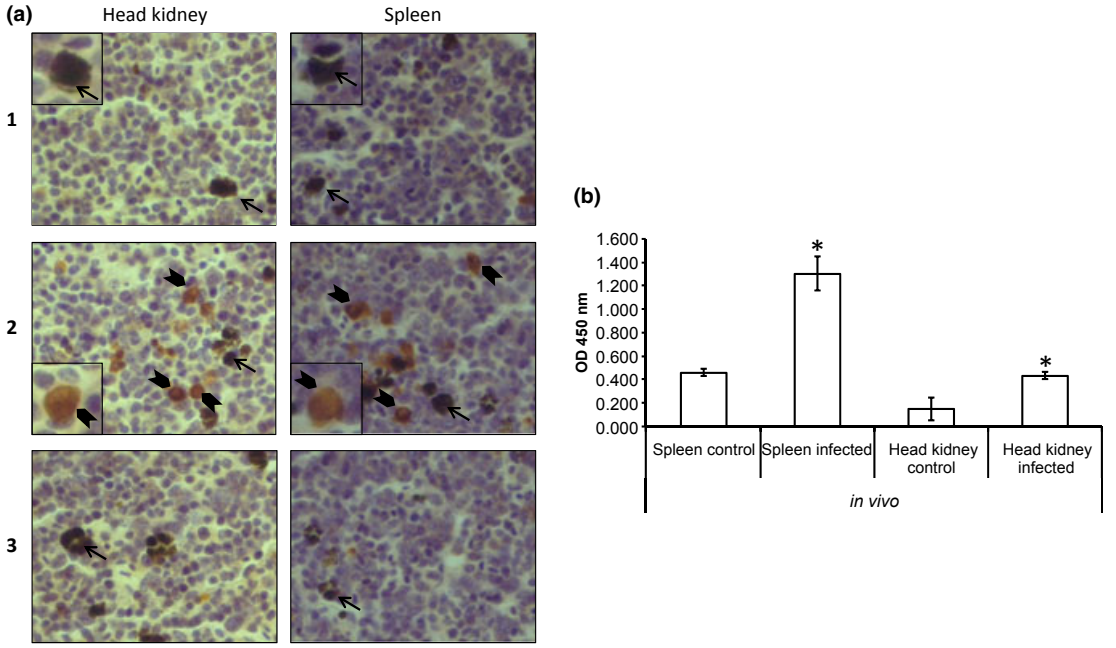

Figure 3 Detection of natural killer enhancing factor (NKEF)-like protein in head kidney and spleen from rainbow trout infected with *Caligus*. (a) Immunohistochemistry; (1) non-infected control, (2) infected fish showing positive peroxidase staining cells (➡), (3) negative control without primary antibody. Marked areas show positive cells differentiated from melanomacrophages. (b) Indirect ELISA of natural killer enhancing factor (NKEF) (*$P < 0.05$).

antibody recognition (Kulkarni-Kale, Bhosle & Kolaskar 2005; Liang *et al.* 2009; Sun *et al.* 2009); moreover, the presence of hydrophobic residues such as cysteine and valine in surface-exposed regions promotes antigenicity (Kolaskar & Tongaonkar 1990). The chosen sequence 169-HGEVCPAGWKPGSDTIKPD-187 of C-terminus does not acquire secondary structure after chemical synthesis, maintaining the loop structure as shown by the circular dichroism, connecting two helical structures as in the complete molecule, increasing the likelihood for the antibody to recognize the entire protein (Kolaskar & Tongaonkar 1990).

The versatility of the antibody was demonstrated in different assays, using both cell cultures and lymphoid tissues. The results indicated that primary cell cultures (HKL) and cell lines (RTS-11) can be used as models to study the expression of NKEF in response to PAMPs. At a systemic level in salmonids challenged with *Caligus* sp., NKEF protein expression is clearly detected by ELISA and visualized by immunohistochemistry. An *in vitro* study of NKEF expression in turbot embryonic cell line (TEC) by RT-PCR demonstrated a significant increase in NKEF 48 h after infection with *Vibrio anguillarum* (Chen *et al.* 2006). This study also showed that NKEF expression gradually increased in liver, spleen and head kidney of turbot 24 h after intraperitoneal injection with the same bacterium; moreover, the mRNA expression of NKEF-B increased 2-5 times in eight tissues analysed in ayu, *Plecoglossus altivelis* (Temminck and Schlegel), infected intraperitoneally with *Aeromonas hydrophila* (1.1×10^6 CFU) (Chen *et al.* 2009), showing that NKEF levels increase in fish infected with bacteria.

In head kidney and spleen tissues from rainbow trout infected with *Caligus rogercresseyi,* a significant increase ($P < 0.05$) of NKEF-like protein expression was detected by indirect ELISA. This condition was also observed in the immunohistochemical assay, with an increase in positive immunoperoxidase staining cells. Considering the biological model used in this study, *Caligus* infection causes the destruction of skin epithelium and may also inoculate pathogens into fish, which creates a condition of high infection, triggering a systemic immune response. This affects internal lymphoid organs including head kidney and spleen. The head kidney serves as a secondary lymphoid organ in the elaboration of immune responses and immune–endocrine interactions (Tort, Balasch & Mackenzie 2001), participating also in the clearance of soluble and particulate antigens from the circulation, as the

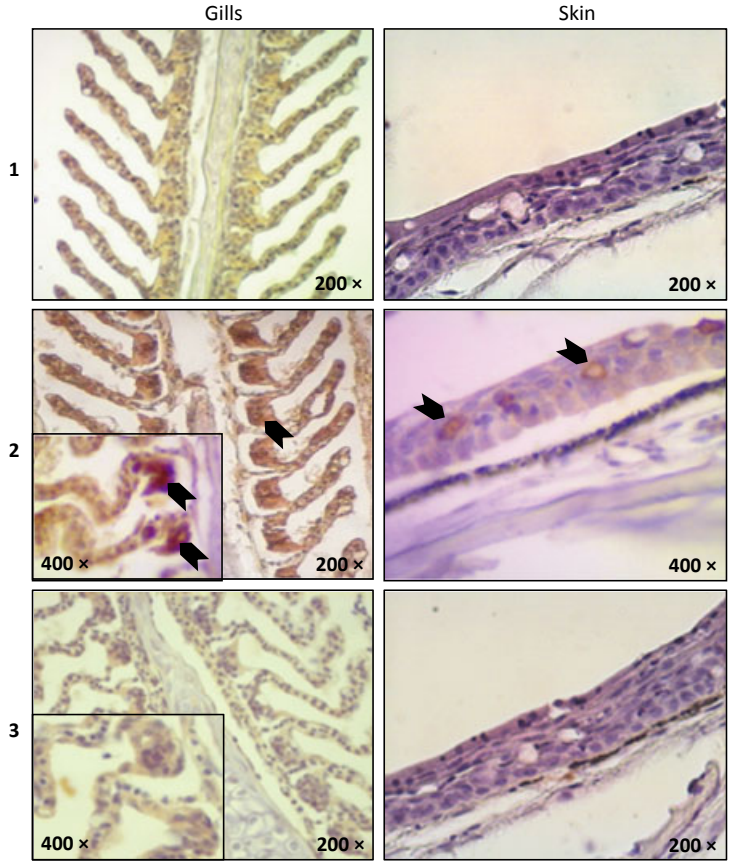

Figure 4 Immunohistochemistry of natural killer enhancing factor (NKEF)-like protein in gills and skin of Atlantic salmon infected with *Caligus*. (1) Non-infected control, (2) infected fish showing positive peroxidase staining cells (➡), (3) negative control without primary antibody. Marked areas show amplified region at the bases of secondary lamellae.

major site of MMΦ that are able to retain antigens for long periods of time (Whyte 2007). The spleen of teleosts is implicated in the clearance of blood-borne antigens, immune complexes and also has a role in the antigen presentation and initiation of the adaptive immune response (Alvarez-Pellitero 2008). Some studies have shown a higher expression of NKEF at mRNA level, as in the spleen and head kidney of turbot after challenge with *Vibrio anguillarum* (Chen *et al.* 2006), in the spleen of LPS-stimulated pufferfish (Dong *et al.* 2007), in channel catfish, a slight increase was observed after 8 and 24 h exposure to LPS (Li & Waldbieser 2006), and in scleroglucan stimulated carp, the enhancement in NKEF expression could be related to the activation of leucocytes in the spleen (Shin *et al.* 2001).

In the first stages of *Caligus sp.* infection (4 days p.i) in Atlantic salmon, skin mucus and gill tissue were affected. The immunoperoxidase-positive staining cells observed in skin could correspond to immune cells like neutrophils and macrophages that recruit during inflammatory reactions of the superficial tissue layers (Buchmann 1999). Other work has shown that Atlantic salmon infected with *Lepeophtheirus salmonis*, a crustacean parasite like *Caligus*, show changes in skin and gill epithelia such as increased apoptosis, necrosis and leucocyte infiltration (Fast *et al.* 2002). Macrophage ROS production probably stimulates the secretion of NKEF as a protection against oxidative stress. The positively stained inflamed group of cells in the base of the gill lamella could correspond to intraepithelial lymphoid tissue where the presence of MHC class II-positive cells and TCR expression was identified by Haugarvoll *et al.* (2008). These cells could correspond to antigen-presenting cells like T cells and macrophages that present MHC class II. Additionally, some cells presenting MHC class II were observed surrounding chloride cells in Atlantic

salmon affected with amoebic gill disease (Morrison *et al.* 2006).

As evidence of the usefulness of the antibody developed in this study, preliminary work showed that LPS increased the expression of NKEF-like protein in the RTS-11 cell line, while infection with ISA virus produced a lower detection of the molecule. Proteomic analysis demonstrated an immunomodulatory role for infectious haematopoietic necrosis virus (IHNV), by the down-regulation of natural killer enhancement factor in Atlantic salmon (Booy *et al.* 2005); thus, anti-epitope of NKEF-like protein could be useful for detecting fish capable of overcoming the mechanisms of evasion of the immune response caused by viruses.

ClustalW of NKEF sequences of other fish species shows that the region of the peptide is highly conserved among different species with an identity over 90%; thus, the developed antibody could be useful in the study of other fish models.

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