



Molecular and cellular characterization of European sea bass CD3 ϵ ⁺ T lymphocytes and their modulation by microalgal feed supplementation

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Abstract

The CD3 coreceptor is a master T cell surface marker, and genes encoding CD3 ζ , $\gamma\delta$, and ϵ chains have been reported in several teleost fish. Here, a complete cDNA sequence of CD3 ϵ chain was identified from a sea bass (*Dicentrarchus labrax* L.) gill transcriptome. Its basal expression was quantified in both lymphoid and non-lymphoid organs of sea bass juveniles with real-time qPCR analysis. After either in vitro stimulation of head kidney leukocytes with the T-cell mitogen phytohaemagglutinin or in vivo stimulation with an orally administered *Vibrio anguillarum* vaccine, CD3 ϵ expression levels increased in head kidney leukocytes, confirming that CD3 ϵ T cells may play important roles in fish systemic protection against pathogens. Further, three peptides were designed on the CD3 ϵ cytoplasmic tail region and employed as immunogens for antibody production in rabbit. One antiserum so obtained, named RACD3/1, immunostained a band of the expected size in a western blot of a sea bass thymocyte lysate. The distribution of CD3 ϵ ⁺ lymphocyte population in the lymphoid organs and mucosal tissues was addressed in healthy fish by IHC. In decreasing percentage order, CD3 ϵ ⁺ lymphocytes were detected by flow cytometry in thymus, peripheral blood leukocytes, gills, head kidney, gut, and spleen. Finally, a significant in vivo enhancement of CD3 ϵ ⁺ T intestinal lymphocytes was found in fish fed on diets in which 100% fish meal was replaced by the microalgae *Nannochloropsis* sp. biomass. These results indicate that CD3 ϵ ⁺ T cells are involved in nutritional immune responses.

Keywords CD3 ϵ · T lymphocytes · Sea bass · Acquired immunity · Lymphoid organs

Introduction

The T cell receptors (TCRs) of all jawed vertebrates are heterodimers found on the surface of all T cells (Parra et al. 2012). They are formed by the pairing of α and β polypeptide chains ($\alpha\beta$ TCR) or of γ and δ chains ($\gamma\delta$ TCR). TCR molecules are associated with a protein

complex, namely CD3, which in mammals is composed of γ , δ , ϵ , and ζ subunits. The TCR/CD3 complex of conventional T cells recognizes specific antigens bound to the major histocompatibility complex (MHC) and elicits the characteristic, critical process termed T cell activation (Davis and Chien 2003; Birnbaum et al. 2014), which in turn ensures adaptive immune response. In teleost fish, the function of the TCR/CD3 complex is similar to the mammalian one, although only three kinds of CD3 chains are present, i.e., ζ , also known as CD247, ϵ and $\gamma\delta$, with the latter considered as a forerunner of the corresponding CD3 γ and CD3 δ chains found in mammals (Liu et al. 2008). To date, CD3 homologue genes have been described in different teleost fish such as Japanese flounder (*Paralichthys olivaceus*) (Park et al. 2001, 2005), common carp (*Cyprinus carpio*) (Shang et al. 2008), fugu (*Takifugu rubripes*) (Araki et al. 2005), Atlantic salmon (*Salmo salar*) (Liu et al. 2008), Atlantic halibut (*Hippoglossus hippoglossus*) (Øvergård et al. 2009), and mandarin fish (*Siniperca chuatsi*) (Guo and Nie 2013).

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In sea bass (*Dicentrarchus labrax*), CD3 γ/β homologue was cloned, molecularly characterized, and analyzed in its expression levels in both lymphoid and non-lymphoid organs, where a gene regulation in response to in vitro stimulations was demonstrated (Randellin et al. 2011). Other CD3-functionally related molecules have been characterized: CD3 ζ (Azeredo et al. 2015), TCR α , β and γ (Buonocore et al. 2012), CD4 (Buonocore et al. 2008), CD8 (Buonocore et al. 2006, 2008; Picchiatti et al. 2009), MHC I (Pinto et al. 2013), and MHC II (Buonocore et al. 2007b; Picchiatti et al. 2015). These molecules of the T cell system showed expression patterns similar to their mammalian counterparts (Toda et al. 2009, 2011; Castro et al. 2011; Laing and Hansen 2011) and indicated that certain features of fish adaptive immune system are actually highly conserved in almost all gnathostomes (jawed vertebrates), although others are plastic, differing in gene numbers, domain organization, and function (Flajnik 2018). The CD3 ϵ sequence is quite conserved among different fish species and shows low homology with mammalian CD3 ϵ (Cook et al. 2001; Liu et al. 2008; Maisey et al. 2011; Ronza et al. 2011).

The aim of this study was to extend the characterization of T cell markers to deeply dissect the adaptive immune pathway in sea bass. To this purpose, we identified a CD3 ϵ sequence in a sea bass gill transcriptome and investigated its basal expression in lymphoid organs, peripheral blood leukocytes (PBLs), and tissues of juvenile fish. Transcripts were also quantified in head kidney (HK) leukocytes following stimulation with the mitogen agent phytohaemagglutinin (PHA) and oral vaccination with *Vibrio anguillarum*. Moreover, the cytoplasmic tail region was chosen as a template for designing three peptides for producing a polyclonal antiserum, named RACD3/1, against the CD3 ϵ chain. We used this antibody to address the normal distribution and number of CD3 ϵ^+ lymphocyte populations in lymphoid organs (thymus, HK, and spleen), mucosal tissues (intestine and gills), and PBL. Finally, the in vivo modulation of CD3 ϵ intestinal lymphocytes was investigated in fish fed with diets containing the marine microalgae *Nannochloropsis* sp., a functional ingredient with great potentialities in aquafeeds.

Materials and methods

CD3 ϵ identification in sea bass: cloning of coding region of CD3 ϵ and synteny analysis

A CD3 ϵ sequence (accession number KX231274) was identified in a sea bass gill transcriptome (Nuñez Ortiz et al. 2014). The sequence was analyzed for the presence of a signal peptide using SignalP software, and of N-linked glycosylation sites with the NetNGlyc 1.0 Server. The cytoplasmic region of the CD3 ϵ was evidenced as a result of a multiple sequence alignment among teleost sequences.

This was performed with Clustal Omega and used to design peptides for antibody production (see Sect. [Production of antisera](#)). A phylogenetic tree was constructed using the multiple sequence alignment as an input for a neighbor-joining method-based clustering, carried out with the MEGA 7 software (Kumar et al. 2016). The evolutionary distances were computed using the JTT matrix-based method, with all ambiguous positions removed for each sequence pair. The sea bass amino acid (aa) CD3 ϵ sequences were compared to each other and to counterparts in other species with the EMBOSS Pairwise Alignment tool.

The CD3 ϵ gene of sea bass *Dicentrarchus labrax* was manually re-annotated based on the alignment of the full-length mRNA sequence, obtained as described above, to the genomic DNA sequence with MUSCLE (Edgar 2004). Donor and acceptor splicing sites were subsequently refined with gene boundaries with NNSPLICE v. 0.9 (Reese et al. 1997). The sea bass genome sequence was obtained from NCBI Genomes (GCA_000689215.1, seabass_V1.0) (Kuhl et al. 2011).

The position of CD3 ϵ gene was identified through a tBLASTn approach (with e-value threshold set to $1E^{-5}$) and neighboring genes annotated at the 5' and 3' ends of the locus were inspected. Finally, its order and orientation were compared with the genome architecture found in sea bass.

Basal expression analysis of CD3 ϵ transcripts in lymphoid organs, peripheral blood leukocytes, and tissues

To investigate the basal expression levels of sea bass CD3 ϵ transcripts, three sea bass specimens (fed with commercial diet) of 100 ± 15 g in body weight (BW) (approximately 7 months old) were obtained from a local fish farm (Civitavecchia, Italy). Fish were acclimatized in aerated aquarium and fed with a commercial diet for 10 days prior to experimentation. Organs and tissues (muscle, liver, spleen, HK, thymus, gills, PBL, gut, and brain) were dissected out following temporary immobilization with 1 mg/ml tricaine methanesulfonate (MS-222) (Sigma) in aquarium water, buffered with 0.6 g/L sodium bicarbonate. Anesthesia was followed by exsanguination or decapitation.

Total RNA was isolated from each tissue separately with TRIsure (Bioline), resuspended in DEPC-treated water and used for real-time qPCR without pooling fish samples. DNA contamination was assessed by amplifying with β -actin primers that bracket an intron (see Table 1). For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used as per previously described protocol (Buonocore et al. 2007a). The expression level of CD3 ϵ transcripts was determined with a Mx3000P qPCR System (Agilent Technologies, Inc.) as per Buonocore et al. (Buonocore et al. 2007a). Specific PCR primers were

Table 1 Primers

Gene	Primers sequence 5'–3' (forward, FW, and reverse, RV)	Accession number
β -Actin	ATGTACGTTGCCATCC (FW) GAGATGCCACGCTCTC (RV)	AJ493428
18S ribosomal RNA	CCAACGAGCTGCTGACC (FW, real-time PCR) CCGTTACCCGTGGTCC (RV, real-time PCR)	AY831388
CD3 ϵ	CCTTACCACTGTAAATATGAGGACG (FW, real-time PCR) CAGGTTGACTCCGGGCTGCTG (RV, real-time PCR)	KX231274

designed for the amplification of a 240-bp product from CD3 ϵ (Table 1). A relative quantification was performed by comparing the levels of the target transcript (CD3 ϵ) to a reference transcript (calibrator, i.e., the tissue with the lowest CD3 ϵ expression, in this case the muscle). A normalizer target (18S ribosomal RNA, see Table 1) was included to correct for differences in total cDNA input between samples. Results were calculated by averaging expression values from the three biological replicates with duplicate samples for each, and expressed as mean \pm SD. Statistical significance was tested with a one-way ANOVA followed by the Bonferroni *post-hoc* test, with $p < 0.05$

CD3 ϵ expression analysis following in vitro stimulation with PHA and after in vivo immunization with *Vibrio anguillarum* on head kidney leukocytes

The CD3 ϵ expression was studied in vitro after stimulation of HK leukocytes isolated from three healthy sea bass specimens (fed with a commercial diet) obtained from a local fish farm (Civitavecchia, Italy). Cells were cultured in L-15 medium (Gibco®, Invitrogen) containing 10% fetal calf serum (FCS, Gibco®, Invitrogen), adjusted to a concentration of 1×10^5 cells/ml and incubated at 22 °C with 10 μ g/ml of phytoagglutinin from *Phaseolus vulgaris* (PHA, Sigma) for 4 h and 24 h. Control samples were incubated with L-15 medium alone (see Stocchi et al. 2017).

Sample design, total RNA isolation method, primers, and real-time qPCR conditions were maintained the same as described above, except that the calibrator was one of the controls at time 0. Results were expressed as mean \pm SD of the three biological replicates (each in duplicate). Statistical significance was tested with a two-way ANOVA followed by the Bonferroni's *post hoc* test, with $p < 0.05$.

The in vivo CD3 ϵ expression analysis was performed on fish that were orally vaccinated against the sea bass bacterial pathogen *Vibrio anguillarum* (see Galindo-Villegas et al. 2013; Stocchi et al. 2017). For gene expression analyses, 7 fish/group/time point (0, 6, 24, and 72 h post-vaccination) were individually sampled to obtain leukocytes from HK. RNA extraction, cDNA synthesis, and real-time qPCR analysis were performed as described above, using one of controls at 0 h as

calibrator. Results were expressed as mean + SD of the three biological replicates (each in duplicate). Statistical significance was tested with a two-way ANOVA followed by the Bonferroni *post-hoc* test, with $p < 0.05$.

Production of antisera raised against sea bass CD3 ϵ peptides

Three immunogenic peptides (NH₂-KSSAGLNHTPKLPARSAC-COOH; NH₂-ARSQPASSSDYEQLNQC-COOH; NH₂-CRTRSQETYSVVNRTG-COOH) were selected based on the CD3 ϵ cytoplasmic tail region and synthetically produced (Primm Srl, Milano, Italy). For the antisera production, two New Zealand rabbits received subcutaneous immunization with a mix of the three synthetic peptides conjugated to KLH in 0.1 M phosphate-buffered saline (PBS) as described in Picchiatti et al. (2017). Complete Freund's adjuvant (Serva, Heidelberg, Germany) was added in the first two inocula. Blood samples were collected both from preimmune and immune animals. The obtained polyclonal antibody (PAb) was named RACD3/1.

Western blotting analysis

For western blotting, 5×10^7 thymocytes obtained from a juvenile sea bass thymus were lysed with 0.5 ml lysis solution (Lysis-M, Roche) and the lysate was centrifuged at 14,000 rpm for 10 min. The total content of proteins in the supernatant was estimated in 20 mg, and 20 μ g was loaded in each lane of a 12% SDS-PAGE later stained with Coomassie R-250. As references, 5 μ g/lane of standard ladder proteins (Pharmacia Biotech) ranging from 10 to 170 kDa were used. Some lanes were electroblotted onto nitrocellulose (BA85 Schleicher & Schuell, Germany) at 100 mA per gel (30 cm²) for 45 min in a semi-dry apparatus. Immunodetection was performed as described previously (Picchiatti et al. 2017), using PAb RACD3/1 as primary antibody or pre-immune serum, both diluted 1:350 in blocking buffer.

Flow cytometry and immunofluorescence analyses

The lymphoid organs (thymus, HK, spleen), mucosal tissues (gills and gut), and PBL were sampled from 3 sea bass juveniles (fed with a commercial diet and obtained from a local fish farm, Civitavecchia, Italy) and immersed in cold Hank's

balanced salt solution without calcium and magnesium (HBSS). Culture media and solutions were adjusted to sea bass osmolarity (350 mOsm/kg). A minimum of 5×10^6 leukocytes from each tissue were obtained following a previously described protocol (Nuñez-Ortiz et al. 2016) with some modifications for gut tissue as described by Picchiatti et al. (2017).

Flow cytometry (FCM) analysis was performed on single-labelled leukocytes. Leukocytes were fixed with 0.4% paraformaldehyde in PBS for 15 min on ice, washed in PBS by centrifugation, and incubated for 1 h at 4 °C with pre-immune serum or with RACD3/1 at a 1:300 dilution in PBS, incubated for 45 min with the secondary antibody anti-rabbit IgG conjugated with Alexa Fluor® 488. Fluorescence was read in a Beckman-Coulter Epics XL-MCL flow cytometer and analyzed by acquiring 10,000 events with the Expo32 system software; cells were also observed under a Zeiss microscope equipped with a color 8 video camera (Axio Cam MRC) and a software package (KS 300 and AxioVision).

For the double-labelling experiment, PFA-fixed splenocytes were incubated for 1 h at 4 °C on glass slides with RACD3/1 and with either a monoclonal antibody against sea bass IgM light chain (DLIg3, as undiluted culture supernatant) (Scapigliati et al. 1996) or with a monoclonal antibody against thymocytes (DLT15 at a 1:300 dilution in PBS) (Scapigliati et al. 1995; Abelli et al. 1997). After washing with PBS, cells were incubated for 45 min with the secondary antibody anti-rabbit IgG or anti-mouse IgG conjugated with Alexa Fluor® 488 and Alexa Fluor® 594 (Molecular Probes, USA), respectively. Double-labelled cells were observed under the Zeiss LSM 710 confocal microscope. Selected images were sized and optimized for contrast and brightness using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

In both experiments, negative controls were incubated with the secondary conjugates only.

Immunohistochemical investigations

Immunohistochemistry (IHC) was carried out to characterize the tissues distribution of the CD3e T lymphocytes in sea bass juveniles (fed with a commercial diet obtained from a local fish farm, Civitavecchia, Italy). Dissected tissues, including thymus, spleen, HK, and gills were fixed in ice-cold Bouin's fixative for 7 h at 4 °C. In each specimen, ten sets of five serial sections (7 µm thick) were processed for IHC performed by ABC-peroxidase method as previously reported by Picchiatti et al. (2017). The rabbit polyclonal antiserum RACD3/1 diluted 1:800 in PBS containing 5% normal goat serum (Sigma) and 0.1% sodium azide was used as primary antibody. Thereafter, sections were incubated with biotinylated goat anti-rabbit IgG serum (Vector Labs., Burlingame, USA) as secondary antibody, followed by incubation with avidin biotinylated peroxidase complex (ABC, Vectastain Elite, Vector). The staining was performed with 3,3'-Diaminobenzidine (DAB) Liquid Substrate

System tetrahydrochloride (Sigma). As negative control, pre-immune serum replaced the rabbit anti-CD3e polyclonal antibody. In each specimen, ten sets of five serial sections (7 µm thick) were processed for immunohistochemistry and stained with May-Grünwald/Giemsa (MGG) for general histology. The sections were examined under a Zeiss microscope equipped with a colour 8 video camera (Axio Cam MRC) and a software package (KS 300 and AxioVision).

Feeding trial and gut sample collection

The trial was carried out at the experimental facilities of the Marine Zoology Station of the Faculty of Sciences, University of Porto, Portugal. The trial was run by trained and certified scientists (following FELASA category C recommendations).

Diets

Four isoproteic (46.3% crude protein as fed basis) and isolipidic (17.5% crude lipids as fed basis) experimental diets were formulated. A positive control (PC) was formulated with 30% fish meal (FM) and fish oil as the only lipid source. A negative control (NC) diet based on plant ingredients and low fishmeal (15%) level was also formulated, including a mixture of fish and vegetable oil (40/60) as lipid source. Tested diets were formulated similarly to the NC, replacing 50% and 100% FM with *Nannochloropsis* sp. biomass, and named NANNO 7.5 and NANNO 15, respectively. The microalgae biomass was provided lyophilized by Buggy power, Lda. (Porto Santo, Portugal), a Portuguese company that produces microalgae in a marine environment by using completely autotrophic closed production systems (photobioreactor) that captures and fixes CO₂ to grow microalgae. Experimental diets were produced using extrusion, by SPAROS (Olhão, Portugal), and each diet was tested in duplicate.

Diets trial

Sea bass juveniles, with an average initial body weight of 24 ± 1 g, obtained from the commercial hatchery Acuinuga (Spain), were used in the diets trial. Fish were transported to the experimental facilities and kept in quarantine for approximately 3 weeks. During this period, fish were fed a commercial diet suitable for sea bass juveniles (AquaGold, Aquasoja; Sorgal, S.A., Portugal).

At the end of the quarantine period, 8 homogeneous groups of 25 fish were constituted and randomly distributed to each tank. Each experimental diet was randomly assigned to duplicate groups of animals. Fish were hand-fed twice a day, 6 days a week, until apparent visual satiation, for 11 weeks. Utmost care was taken to ensure that all feed supplied was consumed. The diets trial was conducted in

a thermo-regulated recirculating water system, equipped with 8 square-shaped fiberglass 100-L tanks, supplied with continuous flow of filtered seawater aerated by diffusion through air stones. During the trial, water temperature averaged 23.0 ± 0.5 °C, salinity was maintained at 35.0 ± 1.0 g L⁻¹, and dissolved oxygen was kept near saturation (7.0 mg L⁻¹). The photoperiod regime adopted was 12:12 h light/dark, provided by artificial illumination.

Sampling

Three fish from each tank were randomly collected, euthanized by anesthetic overdose (ethylene glycol monophenyl ether; Merck, Whitehouse Station, USA), and weighed. Fish were sampled 3–4 h after the last feed to ensure digestion had occurred throughout the entire gut length. Sampled fish were dissected on chilled trays, and the digestive tract excised and freed from adjacent adipose and connective tissues. For IHC purposes, the mid intestine (MI; considered the portion located mid length from the pyloric caeca to the anus) and posterior intestine (PI; distinguishable by an enlarged diameter and darker mucosa) were fixed in Bouin's reagent and subsequently processed as described above (see Sect. [Immunohistochemical investigations](#)).

Statistical analysis

In each specimen, multiple sets of consecutive sections were immunostained with the antibody RACD3/1. Counts of RACD3/1-positive cells (nucleated only) in 100,000 μm² areas (from fifteen non-consecutive sections per specimen) were performed by an observer unaware of treatments, using a computer-assisted image analysis system which includes a Zeiss microscope equipped with a color video camera (AxioCamMRC, Aresé, Milano, Italy) and a software package (KS 300 and AxioVision). GraphPad Prism 8 statistical package was used for data analysis. Numerical results were expressed as the mean + SD. One-way ANOVA followed by Bonferroni's multiple comparison test (selected pairs) were used to determine differences among groups. The level for accepted statistical significance was $p < 0.05$.

Results

CD3ε sea bass sequence analysis

The CD3ε sea bass sequence (GenBank accession number KX231274) identified from the transcriptome (Nuñez Ortiz et al. 2014) was confirmed by cloning the entire coding region from gill cDNA (data not shown). It consists of 174 amino acids with a predicted signal peptide of 21 amino acids and

two potential N-glycosylation sites. The sequence contains an immunoglobulin superfamily domain (IgSF) (Fig. 1a) in its extracellular region with conserved cysteine residues critical for the tertiary structure of the molecule (Pettinello et al. 2017). The CXXCXE motif, involved in the TCR complex assembly and signal transmission, is also conserved in this domain. An ITAM motif, fundamental for the transmission of intracellular signals, is conserved in the cytoplasmic tail (Fig. 1a), although the motif PXXDY, relevant for the binding of the noncatalytic region of tyrosine kinase (NcK), is not present as in the other teleost CD3ε sequences. In the phylogenetic tree analysis (Fig. 1b), the sea bass CD3ε molecule clustered with all other fish CD3ε sequences, with mammals found in a different branch. Sea bass coreceptor is closely related to other Perciformes counterparts, but Salmoniformes sequences are in a different clade. This aspect is quite clear taking into consideration also the percentages of amino acid identity and similarity of sea bass CD3ε molecule with the other fish CD3ε evidenced in Table 2: the highest values have been found for the Chinese perch (*Siniperca chuatsi*).

The CD3ε gene is composed of five exons and four introns, and it was found in the sea bass chromosome LG13. The neighboring genes are myelin protein zero like 2 (MPZL2), on one side, and CD3γδ on the other, with the latter gene flanked by transmembrane protein 25 (TMEM 25).

CD3ε expression at the mRNA level in lymphoid organs, mucosal tissues, as well as peripheral blood leucocytes, liver, and brain of healthy fish

The constitutive expression of sea bass CD3ε was investigated in different tissues of healthy fish (Fig. 2). CD3ε was predominantly expressed in thymus, gut, and spleen, followed by HK, brain, and PBL. The lowest CD3ε transcription was found in gills, muscle, and liver.

Transcription levels of CD3ε in leukocytes following stimulation in vitro with PHA and after immunization in vivo with *Vibrio anguillarum*

To investigate the modulation of CD3ε expression, we performed an in vitro stimulation of HK leukocytes with PHA, a T cell mitogen (Fig. 3a). PHA stimulated the expression of CD3ε at both 4 and 24 h.

To study the involvement of CD3ε in the sea bass immune response following oral vaccination, we detected its gene expression after administration of a commercial vaccine against *Vibrio anguillarum*. The analysis revealed a significant increase of CD3ε expression after 6 h. After 24 and 72 h, mRNA quantities are not statistically dissimilar from the control (Fig. 3b).

Fig. 1 (a) Amino acid sequence alignment of the predicted sea bass CD3 ϵ with selected CD3 ϵ molecules. Conserved amino acids are indicated with an “*,” while “.” and “:” show amino acids with conserved physical and/or chemical properties. The signal peptide, the IgSF domain, the transmembrane region and the cytoplasmic tail are indicated above the sequence. The conserved cysteine residues are highlighted in bold, the CXXCXE motif is underlined and highlighted in red; the ITAM motif is in italic type and evidenced in green. Accession numbers: *Salmo salar* NP_001117094; *Oncorhynchus mykiss* NP_001182103.1; *Takifugu rubripes* NP_001033071.1; *Paralichthys olivaceus* BAC87847; *Oreochromis niloticus* XP_003449345; *Siniperca chuatsi* ADV78596. **(b)** Phylogenetic tree analysis of selected CD3 ϵ sequences. The phylogenetic tree was constructed using amino acid multiple alignments and the neighbor-joining method within the MEGA7 program. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) was shown next to the branches; 0.10 represents the genetic distance. Accession numbers are the same of **a** except for *Homo sapiens* NP_000724 and *Mus musculus* NM_007648.5

Specificity of antibody against sea bass CD3 ϵ

The antiserum RACD3/1 was employed in western blots using thymus leukocyte lysates as antigens, as shown in Fig. 4. A 12% SDS-PAGE loaded with 20 μ g of whole thymus cell lysate (Fig. 4a, lane b) and stained with Coomassie is shown with respect to a standard MW ladder (Fig. 4a, lane a). Different bands at various MW (Fig. 4a, lane b) can be observed in the whole lysate together with a band at 17 kDa. After electroblotting of gels in reducing conditions, the nitrocellulose was probed with RACD3/1 and the results showed an immunoreactive band at 17 kDa (Fig. 4b, lane d). This band well corresponds to the putative molecular weight of the sea bass mature CD3 ϵ chain. No bands were detected with the pre-immune serum when probed on thymus leukocytes (Fig. 4b, lane e).

Table 2 Percentages of amino acid identity and similarity of sea bass CD3 ϵ with other selected CD3 ϵ sequences. The highest percentage values are highlighted in bold. For accession numbers, see Fig. 1

	Amino acid identity	Amino acid similarity
<i>Siniperca chuatsi</i>	55.6	66.9
<i>Oreochromis niloticus</i>	47.4	64.6
<i>Takifugu rubripes</i>	46.5	60.0
<i>Paralichthys olivaceus</i>	42.7	57.3
<i>Oncorhynchus mykiss</i>	40.8	55.7
<i>Salmo salar</i>	39.8	53.8
<i>Mus musculus</i>	26.5	44.9
<i>Homo sapiens</i>	26.0	40.9

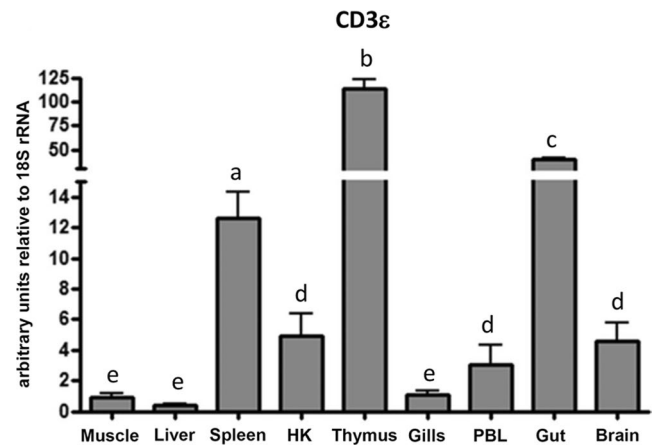


Fig. 2 Basal expression of sea bass CD3 ϵ in different tissues. Sea bass CD3 ϵ mRNA levels were normalized to that of 18S rRNA in the same tissue after real-time PCR analysis and expressed as arbitrary unit using the expression level in one muscle as a calibrator (1 unit). Data were expressed as the mean + SD of three healthy sea bass juveniles. Different letters indicate tissues for which the differences in expression levels are statistically significant ($p < 0.05$)

Specificity of RACD3/1 for the ϵ -chain of CD3 and staining of cells

To identify whether RACD3/1 could specifically recognize T cells, splenocytes layered onto a glass slide were labelled with both RACD3/1 and DLT15 or DLIg3 antibodies and revealed with fluorescent-dye conjugated secondary antibodies (green for RACD3/1; red for DLT15 or DLIg3). Dual staining allowed to identify CD3 ϵ expression on splenic T cells and confocal microscopy was suggestive of a high co-localization, as indicated by yellow staining (Fig. 5a). When RACD3/1 was matched with DLIg3, no detectable double labelling of immunoreactive cells was observed in the spleen. The fluorescent images revealed distinct staining of both CD3 ϵ ⁺ and DLIg3⁺ lymphocytes (Fig. 5b). No fluorescence could be seen in the negative control obtained by incubating the cells with only Alexa-conjugates (data not shown).

The results obtained by employing RACD3/1 as single-labelling for FCM of PFA-fixed cells from sea bass tissues are given in Fig. 6. The highest percentage of RACD3/1-positive cells was found in the thymus ($67.4 \pm 4.8\%$) (Fig. 6a, a'), followed by PBL ($37.9 \pm 2.5\%$) (Fig. 6c, c'), gills ($27.9 \pm 7.5\%$) (Fig. 6e, e'), HK ($26.0 \pm 9.6\%$) (Fig. 6d, d'), gut ($22.7 \pm 4.6\%$) (Fig. 6b, b'), and spleen ($15.9 \pm 3.6\%$) (Fig. 6f, f'). The figure shows FL1 histograms of cells (a', b', c', d', e', f') contained in the gates shown in corresponding FS/SS dot-plots (a, b, c, d, e, f). The morphology and the membrane staining of the immunoreactive cells can be appreciated from the phase contrast (Fig. 6g) and corresponding fluorescence microscopy pictures (Fig. 6h, i, j).

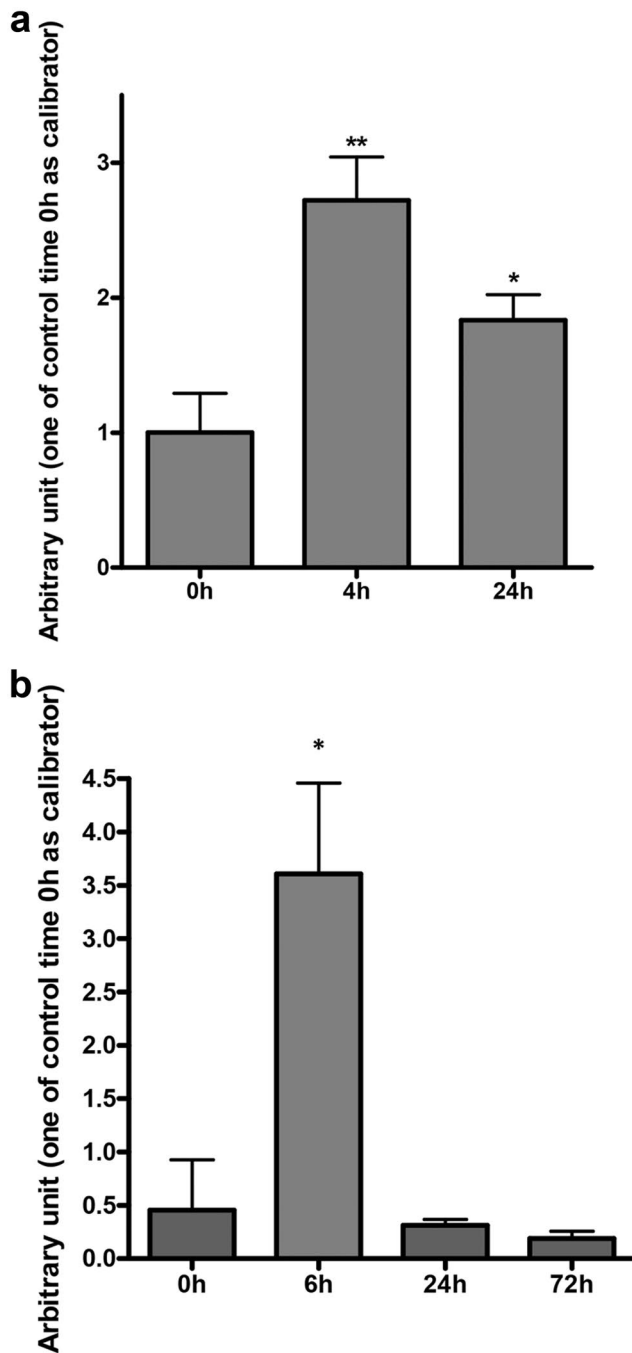


Fig. 3 (a) Expression of sea bass CD3 ϵ after in vitro stimulation with PHA. mRNA levels of sea bass CD3 ϵ were normalized to that of 18S rRNA in the same samples after real-time PCR analysis of HK leukocytes stimulated with L-15 medium (control) or with 10 μ g/ml of PHA for 4 h and 24 h, and expressed as arbitrary unit against one of the non-stimulated 0 h control. Data were expressed as the mean + SD. * p < 0.05 with respect to the time 0 control; ** p < 0.01 with respect to the time 0 control; N = 3. (b) Sea bass CD3 ϵ expression analysis after oral vaccination against *Vibrio anguillarum*. Sea bass CD3 ϵ mRNA levels were expressed as a ratio relative to rRNA 18S in the same samples after real-time PCR analysis of HK leukocytes of three fish vaccinated against *Vibrio anguillarum*. Data were expressed as the mean + SD. * p < 0.05 with respect to the time 0 control

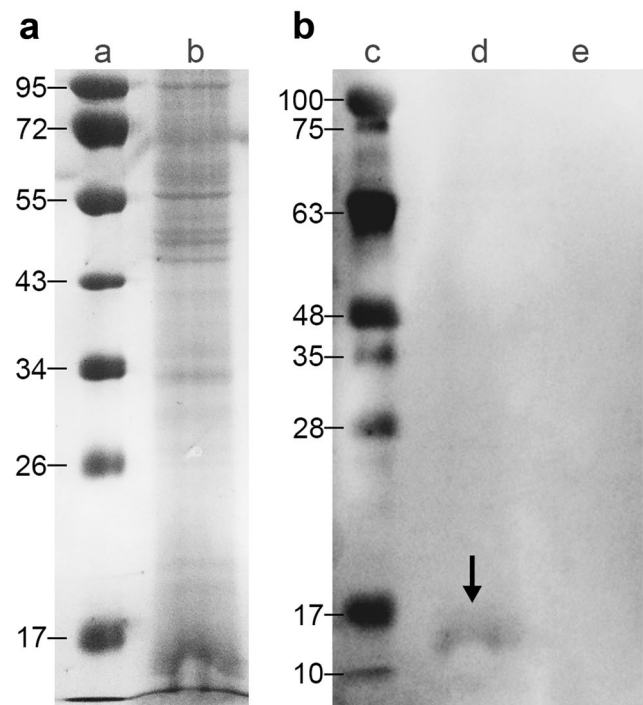


Fig. 4 SDS-PAGE and Western blotting. (a) The figure shows a 12% SDS-PAGE gel, loaded with 20 μ g of thymocyte lysate (lane b) in reducing conditions and stained with Coomassie R-250. (b) The gels were transferred onto nitrocellulose and probed with RACD3/1 diluted 1:350 (lane d) or with pre-immune serum 1: 350 (lane e). Molecular weight markers are reported in lanes a and c

Tissue distribution of CD3 ϵ ⁺ lymphocytes

In juvenile sea bass, the thymus was extensively lobulated and the cortex and medulla regions could be recognized. A compartmentalization of high numbers of CD3 ϵ ⁺ cells was evident in each thymic lobule. Weak staining was observed in the cortical thymocytes, while a strong signal was observed in cells of the medullary zone (Fig. 7a–e). Although less numerous, positive cells were also detected in the subcapsular zone of the thymus, as well as in the connective septa (Fig. 7a, c). No signal was found in subcapsular and cortical epithelial cells. CD3 ϵ ⁺ cells were also widely distributed in other lymphoid organs such as HK and spleen (Fig. 8a, b) and in gill-associated lymphoid tissue (Fig. 8c, d). In particular, several strongly marked CD3 ϵ ⁺ cells were distributed in the epithelium of gill filaments and parallel branchial lamellae (Fig. 8c). Pre-immune serum did not produce any staining (Fig. 8d).

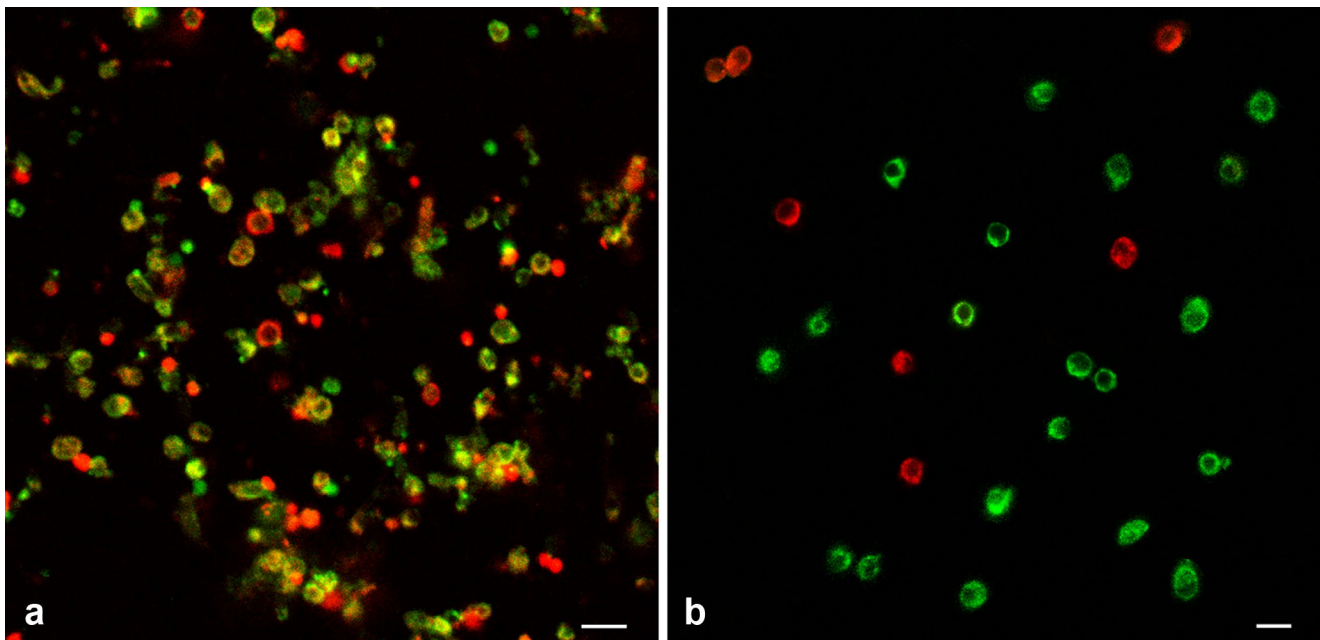


Fig. 5 Immunofluorescence of double-labelled spleen leukocytes. **(a)** Merged staining of cells visualized with anti-CD3 ϵ and anti-T cells antibodies followed by anti-rabbit Alexa fluor 488 and anti-mouse Alexa fluor 594, respectively. Note the almost complete co-localization. **(b)** Cells showing membrane fluorescence, visualized with anti-

CD3 ϵ antibody followed by anti-rabbit Alexa fluor 488 and anti-IgM antibody followed by anti-mouse Alexa fluor 594. Fluorescent images revealed distinct staining of both CD3 ϵ ⁺ and DLlg3⁺ lymphocytes. Bars 5 μ m

Increased CD3 ϵ intestinal lymphocytes after dietary inclusion of the marine-water microalgae *Nannochloropsis* sp.

The effects of diets on intestinal CD3 ϵ ⁺ lymphocytes were evaluated after the dietary inclusion of the marine microalgae *Nannochloropsis* sp. Four isoproteic and isolipidic experimental diets were formulated, and the localization and abundance of CD3 ϵ ⁺ intestinal lymphocytes were analyzed in the mid and posterior intestinal segments (Figs. 9a–h). In all experimental groups, CD3 ϵ ⁺ lymphocytes were rarely located in the intestinal epithelium, while they were abundantly detected in the lamina propria of both mid and posterior intestine. A significant increase in the density of CD3 ϵ ⁺ cells was found in the mucosa of the mid intestine of fish fed on NANNO 15 compared to the posterior segment of the same experimental group and to the mid intestine of the NC (Fig. 10). The density of CD3 ϵ ⁺ cells was not significantly affected in NC, PC, and NANNO 7.5 mid and posterior segments (Fig. 10).

Discussion

The T lymphocyte system was reported to be similar between fish and mammals (Nakanishi et al. 2015). The development and activation of T lymphocytes see the

central involvement of the CD3 antigen (Gouaillard et al. 2001), which is a master T cell marker in higher vertebrates (Keresztes et al. 1996). In the present study, a complete cDNA sequence of CD3 ϵ chain was identified from a sea bass gills transcriptome. Amino acid identity among CD3 ϵ chain sequences of sea bass and other teleost species ranged between 40 and 55% (Table 2), but most of the residues fundamental to the correct folding and functional activity were conserved in all sequences (Boardman et al. 2012). Sea bass CD3 ϵ gene organization is maintained as in Japanese flounder (Park et al. 2005) and Atlantic salmon (Liu et al. 2008). Moreover, CD3 ϵ and CD3 $\gamma\delta$ are closely related in the same locus, and they are oppositely transcribed as in Japanese flounder (Park et al. 2005).

The CD3 ϵ sequence served for designing specific primers to be used in real-time PCR for investigating the basal transcriptional levels in tissues and lymphoid organs of sea bass juveniles. Moreover, in order to obtain information on CD3 ϵ protein expression and localization, three peptides were selected within the cytoplasmic tail region and used to produce a polyclonal antiserum, named RACD3/1.

The antiserum was probed in WB against a thymocyte lysate, being the thymus a primary lymphoid organ. A 17 kDa peptide was recognized in reducing conditions in good agreement with CD3 ϵ predicted molecular weight. The thymus showed the greatest CD3 ϵ transcription

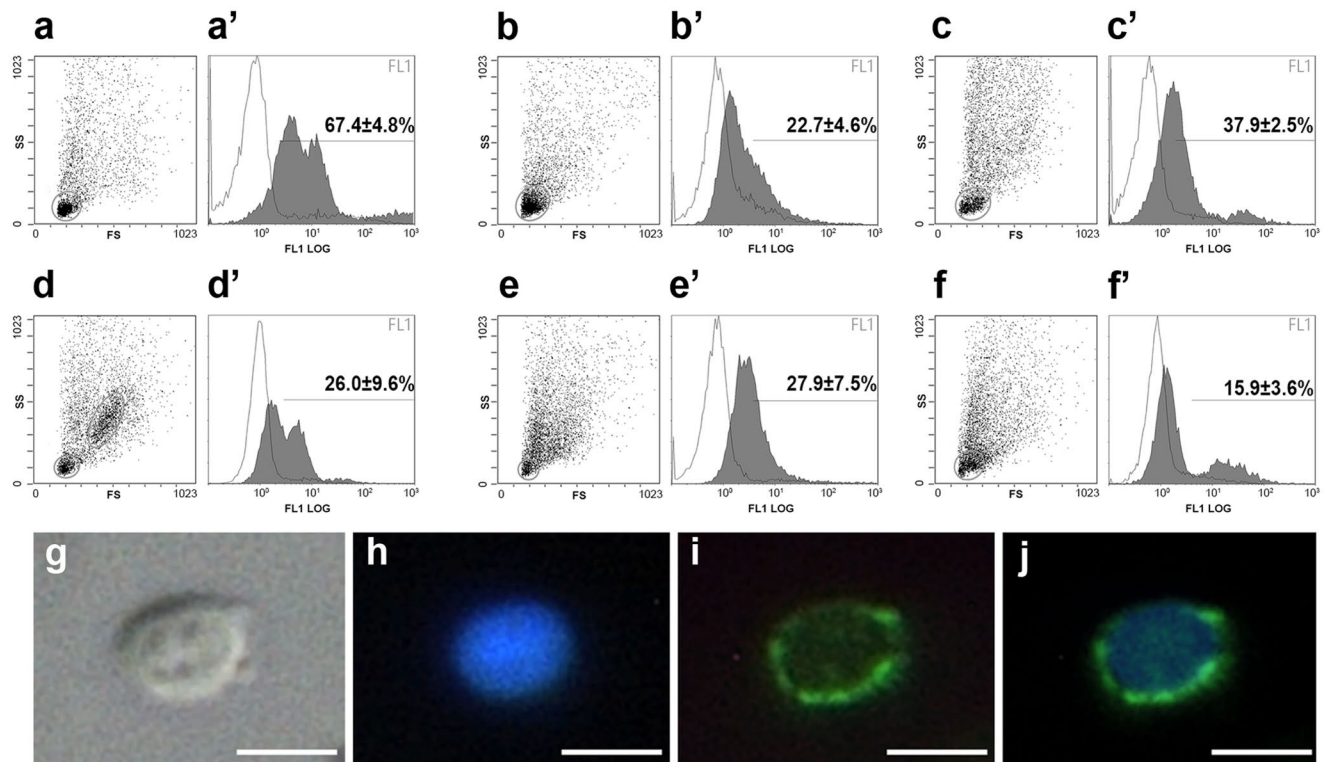


Fig. 6 Flow cytometry of RACD3/1⁺ cells. The counting of PFA-fixed leukocytes positive to RACD3/1 by FCM as the percentage ± SD of cells from three individuals (grey peaks) subtracted of pre-immune serum positivity (white peaks). (a, a') Thymus; (b,

b') gut; (c, c') PBL; (d, d') HK; (e, e') gills; (f, f') spleen. The IIF pattern of thymic lymphocytes with typical membrane staining and corresponding interferential contrast images is shown in the lower panel (g–j). Bars of g–j = 2.5 μm

among all investigated tissues, as evidenced by qPCR, and the highest percentage of CD3ε⁺ cells, as per single-labelling FCM analysis. Because positivity percentages obtained with RACD3/1 and the previously-produced anti-thymocytes MAb DLT15 (Scapigliati et al. 1995) almost matched, we suggest that RACD3/1 may be considered a useful immunophenotypic T cell marker in sea bass.

Tissue quantification of CD3ε protein expression was reported for several fish species, with considerably high percentages of CD3ε⁺ cells found in the thymus, suggesting that this organ, like in mammals, is a major site of T cell production and development. Nevertheless the organ showed great variability with respect to T cell numbers and anatomical zone organization among fish species when addressed with different T cell markers (Rombout et al. 2005; Picchietti et al. 2008; Koppang et al. 2010; Ge and Zhao 2013). In sea bass thymus, the distribution pattern of CD3ε lymphocytes was in agreement with the localization established in mammals and birds (Bertram et al. 1996)—weak staining in the cortex, where immature lymphocytes are located—whereas increasing amount of CD3 molecules occurred during maturation and migration to the medulla, before leaving the organ (Yang et al. 1996; Pearse 2006). This is directly related

to the expression of CD3 molecules in the membrane, which varies according to the degree of lymphocyte maturation. In mice, CD3 chains play critical roles in mature T cell activation (Hayes et al. 2003; Brodeur et al. 2009) and in the various phases of thymocyte development and signaling. For instance, the removal of CD3ε led to a complete impairment of thymocyte development at the DN (double negative) stage and it has been demonstrated that among the three CD3 dimers, CD3γε is fundamental for DN-to-DP transition (Dave 2009). Differently, for the subsequent stages of thymocyte differentiation, including thymic selection, all three CD3 dimers seem to be important, and the maturation and subsequent activation of peripheral lymphocytes depend on the recognition of peptides presented by the MHC molecules (Yates 2014). In this context, the compartmentalization in the sea bass thymus, as previously described by the highest abundance of MHC-II⁺ cells in its cortico-medullary border and in the medulla (Picchietti et al. 2015), was positively correlated to the abundance of CD3ε⁺ cells (Fig. 7a–d). This is in agreement with findings in mammalian thymus, where high MHC class II expression was detected in the medulla together with mature T cells (Douek and Altmann 2000), identified by their strong TCR/CD3 expression and high cytoplasm/nucleus ratio.

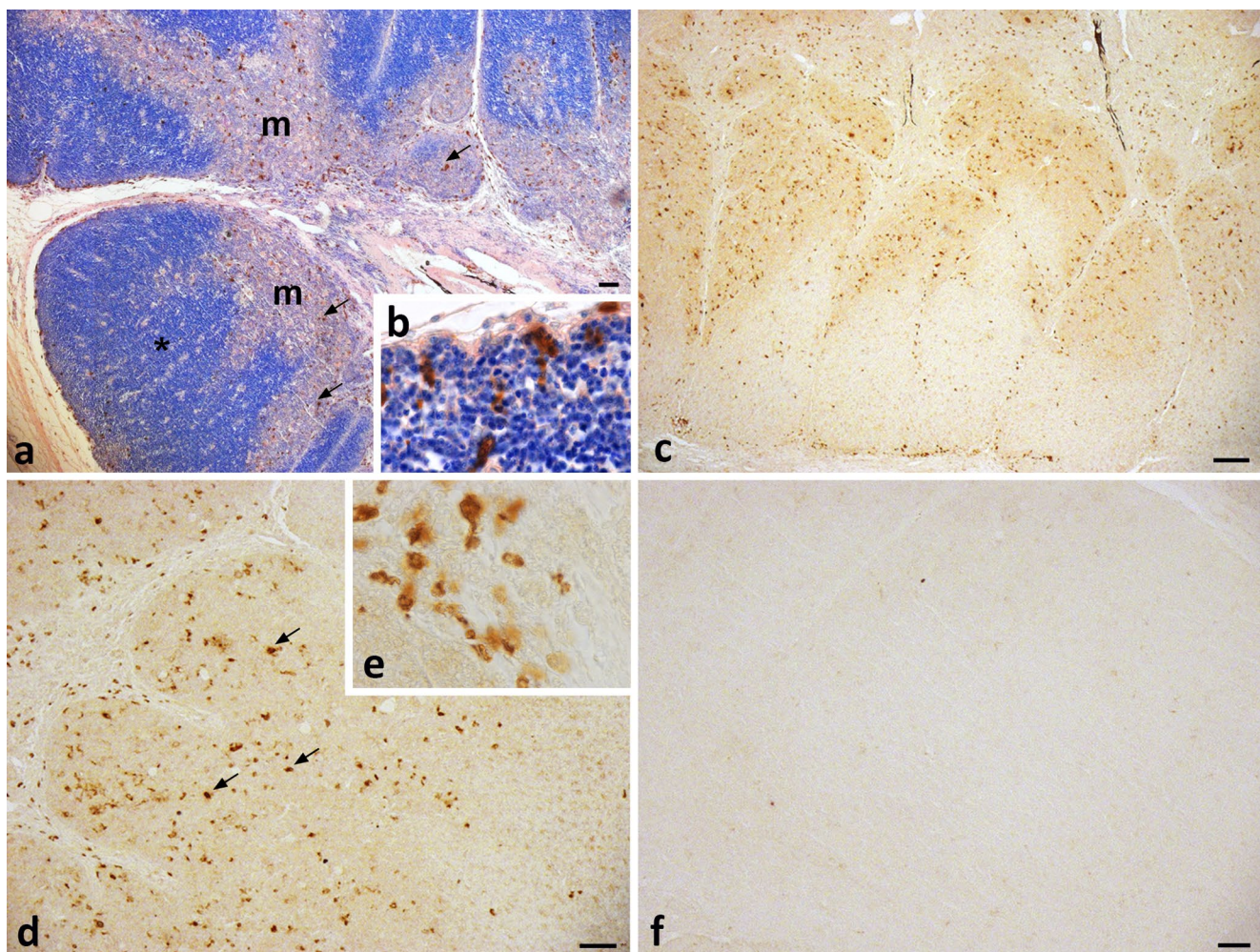


Fig. 7 Immunohistochemistry on thymus. The figure shows 7 μm sections of thymus fixed and immunostained with RACD3/1 (ABC-peroxidase method) (a–e) or with pre-immune rabbit serum (f). Thymus extensively lobulated stained with May-Grünwald/Giemsa (MGG) and immunodetection of RACD3/1⁺ cells (arrows). * = cortex; m = medulla (a). CD3 ϵ ⁺ cells in the subcapsular zone of

the thymus (b). Immunoreactive cells mainly located in the thymic medulla (c). Higher magnification of medullary CD3 ϵ ⁺ cells (arrows) (d) and presence of immunoreactive cells in the connective tissue (e). Pre-immune serum in (f). Bars: a = 50 μm ; b = 5 μm ; c = 100 μm ; d = 50 μm ; e = 5 μm ; f = 20 μm

In the head kidney, CD3 ϵ ⁺ cells were seen scattered throughout the tissue with no characteristic patterns (Fig. 8a) and CD3 transcripts responded vigorously to PHA in vitro stimulation (Fig. 3a). This mitogen has been proven to bind the TCR and to act on different populations of mammalian T lymphocytes (Miller et al. 1975; Miyara and Sakaguchi 2007), demonstrating that CD3 ϵ ⁺ cells identified in sea bass HK were indeed T cells. Nevertheless, this organ is not as important as the thymus for the production of T lymphocytes. This is reflected by the qPCR and FCM analyses, which well correspond to one another, although species-specific differences in CD3 ϵ protein expression and physiological features were previously reported for different fish species (Koppang et al. 2010; Boardman et al. 2012; Maisey et al. 2016; Xing et al. 2017; Miyazawa et al.

2018). Moreover, sea bass HK CD3 ϵ transcripts significantly responded to an orally administered *Vibrio anguillarum* vaccine, confirming that T cells may play important roles in fish systemic protection against pathogens (Xing et al. 2020).

The IIF analysis of spleen leukocytes revealed the absence of double-positive (DP) cells with DLIg3 and, on the contrary, the almost complete co-localization on DLT15⁺ T cells. This organ, composed of splenic ellipsoids and lymphoid tissue, is rich in scattered T and B cells (Scapigliati et al. 2003) and operate as a secondary lymphoid organ that is important in the induction and elaboration of teleost immune responses (Kaattari and Irwin 1985). It was previously speculated that developing B cells mature in the HK and then migrate to the spleen and the mid-kidney for their activation (Zwollo et al. 2005): it is thus reasonable that the teleost

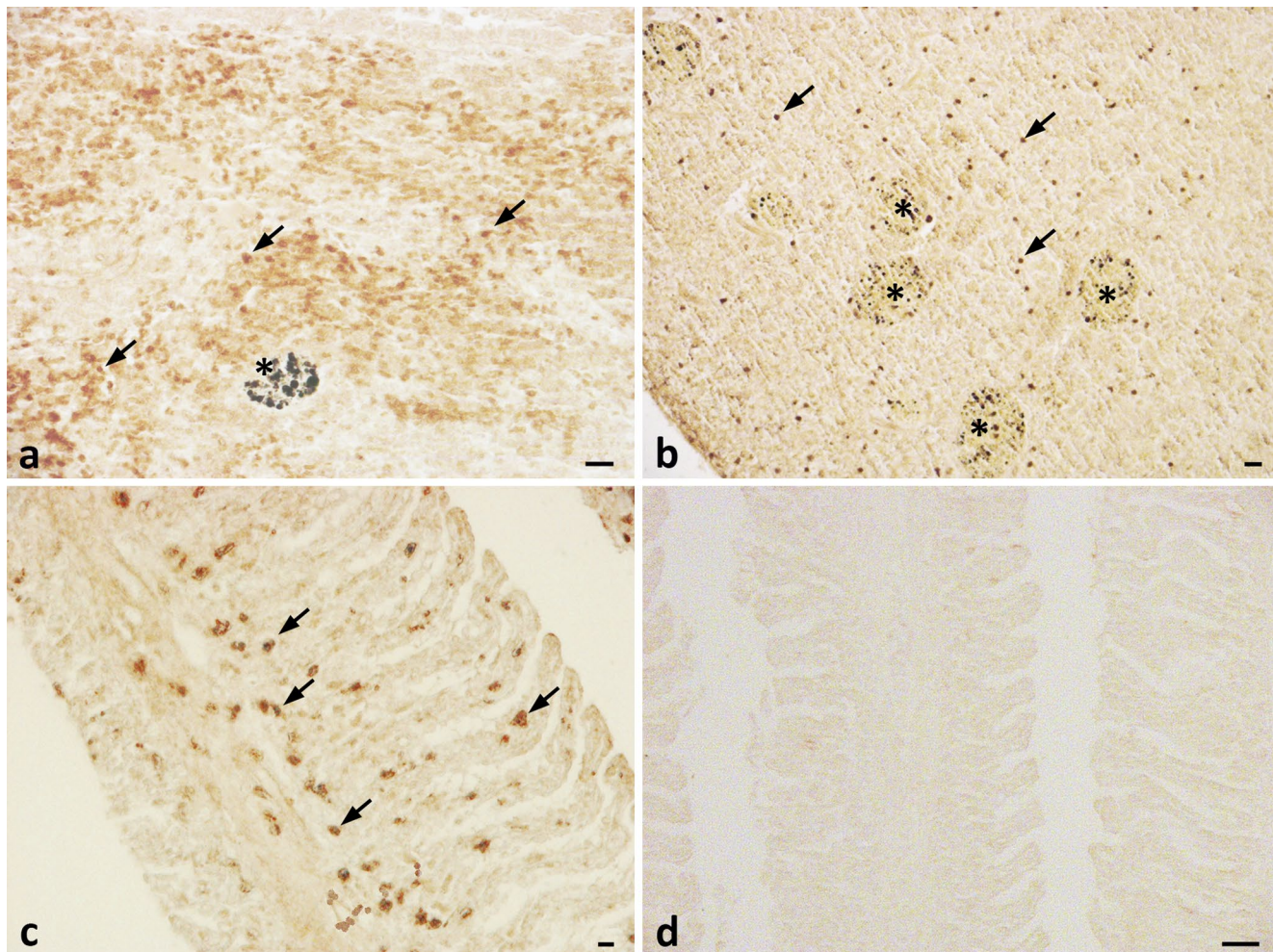


Fig. 8 Immunohistochemistry on head kidney, spleen, and gills. Sections of HK (a), spleen (b), and gills (c) fixed and immunostained with RACD3/1 (ABC-peroxidase method) or with pre-immune rabbit

serum (d). Rounded clusters of melano-macrophages are marked with asterisks. $CD3\epsilon^+$ cells are indicated with arrows. Bars: a, b = 20 μ m; c = 10 μ m; d = 50 μ m

peripheral phase of B-lymphocyte maturation could require the presence of T cells, as already demonstrated in mammals (Milićević et al. 2005).

Although modest CD3 gene transcription was found in the spleen, FCM quantified the lowest positivity percentage of $CD3\epsilon^+$ cells with respect to all tissues/lymphoid organs investigated. This may be due to the fact that the methodologies are directed towards different molecules and transcript levels do not necessarily correlate to protein levels; Vogel and Marcotte (2012), for instance, reported that the correlation can be as little as 40% and can be affected by several aspects such as protein stability, protein half-life and mRNA degradation rate. The same explanation may be used for the gills, where instead low mRNA transcription was paralleled by the $27.9 \pm 7.5\%$ $CD3\epsilon^+$ positivity.

The gills of sea bass hosted $CD3\epsilon^+$ cells distributed in the epithelium of filaments and parallel branchial lamellae (Fig. 8c) and was demonstrated to contain an entire set of T

cell-specific expressed genes that functionally defines active T cell subpopulations. Also, gills were demonstrated to host T cells undergoing in vitro proliferation in the presence of ConA and PHA and displaying a substantial increase in transcription of T cell-related genes (Nuñez Ortiz et al. 2014). These data suggest that the gills, as reported for other fish species (Nakanishi et al. 2015), may work as an effector mucosal tissue; because of its direct contact with the water environment, the tissue occupies a strategic position that ensures efficient antigen surveillance. The peculiar interbranchial lymphoid tissue described in salmonids (Koppang et al. 2010) further corroborates this hypothesis.

In the intestinal mucosa, numerous $CD3\epsilon$ lymphocytes were predominantly localized in the lamina propria and only rarely in the epithelium. Numerous $CD8\alpha$ - and $TCR\beta$ -expressing T cells were highlighted by ISH in the lamina propria of the intestinal mucosa (Picchiatti et al. 2011), confirming this site as key for adaptive immune responses.

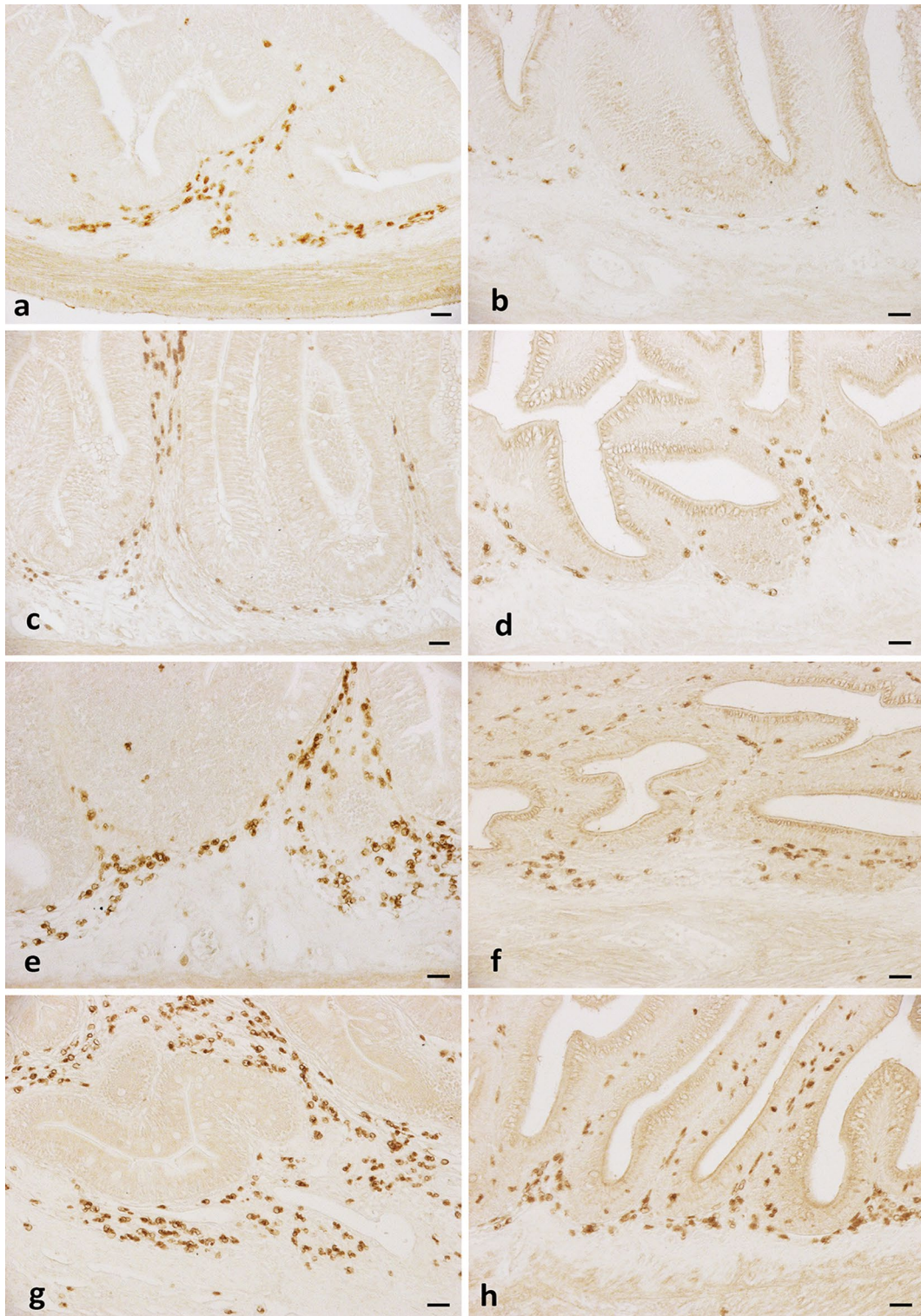


Fig. 9 Immunohistochemistry on mid and posterior intestine after dietary inclusion of *Nannochloropsis* sp. RACD3/1⁺ lymphocytes mainly located in the lamina propria of the mid (a, c, e, g) and

posterior intestine (b, d, f, h). NC group (a, b); NANNO 7.5 fed group (c, d); NANNO 15 fed group (e, f); PC group (g, h). Bars: a–h = 20μm

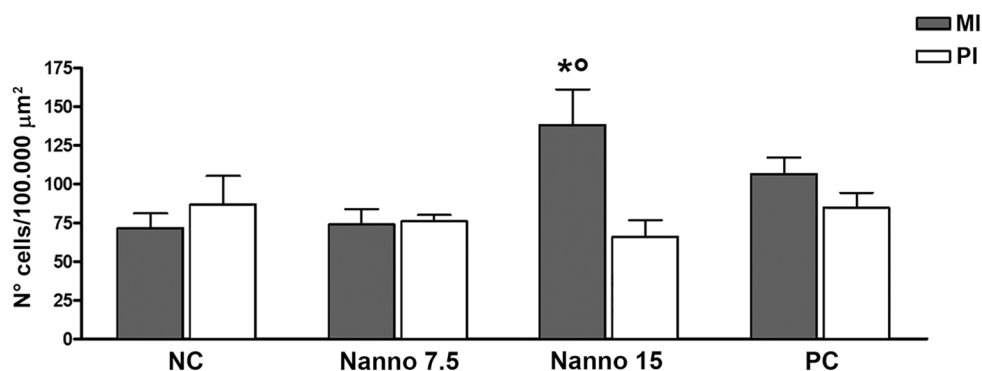


Fig. 10 Relative density (number of cells/10,000 μm^2) of CD3 ϵ^+ cells in sections from mid and posterior segments of the intestine. Each number is the mean \pm SD of results obtained in three animals. *Significantly different from NC of MI (mid intestine) ($p < 0.05$);

°Significantly different from NANNO 15 of PI (posterior intestine) ($p < 0.05$). NC=negative control diet; Nanno 7.5 and Nanno 15=diet formulations with 50% and 100% fish meal replacement by *Nannochloropsis* sp. biomass, respectively; PC positive control diet

Following the thymus, the intestine displayed the second highest CD3 mRNA abundance, in line with other teleosts like salmon (Liu et al. 2008) and Japanese flounder (Park et al. 2005). CD3 ϵ positivity by FCM was inferior to that of thymus but similar to HK and gills. Likewise, research on Atlantic salmon from Koppang et al. (2010) reported high numbers of CD3 ϵ^+ T cells in the intestinal tract as well as in the thymus and gills. Such data reinforces the notion that this mucosal site plays an important role in fish immunology.

Novel feeds are able to modulate fish immunity and increase the intestinal health (Dawood 2020). On this regard, microalgae are an emerging product with a great potentiality in aquafeeds, not only because of their nutritional value in terms of protein and lipid contents (including long-chain polyunsaturated fatty acids) but also because they include fractions possessing immunostimulatory, antioxidant, or anti-inflammatory activities (Nakagawa and Montgomery 2007; Becker 2007; Burnell and Allan 2009). It was proven that an alternative feed replaced of fish meal by the microalgae *Spirulina platensis* affected the intestinal micromorphology of barramundi *Lates calcarifer* (Van Vo et al. 2020). Orally administered microalgae *Nannochloropsis gaditana*, *Tetraselmis chuii*, and *Phaeodactylum tricornutum* enhanced defense activity or innate immunity of gilthead seabream (Cerezuela et al. 2012) and rainbow trout (Amar et al. 2004). Because microalgae also induced a change in the intestinal microbiome diversity (Nayak et al. 2020) and the microbiome itself can modulate the numerosity of T cells (Bakke-McKellep et al. 2007), the significant increase of mucosal CD3 ϵ^+ T cells in the mid intestine of sea bass fed with 100% *Nannochloropsis* sp. (NANNO 15) may reflect local proliferation-inducing effects or a possible microbiome-driven adaptive immune modulation. In either case, T cells appear to be involved in nutritional immunity.

In conclusion, the present study generated data and tools on the CD3 ϵ molecule that allowed a deeper characterization of T cell-mediated immunity in the European sea bass. Such knowledge is of relevance because it can benefit additional teleost species, with particular regards on commercially important ones. The functional study performed using novel feeds suggested that T lymphocytes may be differentially modulated among intestinal segments by a microalgal species. This finding could be further explored in pathogen challenge trials aimed at assessing whether microalgae-supplemented feeds confer increased fish survival, in view of sustainable yet efficient zootechnical strategies.

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Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Animal manipulation during the diet experiments complied with the guidelines of the European Union Directive (2010/63/EU) and Portuguese legislation (DL113/2013) for animal treatments. The Organism for Animal Welfare of CIIMAR and the Portuguese Directorate-General for Food and Veterinary approved all the protocols performed in the present study (approval 0421/000/000/2017 DGAV). Fish handling was performed under ethylene glycol monophenyl ether anesthesia (0.3 mL/L; ref.: 8.07291.2500, Merck, Whitehouse Station, USA), and discomfort, stress, and pain to the experimental animals was avoided, as much as possible, along the experiment. For sampling, fish were euthanized by anesthetic overdose (ethylene glycol monophenyl ether; ref.: 8.07291.2500, Merck, Whitehouse Station, USA).

For the experiments performed in Italy, ethical approval by ethics committee was not required, as per the Explanatory note of the Italian Ministry of Health's Directorate-General for Animal Health and Veterinary Medicinal Products (DGSAF) of 26 July 2017. Organs and tissues were dissected out following temporary immobilization with 1 mg/ml tricaine methane sulfonate (MS-222) (Sigma) in aquarium water, buffered with 0.6 g/L sodium bicarbonate. Anesthesia was followed by exsanguination or decapitation. All efforts were made to minimize suffering. The study was carried out in strict accordance to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and on National Legislative Decree 26 of 4 March 2014 "Attuazione della Direttiva 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici."

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