Molecular characterization of the T cell costimulatory receptors CD28 and CTLA4 in the European sea bass

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ABSTRACT:

For the activation of T cells, it is necessary the specific recognition of the peptide by the T cell receptors (TCR) in the surface of antigen-presenting cells (APCs) and additional signals delivered by costimulatory receptors. In fish, knowledge about the presence of these costimulatory signals is limited and functional evidence almost absent. Thus, in this study, we have identified the stimulatory CD28 and the inhibitory cytotoxic T-lymphocyte-associated protein 4 (CTLA4) coreceptors in the European sea bass (*Dicentrarchus labrax*), and evaluated their transcription. In parallel, the transcription encoding for the T cell markers CD8α and CD4 was also evaluated. Both coreceptors showed the canonical architecture including a signal peptide, an immunoglobulin domain, a transmembrane region and a cytosolic tail. Protein predictions and phylogenetic tree identify them as true mammalian orthologues of CD28 and CTLA4. We found these genes constitutively expressed in all studied organs of European sea bass with high expression in lymphoid organs (thymus, spleen and head-kidney) and liver. The molecular expression pattern of these genes was up-regulated in head-kidney leucocytes stimulated with T mitogens as concanavalin A and phytohemagglutinin (PHA), but not with the B cell mitogen lipopolysaccharide (LPS). Fish challenged with nodavirus (NNV) evidenced a differential and opposing regulation of the *cd28* and *ctla4* transcription levels in the brain, the target organ for viral replication, and head-kidney. While *cd28* transcription tends to decrease over the infection time in both organs the expression of the *ctla4* gene tends to increase. Interestingly, the coreceptor expression is highly and significantly correlated to the transcription of the T cell markers. Our results highlight the important role of CD28 and CTLA4 as costimulatory receptors of T cells in European sea bass but further studies are deserved.

Keywords: Costimulatory receptors; T lymphocytes; CD28; CTLA4; Nodavirus; European sea bass; Fish immunity.

1. Introduction

The cellular adaptive immunity depends on the proper activation of T cells, which comprises two main populations: cytotoxic (CTLs) and helper (Th) T lymphocytes. This activation is based on the specific recognition and binding of the T cell receptor (TCR) with its major histocompatibility complex (MHC)-peptide partner on the surface of antigen-presenting cells (APCs) [1]. However, this is not enough and the regulation and activation of T cells needs the simultaneous binding of other coreceptors, being CD8 and CD4 the canonical surface markers for CTLs and Th cells, respectively. But other coreceptors are still necessary between T cells and APCs. In mammals, up to five different costimulatory receptors have been identified in T cells: two positive costimulatory (CD28 and inducible costimulatory signal (ICOS)) and three negative costimulatory or inhibitory (cytotoxic T-lymphocyte-associated protein 4 (CTLA4 or CD152), programmed cell death-1 (PD-1) and B- and T-lymphocyte attenuator (BTLA)) molecules [2]. Within this group, in mammals, particular attention has been paid to CD28 and CTLA4 [3]. Both receptors are transmembrane protein members of the immunoglobulin gene superfamily containing a single extracellular `V-like' domain [4]. While CD28 is found in both naïve and activated T cells, CTLA4 expression is limited to activated cells. Human CD28 is expressed in 80% of $CD4^+$ T cells and in 50% of $CD8^+$ T cells. However, it shows minor expression in other cell populations including bone marrow stromal cells, plasma cells, neutrophils, and eosinophils, but no clear functions have been documented on them. By its part, CTLA4 is more expressed in activated $CD4^+$ T than in $CD8^+$ T cells, mainly in the regulatory subpopulation of Th cells (Treg), though other cell types such as monocytes, dendritic cells or even tumor cells might also express it. CD28 and CTLA4 are highly homologous and compete for the same ligands expressed on the surface of APCs: CD80 (B7-1) and CD86 (B7-2) [5]. CD28 and CTLA4 have opposing effects on T cell stimulation, which requires an equilibrium between the binding of CD80/CD86 ligands to CD28/CTLA4 receptors [4]. CTLA4 has higher affinity than CD28 for these ligands, while CD86 has a relative preference for CD28 and CD80 binds very strongly to CTLA4. Thus, when APCs are activated by an stimulus they firstly express CD86, that is preferentially recognized by CD28 in naïve T cells and triggers the proliferation of effector T cells, their cell cycle progression and production of interleukin (IL)-2 and interferon (IFN)-γ [4]. Later, when the immune response is progressing and strong enough, APCs increase the expression of CD80, which binds very strongly to CTLA4 in activated lymphocytes and drives the inhibition of the above functions to control the immune response and avoid it in excess. The exact profile of both CD80/CD86 and CD28/CTLA4 is tightly regulated and drives to the immunosuppressive or immunostimulatory signals, and this regulation is under continuous evaluation and elucidation.

Molecular and functional studies strongly confirm that both $CD4^+$ T and $CD8^+$ T cells, as well as APCs, exists in fish. It is known that the fish CTLs, or $CD8^+$ T cells, exert the cytolytic activity in a MHC-I restricted manner [6,7]. However, the restriction of $CD4^+$ T cells to the MHC-II in APCs and the molecular interactions are not well detailed in fish. As in mammals, fish Th cells ($CD4^+$) seem to contain several subpopulations including Th1, Th2, Th17 or induced T regulatory (Treg), based on the main cytokines produced and functions, but they are very poorly characterized [8–11]. Focusing on the first step during costimulation, fish APCs consist on monocyte-macrophages, dendritic cells, granulocytes and B lymphocytes, which express a single receptor CD80/86 that could be able to interact with both CD28 and CTLA4 on T cells [12,13], suggesting subtle differences in the biology of the fish CD80/86, and its ligands, with

respect to the mammalian counterparts. While it has been demonstrated that Nile tilapia (*Oreochromis niloticus*) CD28 interacts with the CD80/86 [14] other members of the B7 family have been also described in fish, which are able to bind to activated T cells and produce either increments in lymphocyte proliferation and production of IL-10 and IFN-γ or inhibit IL-2-mediated proliferation [13,15,16]. Regarding the costimulatory molecules on the surface of fish T cells little is still known. Among the positive stimulators, only CD28 has been clearly documented in several fish species including Nile tilapia, rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*), tongue sole (*Cynoglossus semilaevis*), European sea bass (*Dicentrarchus labrax*) and zebrafish (*Danio rerio*) [14,17–23] while ICOS has not been found. In these studies, apart from the molecular characterization, it has been demonstrated that its transcription is up-regulated by phytohemagglutinin (PHA), a well-known T cell mitogen, and bacterial infection, and up-/down-regulated by viral infections, and that antibody-binding increased lymphocyte proliferation. In addition, among the inhibitory coreceptors, only CTLA4 and BLTA have been evaluated, being CTLA4 the most studied, but still less than CD28. Thus, CTLA4 has been identified in some fish species such as rainbow trout and Kelp grouper (*Epinephelus moara*) and shown to be up-regulated by PHA or viral infections [19,22]. Very interestingly, the cytosolic domain of fish CTLA4 is quite different to that of mammals, and the inhibitory role is under debate [18,19,24]. CTLA4 is expressed in trout $CD4^+$ [10] and $CD8^+$ T lymphocytes [25]. Thus, further investigation on the identification and functions of fish CD28 and CTLA4 in induction and maintenance of T cell responses deserves further efforts.

In the present study, we aimed to investigate the presence and function of CD28 and CTLA4 coreceptors in the European sea bass (*Dicentrarchus labrax*), the most important cultured fish species in the Mediterranean area. Sequences were retrieved from databases and transcription in naïve organs as well as regulation in head-kidney leucocytes by mitogens as well after *in vivo* infection with nodavirus (NNV), the most pathogenic virus for this fish species, was evaluated. In parallel, the transcription of the T cell markers CD8α and CD4 was analysed and correlated with that of the coreceptors. This study will through some light into the fish biology of T cells, and in the European sea bass in particular.

2. Material and methods

2.1. Animal maintenance

Adult European sea bass (*Dicentrarchus labrax*) were bred at the *Instituto Español de Oceanografía* (IEO) installations and transported to the University of Murcia for further experiments. Animals were housed in 450–500 L running seawater (28‰ salinity) aquaria at 24 ± 2°C with a 12 h light:12 h dark photoperiod and fed with 1 % of biomass of commercial diet (Skretting). Animals were fasted 24 h, sacrificed by an overdose of clove oil (40 ppm) and sampled by dissection after blood withdraw. Animal experimentation was approved by the Bioethical Committee of the University of Murcia and followed the European Union regulations.

2.2. In vitro and in vivo exposures

In order to analyze the constitutive gene expression in naïve conditions, brain, gill, liver, skin, gonad, gut, head-kidney (HK), spleen, thymus, and blood from 3 healthy fish specimens were sampled and immediately frozen in TRIzol® Reagent (Life Technologies) and kept at −80°C.

In vitro exposure of European sea bass isolated head-kidney leucocytes (HKLs) to different stimuli was done as previously [26]. Briefly, HKLs (n=5 fish) were individually isolated and maintained in Leibovitz's L-15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 20 mM HEPES (Gibco). Then, HKLs were exposed through incubation of 10^7 HKLs/mL in 48-well microtiter plates (Nunc) at 22 ºC during 24 h with: culture L-15 medium (control treatment), 5 µg/mL concanavalin A (ConA; Sigma-Aldrich), 5 µg/mL lipopolysaccharide (LPS; Sigma-Aldrich), 10 µg/mL phytohemagglutinin (PHA; Sigma-Aldrich) and 10^6 tissue culture infective dose $(TCID)_{50}$ NNV/mL. After exposure, HKLs were washed with phosphate buffer saline (PBS) and conserved in TRIzol® Reagent at -80°C until sampling processing.

Samples from an *in vivo* viral infection were also used [27]. In brief, infection was carried out by intramuscular injection with 100 µL containing 10^6 $TCID_{50}$ of NNV/fish (strain 411/96, genotype RGNNV), while the other group of fish served as a control and was injected with 100 µL of cell culture supernatant [27]. Fish were sampled at 1, 7, and 15 days post-infection and the brain and HK of each fish (n = 4–6 fish) was extracted and immediately frozen in TRIzol® Reagent and kept at −80°C. Fish mortality was recorded and reached a 55% of the specimens [27].

2.3. Genetic analysis

Sequences for European sea bass costimulatory receptors CD28 and CTLA4 were identified in RNA-seq studies performed in our lab and further confirmed within the European sea bass genome project (http://seabass.mpipz.mpg.de/). Exon-intron structure was generated by the Exon-Intron Graphic Maker (http://wormweb.org/exonintron). Predicted proteins were used to search for conserved domains and alignment using ExPASy tools. Phylogenetic analysis was performed by MEGA software [28].

To evaluate the transcription levels, total RNA was isolated from TRIzol® Reagent frozen samples, treated with DNAse I (Promega) and the first strand of cDNA synthesized by reverse transcription using the Superscript III reverse transcriptase (Life Technologies) according to the manufacturer's instruction. Real-time PCR was performed using a 7500 Fast Real Time PCR System (Roche Applied Science) and SYBR Green PCR Core Reagents (Applied Biosystems) [27]. Reaction mixtures were incubated at 95 ºC for 10 min, followed by 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC, and finally 15 s at 95 ºC, 1 min at 60 ºC and 15 s at 95 ºC. The gene expression was corrected and normalized by the geometric mean of the elongation factor 1 alpha (*ef1a*) and ribosomal 18S (*rps18s*) expression as house-keeping genes following the $2^{-\Delta CT}$ method: $2^{-(CT\ target\ -\ CT\ house\text{-}keeping)}$ [29]. C_T values lower than 40 were used for calculations. Samples in which the C_T was undetermined the $2^{-\Delta CT}$ value for calculations was assumed as 0. The primers are listed in Supplementary Table S1. Negative controls with no sample were always included in the reactions.

2.4. Statistical analysis

Figures are presented as mean ± SEM of the data. Statistical differences between groups were analyzed by either one- or two-way analysis of variance (ANOVA; $p \leq 0.05$) using STATGRAPHICS centurion XVII (Statpoint Technologies), followed by the Tukey's comparison of means when applicable. In addition, non-parametric Pearson correlation tests were applied to test relations among costimulatory and T cell marker receptors. The antiviral *mx* gene was used as unrelated gene to confirm the correlations.

3. Results

3.1. Molecular organization

European sea bass sequences for *cd28* and *ctla4* genes were identified in internal RNA-seq studies and then retrieved from the genome project (http://seabass.mpipz.mpg.de/). Both genes show the same genome organization with 4 exons and 3 introns. Thus, sea bass *cd28* introns are of 950, 78 and 661 nt of length (Fig. 1A) whilst those for *ctla4* are of 1942, 1434 and 121 nt (Fig. 2A). One single copy of *cd28* and *ctla4* sequences is found in chromosome 24 and chromosome 15, respectively though the sea bass genome is not complete.

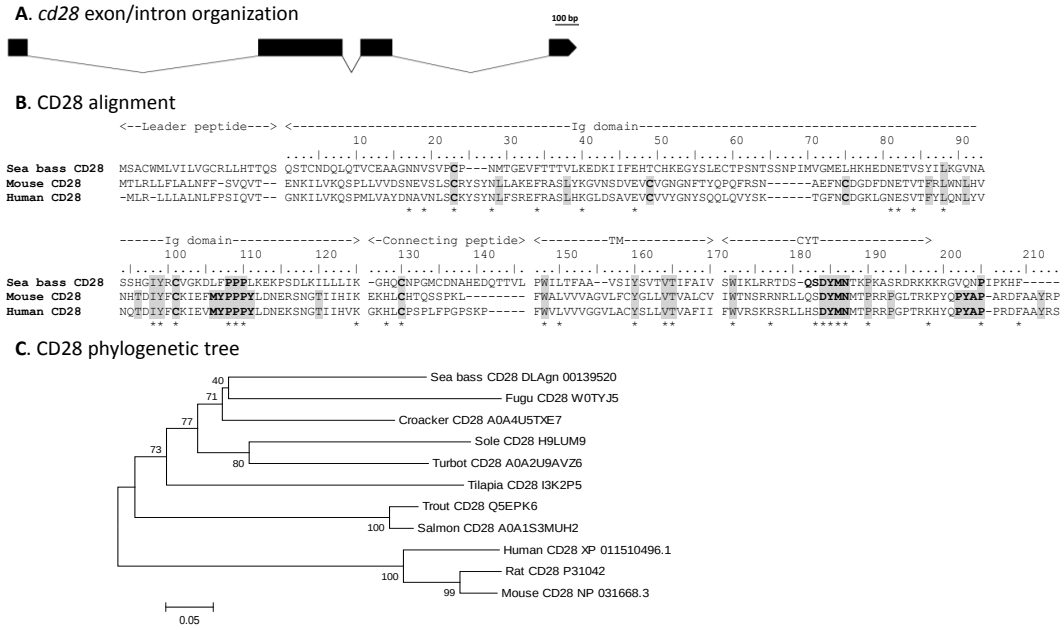


Figure 1. European sea bass CD28. A. Exon-Intron organization, in scale, of the European sea bass *cd28* coding genes. **B**. Predicted putative sea bass CD28 protein was aligned with the mouse and human counterparts using ClustalW. Numbering was according to the sea bass mature protein. Gaps are introduced to strength the alignment. Important residues are shadowed. Asterisk denoted conserved residues. **C**. Phylogenetic tree including the European sea bass CD28 putative protein was constructed using the Neighbour-Joining method, where genetic distances were calculated based on protein differences (p-distance) with pairwise deletion. The number at each node indicates the percentage of bootstrapping after 1,000 replications. GenBank accession numbers are shown.

Sea bass CD28 putative protein is complete with 224 residues while CTLA4 sequence is partial, with 234 residues, and lacks few amino acids in each extreme. Both proteins were searched for domain architecture by InterPro (https://www.ebi.ac.uk/interpro/) and possess the same structure: a signal peptide, an extracellular immunoglobulin (Ig) domain, a transmembrane region and a cytoplasmic tail (Fig. 1B, 2B; Supplementary Fig. S1). Sea bass CD28 and CTLA4 putative proteins were then aligned to the respective mouse and human orthologues. Sea bass CD28 has 34% similarity and 18% identity to human CD28 while the sea bass CTLA4 partial protein has 35% similarity and 20% identity to its human counterpart. Curiously, sea bass CTLA4 is larger than the mammalian counterparts due to the residue inclusion in both the Ig-V and transmembrane regions (Fig. 1B). Alignment of the sea bass CD28 proteins (Fig. 2B) also shows perfect conservation for the canonical Cys (positions 23 and 102) involved in Ig-domain disulphide bond and the Cys^{130} potentially involved in CD28 dimerization. Similarly, sea bass

CTLA4 putative protein also shows good conservation (Fig. 2B) of the 4 Cys (positions 22, 66, 89 and 115) involved in the Ig-V region disulphide bonds and the extra Cys^{145} potentially involved in CTLA4 dimerization. Important and major glycosylation residues are partly conserved in both sequences. The conserved MYPPPY motif involved in CD80/CD86 binding is not completely conserved, being LFPPPY in CD28 and FYPPPY in CTLA4 of sea bass (Fig. 1B, 2B). Sea bass CD28 DYMN motif in the cytoplasmic tail is well conserved but lacks the PYAP motif, the two involved in mammalian signalling transduction. By contrast, the cytoplasmic tail of the sea bass CTLA4 does not show conservation of the residues involved in mammalian signal transduction (YVKM and YFIP) though has 2 Tyr residues. Phylogenetic analysis for the individual proteins confirmed the good conservation and revealed that all fish proteins were grouped in the same branch and close to their respective mammalian counterparts (Fig. 1C, 2C). Among fish, proteins belonging to the order *Perciformes* were in the same branch and separated to the branches formed by members of the orders *Cypriniformes* and *Salmoniformes*.

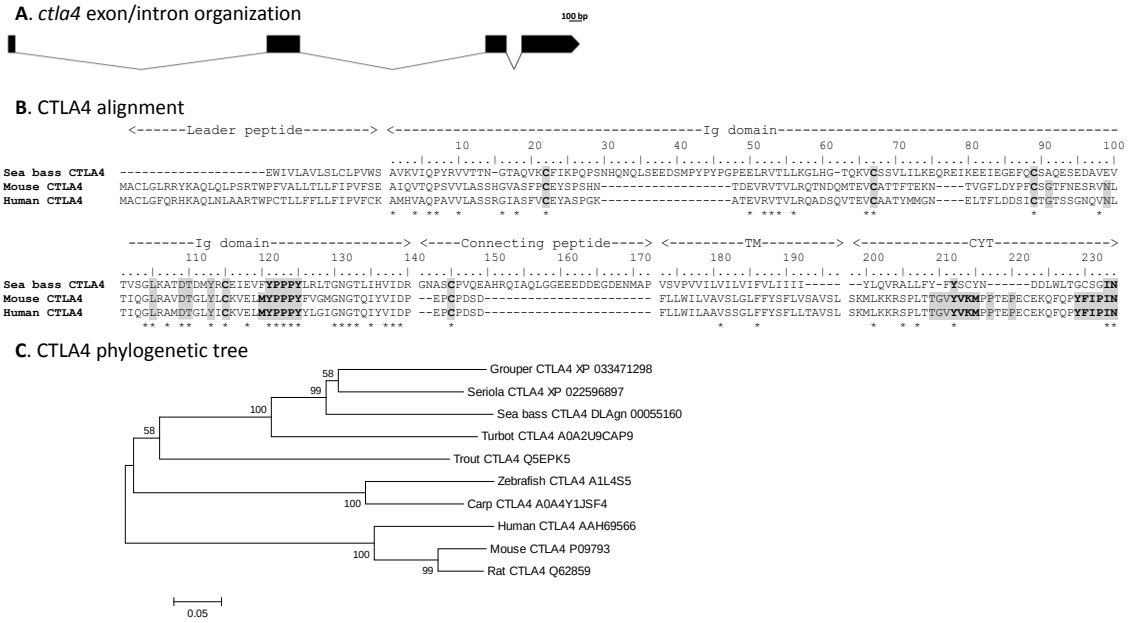


Figure 2. European sea bass CTLA4. A. Exon-Intron organization, in scale, of the European sea bass *ctla4* coding genes. **B**. Predicted putative sea bass CTLA4 protein was aligned with the mouse and human counterparts using ClustalW. Numbering was according to the sea bass mature protein. Gaps are introduced to strength the alignment. Important residues are shadowed. Asterisk denoted conserved residues. **C**. Phylogenetic tree including European sea bass CTLA4 putative proteins was constructed using the Neighbour-Joining method, where genetic distances were calculated based on protein differences (p-distance) with pairwise deletion. The number at each node indicates the percentage of bootstrapping after 1,000 replications. GenBank accession numbers are shown.

3.2. Coreceptors show high transcription in lymphoid organs and liver

Mature T cells can be found throughout the body, and particularly, in lymphoid organs. Our results show that the *cd28* gene is predominantly expressed in the lymphoid organs spleen and thymus, and in liver (Fig. 3A). Sea bass *ctla4* transcript follows very similar pattern than *cd28* except the low expression in the spleen (Fig. 3B). In addition, we also evaluated the transcription of the T cell markers *cd8a* and *cd4*. While *cd8a* is mainly detected in the thymus, spleen and head-kidney (Fig. 3C) the transcription of *cd4* is mainly detected in the thymus and intestine (Fig. 3D). Interestingly, the correlation analysis detected good and significant

correlations between *cd28* and *ctla4* transcription, and of *ctla4* with the CTL marker *cd8a* (Table 1).

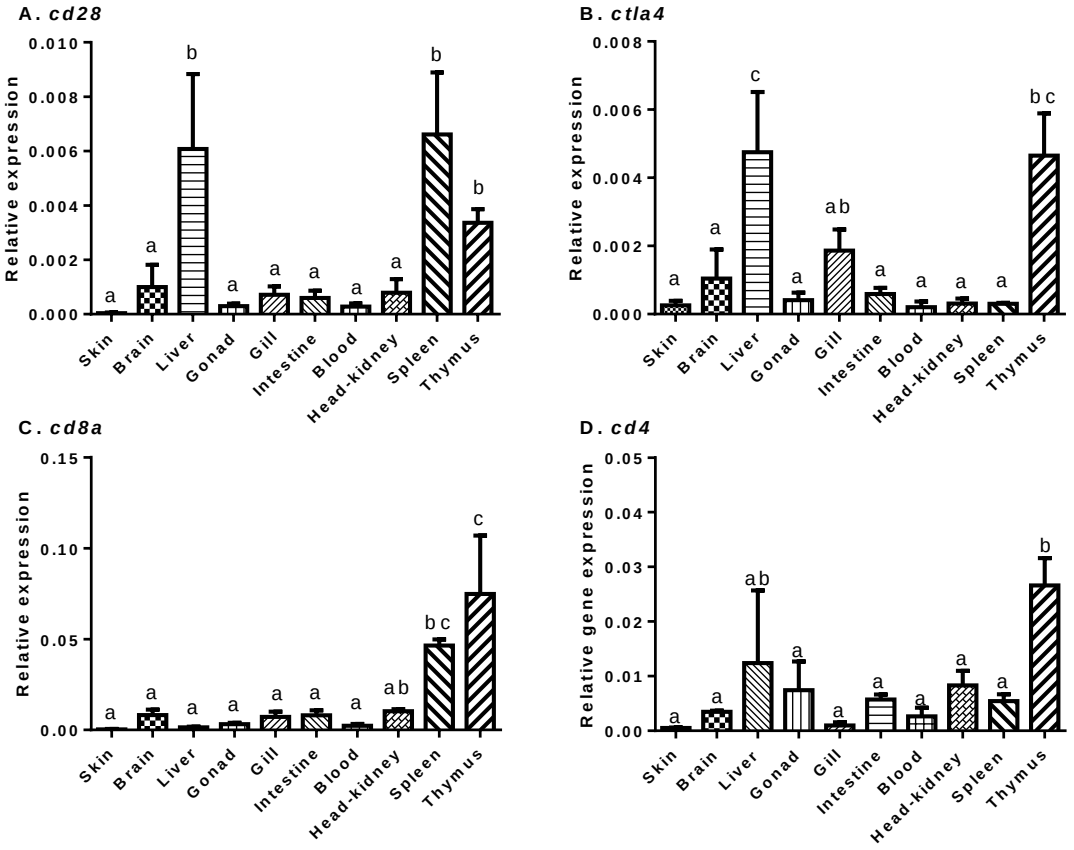


Figure 3. European sea bass *cd28* and *ctla4* genes are widely and constitutively expressed in the organs of naïve European sea bass specimens. Organs were obtained from naïve fish specimens and the expression of *cd28* (**A**), *ctla4* (**B**), *cd8a* (**C**) and *cd4* (**D**) genes evaluated by real-time PCR. Data are presented as means (n= 3) ± SEM relative to the expression of the endogenous controls. Different letters indicate differences between organs according to ANOVA and Tukey's post-hoc tests (p<0.05).

3.3. Transcription of costimulatory receptors is primed by T mitogens, as the T cell markers

We wanted to know if the transcription of both coreceptors is primed in HKLs upon immune *stimuli*. In our study, we observed an up-regulation of the relative expression of both coreceptors upon stimulation of HKLs with T cell mitogens (Fig. 4). The relative transcription of *cd28* in HKLs treated *in vitro* with PHA was increased 2.5-fold compared to controls (Fig. 4A) whilst the transcription of *ctla4* did it 8- and 9.3-fold upon stimulation with ConA and PHA (Fig. 4B), respectively. Regarding the T cell markers, *cd8a* gene expression was not significantly altered in the HKLs (Fig. 4C) though the transcription of *cd4* was significantly up-regulated by stimulation with ConA and PHA (Fig. 4D). The B cell mitogen LPS, as well as NNV particles, failed to regulate the transcription in sea bass HKLs. Again, the transcription of both costimulatory receptors showed good and significant correlation between them, which were also correlated with the T cell markers (Table 1), being highest for *ctla4*.

Sample	Gene	*cd28*	*ctla4*	*cd8a*	*cd4*	*mx*

Naïve organs	*cd28*		**0.721**	0.355	0.251	-0.26
			0.000	*0.055*	*0.181*	*0.166*
	ctla4	**0.721**		**0.477**	0.345	-0.274
		0.000		***0.008***	*0.062*	*0.143*
HKLs *in vitro*	*cd28*		**0.634**	**0.424**	**0.496**	-0.198
			0.002	***0.039***	***0.014***	*0.353*
	ctla4	**0.634**		**0.79**	**0.687**	-0.021
		0.002		***0.000***	***0.000***	*0.926*
NNV infection: Brain	*cd28*		-0.157	-0.237	-0.159	-0.155
			0.496	*0.328*	*0.504*	*0.538*
	ctla4	-0.157		**0.712**	0.250	-0.045
		0.496		***0.001***	*0.289*	*0.858*
NNV infection: HK	*cd28*		-0.055	0.258	0.415	-0.148
			0.814	*0.259*	*0.061*	*0.522*
	ctla4	-0.055		0.125	-0.278	0.312
		0.814		*0.590*	*0.223*	*0.168*

Table 1. Correlation observed between the transcription of costimulatory receptors *cd28* and *ctla4* genes with the T cell markers *cd8a* and *cd4* in European sea bass naïve organs, HKLs stimulated *in vitro* and in the brain and HK after infection with NNV. The first number corresponds to Pearson coefficient of correlation and the second to the *p* value. Written in bolds are the parameters correlated and significant at $p<0.05$. The T cell unrelated gene *mx* was included. HKL, head-kidney leucocytes; HK, head-kidney; NNV, nodavirus.

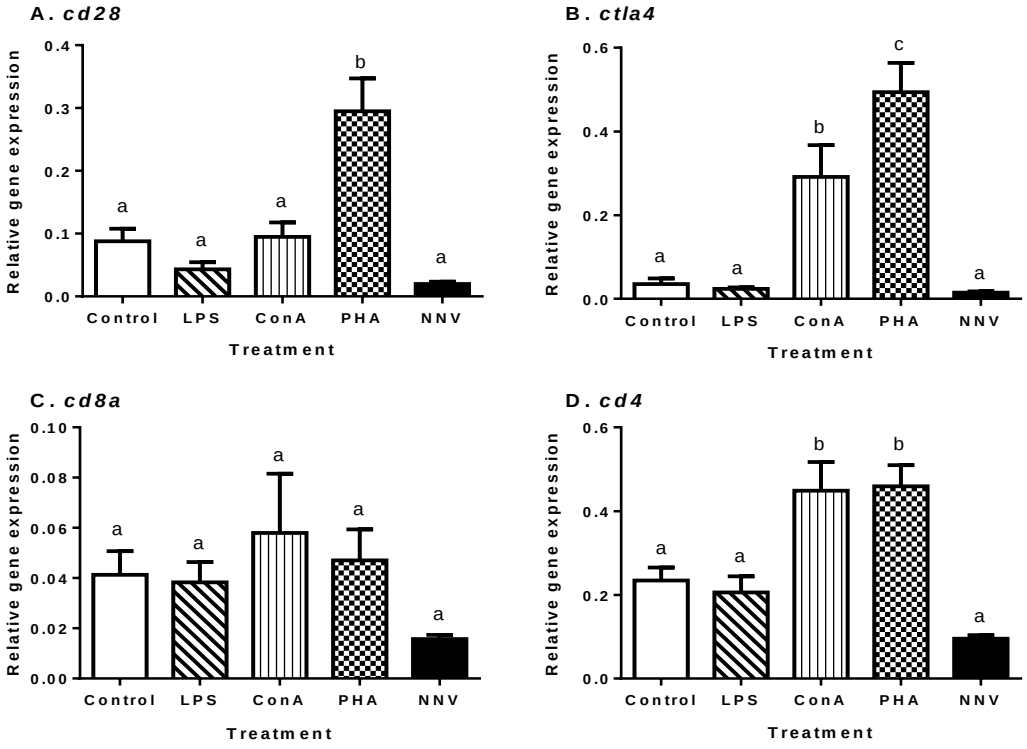


Figure 4. European sea bass coreceptor gene expression is up-regulated by T cell mitogens. Head-kidney leucocytes were isolated from European sea bass and stimulated for 24 h and the expression of

cd28 (**A**), *ctla4* (**B**), *cd8a* (**C**) and *cd4* (**D**) genes evaluated by real-time PCR. Data are presented as means (n= 5) ± SEM relative to the expression of the endogenous controls. Different letters indicate differences between groups according to ANOVA and Tukey's post-hoc tests (p<0.05). LPS, lipopolysaccharide; PHA, phytohemagglutinin; ConA, concanavalin A; NNV, nodavirus.

3.4. NNV infection has opposing profile in the transcription of *cd28* and *ctla4* coreceptors

After the stimulation *in vitro*, we aimed to investigate whether the transcription of both coreceptors is modulated by NNV, the most devastating virus for European sea bass. The transcription of both coreceptors shows very low expression in both tissues, brain (Fig. 5) and head-kidney (Fig. 6). Two-way ANOVA shows that there is a significant effect of the NNV infection in the transcription of *cd28*, *ctla4* and *cd8a* in the brain and of *cd28* and *ctla4* in the head-kidney (Table 2). Regarding the infection time as factor, there is a significant effect in the transcription of *ctla4* and *cd8a* in the brain and of *cd28* and *cd4* in the head-kidney (Table 2). Interestingly, we observe a significant interaction between NNV infection and the infection time in the transcription of *ctla4* and *cd8a* in the brain as well as in the *cd28* in the head-kidney.

		Brain			Head-kidney		
Gene	**Factor**	**DL**	**F-value**	***p-value***	**DL**	**F-value**	***p-value***
cd28	Infection	1	5.474	**0.0374**	1	14.63	**0.0024**
	Time	2	3.139	0.0801	2	4.316	**0.0387**
	Interaction	2	2.599	0.1154	2	4.820	**0.0291**
ctla4	Infection	1	6.954	**0.0217**	1	5.278	**0.0404**
	Time	2	9.219	**0.0038**	2	1.083	0.3695
	Interaction	2	8.586	**0.0048**	2	1.240	0.3239
cd8a	Infection	1	7.875	**0.0159**	1	1.837	0.2003
	Time	2	5.402	**0.0212**	2	0.3981	0.6801
	Interaction	2	5.578	**0.0194**	2	2.342	0.1385
cd4	Infection	1	3.996	0.0688	1	4.249	0.0616
	Time	2	0.3221	0.7307	2	13.28	**0.0009**
	Interaction	2	2.125	0.1621	2	2.375	0.1352

Table 2. Two-way-ANOVA analysis of European sea bass transcription of genes in the brain and head-kidney upon nodavirus infection. DL, degrees of freedom.

Looking at the transcription in the brain, *cd28* (Fig. 5A) was greatly increased after 1 day of NNV infection but did not reach significance (p=0.0921). Thereafter, it shows a decreasing tendency along the infection time to be undetectable in NNV-infected fish at 15 days (p=0.51 between the specimens infected with NNV after 1 and 15 days). By sharp contrast, brain *ctla4* gene expression was very low in general but sharply and significantly up-regulated in NNV infected specimens after 15 days (Fig. 5B). In addition, a significant increase was observed among NNV infected fish with the infection time. Regarding the T cell markers, NNV infection also increased to a significant extent the transcription levels of *cd8a* after 15 days of infection (Fig. 5C) while in the case of *cd4* (Fig. 5D) the changes were not significant. Strikingly, correlation analysis showed strong and significant correlation between the transcription of *ctla4* and *cd8a* in the brain (Table 2).

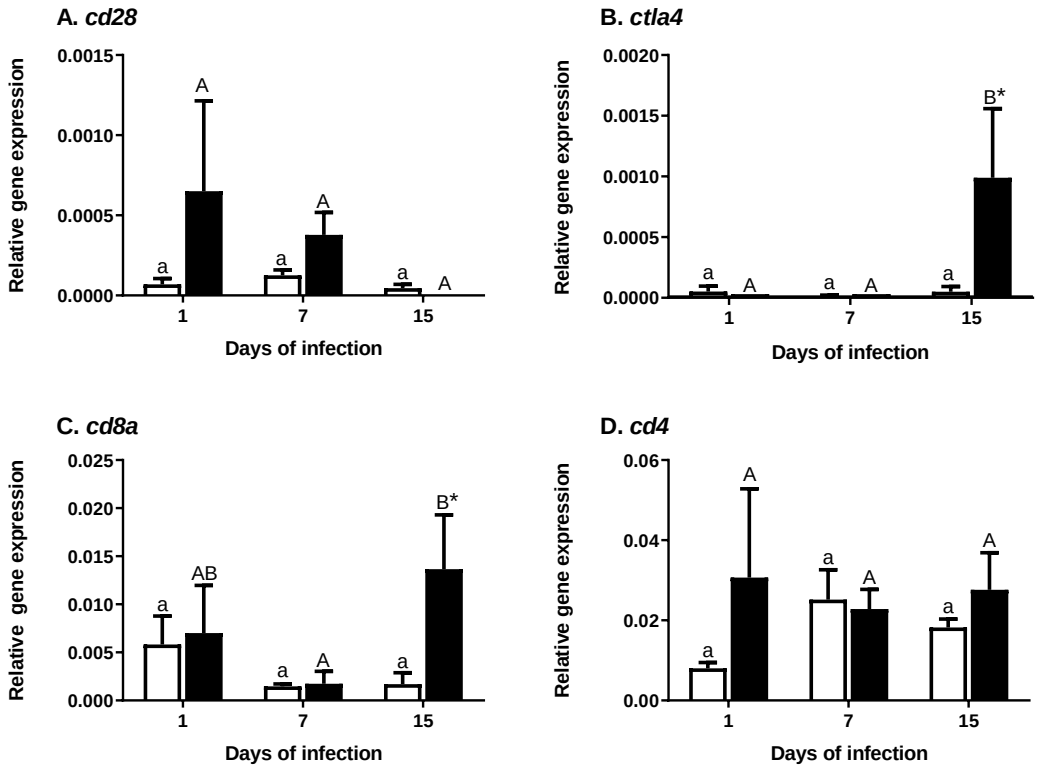
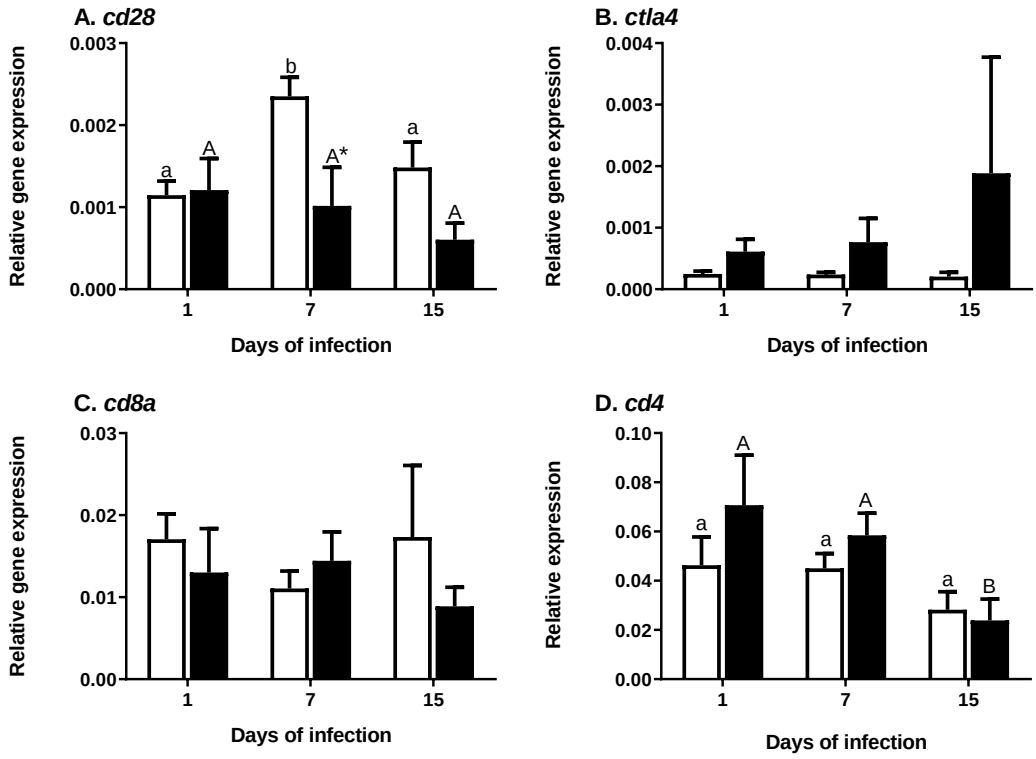


Figure 5. **NNV infection up-regulates the transcription of *cd28* and *ctla4* coreceptors in European sea bass brain.** Specimens were intramuscularly injected with culture medium (mock-infected or control; white bars) or with 10^6 $TCID_{50}$ of NNV/fish (black bars) and after 1, 7 or 15 days of infection the brain was sampled to evaluate the expression of *cd28* (**A**), *ctla4* (**B**), *cd8a* (**C**) and *cd4* (**D**) genes by real-time PCR. Data are presented as means (n= 4-6) ± SEM relative to the expression of the endogenous controls. Asterisks denote significant differences between control and NNV-infected fish according to ANOVA and Tukey's post-hoc tests (p<0.05). Different letters among control (lower case) or NNV-infected (capital letters) groups denote significant differences with the infection time according to ANOVA and Tukey's post-hoc tests (p<0.05).

Regarding the head-kidney, *cd28* transcription was significantly reduced in infected specimens after 7 days compared to controls (Fig. 6A). Although *ctla4* gene expression showed an increasing trend in NNV-infected specimens as the infection progresses this failed to reach significance compared to the respective controls (Fig. 6B). No significant variations after NNV infection were observed in the transcription of the sea bass T cell markers *cd8a* and *cd4* in the head-kidney (Fig. 6C, 6D). The transcription of the genes evaluated in the head-kidney fails to show significant correlations (Table 1).



315

316 **Figure 6**. **NNV infection has opposite regulatory effects in the transcription of *cd28* and *ctla4***
317 **coreceptors in European sea bass head-kidney.** Specimens were intramuscularly injected with culture
318 medium (mock-infected or control; white bars) or with 10^6 $TCID_{50}$ of NNV/fish (black bars) and after 1, 7
319 or 15 days of infection the head-kidney was sampled to evaluate the expression of *cd28* (**A**), *ctla4* (**B**),
320 *cd8a* (**C**) and *cd4* (**D**) genes by real-time PCR. Data are presented as means (n= 4-6) ± SEM relative to the
321 expression of the endogenous controls. Asterisks denote significant differences between control and
322 NNV-infected fish according to ANOVA and Tukey's post-hoc tests (p<0.05). Different letters among
323 control (lower case) or NNV-infected (capital letters) groups denote significant differences with the
324 infection time according to ANOVA and Tukey's post-hoc tests (p<0.05).

325

326 **4. Discussion**

327 The expression of costimulatory responses is essential for the initiation of T cell maturation.
328 Coreceptors play important roles in determining the outcome of the T cell responses by
329 determining the balance of stimulatory and inhibitory co-signals in the regulation of
330 inflammation versus autoimmunity [30]. In fish, though both, the stimulatory CD28 and the
331 inhibitory CTLA4 coreceptors have been identified, only one partner CD80/86 has been
332 identified, suggesting that the fish T and APC interaction and regulation might have some
333 peculiarities and differences respect to the mammalian counterparts. In an effort to throw
334 some light into this we searched both T cell coreceptors in the European sea bass.

335 First, we performed an *in silico* evaluation of the European sea bass CD28 and CTLA4
336 sequences. Both genes have the same exon-intron organization in all fish evaluated and
337 mammals suggesting they belong to the same family. In addition, as in other fish, sea bass
338 *cd28* and *ctla4* genes are located in separate chromosomes in comparison to the linked
339 position in tetrapods, probably due to the genome duplication and posterior deletion events
340 during fish evolution [18]. The extracellular domain of both sea bass predicted coreceptors

contains an Ig-like superfamily domain with 4 Cys involved in intra-Ig domain disulphide bonds and an extra Cys considered to form the homodimers, as their vertebrate counterparts. The MYPPPY residues important for binding to APC receptors CD80/CD86 are well conserved for sea bass CTLA4 but not for CD28, as in other teleost fish such as fugu, zebrafish, pufferfish (*Tetraodon nigroviridis*), medaka, grouper or sole [17]. This would suggest higher affinity/binding of the inhibitory CTLA4 than the stimulatory CD28 for fish CD80/86, which has not been evaluated so far. However, the existence of other members of the B7 family observed in fish [12,15] might also act as partners for fish CD28 and CTLA4. For example, fugu B7-H1/DC inhibited T cell proliferation concomitant with increasing levels of both IL-10 and IFN-γ expression whereas B7-H3 and B7-H4 induced T cell proliferation following IL-2 induction and the suppression of IL-10, resembling the functions induced by CTLA4 and CD28, respectively [15]. However, whether these B7 receptors bind to CTLA4 and CD28 is unknown.

Upon engagement of CD28 or CTLA4 receptors to the respective receptors in the APCs, the cytosolic tail regulates, by phosphorylation-dephosphorylation of important Tyr residues, their stimulatory or inhibitory responses. Thus, sea bass CD28 shows well conservation of the YMNM sequence, in which the Tyr residue is phosphorylated upon CD28 activation leading to the recruitment and binding of SH2 domains from PI3K p85, Grb2 and GADS to the last NM residues [31]. Although the Y residue is well conserved in fish the presence of one or the two NM residues lack in most of the fish species analysed so far [17,19]. However, fish lack the PYAP motif used in mammals for recruitment of SH3 protein kinases as Lck. While the presence of this PYAP motif seems essential to induce IL-2 production by activated T cells it has been demonstrated that cytosolic trout CD28, lacking this motif, is also able to transduce the IL-2 production [19] demonstrating that it is not strictly necessary for fish. Regarding mammalian CTLA4, the cytoplasmic tail contains the YVKM and YFIPIN motifs, which serve as docking residues for AP-1 and AP-2 that mediate clathrin-mediated endocytosis of surface CTLA4 [32]. The balance of surface-endosome CTLA4 molecules is fundamental for T cell activation and the cytosolic domain of vital importance for its function. Therefore, the absence of these two motifs in fish CTLA4 proteins might suggest that CTLA4 regulation does not involve the same cell membrane-endosome trafficking as it happens in mammals. In this line of evidence, trout CTLA4 cytoplasmic tail has a YXXF motif that is less efficient in the internalization leading to a higher presence of surface CTLA4 [24]. This corroborates previous results demonstrating that the cytoplasmic tail of trout fails to trigger the phosphorylation of ERK and production of IL-2 upon anti-CD28 cross-linkage and anti-CD3 treatment, suggesting different signalling pathways in fish [19]. This YXXF motif is found in salmonid and cyprinid species while others such as European sea bass and amberjack (*Seriola dumerili*) shows an YXXD motif. Therefore, further studies are needed to understand the molecular mechanisms behind the CD28 and CTL4 signalling in fish and whether and how these coreceptors counterbalance the fish T cell status.

In humans, CD28 and CTLA4 are mainly identified in lymphoid tissues with highest expression in the thymus, and concretely in $CD4^+$ T cells, followed by $CD8^+$. Our data also found that the highest transcription is in the sea bass thymus and/or spleen, coinciding with high presence of CD4 and CD8 cells, in line with previous observations in fish [14,17–20]. Interestingly, sea bass liver shows very high transcription of both *cd28* and *ctla4,* which is supported to the medium expression levels observed for sea bass *tcr*, *cd8* and *cd4* genes previously observed in this tissue [33]. Strikingly, the liver of resting pufferfish showed the highest *cd28* transcription [21], where this was also related to liver inflammatory response after toxic-induced injury. Strikingly, tilapia liver also showed quite high transcription levels of *fox3p* [34], the marker for Treg cells, in which the expression of CD28 and CTLA4 is highest in humans. Further evaluation

of T cells in fish typical lymphoid tissues, but also in others such as liver, is necessary to have a wider perspective of fish immunity and their regulation.

CD28 is a crucial player for immunological synapse organization, leading to the increase of TCR signalling events necessary for efficient cytokine production, cell cycle progression, survival, regulation of metabolism and T cell responses [35]. It has been demonstrated that, under low levels of costimulation for brief periods, T cells may preferentially engage B7 receptors by the high affinity receptor, CTLA-4, and become anergic [36]. Thus, upon maintained stimulation, the CD28-CTLA4 balance is moved towards the CTLA4 functions in an effort to avoid excess of immune response, including inflammation, favouring tolerance and reducing autoimmunity. Thus, we evaluated the regulation of European sea bass *cd28* and *ctla4* transcripts *in vitro* and *in vivo*. Regarding the B cell mitogen LPS, it failed to significantly alter the sea bass *cd28* and *ctla4* transcripts, in a similar way to that observed for human lymphocytes [37]. Sea bass HKLs showed increased transcription of both genes upon incubation with the T mitogens ConA and PHA, which was correlated to the expression of both $CD4^+$ and $CD8^+$ T cell markers. Very little is known at this respect in fish. Tilapia *cd28* gene expression in HKLs was decreased or not affected upon incubation with inactivated bacteria [14], similar to our data using LPS, inactivated bacteria (data not shown) or NNV (it does not replicate in HKLs). Strikingly, in trout pronephric leucocytes a decreasing CD28:CTLA4 ratio was observed during the PHA time-course stimulation indicating that leucocyte activation moved the balance to the CTLA4 response [19], which is in agreement with the observed time-dependent increase of *fox3p* transcription upon PHA stimulation in tilapia [34] and of the IL-17 transcripts in sea bass [38]. These data strongly suggest that PHA preferentially stimulates the fish Treg and Th17 lymphocyte populations, which are the main producers of CTLA4 and CD28 in humans. If we calculate the sea bass *cd28*:*ctla4* ratio, this is 2.5 in control HKLs but sharply drops to 0.13, 0.72, 0.24 and 0.54 in LPS, ConA, PHA or NNV treated HKLs, respectively, suggesting a T cell immunosuppression to avoid an excessive immune response. However, this hypothesis is controversial since fish T cells, in general, and sea bass T and $CD45^+$ lymphocytes in particular, proliferate upon ConA or PHA stimulation [23]. All these suggest an important regulatory role for fish CD28 and CTLA4 in the T cell biology, but other players might also be relevant, what merits further and deeper characterization before we can get a consensus mode of action in lower vertebrates.

Under viral infection, T cells have a pivotal role due to the killing capacity of the $CD8^+$ CTLs and the regulatory roles of $CD4^+$ lymphocytes. In humans, CD28 is up-regulated during initial T cell activation events but once it binds its ligand on APCs, it is rapidly down-regulated [4]. Contrary, CTLA4 is expressed at low levels within T cells, but it is rapidly up-regulated once CD28-B7 occurred. Although the role of CD28 and CTLA4 against virus is still controversial in humans almost nothing is known in fish. Thus, we investigated the *cd28* and *ctla4* transcription in sea bass infected with a lethal dose of NNV. In the brain, the main target for NNV replication and pathological alterations, we observed a clear tendency in which the maximum expression of *cd28* is at 1 day of infection and decreased afterwards, concomitant with increasing *ctla4* transcription, suggesting a shift from T cell proliferation to inhibitory microenvironment. However, an increase of $CD8^+$ cells is evidenced in the brain as seen herein by gene expression but also by immunohistochemistry (unpublished data) pointing to the regulation of local CTLs. In fact, sea bass *cd28* transcription decreases with the NNV infection while $CD8^+$ cells increase, which is in agreement with the evidences demonstrating that human memory $CD8^+CD28^-$ T cells are increased under chronic infections with several viruses [39]. In the head-kidney, a similar trend to the brain was observed upon NNV infection, favouring an inhibitory

environment. Interestingly, these data are supported by the decreased transcription of sea bass *cd28* in HKLs under the cell-mediated cytotoxic activity against allogenic tumour cells, mock- or NNV-infected [40]. These data are also in agreement with those reporting non-altered *cd28* and up-regulation of *ctla4* transcription in rainbow trout upon viral haemorrhagic septicaemia virus (VHSV) infection [19]. However, NNV infection resulted in increased transcription of both *cd28* and *ctla4* transcription in the head-kidney of groupers [22]. All this information is partly parallel to the general notion that human under chronic viral infections results in decreased CD28 and increased CTLA4 [39] though differences in tissue levels, timing and virus pathogenicity are of vital importance. NNV infection in sea bass is characterized by an inflammatory response since it has been proposed to be responsible for the intense degeneration, mainly vacuolation, observed in brain, retina and spinal cord of affected animals [33,41,42]. The up-regulation of pro-inflammatory cytokines and chemokines is particularly relevant in the context of inflammatory diseases. In particular, the activation of Th17 cells regulate inflammatory gene expression of IL-17 [43,44], and up-regulation of IL17C1 and IL17D coding genes in the brain of NNV-infected sea bass has been observed as a response to control the inflammatory process in the brain [38]. These results may suggest that CD28 may modulate the metabolic processes, which regulate specific pro-inflammatory T cell responses and the amplification of Th17 cells in inflammatory diseases in fish as has been previously reported in mammals [45]. After exposure to inflammatory stimuli, B7-1/B7-2 expression on APCs is up-regulated, and B7-CD28 signals promote T cell activation in concert with signals through the TCR. Later, following induction of CTLA-4, B7-CTLA4 interactions down-regulate T cell responses [1] to control tissue damage. Thus, CTLA4 might down-regulate T-cell activation to maintain peripheral tolerance and display important and complex roles in the control of immune homeostasis in fish. The understanding of T-cell activation mechanisms in fish is essential to develop strategies to fight against pathogenic infections in aquaculture.

In conclusion, our *in silico* results validate the existence of CD28 and CTLA4 costimulatory receptors in the European sea bass, which are constitutively expressed, mainly in immune relevant organs and liver, and their transcription is increased by stimulation with the T cell mitogens ConA and PHA. In addition, infection with the lethal NNV tends to decrease the transcription of *cd28* as the infection progresses, concomitant with an increment trend for *ctla4* gene. This might result first in the T cell stimulation followed by an inhibitory state to control and limit the immune response and homeostasis in fish upon NNV infection. Our results suggest that, as occurred in mammals, these two costimulatory molecules have an important role in T cell activation during pathogen infections, which is essential in fish immune response.

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