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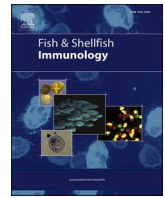
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Full length article



Molecular characterization of the cytotoxic and regulatory T cell coreceptor (CRTAM), and its ligand CADM1, in the European seabass and gilthead seabream

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ABSTRACT

T cell activation is a multifaceted process that depends on the activation of the T cell receptor (TCR). However, other coreceptors are also strictly necessary to provide co-signals and modulate the immune response. However, to date, most of these coreceptors are unknown in fish or their information is very limited. Therefore, in this work, we have identified the cytotoxic and regulatory T cell molecule, CRTAM, and its ligand, the cell adhesion molecule 1, CADM1, in European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*); and evaluated their transcriptional levels. Both putative proteins showed the canonical architecture observed in mammals, where CRTAM exhibited two immunoglobulin domains and CADM1, both the a and b forms, exhibited three of these domains. In addition, phylogeny and synteny analyses showed their conservation throughout vertebrate evolution. We found constitutive expression of all three genes, with *crtam* and *cadm1a* being predominant in immune tissues such as spleen, thymus and head-kidney (HK), while *cadm1b* expression was more limited to the brain. *In vitro*, only the T cell mitogen phytohemagglutinin (PHA) up-regulated the transcription of *crtam* and *cadm1a* in HK leucocytes. Nodavirus (NNV) infection elicited an up-regulation of *crtam* and *cadm1a* in brain and HK, appearing earlier in seabream than in seabass, which could explain the resistance of seabream to the development of nodavirus disease. In addition, they are up-regulated during the innate cell-mediated cytotoxic response in seabream but not in seabass. Altogether, our data seem to indicate that CRTAM is more related to the innate cytotoxicity in seabream and more in the specific and T cell-mediated cytotoxicity in seabass. Our results highlight the importance of CRTAM and CADM1 as important molecules in the activation of T lymphocytes in seabass and seabream, but further studies are needed.

1. Introduction

The presence of an adaptative immune system is unique to vertebrates, first appearing in jawed fish almost 500 million years ago, suggesting a clear evolutionary advantage due to its high specificity and immunological memory capacity against numerous pathogens. Among its components, T lymphocytes are one of the most important, with two main populations: cytotoxic (CTL) and helper (Th) T lymphocytes, the latter with different subpopulations. Participation of T lymphocytes in the mammalian immune response requires their activation based on the binding of the T cell receptor (TCR) with the antigen-presenting cell (APC) through a pathogen-derived peptide bound to its major

histocompatibility complex (MHC) proteins. However, it is also necessary the participation of several coreceptors on the T cell membrane for proper activation, regulation and cessation of activity, with CD8 and CD4 being the classical coreceptors of CTL and Th populations, respectively [1]. In mammals, numerous coreceptors that induce the activation or inhibition of T cells have been characterized [2]. CRTAM (cytotoxic and regulatory T cell molecule or Class-I MHC-restricted T Cell-associated molecule) is an important coreceptor belonging to the immunoglobulin (Ig) superfamily due to the existence of two, V- and C1-type, Ig-like domains in the extracellular region of the protein, whose expression is mostly restricted to MHC-class I-activated cells such as NK (natural killer) cells and CD8⁺ T lymphocytes [3], but also in cytotoxic

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CD4⁺ T lymphocytes [4]. CRTAM is highly expressed in tissues with strong immune function such as spleen, thymus, intestine or peripheral blood leukocytes as well as in Purkinje cells from the cerebellum [5]. Immune function of CRTAM is triggered by its binding to the cell adhesion molecule 1 (CADM1 or NECL2) receptor in the target cell surface [6,7]. This protein belongs to the nectin-like protein (Nect) family and is composed by one V-type and two C2-type Ig-like extracellular domains, whereas the cytoplasmic region contains a C-terminus type II PDZ-binding domain and a juxtamembrane protein 4.1 binding motif [8], and are widely distributed along tissues, having high expression in the nervous system, testis, liver or skin [9]. CRTAM/CADM1 complex has been shown to promote NK cell cytotoxicity and production of interferon (IFN)- γ *in vitro*, NK rejection of NECL2-expressing tumour cells or release of interleukin (IL)-22 by cytotoxic lymphocytes, promoting innate immunity and tumour suppression [6,7]. In addition, CRTAM/CADM1 interaction controls the retention of CD8⁺ T cells in lymph nodes, CD4⁺CD8⁺ T cells in the intestine, and even CD4⁺ Th17 T cells during parasitic infections [10]. Furthermore, CRTAM^{-/-} mice resulted in decreased protection against viral infections [11]. All these data demonstrate the importance of the CRTAM/CADM1 in the development and functional activity of the cell-mediated cytotoxic (CMC) response against tumours and viral infections though more studies are still needed to fully understand their roles in immunity and disease.

The presence and response of CD4⁺ and CD8⁺ T cells has been confirmed in several fish species showing similar function to mammalian T cells. Nevertheless, although the presence of fish CTLs, Th1, Th2, Th17 and regulatory T lymphocyte subpopulations has been postulated due to the expression pattern of cytokines they have been poorly characterized [12,13]. Regarding coreceptors, apart from their identification in genome and transcriptome projects, their knowledge is negligible compared to mammals. In relation to fish CRTAM and CADM1, little information is available. CRTAM was found to be up-regulated *in vitro* during the innate CMC response of head-kidney leucocytes (HKLs) from European seabass (*Dicentrarchus labrax*) [14] whilst 6 members of the CADM family, including CADM1, have been characterized in zebrafish (*Danio rerio*), observing the gene expression of all of them and presence of proteins in the nervous system during development and in the adult [15,16]. Therefore, it is necessary to further study CRTAM and CADM1 genes in fish to clarify their participation in immunity, and in the CMC response in particular.

Thus, the main objective of this study was to identify and characterize the regulation of CRTAM and CADM1 coding genes in European seabass and gilthead seabream (*Sparus aurata*), the two most important species within the Mediterranean aquaculture industry. The sequences were obtained from databases and their transcription evaluated in naïve organs, stimulated head-kidney leucocytes, innate CMC response, and nodavirus (NNV)-infected specimens *in vivo*. Involvement of the CRTAM/CADM1 in fish immunity, mainly related to the cell-mediated cytotoxic response and viral diseases are discussed.

2. Material and methods

2.1. Bioinformatics and genetic analysis

European seabass and gilthead seabream *crtam* sequences were identified in RNA-seq studies carried out in our laboratory and further confirmed within the European seabass genome project (<http://seabass.mpipz.mpg.de/>) and assembled reference genome of gilthead seabream (GCA_900880675.1, NCBI Genome), while seabass and seabream *cadm1a* and *cadm1b* were obtained from the Ensembl database (<https://www.ensembl.org/index.html>). Exon-intron structure was generated by the Exon-Intron Graphic Maker (<http://wormweb.org/exonintron>). Genomic synteny was performed in Genomicus 101.01. Predicted proteins were used for multiple sequence alignments using the CLUSTALW program and phylogenetic analysis were performed with MEGA

software version 6 [17]. Protein domain architecture was searched by InterPro (<https://www.ebi.ac.uk/interpro/>). 3D modelling of proteins structure was conducted using AlphaFold 2 [18].

2.2. Animals

European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) specimens were bred and maintained at the aquaria of the Oceanographic Centre of Murcia, Spanish Institute of Oceanography (COM-IEO) in Mazarrón (Spain) at 16.2 \pm 1.6 °C water temperature, flow-through circuit, suitable aeration, filtration systems and natural photoperiod. The environmental parameters, mortality and food intake, were recorded daily. Specimens of seabass and seabream of 300–500 g and 100–200 g body weight (bw), respectively were used for the study in naïve conditions and *in vitro* stimulations (see below). Seabass and seabream specimens of 305 \pm 77 g and 125 \pm 25 g bw, respectively were transported to the University of Murcia (Spain) aquaria to perform the *in vivo* infection with NNV (see below).

The handling of the specimens was always performed in accordance with the Guidelines of the European Union Council (2010/63/UE) and the Bioethical Committees of the IEO (Permit Number 2010/02) and of the University of Murcia (Permit Number A13150104). In all cases, fish were sacrificed with 40 μ L/L of clove oil, completely bled and rapidly decapitated before sampling.

2.3. *In vitro* and *in vivo* exposures

We have evaluated the mRNA levels and their regulation in both European seabass and gilthead seabream samples previously obtained and tested [14,19–21], considering the 3 Rs principle.

Constitutive gene expression in naïve conditions was analysed in brain, liver, gill, gonad, skin, gut, head-kidney, spleen, thymus, and blood from 3 healthy and independent fish specimens. Samples were obtained and immediately frozen in TRIzol® Reagent (Life Technologies) and kept at – 80 °C.

For the *in vitro* experiments, head-kidney leucocytes (HKLs) from 5 fish were individually isolated and 10⁷ HKLs/mL were incubated in 48-well microtiter plates (Nunc) at 22 °C during 24 h with: culture L-15 medium (control treatment), 5 μ g/mL concanavalin A (ConA; Sigma-Aldrich), 5 μ g/mL lipopolysaccharide (LPS; Sigma-Aldrich), 10 μ g/mL phytohemagglutinin (PHA; Sigma-Aldrich) or 10⁶ tissue culture infective dose (TCID)₅₀ NNV/mL [20]. Regarding the CMC assays, HKLs (n = 3) were incubated for 4 h with the seabream SAF-1 cell line (ECACC 00122301), mock- or NNV-infected during 24 h, as target cells [14,21]. After exposure, HKLs were washed with phosphate buffer saline (PBS) and stored in TRIzol® Reagent at – 80 °C.

For the *in vivo* study, fish received a single intramuscular injection of 100 μ L with culture medium (mock-infection) or containing 10⁶ TCID₅₀ of NNV/fish (strain 411/96, genotype RGNV). One, 7 and 15 days post-infection (dpi) the brain and HK of each fish (n = 4–6 fish) were sampled and stored in TRIzol® Reagent at – 80 °C [19]. Seabass mortality reached a 55% while all the seabream specimens survived the infection.

2.4. Gene expression

Total RNA was isolated from TRIzol Reagent and cDNA synthesized by the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) according to manufacturer's instructions. Real-time PCR was performed using an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C. All the gene expressions were corrected and normalized by the geometric mean of the elongation factor 1 alpha (*ef1a*) and ribosomal 18S (*rps18*) expression as house-keeping genes following the 2^{- Δ CT} method: 2^(CT target – CT house-keeping) [22]. In the *in vivo* experiment, after normalization,

gene expression was normalized by the expression in the control group following the $2^{-\Delta\Delta CT}$ method. 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. Primers are shown in Table 1. Negative controls with no sample were always included in the reactions.

2.5. Statistical analysis

Figures are presented as mean \pm SEM of the data. Statistical differences between groups were analysed by one-way analysis of variance (ANOVA; $p \leq 0.05$), followed by the Tukey's comparison of means, when applicable. Data and figures are processed by either SPSS or GraphPad software.

3. Results

3.1. Genomic organization

Within the genome databases, we found complete sequences for *crtam*, as well as two *cadm1* forms, a and b, in both fish species. A summary of the genomic, mRNA and protein molecules is presented in Table 2. Analysis of the genomic structure predicts the presence of 11 exons in seabass and seabream for *crtam*, 12 in seabass and 9 in seabream for *camd1a* and 11 in both species for *cadm1b* (Fig. 1a, Table 2). Even the genomic structure might show some variations between the two fish species, their synteny present a good conservation of organization in the genome from *Actinopterygii* ancestor (Fig. 1b). Curiously, *cadm1a* is preceded by *ncam1a* whilst *camd1b* is by *ncam1b* suggesting that these genes duplicated and evolved together. mRNA transcripts resulting from these genes contained a complete open reading frame (ORF), except the partial *cadm1a* of seabass, with little variations between the two species though the 3' and 5' UTRs were variable in length (Table 2).

3.2. CRTAM and CADM1 belong to the Ig superfamily of proteins

Predicted putative CRTAM proteins have a size of 485 and 477 amino acids (aa) in seabass and seabream, respectively (Table 2). Domain architecture of CRTAM protein identifies a signal peptide, extracellular, with an Ig domain, transmembrane and intracellular regions (Fig. 2A). Their alignment with the human CRTAM shows low similarities of 16 and 19% for seabass and seabream, respectively (Table 2, Fig. 2A); being the greatest differences observed in the extracellular region, apart from the Ig domain, and in the cytoplasmic tail. Strikingly, while human

CRTAM contains the two canonical Ig domains in the extracellular region, the second one is not identified by InterPro or PROSITE in seabass or seabream CRTAM. However, Cys involved in the Ig formation are present at positions 40, 105, 148 and 203 and clearly well conserved (Fig. 2A). In fact, the 3D modelling of seabass and seabream CRTAM proteins identifies the two Ig domains (Fig. 3A) with a high degree of confidence, as in humans, probably supported by the presence and conservation of key residues such as the Cys, among others (Fig. 2A).

Regarding CADM1 proteins, CADM1a have a full size of 417 aa in seabass and a partial of 448 aa in seabream, respectively, while CADM1b is of 437 and 420 aa for seabass and seabream, respectively (Table 2). They contain a signal peptide, except for the partial seabass CADM1a, whereas both contain an extracellular region, with three Ig domains, transmembrane and cytoplasmic regions (Fig. 2B), as in humans, with similarities ranging from 58 to 64% (Table 2). Canonical Cys residues needed for three Ig domains, at positions 64, 124, 166, 221, 268 and 309 are all present and conserved. In these cases, 3D modelling of seabass and seabream CADM1a and CADM1b showed the three Ig domains with a high degree of confidence (Fig. 3B and C).

Phylogenetic analysis performed with predicted CRTAM, CADM1a and CADM1b proteins confirmed the good conservation of each protein (Fig. 4). In all cases, mammalian clusters were separated with respect to the fish counterparts. Also, seabass and seabream proteins were well related, indicating a high conservation between both species, and supporting gene and protein relatedness identified and described above, probably due to their classification as Ig superfamily proteins.

3.3. *Crtam* and *cadm1* distribution in naïve organs

In European seabass, the three genes have constitutive basal expression in all organs except *cadm1a* in the blood and *cadm1b* in the liver (Fig. 5). In the case of gilthead seabream, transcription levels are, in general, lower than in seabass, being *cadm1b* undetected in the blood. *crtam* shows the highest transcription in the seabass lymphoid organs, thymus and spleen, followed by brain and HK whilst in seabream the highest organ was gills followed by spleen, thymus and HK (Fig. 5). By its part, *cadm1a* presents the highest transcription in the seabass liver, brain, thymus and spleen but in seabream is in the brain, followed by gills, gonad and thymus. Finally, *cadm1b* mRNA is highest in the brain, followed by the skin, for both fish species.

3.4. T cell mitogens up-regulate the seabass *crtam* transcription in HKLs

We also investigated if the expression of the three genes was

Table 1
Primers used for real-time PCR analysis.

Gene name	Gene abbreviation	Specie	Accession number	Primer sequence (5' → 3')
Elongation factor 1 alpha Ribosomal protein S18	<i>ef1a</i> <i>rps18</i>	Seabass	AJ866727	CGTTGGCTTCAACATCAAGA GAAGTTGTCTGCTCCCTTGG
		Seabream	AF184170	CITCAACGCTCAGGTCATCAT GCACAGCGAAACGACCAAGGGGA
		Seabass	AY831388	TTCCITTTGATGCTCTTAACG TCTGATAAATGCACGGCATCC
		Seabream	AM490061	AGGGTGTGGCAGACGTTAC CGCTCAACCTCCTCATCAGT
Cytotoxic and regulatory T cell molecule	<i>crtam</i>	Seabass	DLAgn_00038,630	CCCACTGGATTTCTCTGAA ATTCTCTGCTGGTTGAGT
		Seabream	XP_030,294,706	AAGGACAAAGCGGAAGAGGG TCTGTTGCTGGCATCACAGT
Cell adhesion molecule 1A	<i>cadm1a</i>	Seabass	ENSDLAG00005007417	AATGTGTCCGGTGGTGGAG AAAGCGAGCGTCTTTCAAT
		Seabream	ENSSAUG00010012903	GATAAAGAGCTTCCAGGAAAG GTTCTCTCCTTCCCTGGTC
Cell adhesion molecule 1B	<i>cadm1b</i>	Seabass	ENSDLAG00005034349	TGTCACCAAAGAGGATGATG CTCAGCCAGTTGATCTGATG
		Seabream	ENSSAUG00010016572	GAACTGACAGGTGATGCAG TTCAGGATACGTCAACA

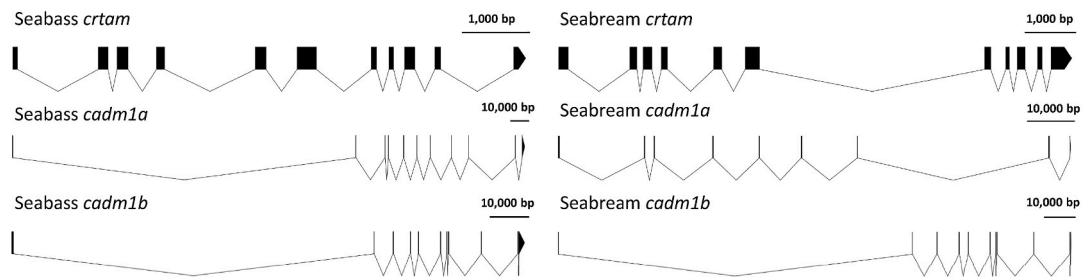
Table 2
Summary of the main genomic, mRNA and protein properties.

Molecules	Fish species	Genomic			mRNA		Protein		
		Acc. Number	Length (bases)	Exon/Intron	Length (bases)	ORF (bases)	Length (Aa)	MW (kDa)	% identity/similarity ^a
CRTAM	Seabass	DLAgN_00038,630	7719	11/10	1689	1458	485	53.54	16/80
	Seabream	XP_030,294,706	9700	11/10	1640	1434	477	51.35	19/80
CADM1a	Seabass	ENSDLAG00005007417	272,180	12/11	2535	1254	417 ^{&}	45.98	64/97
	Seabream	ENSSAUG00010012903	109,270	9/8	3697	1347	448	49.96	60/96
CADM1b	Seabass	ENSDLAG00005034349	130,300	11/10	2953	1314	437	47.83	58/98
	Seabream	ENSSAUG00010016572	163,500	11/10	1748	1263	420	45.87	60/100

CRTAM, Cytotoxic and regulatory T cell molecule; CADM, Cell adhesion molecule; ORF, open reading frame; MW, molecular weight.

^a Compared to their human counterparts; &, incomplete.

A. Exon/intron organization



B. Synteny

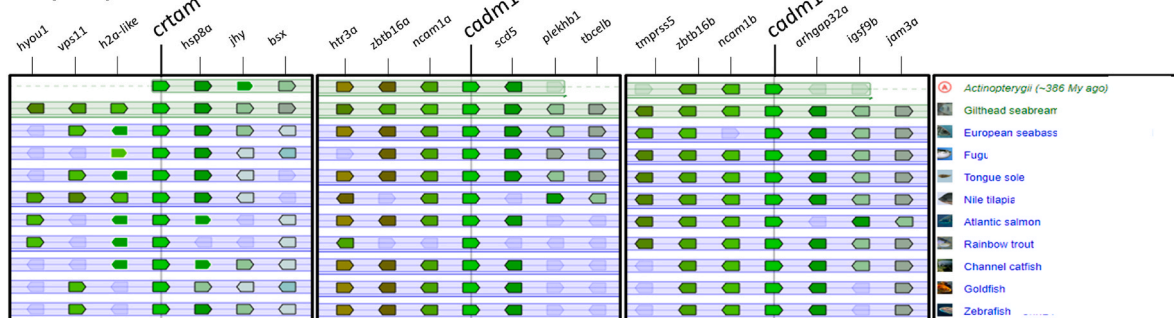


Fig. 1. Genomic organization of the European seabass and gilthead seabream *crtam*, *cadm1a* and *cadm1b* genes. **A.** Exon-intron organization. **B.** Synteny of genes in teleost fish from *Actinopterygii* ancestor. Pentagons represent the genes and the direction of the complementary strand. *crtam*, cytotoxic and regulatory T cell molecule; *cadm*, cell adhesion molecule.

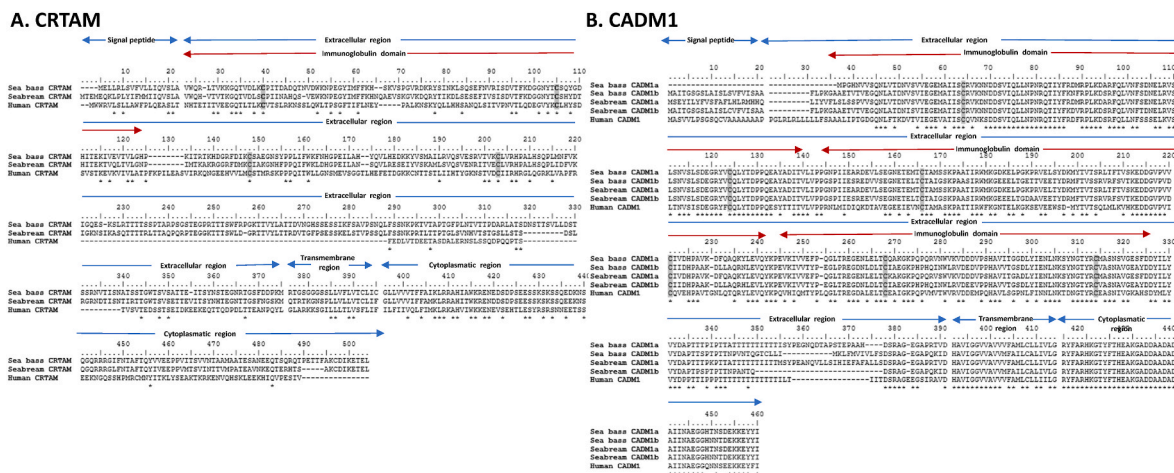


Fig. 2. Alignment of the predicted putative European seabass and gilthead seabream CRTAM (A) and CADM1a and CADM1b (B) proteins with their human counterparts using ClustalW. Numbering was according to the seabass mature protein. Gaps are introduced to strength the alignment. Asterisks indicated conserved residues. Important residues are in bold and shadowed. Different regions in the mature protein are denoted by red/blue arrows.

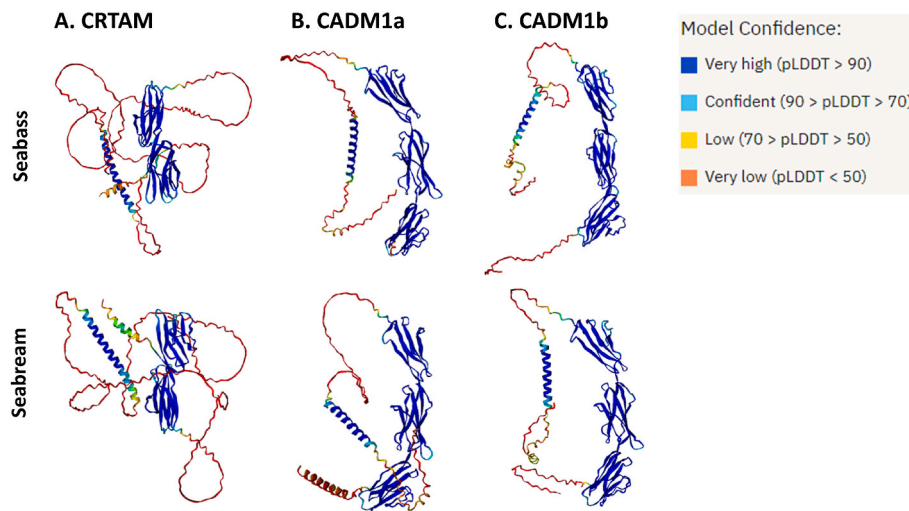


Fig. 3. 3D prediction of European seabass and gilthead seabream putative CRTAM (A), CADM1a (B) and CADM1b (C) proteins from its amino acid sequence using AlphaFold 2. Per-residue confidence score (pLDDT) designate the estimation of confidence on a scale from 0 to 100.

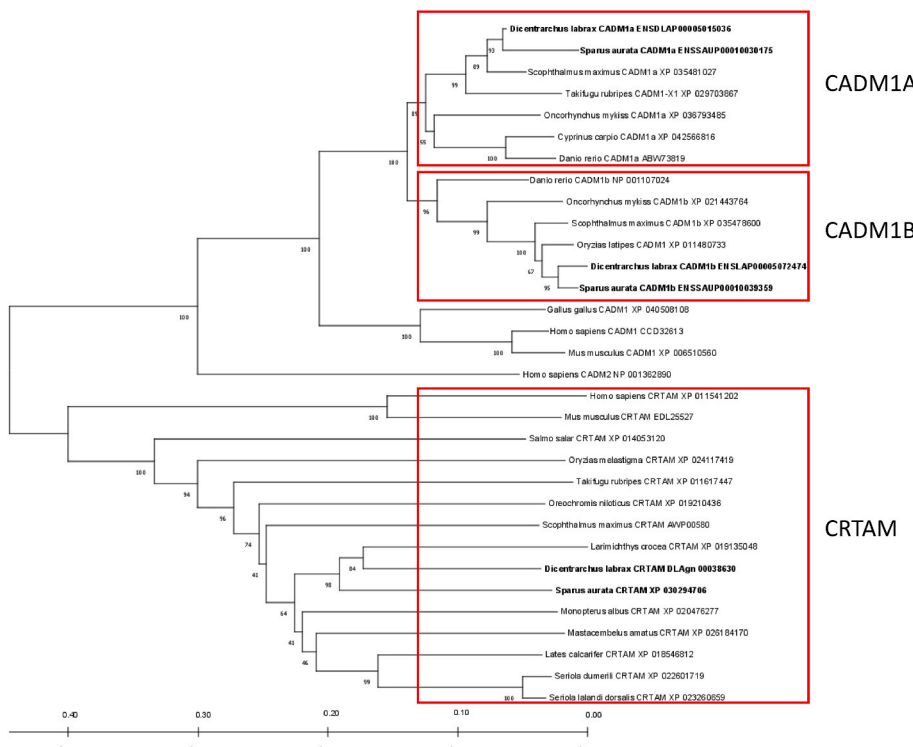


Fig. 4. Phylogenetic tree including the European seabass and gilthead seabream CRTAM, CADM1a and CADM1b putative proteins was constructed using the Neighbour-Joining method, where genetic distances were calculated based on protein differences (p-distance) with pairwise deletion. The number of each node represent the percentage of bootstrapping after 1000 replications. GenBank accession numbers are shown. Seabass and seabream putative proteins are in bold. Fish clades are indicated in red squares.

regulated in HKs after incubation with different *stimuli*. The results show that seabass *crtam* is significantly up-regulated by the T cell mitogens PHA and ConA (Fig. 6A) whereas for seabream the levels were lowered by LPS when compared to untreated control HKs. Interestingly, seabass *cadm1a* was also up-regulated by PHA treatment (Fig. 6B) whilst *cadm1b* was not significantly altered (Fig. 6C).

3.5. Nodavirus infection elicits *crtam* in the susceptible European seabass suggesting activation of T cells

We then evaluated the gene expression in seabass and seabream upon infection with nodavirus, which results pathogenic and lethal for seabass but not for seabream [19]. Regarding seabass *crtam* (Fig. 7A), we found a significant up-regulation at 15 dpi, in both the HK and brain,

compared to control or mock-infected specimens, whilst it was decreased in the brain at 1 dpi (Fig. 7A). However, seabream *crtam* levels were decreased in HK at 1 dpi but increased in brain at the same time point. Concerning *cadm1a*, in seabass it presented a down-regulation at 1 dpi in HK (Fig. 7B). In contrast, in seabream it suffered an up-regulation at 1 dpi in HK and brain but decreased at 4 dpi in brain. In the case of *cadm1b*, its expression was not modulated by NNV infection (Fig. 7C).

3.6. CRTAM/CADM1a might be involved in the seabream innate cell-mediated cytotoxic response

Finally, we studied the level of *crtam*, *cadm1a* and *cadm1b* transcripts in HKs during the innate CMC response against the SAF-1 cell line as target cells, mock- or NNV-infected. The results revealed that *crtam*

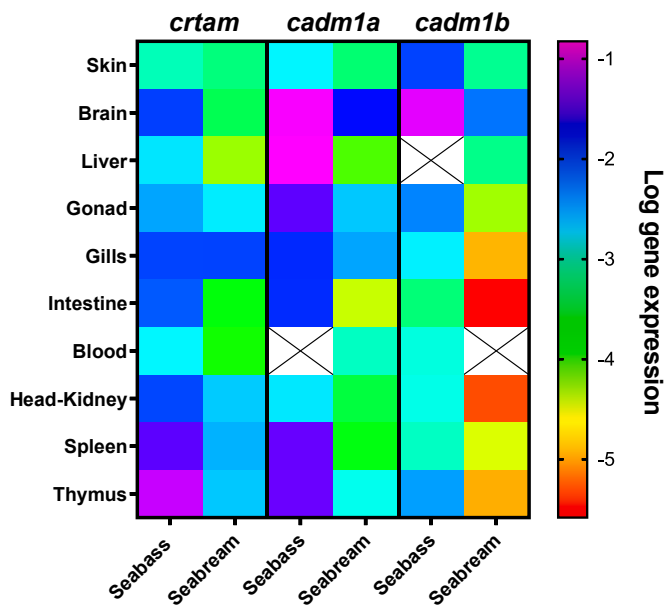


Fig. 5. Heatmap presenting the transcription of *crtam*, *cadm1a* and *cadm1b* in different organs in naïve European seabass and gilthead seabream specimens using real-time PCR. Data are log-transformed and the mean (n = 3) target gene expression relative to the expression of endogenous control *ef1a* and *rps18* genes.

(Fig. 8A) and *cadm1a* (Fig. 8B) were increased during the CMC response only in seabream, but not in seabass HKLs. Levels of *crtam* mRNA experienced a significant increase during CMC against both mock- or NNV-infected SAF-1 cells, whereas *cadm1a* levels only increased against NNV-infected SAF-1 targets. For *cadm1b*, the expression was undetected

for both species.

4. Discussion

For the adaptive immunity, the presence and regulation of T cell coreceptors is crucial for proper activation and functioning of T lymphocytes. In mammals, there are numerous studies on these coreceptors, but in fish their characterization is in its infancy. In order to shed additional knowledge on this subject, we searched CRTAM coreceptor and its ligand, CADM1, in the European seabass and gilthead seabream. So far, there is only a single description of CRTAM from an European seabass RNAseq study as well as gene/protein studies of zebrafish CADMs, including CADM1 [14–16,23]. However, to our knowledge, this is the first time that CRTAM-CADM1 have been analysed and characterized together in teleost fish.

First, we performed an *in silico* analysis of CRTAM and CADM1. We found one *crtam* and two *cadm1* genes, named *cadm1a* and *cadm1b*, in European seabass and gilthead seabream. Bioinformatic analysis reflects that genomic organization of *crtam* and *cadm1* genes is similar and well conserved. Interestingly, *cadm1* synteny analysis seems to support the idea that both versions arose from a genome duplication, a process which has occurred often in fish evolution [24], since the same situation is found for *zbtb16* and *ncam1* genes. At protein level, it is noteworthy that domain predictions for CRTAM reveals only one Ig domain in its extracellular region, both in seabass and seabream. However, their alignment with human CRTAM show a perfect conservation of the two cysteines involved in the second Ig domain, predicted in human CRTAM but not in seabass or seabream. Going further into this, 3D structure prediction with the AlphaFold 2 tool demonstrated that CRTAM of seabass and seabream also present the second Ig domain with a high degree of confidence. Further experimental studies are needed to confirm the existence of the second Ig domain in seabass and seabream CRTAM. On the other hand, for CADM1a and CADM1b proteins,

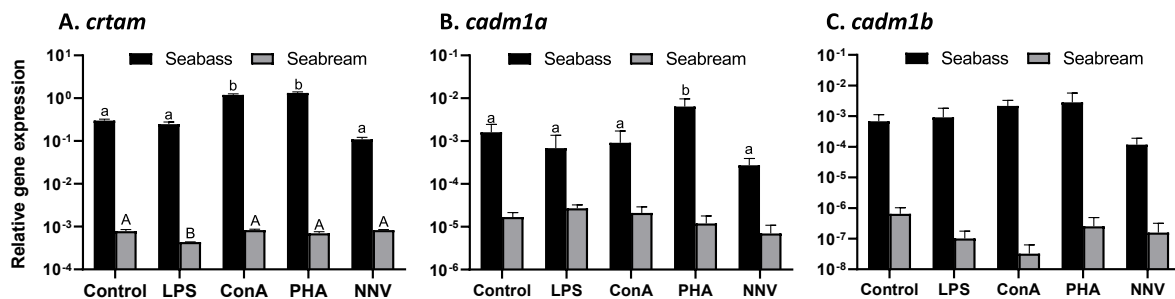


Fig. 6. Transcription of *crtam* (A), *cadm1a* (B) and *cadm1b* (C) in European seabass and gilthead seabream head-kidney leucocytes (HKLs) stimulated for 24 h. Data are presented as means ± SEM (n = 5) relative to the expression of the endogenous controls. Different letters indicate differences between groups according to ANOVA and Tukey’s post-hoc tests ($p < 0.05$). LPS, lipopolysaccharide; ConA, concanavalin A; PHA, phytohemagglutinin; NNV, nodavirus; ND, not detected.

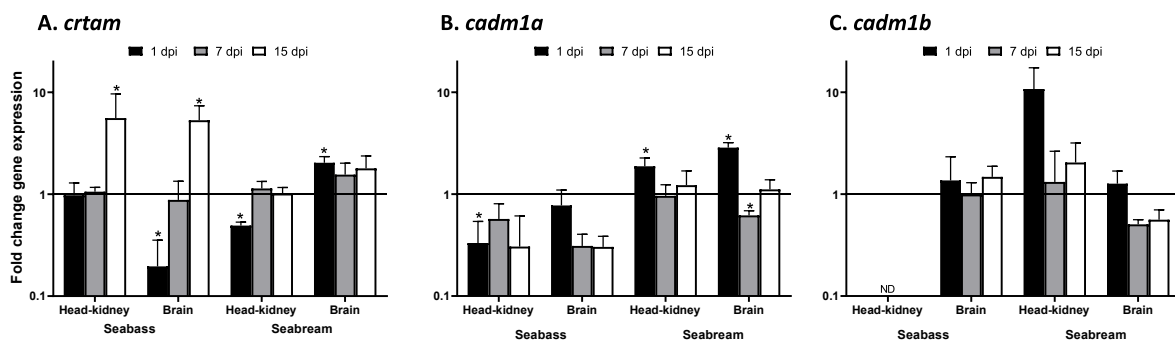


Fig. 7. Transcription of *crtam* (A), *cadm1a* (B) and *cadm1b* (C) in European seabass and gilthead seabream brain and head-kidney tissues upon infection with 10^6 TCID₅₀ of NNV/fish after 1, 7 and 15 days of infection (dpi). Data are presented as fold change in gene expression ± SEM (n = 4–6) in infected groups compared with the controls. Asterisks denote significant differences between control and NNV-infected fish at each infection time according to the *t*-Student test ($p < 0.05$).

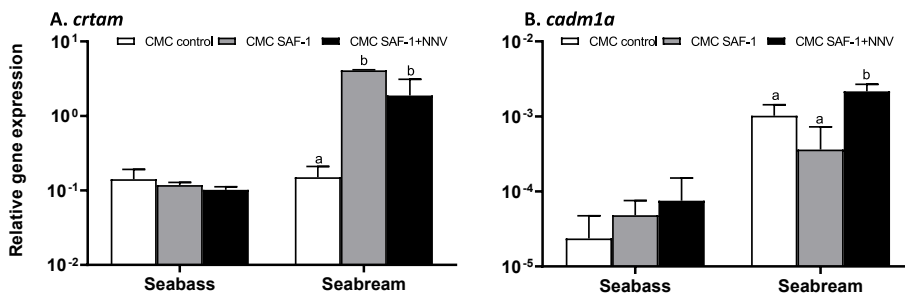


Fig. 8. Expression of *crtam* (A) and *cadm1a* (B) genes in European seabass and gilthead seabream isolated head-kidney leucocytes after the cell-mediated cytotoxic (CMC) response against mock- (CMC SAF-1) and NNV-infected (CMC SAF-1+NNV) SAF-1 cells for 4 h. Samples at time 0 were used as controls (CMC Control). Data are presented as mean \pm SEM ($n = 3$) relative to the expression of the endogenous controls. Different letters indicate differences between groups according to ANOVA and Tukey's post-hoc tests ($p < 0.05$).

alignment, domain and 3D predictions confirm the existence of the three Ig domains in fish and the conservation with the human CADM1. Phylogenetic analysis relates CRTAM and CADM1 proteins due to the existence of shared and conserved Ig domains and also support the conservation between fish and human orthologues.

In mammals, the highest expression of *crtam* is found in the brain followed by the immune tissues. Concretely, it is mainly detected in activated CD8⁺ T cells, although expression has also been observed in NKT, NK and a small proportion of CD4⁺ T cells [3,4]. At tissue level, the highest expression has been reported in immune organs such as thymus and spleen, and in other tissues where lymphocytes usually reside, such as brain, testis or intestine [5]. Regarding seabass, the pattern of expression is similar as in mammals, reaching highest levels of mRNA in thymus, spleen, and head-kidney due to the major presence of lymphocytes. In addition, *crtam* has been found to be highly expressed in the mammalian cerebellum, where it might contribute to neural interactions [5] since inhibition of CRTAM in the cerebellum causes a decrease in cell migration, affecting its development and structure [25]. It is probable that CRTAM in seabass brain has a similar function as in mammals, allowing a better synaptic function and stabilizing brain tissue development. On the other hand, in gilthead seabream, we found expression in immune organs such as head-kidney, spleen and thymus but it is in the gills where the *crtam* is mostly expressed, probably due to the large presence of immune cells as first line of defense to prevent the entry of pathogens present in the water [26]. Regarding *cadm1* gene, in mammals, its expression is found along various tissues due to the involvement in numerous cell-cell junctions. In both seabass and seabream, *cadm1a* has the highest expression in the brain. This may be because CADM1 serves as a synaptic adhesion molecule that drives synaptic assembly, mainly located in the central nervous system [27,28]. In zebrafish, the participation of *cadm* genes has been studied showing an important role in the ontogeny of the central nervous system, neuronal differentiation and synaptogenesis [15] while CADM2a and CADM3 proteins are mainly immunodetected in cells of the central nervous tissue and bind to CADM1 and CADM4 receptors, respectively [16]. Also notable is the high expression in the gonads and gills in both fish species and liver of seabass, where implications in gametogenesis and cell junctions might be relevant [29]. Interestingly, seabass showed high levels of *cadm1a* in the spleen and thymus, correlating with high *crtam* transcription, suggesting their interaction and promoting the activation of CD8⁺ T lymphocytes. In fact, the presence of CADM1 in lymphoid tissues of mammals is due to the presence of CD8 α + dendritic cells, that can bind to CRTAM of lymphocytes present there, promoting their cytotoxic activity [6,30]. In fish, the existence of dendritic cells has been demonstrated [31,32], even the presence of a CD8 α + dendritic cells [33], so it could be possible that the presence of *cadm1a* mRNA in the lymphoid tissues of seabass is the result of the presence of these cell populations. Finally, *cadm1b* gene expression is more limited in seabass and seabream. Curiously, in both species, tissues with high expression are the skin and brain, which might suggest that this gene is more involved in the participation of cell-cell junctions of epithelial cells and less in the immune function. However, further studies are still needed to ascertain the tissue distribution and potential roles in fish physiology of

CRTAM-CADM molecules.

Regarding their regulation in HKLs, seabass *crtam* gene was up-regulated by the T cell mitogens ConA and PHA, suggesting that T lymphocytes are the major cells producing CRTAM. In fact, other coreceptors such as CD4, CD28 and CTLA4 are also activated by T mitogens, but not B mitogens [34]. Strikingly, PHA also up-regulated the *cadm1a* transcription. By contrast, seabream HKLs were not primed and even a low down-regulation by LPS in the *crtam* transcription was noted. Since this is the first study about CRTAM-CADM1 in fish we wanted to go further and evaluated their expression in these HKLs during a CMC response, concretely in an innate CMC response, which is mediated by non-specific cytotoxic (NCC) or NK-like cells. Thus, and very strikingly, we found that *crtam* and *cadm1* genes are not regulated during the innate CMC response of seabass HKLs but do in seabream leucocytes. Curiously, the innate CMC activity of seabass HKLs is much lower than that of seabream leucocytes [14]. Thus, and linked with the regulation by PHA, our data suggest that seabass T cells are the major producing cells for CRTAM and not the NCCs, whilst for seabream the opposite might be occurring. In viral infections, CMC response plays a crucial role in addressing and controlling the viral challenge through NK cells, CD8⁺ cytotoxic and CD4⁺ regulatory lymphocytes. In humans and mice, CRTAM is directly related to T lymphocyte retention and proliferation, production of perforin and granzyme B and viral-specific killing [4,11, 35]. However, to date, no study has assessed the role of CRTAM against viral infections in teleost fish. Thus, we investigated *crtam*, *cadm1a* and *cadm1b* transcription in seabass and seabream against NNV infection. In seabass, there is an initial decrease of *crtam* transcript level in the brain at 1 dpi, but whose expression increases during the course of infection till 15 dpi, showing the same trend as *granzyme A* [21] and *cd8a* [34]. These observations suggest that CMC response might be not operating efficiently in the brain at early infection times, since NNV has a clear tropism towards central nervous system causing rapid vacuolization and destruction, producing high mortality rates from the onset of infection. However, later production of CTLs and CMC response would help to control the virus in the brain and maintain brain functions in survivors, a hypothesis that merits further investigations. Interestingly, *crtam* transcripts also increased at 15 dpi in the head-kidney, which might explain the presence and maturation of T lymphocytes in this organ and their mobilization to the brain. On the other hand, seabream *crtam* levels increased in the brain, while decreased in head-kidney at 1 dpi. This would suggest an effective and rapid CMC response in the brain, which could contribute to the clearance of NNV, and a reason to the lack of histopathological lesions and mortalities [36]. This would suggest that seabream is able to mount an effective local innate CMC response at early infection times by NCC/NK-like cells while in seabass this CMC response is mainly achieved later and specific, involving T lymphocytes, as also hypothesized above. However, seabream *cadm1a* levels are increased in both tissues at the onset of infection. Although *cadm1a* might be involved in cell interactions and reorganization in the infected brain this could not be the case since *cadm1b*, more specialized for cell-cell junctions, is not regulated in any species and tissues. Therefore, we could speculate about that activation of *crtam* and *cadm1a* in the brain of NNV-infected seabream could lead to a more effective innate

CMC response. In fact, this is supported by the *in vitro* CMC response data in seabream against SAF-1 target cells, either mock- or NNV-infected. This CMC response is high and increased against NNV-infected target cells [14], and the *crtam* and *cadm1a* follows the same pattern and might explain the functional observations. However, in seabass, the innate CMC response was low and not increased against NNV-infected cells, and *crtam-cadm1a* seem to be not involved.

In conclusion, our study confirms, *in silico*, the presence of *crtam* gene, and two versions of *cadm1*, named *cadm1a* and *cadm1b*, in European seabass and gilthead seabream. They are constitutively expressed, mainly in lymphoid tissues and brain for *crtam* and *cadm1a*. Regulation of transcription suggest that European seabass CRTAM is mainly expressed in T lymphocytes, since it is increased by T mitogens and at later infection times, being probably involved in the specific CMC response. In the case of seabream, it seems to be more related to the NCC/NK-like cells since it is not increased by T mitogens and is concomitant with high innate CMC response. Our results suggest that, as occurs in mammals, CRTAM and CADM1a have an important role during the CMC response in health and upon nodavirus infection, while CADM1b has specialized in other functions related to cell junctions.

Author contributions

Methodology and investigation, M.Á.G.-Á., C.G.-F.; software and data curation, M.Á.G.-Á. and A.C.; conceptualization and supervision, A.C.; writing—original draft preparation, M.Á.G.-Á.; writing—review and editing, M.Á.G.-Á., C.G.-F., M.Á.E. and A.C.; project administration and funding acquisition, A.C. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Data will be made available on request.

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