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Expression of DC-SIGN-like C-Type Lectin Receptors in *Salmo salar*

Nicolás Ojeda, Carolina Salazar, Constanza Cárdenas, Sergio H. Marshall *

Instituto de Biologia, Pontificia Universidad Catolica de Valparaiso, Valparaiso, Chile

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ABSTRACT

C-Type Lectin Receptors (CTLR) are involved in the activation of innate and adaptative immune responses. Among these receptors, the Dendritic Cell-Specific ICAM-3-Grabbing nonintegrin (DC-SIGN/CD209) has become a hot topic due to its ability to bind and facilitate the infections processes of several pathogens. Although well characterized in mammals, little documentation exists about the receptor in salmonid fishes. Here, we report the sequence and expression analysis of eight DC-SIGN-like genes in *Salmo salar*. Each receptor displays structural similarities to DC-SIGN molecules described in mammals, including internalization motifs, a neck region with heptad repeats, and a Ca^{+2}-dependent carbohydrate recognition domain. The receptors are expressed in multiple tissues of fish, and fish cell lines, with differential expression upon infection with viral and bacterial pathogens. The identification of DC-SIGN-like receptors in *Salmo salar* provides new information regarding the structure of the immune system of salmon, potential markers for cell subsets, as well as insights into DC-SIGN conservation across species.

1. Introduction

The C-Type Lectin (CTL) superfamily includes a large number of members throughout the animal kingdom. Characterized by Ca^{+2}-dependent carbohydrate-binding, they are functionally involved in cell adhesion, cell communication, pathogen recognition and activation of immune responses, among others (Dambuza and Brown, 2015; Weis et al., 1998; Zelensky and Gready, 2005). This superfamily has been classified in 14 groups of proteins, based on their C-type Lectin Domain (CTLD) architecture and phylogeny (Drickamer and Fadden, 2002). Group II contains Asialoglycoprotein Receptors (ASGR) and Dendritic Cell (DC), Macrophage, Langerin, and Kupffer cells receptors. They are type II transmembrane proteins, containing a short cytoplasmatic tail and an extracellular neck region, which varies significantly among different members, which connects to the C-terminal CTLD.

CD209, also known as DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), and its homolog DC-SIGNL/CD209R, are members of the Group II CTL superfamily. DC-SIGN has been identified as an adhesion molecule, involved in the attachment of antigen-presenting cells (APC) to resting T cells, and the aggregation and migration of APCs, as well as inflammatory responses, concomitantly participating in innate and adaptative immunity in mammals (Geijtenbeek et al., 2000; Khoo et al., 2008; Rappocciolo et al., 2008, 2006). Similar to Toll-like receptors (TLRs), DC-SIGN also acts as a pattern recognition receptor (PRR), promoting phagocytosis in macrophages and DCs (Montoya et al., 2009; Serrano-Gómez et al., 2004). Intracellular signaling pathways can be activated indirectly via association with other receptors, or directly, through their own Immunoreceptor Tyrosine-based Activation Motif (ITAM, YxxL/I) (Hoving et al., 2014).

Even though they play an essential role in the defense against a broad range of pathogens, viral recognition by CTLRs can favor infection. Most notably, recognition of the Human Immunodeficiency Virus (HIV) by DC-SIGN in dendritic cells facilitates the viral infection of $CD4^{+}$ cells. CTLRs also aid in the infective process of other viruses, such as Influenza A, Cytomegalovirus, Dengue, Ebola, Hepatitis C, Coronavirus, West Nile, and Measles (Avota et al., 2013; Gillespie et al., 2016; Hillaire et al., 2013; Mesman et al., 2012). Moreover, there is also evidence that these receptors interact with bacterial pathogens and parasites (Appelmelk et al., 2003; Cambi et al., 2003). This condition makes CD209 all the more relevant not only in the context of the immune response but in the

Abbreviations: CTL, C-Type Lectin; CTLD, C-Type Lectin Domain; ASGR, Asialoglycoprotein Receptors; DC, Dendritic Cell; APC, Antigen Presenting Cell; ITAM, Immunoreceptor Tyrosine-based Activation Motif; HIV, Human Immunodeficiency Virus; SsSIGN, *Salmo salar* SIGN; TM, Transmembrane Domain; SERNAPESCA, Chilean National Fisheries and Aquaculture Service; FBS, Fetal Bovine Serum; CBD, Carbohydrate-Binding Domain; dpi, days post infection; MOI, multiplicity of infection.

* Corresponding author.
E-mail address: sergio.marshall@pucv.cl (S.H. Marshall).

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identification of susceptibilities and the design of prevention strategies (de Witte et al., 2008).

DC-SIGN genes have been described in at least three species of fish, including, *Fugu rubripes, Danio rerio,* and *Cynoglossus semilaevis*, based on sequence homology to mammal genes and different functional assays (Jiang and Sun, 2017; Lin et al., 2009; Zelensky and Gready, 2004). In fugu, eight different copies of DC-SIGN were found, a similar condition to the eight mouse genes that encode SIGNs (Powlesland et al., 2006). A significant number of putative gene sequences in the available salmon genome are annotated as DC-SIGN-like genes, with no dedicated or functional analyses involved. Furthermore, even though DC has not been fully isolated in *Salmo salar*, a putative CD209 sequence was detected in and used to characterize a DC-like subtype of cells in this species (Iliev et al., 2019). A dedicated curation of available data may help to identify sequences corresponding to proprietary *Salmo salar* DC-SIGN genes.

In the present study, we describe the characterization of eight novel DC-SIGN/CD209 orthologs from *Salmo salar*, based on available sequences from the genome and ESTs data, as well as expression analysis assays. We termed the genes SsSIGN1 - 8 (*Salmo salar* SIGN1 to 8), retaining the original SIGN acronym, but removing the DC-limited component. The eight genes code for proteins that display remarkable structural similarities to mammalian DC-SIGN proteins, including internalization motifs, a neck region with conserved heptad repeats, and a CTLD. The identified genes are distributed in two groups, containing four genes each, and are located in discrete regions of chromosomes 4 and 8. Differential gene expression in fish tissues and cell lines was detected, as well as specific responses to viral and bacterial pathogens. The presence of similar genes in other salmonid fishes was also identified and further discussed. Our work provides fundamental knowledge about the *Salmo salar* immune system, its response to specific pathogens, as well as novel insights into the DC-SIGN function and conservation across species.

2. Materials and methods

2.1. Database screening and sequence analyses

The ICSASG_v2 RefSeq genome records for *Salmo salar*, annotated by the NCBI Eukaryotic Genome Annotation Pipeline, was screened using "CD209", "DC-SIGN" and "C-Type Lectin" as queries (Davidson et al., 2010). Non-redundant gene results were analyzed for transcription variant, using the *Salmo salar* ESTs database to identify effectively transcribed sequences. Identified protein sequences were further analyzed for the presence of a Transmembrane Domain (TM) (TMHMM Server 2.0, CBS), Heptad Repeats (RADAR, EMBL-EBI), coiled-coil domains (Parcoil2, MIT) and a CTLD at the carboxy end (NCBI, CD-search) (Madeira et al., 2019; Marchler-Bauer et al., 2017; McDonnell et al., 2006; Sonnhammer et al., 1998). Eight genes were identified, coding for DC-SIGN-like proteins in *Salmo salar*. The *Oncorhynchus mykiss* (Rainbow trout) Omyk_1.0 RefSeq, *Salmo trutta* (River trout) fSalTru1.1 RefSeq, *Danio rerio* (Zebrafish) GRCz11 RefSeq genome records, were used for similar analyses, to identify DC-SIGN genes on those species (Pasquier et al., 2016; Zardoya et al., 1995). Putative promoter sequences for each gene were analyzed for the presence of transcription factor binding sites using the TRANSFAC 8.3 database in PROMO (Farré et al., 2003; Messeguer et al., 2002). Sequence alignment was performed using Clustal Omega and visualized in Jalview (Madeira et al., 2019; Waterhouse et al., 2009). Protein structure homology modeling was performed for SsSIGN5 using the Swiss-Model server, with 1fih as a template (Waterhouse et al., 2018).

2.2. Expression analysis of Salmo salar SIGN genes

The expression of *Salmo salar* SIGN genes was assessed *in vivo* using healthy fishes, as well as in infected cell lines.

2.2.1. Animal ethics

Experiments involving live animals were conducted following the regulations of Chile, according to the Chilean National Fisheries and Aquaculture Service (SERNAPESCA). Salmon were cultivated in Centro de Investigación en Acuicultura Curauma at Pontificia Universidad Católica de Valparaíso. Fish were purchased from authorized centers of Sernapesca, and the same organization approved the transfer and the experiment itself.

2.2.2. Tissue collection

Selected organs were recovered from clinically healthy juvenile *Salmo salar* specimens (average weight of 30 g), that previously tested free of bacterial, viral and fungal pathogens, and were cultivated in saltwater conditions. Fish were euthanized with an overdose of benzocaine. Samples from 5 fish were recovered, including blood, kidney, spleen, gill, liver, and brain. Peripheral blood leukocytes (PBL) were recovered from blood samples using a discontinuous Percoll gradient, as previously described (Pettersen et al., 1995).

2.2.3. Cell culture

Atlantic salmon kidney (ASK) cells (ATCC CRL2747), were cultured at 20 °C in Leibovitz's L-15 media with 4 mM glutamine (Gibco) and supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 0.5 μg/ml amphotericin, and 20% Fetal Bovine Serum (FBS) (Gibco). Atlantic Salmon Head Kidney (SHK-1) cells (ECACC 97111106) were cultured at 20 °C in Leibovitz's L-15 media with 4 mM glutamine (Gibco), 10% FBS (Gibco) and 40 μM 2-Me (Gibco). Cells were grown to 80% confluence and sub-cultured accordingly.

2.2.4. In vitro infection assays

To evaluate the expression profile of SsSIGN genes upon infection with relevant pathogens, we selected experimental models of Infectious Salmon Anemia Virus (ISAV) infection of ASK cells, and *Piscirickettsia salmonis* infection of SHK-1 cells (Castillo-Cerda et al., 2014; Gómez et al., 2013).

A field isolate of ISAV corresponding to the HPR7b type was obtained from the Laboratorio de Genética e Inmunología Molecular strain collection (Pontificia Universidad Católica de Valparaíso, PUCV). For viral infection and propagation, an 80% confluent ASK cell monolayer was washed twice with L-15 media and covered with a viral dilution prepared in L-15 media. After 4 h, the virus was removed, and the cells were washed twice with L-15 media and cultured in 2% FBS L-15 media with antibiotics at 17 °C. After 7 days, the cell supernatant was filtered (0.45 μm), and the virus was collected. A plaque assay was used for virus tittering 12 days post infection (dpi), as previously described (Castillo-Cerda et al., 2014).

A *Piscirickettsia salmonis* field isolate, termed Psal-104, was obtained from the Chilean National *Piscirickettsia salmonis* Strain Collection (PUCV), and cultured in BM3 media, with 100 rpm. agitation, at 19 °C, as previously described (Henríquez et al., 2013). Exponentially growing bacteria ($O.D._{600} = 0.6$) was recovered by centrifugation at 5000 x *g*, washed twice, and resuspended in L-15 media. Bacteria were counted using a Petroff-Hausser chamber.

ASK cells were seeded in 6 well plates, at 250.000 cells/well, and incubated for 24 h before removing the culture media and infecting with an ISAV inoculum at a multiplicity of infection (MOI) of 0.5. After a 4-h incubation, the monolayer was washed twice with L-15 media and incubated in antibiotics and 2% FBS supplemented L-15 at 17 °C. Three days post infection culture media was removed, and cells were recovered applying TRIzol™ solution (Invitrogen™) for RNA extraction. An aliquot of the virus, corresponding to the same viral load, was inactivated by incubation at 56 °C for 30 min and used to infect ASK cells; uninfected cells were used as controls (Falk et al., 1997). All infections were performed in triplicates.

SHK-1 cells were seeded in 6 well plates, at 200.000 cells/well, and incubated for 24 h before removing the culture media and infecting with

Table 1
Primers sequences for RT-qPCR of *Salmo salar* DC-SIGN-like receptors. Elongation factor 1 alpha was used as a house-keeping gene for normalization. Chromosomal location is indicated by Chr04 (chromosome 04) and Chr08 (chromosome 08).

Chro.	RefSeq mRNA	Forward	Reverse	Obs.
Chr04	XM_014194757.1	TCAACGATAGGACCAGAACCTG	AGGGTGTCTGCACTGACGTA	SsSIGN1
	XM_014194638.1	GGAGGAGGACTGTGTTGAGC	TGCGTTTGATACCGGTCCAT	SsSIGN2
	XM_014194726.1	AGGCTGGTGGAAGTCATGTG	CTTCTCCCTGCACTGTCCTG	SsSIGN3
	XM_014194753.1	TATATGGCAATGTGGGAGCCT	AGACACACTGCAACAACTAGGA	SsSIGN4
Chr08	XM_014209668.1	TACTGACCACCCCAAGGTACTG	TTGAGTTTGCTGTCGCATGAC	SsSIGN5
	XM_014209793.1	ACCACTGACCACAAAGTATTGGA	GTTGAGAGAGTACCGAGGAGG	SsSIGN6
	XM_014209821.1	GGCTTACCAATCTCGGCTGG	ATCAGGCTGCTTGTCATTCCA	SsSIGN7
	XM_014209791.1	GTCATCAGACACCGGAGCAT	CAGCGAGTTGTCCCCTCTTT	SsSIGN8
	NM_001173967.1	GCTTACAAAATCGGCGGTAT	CTTGACGGACACGTTCTTGA	EF-1a

a *Piscirickettsia salmonis* inoculum at a MOI of 100. After a 4-h incubation, cells were washed five times with L-15 media, and fresh SFB supplemented L-15 media was added. Infected cells were incubated at 20 °C. Five dpi, media was removed, and cells were recovered, applying TRIzol™ solution (Invitrogen™) for RNA extraction. An aliquot of the bacteria, corresponding to the same bacterial load, was inactivated by incubation at 56 °C for 15 min, and used to infect SHK-1 cells; uninfected cells were used as controls (Álvarez et al., 2016). All infections were performed in triplicates.

2.2.5. RNA extraction and cDNA synthesis

RNA was obtained from tissue samples using a combination of TRIzol™ buffer, and E.Z.N.A.® Total RNA Kit I (OMEGA Bio-Tek). 50 mg of tissue, or cells from 1 well from a 6-well plate, were resuspended in 1 ml of TRIzol buffer and disaggregated using a syringe. After following the manufacturer's indications, the aqueous phase from the TRIzol-Chloroform extraction was mixed with 1 volume of 70% ethanol, and RNA extraction proceeded according to the column's recommended protocol. RNA was resuspended in nuclease-free water and quantified using a Nanodrop spectrophotometer (Thermo Scientific). All samples had A_{260}/A_{280} ratios over 1.8. Samples corresponding to 1 μg of total RNA were treated with 1 U of DNase I, RNase-free (Thermo Scientific) for 45 min at 37 °C. After DNase inactivation, cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit, using Random Hexamers as primers (Thermo Scientific).

2.2.6. Quantitative PCR (qPCR)

Specific primers were designed to assess the level of expression of each selected *Salmo salar* SIGN mRNA. Expression of Elongation factor 1 alpha was used as a house-keeping gene for normalization (Table 1) (Salazar et al., 2016).

qPCR reactions were performed using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies), according to the manufacturer's instructions, with 200 nM of each primer, and 2 μl of cDNA as the template for a 20 μl reaction. Reactions were performed in a Bio-Rad CFX96 thermal cycler, with cycling conditions as follows: 1 cycle of 3 min at 95 °C and 40 cycles of 5 s at 95 °C and 10 s at 60 °C. Finally, a dissociation curve was performed according to the instrument settings.

2.2.7. Expression analysis

The fold change of gene expression levels, relative to controls, was assessed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The results are presented as the means ± standard deviations of replicas. The statistical significance of the data was determined by using a Student's *t-test*. P-values < 0.05, <0.01 and < 0.001, are indicated by *, ** and *** respectively, and were considered significant as properly indicated.

2.3. Phylogenetic analysis

Amino acid sequences corresponding to the Carbohydrate-Binding Domains (CBD) of selected DC-SIGN/CD209 molecules were recovered from NCBI (Table 2). Analysis of *Oncorhynchus mykiss*, *Salmo trutta* and

Table 2
Sequences used for phylogenetic analyses. Species, GenBank accession number, and the region corresponding to CTLD are displayed.

Species	Access n°	CTLD	*Species*	Access n°	CTLD	*Species*	Access n°	CTLD
H.sapiens	>Q9NNX6-2	251..398	*G.gallus*	>NP_990815.1	85..203	*O.mykiss*	>XP_021475407.1	156..280
H.sapiens	>AAK20998.1	207..343	*X.tropicalis*	>XP_031755207.1	91..211	*O.mykiss*	>XP_021475400.1	167..291
M.musculus	>AAL13235.1	162..297	*O.niloticus*	>XP_025758281.1	84..224	*O.mykiss*	>XP_021472964.1	136..264
M.musculus	>AAL13236.1	105..240	*S.scrofa*	>NP_001123444.1	112..240	*O.mykiss*	>XP_021475402.1	167..291
M.musculus	>AAL13237.1	101..237	*D.rotundus*	>XP_024416423.1	131..259	*O.mykiss*	>XP_021428956.1	175..297
M.musculus	>AAL13238.1	72..208	*P.walrus*	>XP_004412214.1	121..249	*O.mykiss*	>XP_021428954.1	193..315
M.musculus	>AAL13234.1	103..238	*O.orca*	>XP_004277712.1	150..278	*O.mykiss*	>XP_021428962.1	24..146
M.musculus	>XP_889104.1	72..208	*L.africana*	>XP_023396298.1	130..261	*O.mykiss*	>XP_021428969.1	193..315
M.musculus	>NP_081619.3	61..194	*C.Hircus*	>XP_005682468.1	131..259	*O.kisutch*	>XP_020327037.1	133..251
M.musculus	>NP_081232.2	122..255	*E.asinus*	>XP_014717459.1	107..238	*O.kisutch*	>XP_020312192.1	169..295
P.troglodytes	>AAL89545.1	236..358	*O.cuniculus*	>XP_017193467.1	109..239	*S.trutta*	>XP_029622272.1	166..294
P.troglodytes	>AAL89535.1	314..437	*C.griseus*	>XP_027271607.1	110..238	*S.trutta*	>XP_029621727.1	161..286
P.pygmaeus	>AAL89542.1	256..379	*U.maritimus*	>XP_008709536.1	121..249	*S.trutta*	>XP_029622357.1	179..302
P.paniscus	>ABW34403.1	256..379	*V.pacos*	>XP_015096456.1	121..249	*S.trutta*	>XP_029622352.1	195..321
P.paniscus	>ABW34400.1	268..391	*L.obliquidens*	>XP_026941610.1	177..305	*S.trutta*	>XP_029622368.1	146..272
H.lar	>AAL89538.1	302..425	*P.vampyrus*	>XP_023380689.1	62..193	*S.trutta*	>XP_029622359.1	177..301
H.lar	>AAL89528.1	268..391	*S.salar*	>XP_014050232.1	176..310	*S.trutta*	>XP_029622481.1	191..315
N.leucogenys	>ABW34404.1	256..379	*S.salar*	>XP_014050113.1	231..367	*S.trutta*	>XP_029622476.1	317..441
N.leucogenys	>ABW34402.1	245..368	*S.salar*	>XP_014050200.1	163..287	*D.rerio*	>XP_003197805.3	196..308
S.syndactylus	>AAL89539.1	233..356	*S.salar*	>XP_014050228.1	134..262	*D.rerio*	>XP_017211404.1	195..306
S.syndactylus	>AAL89529.1	291..414	*S.salar*	>XP_014065143.1	137..272	*D.rerio*	>XP_009293464.2	196..307
C.l. familiaris	>NP_001124304.1	118..238	*S.salar*	>XP_014065268.1	146..275	*D.rerio*	>NP_001186302.2	220..343
O.latipes	>ADB55614.1	75..203	*S.salar*	>XP_014065296.1	187..311	*C.semilaevis*	>XP_024920596.1	122..265
T.nigroviridis	>ADB55615.1	47..168	*S.salar*	>XP_014065266.1	373..502			

Table 3
Identified genes coding for SsSIGN proteins. Chromosomal locations for each of them, reveals two clusters of genes located on chromosome 4 and 8, respectively. Internalization Motifs (Int.Motif) are annotated as single (+), double (++) or triple (+++). Predicted transmembrane domains (TM), and Heptad repeats (Hept. Rep.) are indicated. CTLD denotes the position of the C-Type Lectin Domain.

Chro.	Location	RefSeq mRNA	RefSeq Protein	Size (aa)	Int. Motif.	TM	Hept. Rep.	CTLD	Name
Chr04	2.240.891..2.244.441	XM_014194757.1	XP_014050232.1	310	++	83..105	117..170	176..310	SsSIGN1
	3.468.837..3.485.920	XM_014194638.1	XP_014050113.1	367	+	40..62	No	231..367	SsSIGN2
	3.596.403..3.600.814	XM_014194726.1	XP_014050201.1	287	+++	64..86	117..156	163..287	SsSIGN3
	3.706.690..3.710.679	XM_014194753.1	XP_014050228.1	280	++	57..79	109..148	152..280	SsSIGN4
Chr08	19.257.147..19.259.043	XM_014209668.1	XP_014065143.1	272	+++	63..85	92..131	137..272	SsSIGN5
	20.942.043..20.948.139	XM_014209793.1	XP_014065268.1	309	++	59..81	82..177	180..309	SsSIGN6
	22.609.016..22.616.088	XM_014209821.1	XP_014065296.1	311	+++	60..82	99..175	187..311	SsSIGN7
	22.870.914..22.885.313	XM_014209791.1	XP_014065266.1	502	++	50..72	103..198/260..357	373..502	SsSIGN8

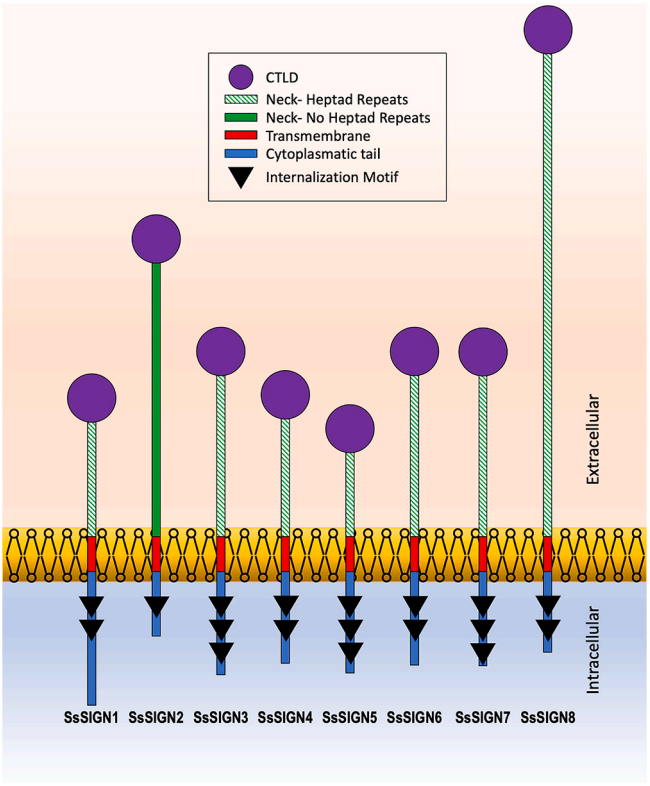

Fig. 1. Domain organization of SsSIGN proteins. CTLD is located at the carboxyl end and connected to the transmembrane domain trough a neck region containing, in most cases, heptad repeats possibly involved in oligomerization. Neck and cytoplasmatic regions primary structure lengths are displayed proportionally for each homolog.

Danio rerio genomes, as previously described, allowed for the identification of DC-SIGN/CD209 orthologous in these species; CBDs were recovered from these sequences and included in further analyses. A phylogenetic tree was constructed by Neighbor-Joining method, using MEGA X (Stecher et al., 2020), based on the amino acid alignment of all these sequences (Clustal Omega) (Madeira et al., 2019). Bootstrap values were calculated from 2000 repetitions. The phylogenetic tree was rendered using iTOL (Letunic and Bork, 2007).

3. Results

3.1. Sequence analysis of DC-SIGN-like genes in Salmo salar

Potential *Salmo salar* orthologs of mammalian DC-SIGN genes were identified on the fish genome. We screened the ICSASG_v2 RefSeq genome for non-redundant gene sequences coding for transmembrane proteins containing canonical features of DC-SIGN receptors, that is: a CTLD, a neck region with heptad repeats, and internalization motifs in the cytoplasmatic region. The genes encoding each of the identified DC-SIGN homologs, termed SsSIGN 1–8, are located in discrete regions (~2 megabases) of chromosomes 4 and 8 (Table 3). Transcriptional variants were selected upon analysis of the *Salmo salar* EST database.

Fig. 1 shows the projected domain organization of the SsSIGN proteins. The sizes of the proteins range from 272 to 502 amino acids, with notable differences among the neck and cytoplasmatic regions of different receptors. All of them display typical internalization signals corresponding to either double leucine, triple acid, or ITAM motifs (Fig. 2). Heptad repeats are absent only on the SsSIGN2 gene, but the neck does conform a theoretical coiled-coil domain (data not shown), which is probably involved in oligomerization. The cytoplasmatic domains contain internalization signals, typical of this type of receptor. Notably, the repeats on the neck region correspond to a semi-conserved sequence ERDQLQYNNLTK, forming a heptad pattern of hydrophobic residues. SsSIGNs feature conserved residues implicated in carbohydrate and Ca^{+2} binding, compared to *Homo sapiens* and *Mus musculus* sequences; the fish CTLD contains only six cysteine residues, possibly involved in three disulfide bridges, unlike mammals DC-SIGNs which are characterized by a fourth bridge.

The CTLD sequence on the SsSIGNs appears highly conserved except for a 35–40 amino acid stretch, which displays high variability among different receptors (Fig. 3). Structural modeling locates these residues in the putative CBD, suggesting different specificities/avidities for each one of the receptors.

A 1000 bp gene sequence upstream of the start codon was analyzed to identify possible Transcription Factor (TF) binding sites described in mammalian homologs (Fig. 4). The promoter regions contain binding sites for Activator Protein 1 (AP-1), Nuclear Factor kappa B (NF- κB), and Transcription Factor SP1 (SP1). Each gene contains different number and distribution of sites, with discrete conservancy between SsSIGN3 and 7.

3.2. Expression analyses of SsSIGNs

The expression of each receptor was evaluated in immuno-relevant tissues of healthy fish, using RT-qPCR. Expression was effectively detected in all samples, with unique distribution patterns for each (Fig. 5). SsSIGN1, 2, 7, and 8, had in general higher expression levels in organs directly involved in immune response (kidney and spleen). Interestingly, SsSIGN 3 was highly expressed in PBL and gill, compared to the kidney, and SsSIGN 4 had the highest levels in the brain. Differential expression of the genes in each tissue suggests specific roles for the receptors in different organs.

Two *Salmo salar* cell lines were tested for expression of the SsSIGN genes. ASK cells correspond to epithelial cells, highly susceptible to ISAV infection; SHK-1 cells display properties of fish macrophages and are susceptible to infection with *Piscirickettsia salmonis*. The expression of the receptors was detected in both cell lines, previous to infection assays (Fig. 6). Both pathogens regulated the expression of the SsSIGNs differentially. ISAV infection induced the expression of SsSIGN 1 and 4

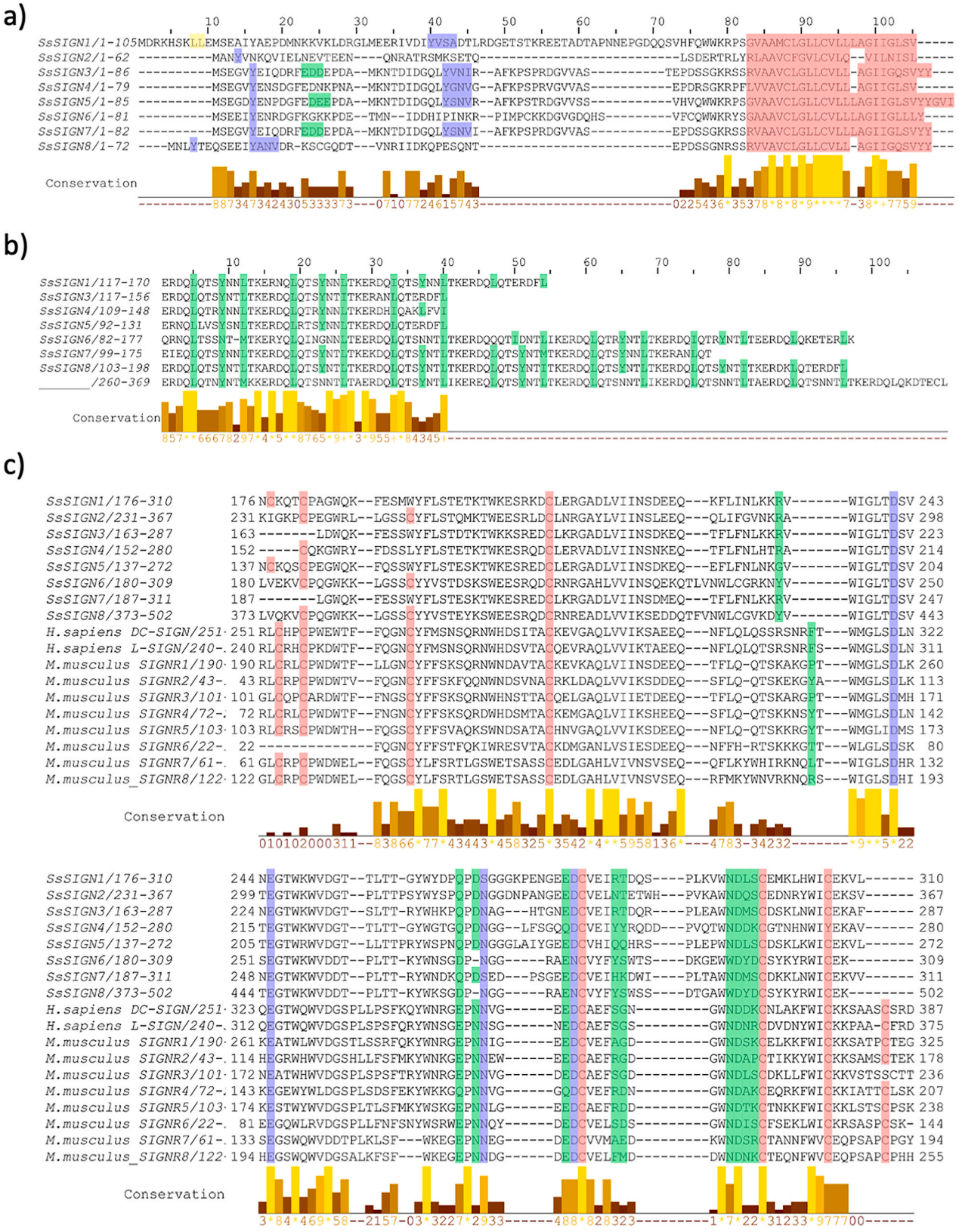

Fig. 2. Multiple sequence alignment, displaying typical features of SsSIGN. A) Alignment for cytoplasmatic regions of salmon SIGN, with color-coded internalization motifs: yellow – double leucine, green-triple acid, and blue ITAM (YXX-I/L or YXXΦ). Residues in red correspond to the transmembrane domain. B) Alignment of neck regions of salmon SIGNs. The sequence ERDQLQYNNLTK appears highly conserved among the different SsSIGNs. Hydrophobic residues, forming the heptad repeats, are highlighted in green. C) Alignment of SsSIGN with sequences from *Homo sapiens* and *Mus musculus* homologous, with highlighted conserved residues. In red, cysteines involved in disulfide bridges in mammals. In green, residues involved in carbohydrate binding. In blue, residues involved in Ca+2 binding. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

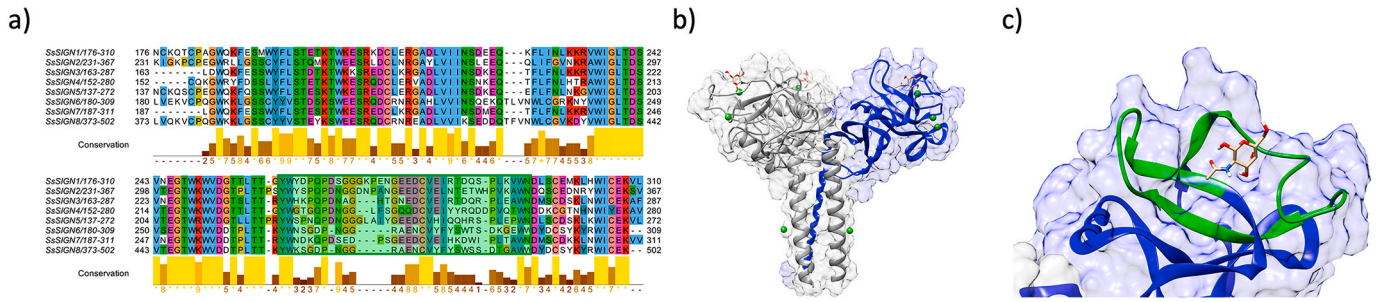

Fig. 3. Features of the CTLD of SsSIGNs. A) Multiple sequence alignment for the different salmon DC-SIGN homologous CTLD. Sequences are conserved among different genes, with a discrete region of particular variability highlighted in green. B) A structural model for a monomer of SsSIGN5 (blue), in the context of a tetramer formed by the template protein, with mannose molecules located at the CBD. C) Structural detail of the putative CBD of SsSIGN5. The variable region from A is highlighted in green and corresponds to residues interacting with the carbohydrate ligand. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and reduced the expression of SsSIGN 3, 6, and 8. The effects were dependent on the active infection (i.e., inactivated virus did not render the effect in the same magnitude), which suggests that the regulation is not only related to the receptor activation but may be enhanced by an integrated immune response to the viral infection. On the other hand, *Piscirickettsia salmonis* infection of the SHK-1 cell line downregulated SsSIGN 1, 3, and 6, with the effect being absent in cells with the inactivated bacteria (Fig. 7).

3.3. Phylogenetic analyses of SsSIGNs

To explore the conservation of DC-SIGN-Like genes in other fishes, we screened the genomes of two other salmonid species, *Oncorhynchus mykiss* and *Salmo trutta*, and the cyprinid *Danio rerio*, for DC-SIGN-like sequences, using the same approach described for *Salmo salar*. Both salmonid species display a similar array of DC-SIGN-like genes, located in discrete regions of their genomes. Particularly, *O. mykiss* has eight homologous, evenly distributed between chromosome 10 a chromosome 19. On the other hand, the 8 DC-SIGN-Like genes of *S. trutta* are located in chromosome 11, with one, 4-gene cluster at ~18 Mb, and two pairs of genes at ~21 and ~16 Mb. The homologous, display the conserved features of this type of gene, with a single copy in both salmonid genomes lacking the heptad repeat domain, similar to SsSIGN2. Moreover, one gene in *O. mykiss* and two in *S. trutta*, lack a TM domain, suggesting a soluble nature, similar to the structure of *M. musculus* SIGNR2 and 6. Finally, we identified only three homologous DC-SIGN genes in the *Danio rerio* genome, located in a discrete region of chromosome 1 (Table 4).

For phylogenetic analysis, a total of 33 DC-SIGN sequences described for mammal species, the 19 DC-SIGN-Like homologous from *O. mykiss*, *S. trutta* and *D. rerio*, 3 CTL Receptors previously described by Soanes (Soanes et al., 2004), DC-SIGN sequences described for *Danio rerio* and *Cynoglosus semilaevis* (Jiang and Sun, 2017; Lin et al., 2009), and the eight SsSIGN sequences, were used to construct an unrooted phylogenetic tree using the neighbor-joining method. The analysis showed that SsSIGN sequences are classified in a cluster formed by salmonid species, except for two *S. trutta* sequences, and separated from the other CTLR from salmon. The three sequences we identified in *D. rerio* form an independent cluster, separated from the previously reported sequence. Among mammals, primates are clustered in a defined group, and *M. musculus* sequences are distributed in all clades. The phylogenetic analysis reflects the variety and diversity of DC-SIGN genes, and its distribution across species (Fig. 8).

4. Discussion

4.1. DC-SIGN receptors

Initially recognized as a receptor for HIV, present in human placenta, DC-SIGN was further characterized as a broad range pathogen-binding receptor, as well as an adhesion molecule that facilitates attachment of Dendritic Cells (DC) to T cells, supporting primary immune responses (Curtis et al., 1992; Garcia-Vallejo and van Kooyk, 2013). DC-SIGN homologs have been described in various species of mammals, where there are at least three family members with conserved functional domains; interestingly, mouse has eight DC-SIGN homologs, clustered in a discrete genomic region (Liu et al., 2004; Powlesland et al., 2006).

Even though DC-SIGN homologs have been described for other fish species, including zebrafish, fugu, and tongue sole, no ortholog has been described in salmonid species. *Salmo salar* is a commercially important farmed fish species, with a continually growing, worldwide industry (Little et al., 2015). Robust expansion on fish farming is based on the development of sanitary measures, which in turn relies on a proper understanding of the fish immune system (Andresen et al., 2020). In this work, we sought to identify and describe putative salmon orthologs of mammalian DC-SIGN receptors, focused on conserved structural features and expression patterns in response to viral and bacterial infections.

4.2. Sequences and features of SsSIGN

Our analysis of the *Salmo salar* genome and EST sequences led us to identify eight putative proteins sequences with characteristics of DC-SIGN receptors, including the canonical CTLD at the carboxy end connected to the transmembrane domain by a neck region, containing heptad repeats, and a cytoplasmatic tail with internalization signals. Even though our screening revealed the presence of several other proteins containing CTLD, only these eight sequences carry the features described for DC-SIGN genes (Table 3).

DC-SIGN molecules are type II transmembrane proteins, characterized by the presence of a C-Type Lectin Domain (CTLD), which interacts with glycans in a Ca^{+2} dependent fashion. The CTLD in SsSIGN displays conserved amino acid residues involved in the interaction with carbohydrates and Ca^{+2}, compared to the *Homo sapiens* and *Mus musculus* sequences (Fig. 2) (Feinberg, 2001). The non-conserved residues in the Receptor Binding Domain (RBD) display high diversity among the salmon DC-SIGN sequences; this is similar to the mouse SIGN receptors, were the eight homologs feature different ligand-binding specificities, with this divergence being, arguably, a product of evolutionary pressure related to the exposure to species-specific pathogens (Garcia-Vallejo and van Kooyk, 2013) (Fig. 3). Furthermore, DC-SIGN typically recognizes fucosylated and high-mannose structures, modulating different cellular

Fig. 4. Promoter regions for SsSIGN genes. Predicted binding sites for transcription factors are highlighted in red for AP-1 sites, green for NF-κB, and blue for SP1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

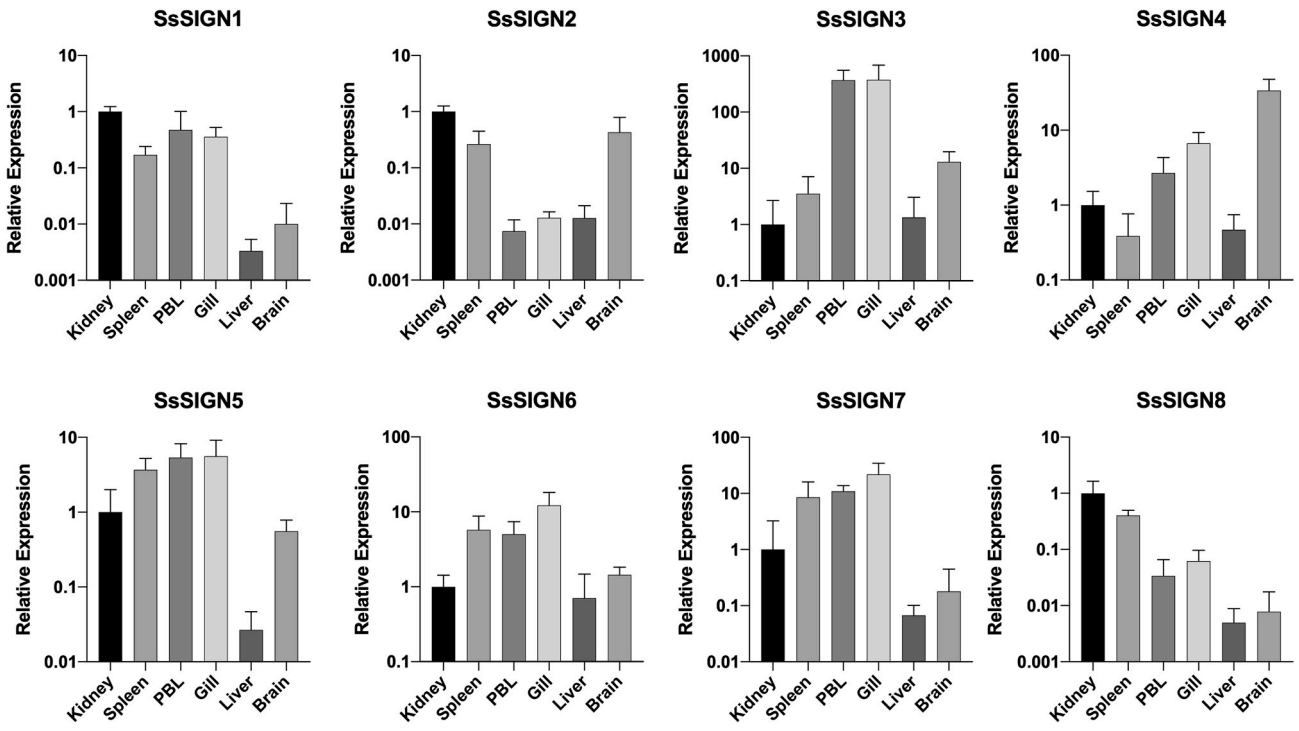

Fig. 5. Expression analysis of SsSIGN in fish organs. Expression levels for each gene in different organs relative to the expression in the kidney. Each gene displays a different pattern of distribution. Results correspond to an n = 5.

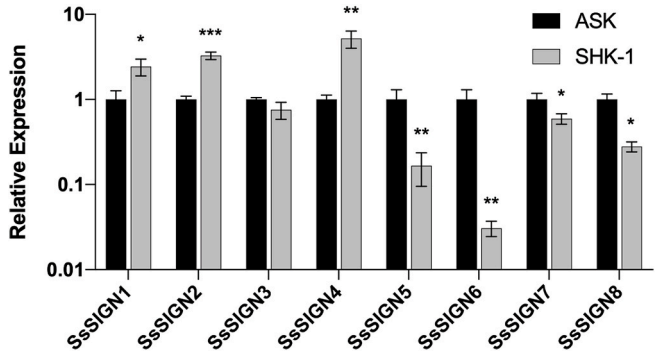

Fig. 6. Expression of SsSIGN genes on two salmon cell lines. Levels are relative to the expression in the ASK line. Results correspond to an n = 3.

responses depending on the bound ligand: high-mannose glycans, expected to occur in higher mammals only in proteins during maturation, trigger a proinflammatory response (i.e., cells damage caused by pathogens). In contrast, fucosylated glycans suppress proinflammatory cytokines (inter-cellular signaling) (Gringhuis et al., 2009). Similarly, SsSIGNs could display different specificities, with relevant functional implications: on the one hand, allowing for the interaction with a broad range of microorganisms (from virus to fungus), and on the other, discriminating between different types of signals coming from other cells.

The heptad repeats in the neck domain in DC-SIGN consist of a heptad pattern of hydrophobic residues in a helical region, which mediates the packing of helices from different DC-SIGN monomers to form a 4-stranded coiled-coil in the neck domain of the DC-SIGN tetramer (dos Santos et al., 2017). According to our analyses, SsSIGN 2 lacks a heptad repeat motif in its neck region, where all the other homologs carry a repeated, semi-conserved sequence (Fig. 2). The lack of heptad repeats is also present in the CD209L receptor, found in some non-human primates, as well as in the zebrafish and tongue sole DC-SIGNs; it has been suggested that this type of SIGN receptor corresponds to the ancestor of the DC-SIGN family (Lin et al., 2009; Ortiz et al., 2008). Furthermore, a monomeric conformation, due to the lack of heptad repeats, may correlate with a specific function for SsSIGN2.

Dendritic Cells (DC) are specialized in presenting antigens for the activation of T cells to initiate an immune response. In these cells, DC-SIGN is involved in the internalization of antigens and pathogens upon ligand binding, and the complex is targeted to late endosomes/lysosomes (Engering et al., 2002). The cytoplasmatic tails of all SsSIGNs possess different internalization signals, including di-leucine motifs and tri-acid clusters which can be involved in the internalization process (Engering et al., 2002; Lin et al., 2009). On the other hand, even though all of the receptors carry tyrosine residues in this region, only SsSIGN3 displays a canonical Hemi ITAM motif (i.e., YxxI/L), with SsSIGN1,4 and 8 displaying a YxxΦ motif (where Φ is a hydrophobic residue), all of which may be involved in intracellular signaling (Guo et al., 2004) (Fig. 2). Concomitantly, DC-SIGN molecules are characterized by the lack of typical ITAM or ITIM motifs but do interact with a sophisticated signalosome inside of DCs (Gringhuis et al., 2009). Moreover, homo or heterotetramerization may play a role in signal transduction, where multiple phosphorylated tyrosine residues are necessary for signaling from a homotetramer, or the activation signal is conducted using an ITAM/ITIM carrying protein, in the context of a heterotetramer (Garcia-Vallejo and van Kooyk, 2013; Haining et al., 2017). As their homologs in mammals, SsSIGNs could be involved in the modulation of the responses initiated by other receptors (Hovius et al., 2008; Rodríguez et al., 2017).

4.3. Expression of SsSIGN and response to infection

Analysis of the upstream sequences of the 8 SsSIGN, revealed the presence of potential binding sites for transcription factors described in the mammalian homologs, including NF-κB, Sp1 and AP-1 sites (Fig. 4) (Liu et al., 2003). DC cells activate distinct sets of transcription factors upon maturation, which will lead to the transcription of different sets of genes as well; furthermore, expression of SsSIGNs in a wider variety of

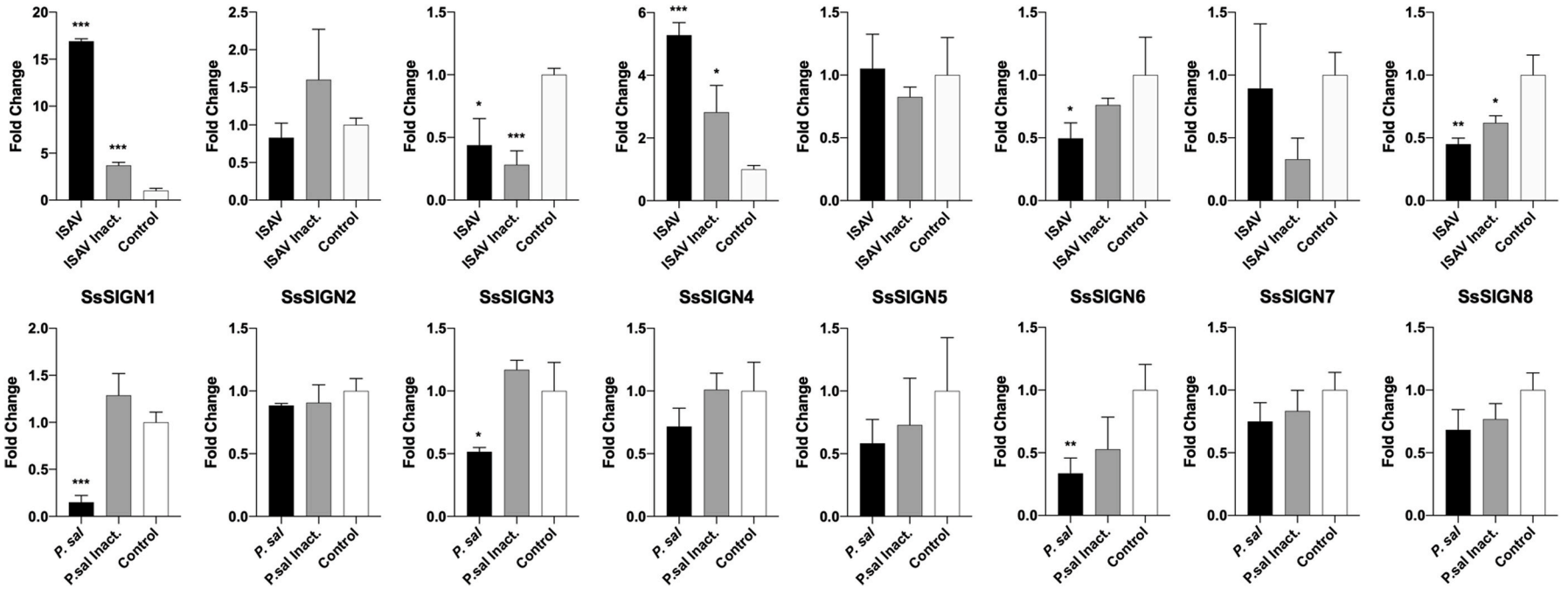

Fig. 7. Expression of SsSIGN genes during viral and bacterial infection in vitro. The upper panel displays the expression levels of SsSIGNs in ASK cells after 72 h infection with ISAV. The lower panel shows gene expression on SHK-1 cells after a 5-day infection with *Piscirickettsia salmonis*. Levels are relative to the uninfected controls. Results correspond to an n = 3.

Table 4
DC-SIGN-Like sequences identified in selected fish species.

Species	Chro.	Location	RefSeq Protein	Int. Motif	TM	Hept. Motif	CTLD
Oncorhynchus mykiss	Chr10	68,316,695..68,351,882	XP_021475407.1	+++	58..80	94..145	156..278
		68,437,277..68,440,058	XP_021475400.1	++	60..82	106..157	167..289
		68,449,595..68,452,375	XP_021472964.1	++	7..29	44..124	136..262
		68,502,845..68,505,833	XP_021475402.1	++	60..82	106..157	167..289
	Chr19	3,359,438..3,415,338	XP_021428956.1	+	63..85	107..160	175..295
		3,418,729..3,424,484	XP_021428954.1	+	62..84	107..179	193..313
		3,426,261..3,427,566	XP_021428962.1	–	–	–	24..144
		3,615,353..3,621,025	XP_021428969.1	++	62..84	107..179	193..313
Salmo trutta	Chr11	16,730,161..16,733,194	XP_029622272.1	+	59..81	77..158	166..292
		16,911,497..16,937,038	XP_029621727.1	–	–	–	161..284
		18,643,399..18,648,784	XP_029622357.1	++	65..87	106..156	179..300
		18,740,308..18,756,197	XP_029622352.1	++	86..108	134..185	195..319
		18,775,740..18,784,853	XP_029622368.1	+	39..61	83..133	146..270
		18,918,066..18,936,039	XP_029622359.1	+	58..80	92..168	177..297
		21,422,649..21,430,498	XP_029622481.1	–	–	3..167	191..315
		21,527,965..21,532,327	XP_029622476.1	+	32..54	97..309	317..437
Danio rerio	Chr01	55,903,808..55,906,907	XP_003197805.3	++	43..65	103..184	196..308
		55,908,572..55,919,463	XP_017211404.1	++	42..64	102..183	195..306
		55,920,864..55,922,977	XP_009293464.2	++	43..65	103..184	196..307

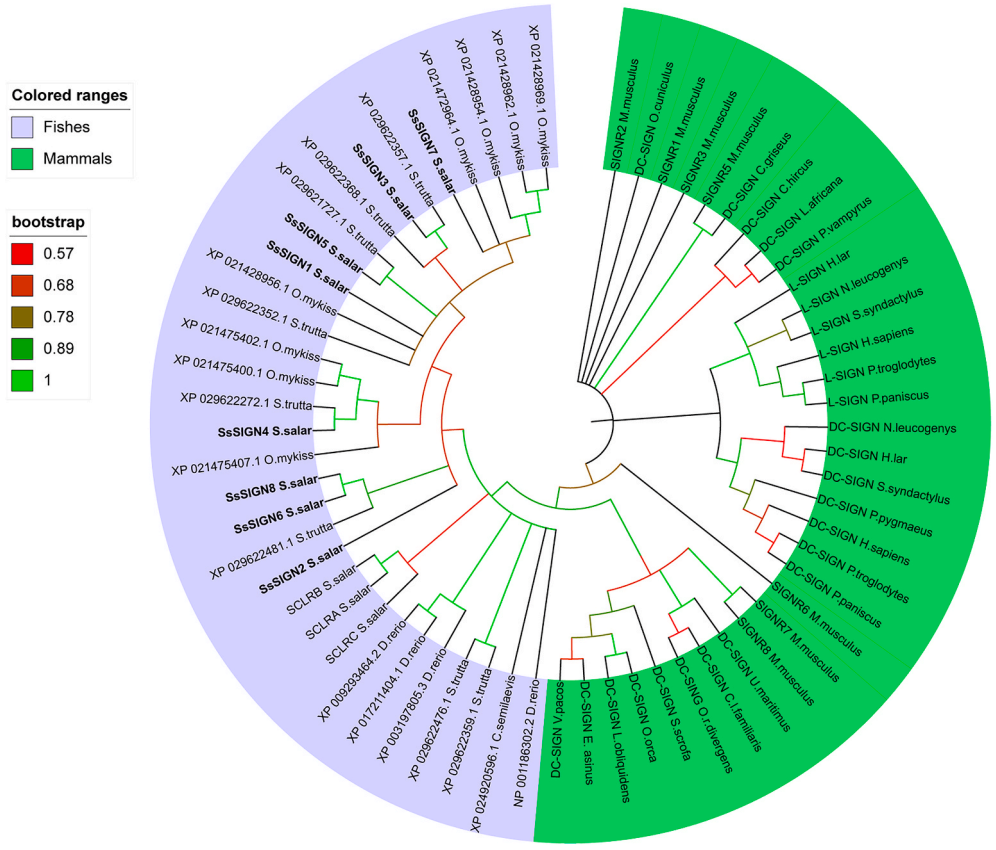

Fig. 8. Phylogenetic tree showing the relationship between SsSIGN amino acid sequences and other species of the DC-SIGN family. The unrooted phylogenetic tree was constructed by the neighbor-joining method, based on the amino acid alignment (Clustal Omega) of CBD of protein sequences. Bootstrap values were calculated from 2000 repetitions.

cell types would lead to a much more sophisticated expression profile, effectively mediated by its promoters (Mizumoto et al., 2005). Interestingly, polymorphism in mammalian DC-SIGN promoters is associated with susceptibility to viral infections (Wang et al., 2011).

SsSIGNs were expressed in all the analyzed salmon tissues, with differential expression patterns for each of them (Fig. 5). Immune system-related tissues (kidney and spleen) were, in general, enriched for the expression of the receptors. The liver displays discrete levels of most genes compared to the kidney. SsSIGN3 was highly expressed in PBL and Gill (over 300 times compared to the expression in the kidney), which suggest homing of a specific subset of SsSIGN3-expressing cells; macrophages and microfold-like cells have been described in rainbow trout gills, featuring antigen-sampling capabilities (Kato et al., 2018). Interestingly, the brain displays relatively high levels of most SsSIGN genes, particularly of SsSIGN4; both macrophages and mast cells, which act as APCs, have been detected in fish brains and could account for SsSIGN4 expression in that organ (Herbomel et al., 2001; Kordon et al., 2018).

Two salmon cell lines were analyzed for the expression of SsSIGN

genes. ASK cells correspond to epithelial cells and SHK-1 to macrophage-like cells. Both cell lines displayed expression of all genes, with differential levels for each of them (Fig. 6). Although DC-SIGN has been canonically associated with APC like macrophages and DCs, expression of SsSIGN in salmon epithelial cells may be related to a role in antigen processing and presentation on this species. Furthermore, the expression of a specific profile of these receptors could increase differential susceptibility to infection and differential responses to specific pathogens to each cell line.

Infectious Salmon Anemia Virus (ISAV) produces an aggressive disease, primarily affecting *Salmo salar* (Vike et al., 2014). The virus is part of the Orthomyxoviridae family, with a segmented single-stranded negative-sense RNA genome and a viral envelope (Krossøy et al., 1999). Attachment to the cell surface is mediated by a viral glycoprotein termed hemagglutinin-esterase (HE), which binds to specific sialic acids (sia) on glycan chains present in the cellular surface proteins; endocytosis of the virion leads to membrane fusion and infection (Aamelfot et al., 2012). Furthermore, the virus codes for at least two proteins with interferon (IFN) antagonistic activities (McBeath et al., 2006; Olsen et al., 2016). We sought to determine the effect of ISAV infection on the expression of SsSIGN genes in vitro, considering the role that these receptors could play in viral binding and the regulation that viral proteins could have over them. ISAV infection of the permissive ASK cell line leads to significant upregulation of SsSIGN1 and 4, with a more pronounced effect on the former. The promoter for SsSIGN1 contains an NF-κB binding site, which is canonically activated by Influenza Virus infection in mammals, suggesting a similar effect for ISAV infection in salmon (Alexopoulou et al., 2001; Schmitz et al., 2014) (Fig. 7).

On the other hand, SsSIGN genes containing AP-1 sites display a tendency to be downregulated, a process that could be mediated by ISAV NS1, in parallel to what is observed in Influenza A Virus (IAV) infection (Ludwig et al., 2002). Moreover, differential effects are observed in ISAV infected and mock (inactivated virus) infected cells, which suggest a direct connection between active infection and cellular responses. Regulation of expression of SsSIGN genes during ISAV infection could play a direct role in cellular susceptibility: IAV is capable of infecting DC-SIGN/L-SIGN expressing cells, in a sia-independent fashion. In that context, SIGN molecules interact with IAV hemagglutinin (HA) glycosylations, acting as actual receptors for the virus (Hillaire et al., 2013). ISAV HE possesses at least two glycosylation sites, and infection has proven to be Ca^{+2} dependent, suggesting a role for CTLRs in the infective process (Fourrier et al., 2015).

Piscirickettsia salmonis (*P. sal*) is a facultative intracellular gram-negative bacteria, the etiological agent of the disease known as piscirickettsiosis, which causes significant economic losses in the aquaculture industry (Rozas and Enríquez, 2014). The bacteria produces an imbalance in the interleukin (IL) 10–12 equilibrium in infected macrophages, leading to an anti-inflammatory response and successful, productive infection in intracellular vesicles (Álvarez et al., 2016). We assessed the effect of *Piscirickettsia salmonis* in the expression of SsSIGN genes in the macrophage-like SHK-1 cell line. Most notably, the SsSIGN1 receptor was significantly downregulated in the productive infection, an opposite effect to what was observed with ISAV (Fig. 7). It has been shown that *P. sal.* induces IκBα expression, inhibiting NF-κB translocation to the nucleus, which would lead to IL-12 and SsSIGN1 downregulation (Soto et al., 2016). On the other hand, Ap-1 and Sp1 binding sites may also be involved in the downregulation of SsSIGN genes, as it has been described for the phylogenetically-related *Francisella tularensis* (Walters et al., 2015).

4.4. Phylogenetic analysis

DC-SIGN genes have been described in a variety of mammalian species, with recent reports for fish homologs, revealing the wide distribution of this type of gene. Even though they share structural features, namely the presence of a CTLD, distinct differences identify specific homologs. Our phylogenetic analysis was based on the sequence corresponding to the CBD of SIGN genes, which represents the pathogen-interaction domain for the receptor. The phylogenetic tree revealed a clade distribution, with mammals and fishes separated, and primates grouped in a defined cluster (Fig. 8). The structure of the CBD is related to the glycan with which it interacts; as previously discussed, species-specific pathogens may influence the evolution of SIGN genes (Garcia-Vallejo and van Kooyk, 2013; Powlesland et al., 2006). Salmon, like what it has been described in mouse, may display multiple versions of SIGN genes in response to exposure to a wide variety of pathogens. Concomitantly, several immune parameters in teleost fish display more diversity than their mammalian homologs (Rebl et al., 2010). Furthermore, the presence of multiple SIGN homologs in salmon may contribute to fine-tuning of the immune response, regulating mechanisms triggered by other PRRs (i.e., TLRs); fish live in intimate contact with a potentially high amount of microorganisms, so a tightly regulated immune response is a must to avoid deleterious inflammatory responses (Novoa et al., 2009).

To complement our observations, we extended our sequence analyses to other fish species with published assembled genomes. We identified SIGN-like genes in the three analyzed genomes (*Oncorhynchus mykiss*, *Salmo trutta*, and *Danio rerio*) coding for proteins with structural features present in mammalian and *Salmo salar* SIGN genes (Table 4). Interestingly, the identified sequences are located in discrete regions on the fish genomes, with both rainbow and brown trout having eight SIGN homologs, identical to what we describe for *Salmo salar*. These findings reinforce our proposal of a "SIGN cluster" in fish species.

5. Conclusions

We described eight homologs for DC-SIGN receptors in *Salmo salar*. The proteins possess conserved structural features compared to their mammalian counterparts and are differentially expressed and induced during infection. Our work is not only relevant for a better description and knowledge of the salmon immune system, but it can also offer new perspectives regarding prophylaxis development for this species: DC-SIGN-targeted vaccines has become a promising strategy to improve antigen immunogenicity (Hossain and Wall, 2019; van Kooyk et al., 2013).

Functional analyses are still necessary to assess the effective interaction of SsSIGNs with specific pathogens, their sub-cellular localization, and induction/regulation of immune responses; these are immediate objectives to our research group.

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