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Identification and expression modulation of a C-type lectin domain family 4 homologue that is highly expressed in monocytes/macrophages in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The C-type lectin domain containing (CLEC) receptors including CD209 are expressed *in vivo* by monocytes, monocyte-derived macrophages and dendritic cells and by *in vitro* generated monocyte-derived cells. This paper reports the cloning and sequencing of a lectin molecule, CLEC4T1, in rainbow trout that is a homologue of the CLEC4 family. The expression pattern of the CLEC4T1 was investigated *in vivo* after infection with a bacterial pathogen and in cultured macrophages after modulation with microbial mimics. Trout CLEC4T1 was highly expressed in spleen and head kidney following infection with *Yersinia ruckeri*. Expression could also be induced in macrophage cultures by LPS but not by Poly I:C, and suggests that the regulation of CLEC4T1 expression in trout varies according to the nature of the stimulant. A polyclonal CLEC4T1 antibody was generated and validated by Western blotting for use in evaluation of $CLEC4T1^+$ cells by flow cytometry analysis. Freshly isolated adherent trout head kidney cultures, potentially containing macrophages and dendritic cell precursors, showed an increase of $CLEC4T1^+$ cells (assessed by flow cytometry) upon stimulation with recombinant interleukin-4/13A. The results suggest that CLEC4T1 is a useful marker for further characterisation of monocyte derived antigen presenting cells in fish.

1. Introduction

In humans, dendritic cells (DCs), monocytes and macrophages are closely related cells derived from a common progenitor cell, the monocyte/macrophage-DC precursor, in the bone marrow (Geissmann et al., 2010). Monocyte/macrophage-DC precursors can develop into either DCs or macrophages. DCs are a heterogeneous population of cells that can be divided into two subpopulations, the myeloid and the lymphoid lineage. The myeloid lineage consists of precursors of Langerhans cells and interstitial DCs, while thymic DCs and plasmacytoid DCs belong to the lymphoid lineage (Ito et al., 1999). The DC-specific ICAM-3-grabbing non-integrin (DC-SIGN or CD209) is expressed *in vivo* by monocyte-derived DCs and DCs found in several tissues such as the mucosa and lymph nodes, and by *in vitro* generated monocyte-derived DCs. The expression of CD209 has also been studied in tissue macrophages found in noninflamed lymph nodes and in specialized macrophages in the placenta and lung (Bleijs et al., 2001; Soilleux et al., 2002; Granelli-Piperno et al., 2005). CD209 is a type II transmembrane protein that is classified as a C-type lectin (CLEC) domain family 4 member based on the presence of a ligand recognising Ca^{2+}-dependent carbohydrate-recognition domain, as seen in other family members (Geijtenbeek et al., 2000). The CLEC4 family consists of multiple members in humans and mice. The carbohydrate-recognition domain of CD209 is responsible for binding to carbohydrate structures present on pathogens, but also functions as an adhesion receptor through binding of self-antigens (Drickamer, 1999). CD209 was first discovered as a cell surface receptor for the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein, expressed by DCs in the skin and at mucosal surfaces. The receptor is responsible for viral transmission to the main target of HIV-1

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replication, $CD4^+$T cells (Cameron et al., 1992). Previous studies show that DC-SIGN/CD209 expressed by DCs mediates transient adhesion to ICAM-3 expressed by resting naive T cells. The stabilization of the contact zone between DCs and T cells subsequently facilitates the proliferation of T cells (Geijtenbeek et al., 2001).

The CD209 gene family in primates includes CD209 (DC-SIGN), CD209L (L-SIGN) and CD209L2, but CD209L2 is not present in humans. CD209 and CD209L are thought to be derived from the duplication of an ancestral precursor gene and share similar functional activity. However, their expression patterns differ in that CD209L, in contrast to CD209, is not expressed on DCs but is highly expressed on endothelial cells in lymph nodes and liver (Bashirova et al., 2001). In mice, five homologues of human CD209 (DC-SIGN or CD209A, SIGN-related gene 1 or CD209B, SIGN-related gene 2 or CD209C, SIGN-related gene 3 or CD209D and SIGN-related gene 4 or CD209E) were identified from initial screening of mouse cDNA libraries (Park et al., 2001). Although the protein structure is well conserved in the carbohydrate-recognition domain, the mouse homologues seem to have various ligand-binding properties and only one of the genes (CD209A) is highly expressed in splenic DCs and is considered to be equivalent to DC-SIGN in humans (Park et al., 2001).

The existence of cells in fish with resemblance to DCs has been described in zebrafish (*Danio rerio*) (Lin et al., 2009; Lugo-Villarino et al., 2010), medaka (*Oryzias latipes*) (Aghaallaei et al., 2010) and rainbow trout (*Oncorhynchus mykiss*) (Bassity and Clark, 2012). Two recent studies also confirm the presence of antigen presenting cells in Atlantic salmon (*Salmo salar*). Lagos et al. (2012) showed that antigen presenting cells can be induced by CD40L stimulation and Haugland et al. (2012) identified a small population of salmon blood leukocytes/putative DC progenitors, with a potent phagocytic capacity by magnetic bead depletion, using the monoclonal antibody C4B6 (reactive with salmon leukocytes) to fractionate peripheral blood leukocytes. In the present study we describe the identification and expression analysis of a CLEC4-like molecule from rainbow trout (termed CLEC4T1) to aid identification of antigen presenting cells in salmonids. The full length cDNA was sequenced and its expression studied in tissues, cell lines and primary leukocytes in response to immune stimuli. We also generated and validated an anti-trout CLEC4T1 polyclonal antibody for immunological studies. The protein expression in tissues and recombinant interleukin (rIL)-4/13A treated adherent head kidney leukocytes was determined by Western blotting or flow cytometry analysis for a better understanding of the distribution and function of trout CLEC4T1 expressing cells.

2. Materials and methods

2.1. Fish

Rainbow trout weighing approximately 100 g, were maintained in 1 m diameter tanks at the aquarium facilities of the Zoology building, University of Aberdeen, in a recirculation unit supplied with a continuous flow of freshwater at 14 °C. The fish were fed twice a day with commercial trout pellets (EWOS). There were no signs of infection and no mortality in the fish. Fish were anaesthetised using 2-phenoxyethanol (0.05%; Sigma–Aldrich) and killed by destruction of the brain (schedule 1 procedure) prior to tissue harvest. All the experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and Home Office Code of Practice guidance.

2.2. cDNA production

Total RNA was isolated from various rainbow trout tissues by extraction in 1 ml TRI Reagent® (Sigma–Aldrich) and treated with RNase-free DNase I Set (Qiagen) according to the manufacturer's instructions. Ten μg of treated total RNA was reverse transcribed to cDNA using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions, with an $Oligo(dT)_{18}$ primer (0.5 μg/μl) (Fermentas) (Suppl. Table 1). Total RNA isolated from trout head kidney and spleen was also used to obtain cDNA for 5′ RACE (random amplification of cDNA ends) with a GeneRacer™ Kit (Invitrogen), used according to the manufacturer's instructions. To obtain cDNA for 3′ RACE, the same procedures were followed as for the cDNA production for 5′ RACE, with the exception that 1 μl Adaptor $(dT)_{17}$ (0.5 μg/μl) replaced the $Oligo(dT)_{18}$ primer.

2.3. Cloning and sequencing of CLEC4T1 cDNA

The protein sequence of human CD209 (GenBank accession number AAK20997) was used as bait to search the EST database of rainbow trout and Atlantic salmon. Several candidate sequences were obtained, including BX912786, BX870419, EG938110 and EG938111. PCR cloning of the trout CLEC4T1 was performed based on the obtained EST sequences using head kidney cDNA with trout primers CLEC4T1_F and CLEC4T1_R (Suppl. Table 1), designed to the untranslated regions (UTRs). During the cloning of the full length trout CLEC4T1, the full length cDNA sequence of the salmon homologue (GenBank accession number NM_001146583) was deposited in the NCBI database.

For cloning, the PCR was run in a standard 50 μl reaction combining 2 μl of each of the primers (10 μM), 2 μl of cDNA (or in some cases 5′ RACE cDNA or 3′ RACE cDNA), 10 μl of 5 × PCR buffer, 1 μl of $MgCl_2$ (50 mM), 1 μl dNTP (2.5 mM each), 31.75 μl of PCR water and 0.25 μl MangoTaq DNA polymerase (5 U/μl) (Bioline). A GeneRacer™ Kit (Invitrogen) was used to amplify the full-length 5′ end by RACE PCR and the full-length 3′ end was obtained using trout head kidney and spleen cDNA modified for 3′ RACE PCR. The full-length ORF was subsequently sequence confirmed by PCR with gene specific primers (Suppl. Table 1). Products of the expected size were ligated into pGEM®-T Easy Cloning Vector (50 ng/μl) (Promega), and following transformation into competent *E. coli* cells (TOP10F' One Shot®, Invitrogen) plasmid DNA from at least 3 independent colonies was purified and sequenced using the vector specific primers SP6 and T7 (Suppl. Table 1). The full length gene sequence was submitted to the EMBL Nucleotide Sequence Database (GenBank accession number FN667662). The sequence was analysed for similarity with other known sequences using the Basic Local Alignment Search Tool (BLAST, Altschul et al., 1990). Comparisons between sequences were performed using the CLUSTAL W multiple sequence alignment package (Thompson et al., 1994). Phylogenetic analysis was conducted using the Maximum Likelihood method (Jones-Taylor-Thornton substitution model) within the MEGA programme (version 6) (Tamura et al., 2011). The signature of the protein families was analysed using the PROSITE database (Falquet et al., 2002) and potential N-glycosylation sites were analysed using NetNGlyc (version 1.0) (http://www.cbs.dtu.dk/services/NetNGlyc/). The primers used for cloning of CLEC4T1 and real-time quantitative RT-PCR (qRT-PCR) detection of gene expression are detailed in Suppl. Table 1. The primers for qRT-PCR were designed to cross an exon-intron boundary to exclude amplification from potential genomic DNA contamination. Splice sites and exon-intron boundaries were predicted by alignment of genomic DNA sequences obtained from the Atlantic salmon Whole Genome Shotgun Project (NCBI AGKD00000000, version 1) with the corresponding rainbow trout protein sequences using the GeneWise (Wise2 version 2.1.20 stable) program, allowing for introns and frame shifting errors.