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Molecular and functional characterization of the scavenger receptor CD36 in zebrafish and common carp

Inge R. Fink[a], Erica L. Benard[b], Trudi Hermsen[a], Annemarie H. Meijer[b], Maria Forlenza[a], Geert F. Wiegertjes[a,*]

[a] Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, PO Box 338, 6700 AH Wageningen, The Netherlands
[b] Institute of Biology, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands

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ABSTRACT

CD36 is a scavenger receptor which has been studied closely in mammals where it is expressed by many different cell types and plays a role in highly diverse processes, both homeostatic and pathologic. It is among other things important in the innate immune system, in angiogenesis, and in clearance of apoptotic cells, and it is also involved in lipid metabolism and atherosclerosis. Recently, in the cephalochordate amphioxus a primitive CD36 family member was described, which was present before the divergence of CD36 from other scavenger receptor B family members, SCARB1 and SCARB2. Not much is known on the Cd36 molecule in teleost fish. We therefore studied Cd36 in both zebrafish and common carp, two closely related cyprinid fish species. Whereas a single *cd36* gene is present in zebrafish, carp has two *cd36* genes, and all show conserved synteny compared to mammalian CD36. The gene expression of carp *cd36* is high in brain, ovary and testis but absent in immune organs. Although in mammals CD36 expression in erythrocytes, monocytes and macrophages is high, gene expression studies in leukocyte subtypes of adult carp and zebrafish larvae, including thrombocytes and macrophages provided no indication for any substantial expression of *cd36* in immune cell types. Surprisingly, analysis of the *cd36* promoter region does show the presence of several binding sites for transcription factors known to regulate immune responses. Overexpression of carp *cd36* locates the receptor on the cell surface of mammalian cell lines consistent with the predicted topology of cyprinid Cd36 with a large extracellular domain, two transmembrane domains, and short cytoplasmic tails at both ends. Gene expression of *cd36* is down-regulated during infection of zebrafish with *Mycobacterium marinum*, whereas knockdown of *cd36* in zebrafish larvae led to higher bacterial burden upon such infection. We discuss the putative role for Cd36 in immune responses of fish in the context of other members of the scavenger receptor class B family.

1. Introduction

Scavenger receptors are soluble or membrane-associated molecules which have in common the ability to bind chemically modified lipoproteins or other poly-anionic ligands (Plüddemann et al., 2007). Each molecule additionally binds to other ligands, and scavenger receptors are thus functionally diverse. They have been grouped together into eight classes (A-H) where members within a class display structural homology. However, between the eight classes the scavenger receptors are structurally very different. CD36 is a member of the scavenger receptor class B family, which also contains scavenger receptor class B member 1 and 2 (SCARB1 and SCARB2) (Calvo and Vega, 1993; Calvo et al., 1995). SCARB1 is important in lipid metabolism as a receptor mediating cholesterol transfer to and from high density lipoprotein HDL (Brundert et al., 2005; Chadwick and Sahoo, 2013). SCARB2 (also referred to as lysosomal integral membrane protein-2, LIMP-2) is required for the normal biogenesis and maintenance of lysosomes and endosomes and as such plays a role in various human diseases (Gonzalez et al., 2014). CD36 is an integral membrane glycoprotein with two transmembrane domains, a large extracellular loop containing multiple glycosylation sites, and two short intracellular tails. In mammals, CD36 is expressed by many different cell types such as platelets,

Abbreviations: CD36, cluster of differentiation 36; dpf, days post fertilization; dpi, days post injection; hpf, hours post fertilization; LDL, low density lipoprotein; LTA, lipoteichoic acid; PBL, peripheral blood leukocytes; RT-PCR, reverse transcription polymerase chain reaction; RT-qPCR, real-time quantitative polymerase chain reaction; SCARB, scavenger receptor class B; TLR, Toll-like receptor.

* Corresponding author. Tel.: +31 317 482732.
E-mail address: geert.wiegertjes@wur.nl (G.F. Wiegertjes).

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monocytes and macrophages, endothelial cells and hepatocytes, and CD36 seems to play a role in highly diverse processes, both homeostatic and pathologic (Febbraio et al., 2001). Mammalian CD36 was originally identified as a macrophage receptor for oxidized LDL (Endemann et al., 1993), but its ligand-binding ability is very extensive and encompasses endogenous as well as exogenous ligands (reviewed in Silverstein and Febbraio, 2009). Among other molecules, CD36 recognizes microbial diacylglycerides such as macrophage-activating lipopeptide 2 from *Mycoplasma pneumoniae* (MALP-2) and lipoteichoic acid from *Staphylococcus aureus* and acts as a co-receptor for these molecules together with Toll-like receptor (TLR) 2/6 heterodimers (Hoebe et al., 2005; Stuart et al., 2005). As a receptor recognizing exogenous pathogen-associated molecular patterns (PAMPs), CD36 is acknowledged as a pattern recognition receptor (PRR) important in innate immunity.

CD36 orthologs have been described in many vertebrate species such as human, mouse (Endemann et al., 1993), rat (Abumrad et al., 1993), cow (Greenwalt et al., 1990) and chicken (Shu et al., 2011). Ancestral homologs are also found in invertebrate species such as *C. elegans* (Means et al., 2009), sea urchin and fruit fly, and a recent study reported several homologs in amphioxus, a basal chordate (Zhang et al., 2013). Presumably due to amphioxus-specific gene duplication events there are five *cd36* paralogs in this species. These genes are thought to represent the primitive form of scavenger receptor class B before the divergence of CD36, SCARB1 and SCARB2. One of the amphioxus genes was cloned and studied in more detail: its expression was up-regulated in the gills upon feeding and down-regulated during fasting. In addition, studies in Atlantic salmon and rainbow trout have focused on fatty acid transport proteins including Cd36 and described gene expression changes regulated by insulin, fasting and dietary composition (Sanchez-Gurmaches et al., 2011, 2012; Torstensen et al., 2011). This suggests a conservation of the role of SCARB family members in at least nutritional control and/or lipid metabolism. Contrary to the mammalian species, knowledge of the immune function of Cd36 in teleost fish is lacking.

In mammals, there is evidence of CD36 interaction with preformed Toll-like receptor TLR2-TLR6 heterodimers, where CD36 recognizes specific lipids or lipoproteins and brings the ligand to the TLRs. Ligand/receptor clusters localize within lipid rafts of the plasma membrane, after which intracellular signalling and phagocytosis is initiated (Triantafilou et al., 2006). The intracellular signalling goes via MyD88 and a series of phosphorylation and ubiquitination events of cytosolic proteins, leading to activation of the transcription factor NFκB and expression of immune related genes (Akira and Takeda, 2004). Of interest, out of eight accessory molecules classified in mammals as important mediators of ligand delivery and/or recognition and required for TLR function (Lee et al., 2012) only four molecules, including CD36, seem to have clear orthologs in fish genomes (Pietretti et al., 2013). Given our interest in the function of TLRs (Pietretti and Wiegertjes, 2014) among which Tlr2 (Ribeiro et al., 2010), we studied Cd36 in zebrafish and common carp, two closely related cyprinid fish species, taking advantage of the complementary tools available for these two species to achieve a more thorough understanding of the function of Cd36 in teleost fish.

We characterize for the first time in detail Cd36 of zebrafish and two Cd36 molecules of common carp finding a molecular structure consistent with mammalian CD36. Phylogenetic analysis as well as a synteny approach allowed us to confidently designate the cyprinid molecules as homologs of the mammalian CD36. Gene expression was studied in tissues and leukocyte subtypes, indicating a complete absence of *cd36* basal gene expression in immune organs and leukocytes of adult carp. However, knockdown of *cd36* in zebrafish embryos by a morpholino approach and subsequent *Mycobacterium marinum* infection indicated a role for Cd36 in controlling bacterial burden. We discuss the possibility that the division of functions between the Cd36 family members Cd36, Scarb1 and Scarb2, all of which are present in teleosts, may not be exactly the same as in their mammalian counterparts.

2. Materials and methods

2.1. Animals

European common carp (*Cyprinus carpio carpio* L.) were reared in the central fish facility Carus, at Wageningen University, Wageningen, Netherlands. Fish were kept at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain) (Irnazarow, 1995). Carp were between 9 and 11 months old at the start of the experiments. All studies were performed with approval from the local animal welfare committee (DEC) of Wageningen University.

Zebrafish were handled in compliance with the local animal welfare regulations, maintained according to standard protocols (zfin.org), and culture was approved by the local animal welfare committee (DEC) of Leiden University. Embryos from the zebrafish AB/TL line were used for the infection experiments. Embryos were grown at 28.5 °C in egg water (60 μg/mL Instant Ocean sea salts). For the duration of bacterial injections and stereo fluorescence imaging embryos were kept under anaesthesia in egg water containing 200 μg/mL tricaine methane sulfonate (Sigma-Aldrich, St. Louis, MO, USA). Embryos used for stereo fluorescence imaging were kept in egg water containing 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich) to prevent melanisation which can produce background fluorescence during imaging. For fluorescence activated cell sorting (FACS) isolation of macrophages and neutrophils from zebrafish larvae, the transgenic lines *Tg(mpeg1:mCherry-F)*^{UMSF001} (Bernut et al., 2014) and *Tg(mpx:egfp)*^{i114} (Renshaw et al., 2006) were used, respectively.

2.2. Organ isolation

Carp aged 9-11 months were euthanized with 0.3 g/L tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.6 g/L $NaHCO_3$. Carp were bled from the caudal vein using a needle and syringe containing cRPMI medium (RPMI 1640 with 25 mM HEPES, (Lonza, Basel, Switzerland) adjusted to an osmolality of 280 mOsm/kg with sterile water) containing 50 U/mL heparin (Leo Pharma, Ballerup, Denmark), 50 U/mL penicillin G (Sigma-Aldrich), and 50 μg/mL streptomycin sulphate (Sigma-Aldrich). For isolation of peripheral blood leukocytes (PBL), the heparinized blood was centrifuged at 100 × *g* for 5 min at 4 °C and then another 5 min at 300 × *g*. The buffy coat was collected, carefully layered on Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) and centrifuged at 800 × *g* for 25 min at 4 °C without brake. The leukocyte layer was collected and washed twice with cRPMI. The obtained PBL were stored at −80 °C until used for RNA isolation. After bleeding the fish, the organs of interest were aseptically removed and immediately frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation.

Zebrafish embryos for RNA isolation were snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. Isolation of leukocyte subtypes

Carp leukocyte subtypes were isolated by density gradient separation and/or magnetic cell sorting using specific antibodies as