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# Immune-relevant thrombocytes of common carp undergo parasite-induced nitric oxide-mediated apoptosis

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ABSTRACT

Common carp thrombocytes account for 30–40% of peripheral blood leukocytes and are abundant in the healthy animals' spleen, the thrombopoietic organ. We show that, *ex vivo*, thrombocytes from healthy carp express a large number of immune-relevant genes, among which several cytokines and Toll-like receptors, clearly pointing at immune functions of carp thrombocytes. Few studies have described the role of fish thrombocytes during infection. Carp are natural host to two different but related protozoan parasites, *Trypanoplasma borreli* and *Trypanosoma carassii*, which reside in the blood and tissue fluids. We used the two parasites to undertake controlled studies on the role of fish thrombocytes during these infections. *In vivo*, but only during infection with *T. borreli*, thrombocytes were massively depleted from the blood and spleen leading to severe thrombocytopenia. *Ex vivo*, addition of nitric oxide induced a clear and rapid apoptosis of thrombocytes from healthy carp, supporting a role for nitric oxide-mediated control of immune-relevant thrombocytes during infection with *T. borreli*. The potential advantage for parasites to selectively deplete the host of thrombocytes via nitric oxide-induced apoptosis is discussed.



## 1. Introduction

Thrombocytes are found in all non-mammalian vertebrates such as birds, reptiles, amphibians and fish. Thrombocytes are thought to be the nucleated equivalents of platelets in mammals; they have haemostatic functions, circulate in the blood and participate in the formation of blood clots through aggregation when the vasculature is damaged. Indeed, teleost fish do not contain anucleated platelets in their vascular system, but thrombocytes (Jagadeeswaran et al., 1999; Khandekar et al., 2012; Rombout et al., 1996). Common carp (*Cyprinus carpio* L.) thrombocytes were described almost two decades ago, when a mouse monoclonal antibody (WCL6) was used to study morphology, function and origin of this cell type. Carp thrombocytes were shown to have a round to spindle-shaped morphology with a cytoplasm that contains numerous vesicles that occasionally open to the cell surface consistent with the presence of a surface-connected canalicular system (Rombout et al., 1996). The latter is also seen in mammalian platelets where the surface-connected canalicular system serves as the pathway for transport of substances into and out of the cells (Escolar and White, 1991). Thrombocytes in carp account for 30–40% of peripheral blood leukocytes (PBL)[1], are also found in head kidney, thymus and intestine and are highly abundant in the spleen. The spleen is considered the thrombopoietic organ in carp (Rombout et al., 1996).

It is becoming increasingly evident that mammalian platelets have immune functions (reviewed in Semple et al., 2011). Platelets can produce antibacterial proteins such as defensins (Krijgsveld et al., 2000), express cytokines and chemokines (McRedmond et al., 2004), interact with leukocytes such as dendritic cells and neutrophils (Clark et al., 2007; Kissel et al., 2006), and express Toll-like receptors (TLRs) through which they can actively bind bacteria and their products (Aslam et al., 2006). Studies in chicken suggest that also thrombocytes have immune functions including reports on the expression of immune genes and the ability to phagocytose (Chang and Hamilton, 1979; St. Paul et al., 2012). In rainbow trout, thrombocytes express genes involved in antigen presentation and immune

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[1] List of abbreviationsAP-SS: *N*-acetyl-D,L-penicillamine disulphide, IL: interleukin, MACS: magnetic-activated cell sorting, MFI: mean fluorescence intensity, MH: major histocompatibility, NO: nitric oxide, PBL: peripheral blood leukocytes, PE: phycoerythrin, RT-qPCR: real-time quantitative PCR, SNAP: *S*-nitroso-*N*-acetylpenicillamine, SOCS: suppressor of cytokine signalling, TLR: Toll-like receptor, w.p.i.: weeks post-infection.

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regulation (Kollner et al., 2004), whereas phagocytosis by thrombocytes was recently claimed a conserved innate immune mechanism in lower vertebrates, including several fish species (Nagasawa et al., 2014). Despite these indications for immune function of thrombocytes, not many studies have described the role of fish thrombocytes during infection.

Carp are natural host to two different but related protozoan parasites, *Trypanoplasma borreli* and *Trypanosoma carassii* that reside in the blood and tissue fluids. Parasitic infections can cause a massive increase in spleen size during infection (Bunnajirakul et al., 2000). Although related, *T. borreli* and *T. carassii* induce fundamentally different immune responses in the carp host (reviewed in Forlenza et al., 2011). For example, in *T. borreli*-infected carp, gene expression profiles show up-regulated expression of cytokines including *ifnγ* (Stolte et al., 2008) and *tnfα* (Forlenza et al., 2009). At the same time, *inos* gene expression is up-regulated in head kidney, spleen, PBL and liver leading to NO production and a dramatic increase in serum nitrite levels and tissue nitration (Forlenza et al., 2008b; Joerink et al., 2006; Saeij et al., 2000, 2002). In contrast, during *T. carassii*-infections, the production of pro-inflammatory cytokines is negligible (Joerink et al., 2006) and NO production is not induced (Saeij et al., 2002). Instead, *T. carassii* infections are characterised by *arginase* gene expression and activity (Joerink et al., 2006), increased numbers of splenic neutrophils, as well as a cytokine profile associated with a Th17-like immune response (Ribeiro et al., 2010b). The possibility to adjust parasite dose and route of injection facilitates tightly-regulated infections in the laboratory, allowing for controlled *in vivo* studies into the effects of experimental infection on the thrombocyte population.

We have used a well-characterised monoclonal antibody to purify populations of carp thrombocytes and determine their immune gene profile by real-time quantitative PCR *ex vivo*. We studied, in particular, the expression of pro-and anti-inflammatory mediators and expression of Toll-like receptors. We used the *in vivo* experimental infection models with *T. borreli* and *T. carassii* to study the differential effect of these parasites on the thrombocyte population in spleen by histology and the effect on thrombocytes in peripheral blood by flow cytometry. To explain the massive depletion of thrombocytes seen during *T. borreli* infections, we studied the effect of NO, as the hallmark immune modulator induced by this parasite, on apoptosis of thrombocytes *ex vivo*. Our data provide evidence for a strategy of *T. borreli* parasites to selectively deplete the host of thrombocytes via, at least, a nitric oxide-mediated apoptosis. Carp thrombocytes are immune-relevant and may play an important role in immune responses to pathogens.

## 2. Material and methods

### 2.1. Animals

European common carp (*Cyprinus carpio carpio* L.) were reared in the central fish facility Carus, at Wageningen University, Wageningen, The Netherlands. Fish were kept at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Sniff, Soest, Germany) daily. R3 × R8 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 of the animal experimental committee of Wageningen University.

### 2.2. Infection of carp with protozoan parasites

Infection of carp with *Trypanoplasma borreli* and with *Trypanosoma carassii* was performed as described previously (Forlenza et al., 2008a; Joerink et al., 2006). At least 2 weeks before the start of the experiment fish were moved to the infection quarantine facilities and acclimatised to 20 °C, the optimal temperature for parasite growth. Carp were injected i.p. with $10^4$ parasites per fish in 100 μL, or with PBS as non-infected controls. At time of sampling, carp were euthanised with 0.3 g/L tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, United States) buffered with 0.6 g/L $NaHCO_3$. Carp were bled from the caudal vein using a syringe containing cRPMI (RPMI 1640 with 25 mM HEPES (Lonza, Basel, Switzerland), adjusted to an osmolality of 280 mOsm/kg with water) containing heparin (50 U/mL, Leo Pharma, Ballerup, Denmark), and the spleen was aseptically removed. Parasitaemia was determined by counting the number of parasites in a small aliquot of each blood sample in a Bürker counting chamber.

### 2.3. Magnetic-activated sorting of thrombocytes

For analysis of immune gene expression and for *ex vivo* assays on thrombocytes, cells were purified from PBL of healthy fish by magnetic-activated cell sorting (MACS) with the help of a well-characterised thrombocyte-specific monoclonal antibody (WCL6 (Rombout et al., 1996)). Thrombocytes were stained with WCL6 (diluted 1:100) followed by phycoerythrin (PE)-conjugated goat anti-mouse (diluted 1:75, DAKO, Glostrup, Denmark) as secondary antibody. After washing twice, 10 μL of magnetic beads (anti-PE MicroBeads, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was added per $10^8$ cells. After 15 min incubation at 4 °C, cells were washed twice and resuspended in cRPMI. Magnetic separation was performed using LS Columns and a MidiMACS Separator (Miltenyi Biotec) according to the manufacturer's instructions. The $WCL6^+$ fraction was analysed by flow cytometry (Beckman Coulter Epics XL-MCL, Brea, CA, United States) to assess their purity, which exceeded 90%. Purified thrombocytes were either lysed in RLT buffer (QIAGEN, Venlo, The Netherlands) for RNA isolation or were seeded in 96-well plates for *ex vivo* apoptosis analysis (see description later).

### 2.4. RNA isolation and cDNA synthesis

Total RNA was extracted using the RNeasy Mini kit according to the manufacturer's protocol (QIAGEN) including on-column DNase treatment with the RNase-free DNase set (QIAGEN). Final elution was performed with 30 μL nuclease-free water. RNA concentrations were measured by spectrophotometry (Nanodrop, Thermo Scientific, Waltham, MA, United States) and stored at –80 °C until use. Prior to cDNA synthesis, 250 ng–2 μg of total RNA was subjected to a second DNase treatment by using DNase I Amplification Grade (Invitrogen, Carlsbad, CA, United States). Synthesis of cDNA was performed with Invitrogen's SuperScript III Reverse Transcriptase and random hexamers, according to the manufacturer's instructions. For all samples a non-reverse transcriptase control was included. cDNA samples were diluted 25 times in nuclease-free water before their use as templates in real-time PCR experiments.

### 2.5. Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed in a Rotor-Gene 6000 with a 72-well rotor (Corbett Research, QIAGEN) with the ABsolute QPCR SYBR Green Mix (Thermo Scientific, Waltham, MA, United States) as detection chemistry. All primers were from Eurogentec (Liège, Belgium) and are listed in Table 1. Some primers were designed for this study based on information from the carp genome PRJNA73579 (Henkel et al., 2012). All primers were used at a 300 nM final concentration. Master-mix for each RT-qPCR run was prepared as follows: per reaction, 2 μL of forward and reverse primer stock was mixed with 7 μL of SYBR Green. To 9 μL of master mix, 5 μL of diluted cDNA was added in a 0.1 mL tube. The following amplification program was used: one denaturation step of 15 min at 95 °C; followed by 40 cycles of a three-step amplification (15 s