Through the looking glass: trypanosome infections in zebrafish

—— Seeing is believing ——

Éva Dóró

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1

CHAPTER

General Introduction

Trypanosomes

Trypanosomes of the *Trypanosoma* genus are unicellular eukaryotic flagellate pathogens causing diseases in many if not all vertebrate species, from fish to mammals. Since their early discovery in the 19th century trypanosomes have become important (model) organisms for cell biology and biomedical studies, owing to their unicellular eukaryotic nature and their importance as disease-causing agents in animals and people worldwide. Trypanosomes are transmitted to their host by a vector, most often an arthropod vector, which requires great adaptation of these parasites to different environments. During their life cycle they are present in epimastigote form in the vector, while in the final host they transform into trypomastigote forms although, depending on the trypanosome subspecies, further forms have been described based on distinct molecular and morphological features. For a long time now, the cell cycle, motility, developmental plasticity and immune evasion mechanisms typical of trypanosomes have attracted the attention of biologists trying to unravel fundamental aspects of trypanosome cell biology and pathogenicity.

Stercorarian trypanosomes are transmitted by a variety of blood-sucking insects and develop in the insect gut until excreted into the faeces (stercus (Latin) = manure, thus stercorarian means 'of faecal origin'). Via the faeces of the insect these parasites can then contaminate the skin of the vertebrate host, penetrate the skin and spread throughout the whole body. The best-known stercorarian parasite is *Trypanosoma cruzi*, the causative agent of Human American Trypanosomiasis, or Chagas' disease. Upon infection, after a short period during which the parasite resides in the blood of the host, these trypanosomes effectively escape detection by the immune system by hiding intracellularly in cells of various tissues. Salivarian trypanosomes on the other hand are trypanosomes that are transmitted to their main host through insect saliva, in this case tsetse flies (*Glossina* spp.). The best-characterised salivarian parasites are of the *Trypanosoma brucei* species, best known for causing the potentially fatal Human African Trypanosomiasis, also called Sleeping Sickness, but also known for causing Animal Sleeping Sickness in livestock. *T. brucei* species has 3 subspecies: *T. b. brucei* (livestock infectious), *T. b. gambiense* and *T.*

b. rhodesiense (human infectious). As the infection progresses parasites enter the central nervous system causing neurological symptoms which give the name to sleeping disease.

Within the family Trypanosomatidae, research shows evidence of multiple independent adaptation events of the hosts to the different intra- and extracellular lifestyles of trypanosomes, resulting in different immune evasion mechanisms. For example, some but not all Salivarian species show antigenic variation, a well-studied immune evasion mechanism based on the sequential switching of expression of the major (variant) surface glycoprotein (VSG). Antibodies generated against the first-expressed VSG typically are ineffective against a sequentially-expressed VSG protein. In contrast, cattle-infective *T. theileri* does not show antigen variation but has a completely different cell surface composition, containing a mixture of proteins encoded by four novel large and divergent gene families and by members of a major surface protease gene family distinct from the uniform VSG coat (Kelly et al., 2017).

In fact, there have been numerous mechanisms implicated to contribute to long-term survival of trypanosomes in the host's blood, including presence of proteases on the trypanosome's surface, secretion of immunosuppressive mediators as well as presence of a thick coat of highly glycosylated GPI-anchored proteins (Stijlemans et al., 2016). Since the vast majority of trypanosomes do not hide intracellularly nor display antigenic variation but are perfectly able to establish and maintain long-term infections in their vertebrate host, there is a continuing interest in the unique mechanisms of immune evasion displayed by specific trypanosomes in different vertebrate hosts.

Figure 1. Phylogenetic tree of kinetoplastids.

Higher-level classification of kinetoplastids was used. Red-blue lines indicate unknown status; circles denote single or a few known representatives of a particular clade; triangles denote several known representatives of a particular clade. In the squares are the clades in which the fish *Trypanosoma carassii* and *Trypanoplasma borreli*, the focus of this thesis, are found. Modified from (Simpson et al., 2006).

There are many different trypanosomes and they can affect virtually all vertebrate species, from warm-blooded mammals and birds to cold-blooded reptiles, amphibians and fish (**Figure 1**). The aquatic clade within the genus *Trypanosoma* includes more than 200 different trypanosomes affecting fresh and marine fishes and includes one of the most studied fish trypanosomes, *Trypanosoma carassii* (Overath et al., 1999; Woo and Black, 1984), a sister species to the Stercorarian and Salivarian clades and a major subject for study in this thesis. *T. carassii* has also been named *T. danilewskyi* and has, similar to other trypanosomes, a single flagellum. Also well-studied in fish but more distantly related is the biflagellate *Trypanoplasma borreli* (Black et al., 1997), which belongs to the 'Fish Cryptobia' clade of the *Parabodonida* (**Figure 1**). Both *T. carassii* and *T. borreli* are extracellular parasites naturally infecting cyprinid fish such as common carp (*Cyprinus carpio*) or goldfish (*Carassius auratus*) but can also infect non-cyprinid fish species; upon transmission by blood-sucking leeches they remain exclusively extracellular, multiplying in the blood and tissue fluids of their host. They cause pathological

features typical of infections with certain mammalian trypanosomes including symptoms such as anaemia, splenomegaly and polyclonal B and T cell proliferation (Katzenback et al., 2013; Saeij et al., 2003, 2002; Woo and Ardelli, 2014). Both parasites can establish and maintain long-term infections without evidence of VSG-mediated antigenic variation (Haag et al., 1998; Overath et al., 1999), (this thesis). Interestingly, some cell surface proteins of *T. carassii* have been identified that may play a role in modulation of immune responses or immune evasion, such as GPI-anchored (mucin-like) glycoproteins, trans-sialidase-like proteins and a member of the major surface protease (MSP), GP63 (Aguero et al., 2002; Oladiran and Belosevic, 2012). The blood-dwelling trypanosomes and (trypanoplasms) found in fish, typically transmitted by leeches, belong to interesting clades in the evolution of trypanosomes with respect to studies into their mechanisms of immune evasion.

Flagellar motility

T. carassii are trypanosomes with a single flagellum whereas *T. borreli* are trypanoplasms with two flagella (**Figure 2**).

Figure 2. Scanning electron microscopy image of *Trypanosoma carassii* and *Trypanoplasma borreli*. Note the difference in body shape and the presence of one cell-attached (recurrent) flagellum in *T. carassii* (left) and two flagella in *T. borreli* (right). In this image, the recurrent, cell-attached flagellum of *T. borreli* protrudes at the top, whereas the non-cell-attached free flagellum protrudes at the bottom.

Flagellar motility was shown to be essential for the survival of mammalian trypanosomes in the blood of the host because indispensable for replication (Broadhead et al., 2006; Langousis and Hill, 2014; Shimogawa et al., 2018). Motility also has been shown essential for progression of the trypanosome life cycle by mediating migration between the gut and salivary glands of the tsetse fly vector (Gibson and Bailey, 2003; Rotureau et al., 2014). And last but not least, besides being required for parasite migration, replication and morphological adaptation to different microenvironments, flagellar motility has been implicated in immune evasion (Krüger and Engstler, 2015; Stellamanns et al., 2014). For example, *in vitro* studies suggest that *T. brucei* can clear antibodies from the surface by using hydrodynamic drag forces generated upon directional swimming, which cause VSG-bound antibodies to accumulate in the flagellar pocket and be eliminated by endocytosis. Therefore, swimming speed associated with flagellar motility was shown to be an important element of antibody clearance (Engstler et al., 2007). Interestingly, our group previously showed that the fish parasite *T. borreli* probably uses a similar method of surface-bound antibody clearance, even though this parasite does not display VSG on its surface (Forlenza et al., 2009b). This suggested that motility-mediated endocytosis of surface-bound molecules could be considered a conserved mechanism implicated in immune evasion and used by different trypanosome parasites. Additional *in vivo* studies addressing flagellar motility, however, showed somewhat contradicting results in that motility-mutant parasites remained infectious (Krüger and Engstler, 2015). No matter what, the importance of the flagella for trypanosome biology should not be underestimated: among the key elements implicated in the pathogenesis of trypanosomes, flagellar motility may be considered one of the most crucial ones.

Several if not most of the arguments above that describe the importance of flagellar motility have been obtained from *in vitro* experiments. *In vivo* models that could directly visualise rather than mimic trypanosome motility could prove highly informative. *In vivo* studies in mice using transgenic parasites expressing fluorescent markers or luciferase, owing to the lack of transparency of the host, have shown more suitable for *in vivo* localization and quantification of parasites than for visualization of

motility and associated swimming behaviours (Silberstein et al., 2018). Yet, these studies showed that the skin represents a significant anatomic reservoir of infection and did lead to the suggestion that there is a need for revision of current diagnostic methods, especially in asymptomatic individuals (Capewell et al., 2016). Recently, using multicolour light sheet fluorescence microscopy, swimming behaviour of *T. brucei* *in vivo* could be visualised in the tsetse fly (Schuster et al., 2017). This real-time imaging approach revealed that the same parasite stage can adopt several swimming behaviours within the same or different tissues of the vector. Further, collective swimming, a phenomenon which was previously observed only *in vitro*, could also be seen *in vivo* in the midgut of the insect vector, confirming the relevance of *in vivo* studies on parasite motility. There is no doubt that *in vitro* studies focusing on flagellar motility have brought a wealth of information on qualitative and quantitative aspects of swimming behaviour of trypanosomes and have suggested potentially novel immune evasion mechanisms. Nevertheless, the possibility to visualize parasite swimming behaviour and host-pathogen interaction *in vivo* could greatly expand our knowledge of trypanosome biology.

Zebrafish

Zebrafish is a cyprinid fish species phylogenetically close to common carp and goldfish (Figure 3), both of which are natural hosts to *T. carassii* and/or *T. borreli*. They all live at the same temperature range and all are fresh water species. Typically, zebrafish larvae, and to some extent also juveniles and adults are transparent, which facilitates visualization of biological processes in live animals, a hallmark that made zebrafish an important vertebrate animal model species. Further, the availability of numerous mutant and transgenic zebrafish lines marking relevant immune cells allows for visualisation of immune responses in live animals, another great aspect of this animal model.

Figure 3. Phylogenetic relationships between fishes. Zebrafish (*Danio rerio*) is a small cyprinid freshwater fish that belongs to the *Danio* genus of the Cyprinidae family in the order of Cypriniformes. Zebrafish is phylogenetically close to common carp and goldfish. Modified from (Ota and Abe, 2016).

There is no doubt that over the past 30 years, zebrafish has become an important laboratory animal species and zebrafish has served as a model for studies on developmental biology (Scholz, 2012), genetics (Becker and Rinkwitz, 2012), toxicology (Dai et al., 2014), neurobiology (Rinkwitz et al., 2011), behavioural studies (Teles and Oliveira, 2016), biomedical research (Tavares and Santos Lopes, 2013) and many others (Das et al., 2013; Quaife et al., 2012; Santoro, 2014; Wilkinson and van Eeden, 2014). This success is due to several features that are uniquely combined in this animal model. Highly relevant for the studies reported here; the immune system develops sequentially, allowing for studies focusing on innate immune responses when using zebrafish larvae up to 5 days post fertilization (dpf), but also allowing for studies focusing

on both innate and adaptive immune responses when using fish older than 3 weeks of age. Further, the availability of a well-annotated genome has allowed for detailed gene expression studies, genetic manipulation using gene knockdown with morpholinos or RNA interference (Sifuentes-Romero et al., 2011; Timme-Laragy et al., 2012), targeted mutagenesis or gene knockout using the latest TALENS or CRISPR approaches, and has allowed for the generation of an ever increasing number of transgenic lines marking immune cells (Ellett et al., 2011)(Renshaw et al., 2006), neurons (Higashijima et al., 2000), endothelial cells (Gore et al., 2012), to name a few. In need, currently over 38.000 lines are reported in the Zebrafish International Resource Center (ZIRC) online database. Finally, and perhaps most importantly, the combination of above-mentioned genetic tractability with the optical transparency of the embryo (and to some extent juvenile and some adult stages) and accessibility for *in vivo* imaging and manipulation (owing to *ex utero* development), make the zebrafish an excellent animal model in many fields of human and animal biology.

Disease models in zebrafish

Several are the examples of how zebrafish (disease) models have already contributed to the biomedical field by leading to the discovery of novel mechanisms of disease pathogenesis, that have now been validated also in humans (reviewed in the special issue <http://dmm.biologists.org/content/7/7>). To briefly illustrate, the group of L. Ramakrishnan used the *Mycobacterium marinum*-zebrafish model (Tobin and Ramakrishnan, 2008) to identify a novel role for Lta4h-dependent lipoxin signalling in mycobacterial pathogenesis (Tobin et al., 2010). Based on these findings, *LTA4H* variants were investigated in human patients showing a clear link between *LTA4H* genotype and disease susceptibility. Such strong correlation paved the way for host genotype-specific tuberculosis therapy (Tobin et al., 2012). Similarly, the group of L. Zon performed a large chemical screen in zebrafish larvae leading to the identification of leflunomide, an inhibitor of pyrimidine synthesis, which was shown to be a strong inhibitor of melanoma cell growth. The combination of

leflunomide and Braf inhibitors in the treatment of advanced metastatic melanoma is now in Phase II clinical trials (reviewed in (Ablain and Zon, 2013)). Such clinical examples formed the scientific inspiration to start the Initial Training Network (ITN) named FishForPharma, funded by the 7th Framework programme of the European Community.

In the period of 2012-2016 the FishForPharma project on 'Zebrafish Infection Models for Pharmaceutical Screens' brought together more than 10 leading European research groups and industrial partners all sharing an interest in using zebrafish to establish or further characterize different infection models. In order to facilitate drug development, for example based on high-throughput drug-screening methods in zebrafish embryos, it was deemed essential to have a better understanding of the underlying mechanisms of diseases. Central to the development of animal infection models in zebrafish was the assumption that zebrafish could provide a complementary platform for scientific questions that would be difficult, impossible or (too) expensive to answer in mammalian animal models based on thorough elucidations of mechanisms of pathogenicity. To this end, 11 PhD students and 3 post-doctoral fellows were recruited to develop and/or study drug-screening methods relevant to infection with bacteria (*M. marinum*, *Escherichia coli*, *Burkholderia*, *Staphylococcus aureus*), viruses (Infectious Hematopoietic Necrosis Virus, Chikungunya virus) or protozoan parasites (*T. carassii* or *T. borreli*, this thesis).

Immune responses against fish kinetoplastids

At the start of the project no zebrafish trypanosome infection model existed, but extensive experience over the past years with common carp as a host to *T. borreli* and *T. carassii* did exist. For example, based on *in vitro* and *in vivo* infections it was shown that whereas infection with *T. borreli* would lead to classical inflammatory responses characterized by the induction and omnipresence of nitric oxide and pro-inflammatory cytokines (Joerink et al., 2006; Ribeiro et al., 2010; Saeij et al., 2002; Wiegertjes and Forlenza, 2010), *T. carassii* would promote an anti-inflammatory state of macrophage activation characterized by increased arginase activity and upregulation of regulatory cytokines (Joerink et al., 2006). Although these

parasites thus triggered substantially different innate immune responses in carp, they both induced polyclonal B cell activation and in both cases the infection seemed to be controlled by a combination of complement and final presence of sufficiently high concentrations of parasite-specific antibodies (Joerink et al., 2007; Katzenback et al., 2013; Saeij et al., 2003).

One of the most remarkable observations was linked to *T. borreli* infection of carp and was related to the discovery of an important protective role of membrane Tumour Necrosis Factor- α (mTnfa) (Forlenza et al., 2009a) (**Figure 4**). Our group showed that soluble Tnfa was involved in both protection and immunosuppression of the host immune system whereas the membrane form of Tnfa played a pivotal role in protection and parasite clearance. Injection in highly infected fish of a TACE (TNFa converting enzyme) inhibitor, supposed to block cleavage of mTnfa, lead to total recovery from severe infection levels within 3 days. However, since TACE can exert effects not only on Tnf, but also on other membrane proteins such as EGFR ligands, proinflammatory cytokines TNF Receptors, adhesion molecules and the amyloid precursor protein (Black et al., 1997; Garton et al., 2003; Lammich et al., 1999; Reddy et al., 2000), the exact events and mediators involved in total recovery have not been described in detail, and exact role of mTnfa remained unknown.

Figure 4. Tumour Necrosis Factor-alpha (Tnfa) signalling. Tnfa is a pleiotropic cytokine secreted by and acting on several immune and somatic cell types. Tnfa is transported to the cell surface where it forms a homotrimer. Tnfa converting enzyme (TACE) cleaves the membrane form of Tnfa which results in the release of soluble Tnfa (sTnfa). TACE is a zinc metalloproteinase, also known as ADAM17. Both, the membrane and soluble forms are bioactive. sTnfa and membrane Tnfa (mTnfa) both can bind to either Tnfr1 or Tnfr2, which triggers specific signalling cascades (direct signalling) in the receptor-expressing cell leading to Apoptosis, cell proliferation or cytokine production. Alternatively, upon interaction with the receptor, mTnfa itself can also deliver a signal in the mTnfa-expressing cell (reverse or bidirectional signalling). The exact mechanisms and signalling molecules involved in this process, are still ill defined. Figure modified from (Horiuchi et al., 2010).

Aim of this thesis

The aim of this thesis was to develop an *in vivo* trypanosome infection model in zebrafish using a fish-specific trypanosome, to study different aspects of trypanosome biology, the interaction between parasite and the host, and for the first time, trypanosome swimming behaviour *in vivo* in the natural environment of a vertebrate host.

Thesis outline

In this thesis, I describe how we set out to establish a host-parasite model for trypanosome infection in zebrafish and how we aimed to take advantage of this well-established animal model and its benefits, which include sequential development of the immune system, transparency of early life stages and availability of transgenic lines marking important immune cell types (**Chapter 1, General introduction**).

Among all elements key to the pathogenesis of trypanosomes, flagellar motility may be one of the most important ones. There is no doubt that *in vitro* studies focusing on flagellar motility have brought a wealth of information on qualitative and quantitative aspects of trypanosome's swimming behaviour and associated novel immune evasion mechanisms. However, the possibility to visualize parasite swimming behaviour *in vivo*, in the host environment, can greatly expand our knowledge of trypanosome biology and behaviour. **Chapter 2** therefore provides a detailed description of *T. carassii* swimming behaviour *in vivo* in zebrafish. This is the first report of trypanosomes swimming in the natural environment of a vertebrate host. In this chapter we do not only report the swimming behaviour of trypanosomes in the complex and dynamic environment of the blood and tissues of the vertebrate host, but report a novel 'whip-like' movement and backward swimming behaviour that allow the parasite to invert direction and finally identify the posterior end of the parasite as a novel anchoring site *in vivo*.

The majority of trypanosomes do not hide intracellularly nor do they display antigenic variation, and *Trypanosoma carassii* is no exception. Yet, these parasites are perfectly able to establish and maintain long-term infections in their vertebrate host, not only in carp but also in zebrafish, stimulating interest in the mechanisms of immune evasion displayed by these blood-dwelling trypanosomes. In **Chapter 3** we provide a first insight into the mechanisms of trypanosome-host immune interaction between *T. carassii* and especially, macrophages of zebrafish. Using various transgenic lines marking neutrophils and macrophages we describe a differential immune response of these two innate cell types and identified foamy macrophages as potentially involved in the pathology and exacerbation of the infection. The aquatic clade within the genus *Trypanosoma* includes several

trypanosome species affecting fish, with *T. carassii* being one of the best-characterized trypanosomes. Under natural circumstances, this extracellular blood-dwelling parasite naturally infects cyprinid fish via transmission by blood sucking leeches. The pathological features naturally associated with experimental infections includes features typical of many other trypanosome infections. **Chapter 4** provides a *de novo* transcriptome assembly and comparative bioinformatic analysis of genes and proteins expressed by *T. carassii*, identifying several important surface proteins potentially involved in immune evasion mechanisms.

Our group previously showed that soluble carp Tnfa was involved in both protection and immunosuppression of the host immune system, whereas the membrane form of Tnfa played a pivotal role in protection and parasite clearance. Injection in highly-infected fish of a TACE inhibitor, supposed to block cleavage of mTnfa, lead to total recovery from severe infection levels within 3 days. The genetically-amenable zebrafish provided a great opportunity to further investigate the role of mTnfa *in vivo*, but these studies were complicated by the fact that zebrafish not only have 2 paralogues of *tnfa* genes located on two different chromosomes (*tnfaa* and *tnfab*; called *tnfa* and *tnfb* in this thesis), and by the fact that the exact cleavage site of TACE in the Tnf sequences was unknown. Because TACE can target several proteins other than mTnfa, a different approach was taken based on the generation of constructs encoding for uncleavable Tnfa. **Chapter 5** describes the technical approach taken to aim at studies that would evaluate *in vitro* Tnf receptor preferences and binding properties of zebrafish Tnfa and Tnfb and evaluate *in vivo* the role of mTnfa during trypanosome infection.

Finally, in **Chapter 6** I discuss how the combination of genetic tractability with optical transparency and accessibility for imaging make the zebrafish an excellent animal model for studies on trypanosomiasis, complementary and sometimes superior to existing animal models. I discuss all approaches taken and described in this thesis and put these in context to the trypanosome infection model of zebrafish.

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2

CHAPTER

Visualising trypanosomes in a vertebrate host reveals novel swimming behaviours, attachment mechanisms and adaptation strategies

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Abstract

Trypanosomes are excellent models to study evolutionary biology of eukaryotic cells and are important disease agents of humans, livestock and cold-blooded species, including fish. Instrumental to their pathogenesis is their morphology, motility and adaptation to the host's environments. Yet, the behaviour of trypanosomes within a vertebrate host is currently unknown. In this study, we describe an infection model in zebrafish (*Danio rerio*) based on experimental infection with *Trypanosoma carassii*. By combining high spatio-temporal resolution microscopy with the transparency of zebrafish, we describe in detail the swimming behaviour of trypanosomes in blood and tissues of a vertebrate host. Besides the conventional tumbling and directional swimming, we report that *T. carassii* can change direction through a 'whip-like' motion or by backward swimming, and identify its posterior end as an anchoring site in vivo. To our knowledge, this is the first report of a vertebrate infection model that allows detailed imaging of trypanosome swimming behaviour in vivo in the natural host environment.

Introduction

Trypanosomes are protozoan flagellate pathogens; most species proliferate in the blood and tissue fluids of their host. Trypanosomes are best known as the causative agents of Human African Trypanosomiasis (Sleeping Sickness) or American trypanosomiasis (Chagas Disease). However, trypanosomes infect animals from all vertebrate classes, including warm-blooded mammals and birds as well as cold-blooded amphibians, reptiles and fish (Simpson et al., 2006).

African trypanosomes, such as *Trypanosoma brucei*, live exclusively extracellular and are continuously exposed to humoral and cellular immune factors. *T. brucei* have become a textbook example of antigenic variation based on a low frequency switches of the expressed variant surface glycoprotein (VSG) that dominates the cell surface and allows the population to escape the host adaptive immune system. In addition, individual cell survival is favoured by a process that cleans the cell surface of low levels of bound immunoglobulins through endocytosis and degradation (Engstler et al., 2007). Motility is an integral part of this process as the hydrodynamic drag on antibody-VSG complexes, caused by the forward swimming motion of the cell, results in accumulation of the complexes at the posterior pole, close to the flagellar pocket where endocytosis occurs (Engstler et al., 2007). In addition, motility is essential for successful cell division, immune evasion and development in the host (Broadhead et al., 2006; Griffiths et al., 2007; Ralston et al., 2006; Shimogawa et al., 2018).

Several recent *in vitro* studies using African trypanosomes have focused on the characterization of qualitative and quantitative parameters of trypanosome morphology and motility and emphasized the ability of trypanosome species to adapt to the various environments of their mammalian hosts (Alizadehrad et al., 2015; Bargul et al., 2016; Heddergott et al., 2012; Krüger and Engstler, 2015). Thus far, detailed analysis of swimming behaviour, morphology and trypanosome-host cell interaction has been restricted to *ex vivo*, using conditions that best mimic the host environment, for example in blood taken from an infected animal (Bargul et al., 2016; Beattie and Gull, 1997; Engstler et al., 2007;

Heddergott et al., 2012; Hemphill and Ross, 1995; Skalický et al., 2017; Sunter and Gull, 2016; Wakid and Bates, 2004). It has not been possible to recreate the complex *in vivo* microenvironment, which, in the case of the vertebrate host, includes the streaming of the blood, vessels with heterogeneous size and endothelium composition, as well as changes occurring during the course of an infection, for example due to the onset of anaemia. It still remains a challenge to mimic *in vitro* the dynamic conditions of the crowded and fast-moving microenvironment of the blood stream in which trypanosomes move. Given the importance of motility for trypanosome survival, analysis of trypanosome swimming behaviour *in vivo*, in a vertebrate host environment, is important to fully understand trypanosome biology and pathogenesis.

Quantitative and qualitative approaches *in vivo* are hindered by the lack of transparency of the mammalian vertebrate hosts of trypanosome species. Nevertheless, using transgenic trypanosomes expressing a luciferase reporter protein it has been possible to monitor overall trypanosome distribution and to quantify total trypanosome load in mice (Burrell-saward et al., 2015; Capewell et al., 2016; Goyard et al., 2014; McLatchie et al., 2013). Fluorescent trypanosomes have been used *in vivo* to visualize the presence of parasites in mouse skin, a possible reservoir of trypanosomes in the late phase of infection (Capewell et al., 2016). Although the sensitivity of both systems allows for indirect visualization of trypanosome location or distribution, low spatio-temporal resolution and the lack of transparency of deep tissues limits the possibility for qualitative and quantitative analysis of trypanosome swimming behaviour *in vivo*. Finally, using a combination of multicolour light sheet fluorescence microscopy and high-speed fluorescence microscopy it has been possible to analyse the infection process and swimming behaviour of *T. brucei* *ex vivo*, in dissected tissues of the partially transparent tsetse fly vector (Gibson and Peacock, 2019; Schuster et al., 2017; WANG and BELOSEVIC, 1994) Nevertheless, to date, no method is available to study with sufficient resolution trypanosome swimming behaviour *in vivo* in a vertebrate host environment.

In the current study, we have used *Trypanosoma carassii* infection of zebrafish, *Danio rerio* to visualise trypanosome movement in the

bloodstream and tissues of a vertebrate host. *T. carassii* infects a broad range of cyprinid fish (Overath et al., 1998) and lives extracellularly in the blood and tissue fluids of the fish (Haag et al., 1998). It can establish a long-term infection characterized by polyclonal B cell activation and recurrent waves of parasitaemia, without the expression of a uniform VSG-like surface coat (Agüero et al., 2002; Joerink et al., 2007; Overath et al., 2001, 1999). Phylogenetically, *T. carassii* belongs to the aquatic clade of *Trypanosoma*, a sister group that diverged from the *Trypanosomatid* lineage prior to the divergence of the Stercorarian and Salivarian trypanosomes that infect mammals (Simpson et al., 2006; Stevens, 2008). Morphologically, *T. carassii* has a single flagellum that emerges from the flagellar pocket at the cell posterior, is attached to the cell body and extends free at the anterior end, defining the anterior-posterior axis. Zebrafish are an important model species for biomedical, developmental biology, and neurobiology research (Asnani and Peterson, 2014; Blackburn and Langenau, 2014; Cronan and Tobin, 2014; Goessling and North, 2014; Miyares et al., 2014; Nguyen-Chi et al., 2014; Phillips and Westerfield, 2014; Renshaw and Trede, 2012; Scharl, 2014; Torraca et al., 2014; Veinotte et al., 2014; Zon and Cagan, 2014) and are a fresh water cyprinid fish closely related to many of the natural hosts of *T. carassii*. The greatest advantage of zebrafish compared to other natural hosts of *T. carassii* is the transparency of the larvae, and of some juvenile and adult stages. Furthermore, an ever-increasing number of mutant and transgenic lines, including those marking blood vessels and relevant immune cell lineages, is currently available (Benard et al., 2015; Bertrand et al., 2010; Ellett et al., 2010; Langenau et al., 2004; Lawson and Weinstein, 2002; Page et al., 2013; Petrie-Hanson et al., 2009; Renshaw et al., 2006). The transparency of the zebrafish allows high-resolution, real-time imaging of trypanosome movement and host-parasite interaction *in vivo*, under conditions that exactly represent the natural environment during infection.

In this study, by exploiting the *T. carassii* infection of transparent zebrafish with high-speed microscopy, we report the first description of trypanosome swimming behaviour *in vivo* in a vertebrate host. Our observations reveal that trypanosome can rapidly adapt their swimming behaviour to the extremely heterogeneous host environment. It was not

possible to assign one preferred swimming behaviour to trypanosomes in either the blood stream, tissues or other body fluids. Conditions such as the presence or absence of the blood flow or of red blood cells, the speed of the flow, the size of the blood vessel, the type of endothelium or epithelium lining the vessels or the tissues, as well as the compactness of the tissues, all influenced the swimming behaviour. Furthermore, we show that trypanosomes can change direction through backwards swimming and through a 'whip-like' motion. Finally, we were able to capture a novel mechanism through which trypanosomes attach to host cells or tissues. These observations greatly expand our knowledge on trypanosome swimming behaviour and show that trypanosomes can rapidly adapt to match the host environment.

Material and methods

Zebrafish lines and maintenance

Zebrafish were kept and handled according to the Zebrafish Book (zfin.org) and animal welfare regulations of The Netherlands. Adult zebrafish were reared at the aquatic research facility of Wageningen University & Research (Carus). Zebrafish embryos and larvae were raised in egg water (0.6 g/L sea salt, Sera Marin, Heinsberg, Germany) at 27°C with a 12:12 light-dark cycle. From 5 days post fertilisation (dpf) until 14 dpf, larvae were fed once a day with *Tetrahymena*. Larvae older than 10 dpf were also fed daily with dry feed ZM-100 (zmsystem, UK). The zebrafish lines used in this study included: wild type AB, optically transparent casper lines (White et al., 2008), transgenic *Tg(fli:gfp)* line marking the vasculature (Lawson and Weinstein, 2002) or crosses thereof. All animals were handled in accordance with good animal practice as defined by the European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). All animal work at Wageningen University was approved by the local experimental animal committee (DEC number 2014095).

***Trypanosoma carassii* culture**

Adult common carp (*Cyprinus carpio*) were infected by intraperitoneal injection of 1×10^4 *Trypanosoma carassii*. Approximately 3 weeks post infection and before the parasitaemia reached $1 \times 10^6/\text{ml}$, carp were euthanized in 0.6 g/l tricaine methane sulfonate (TMS, Crescent Research Chemicals) and bled *via* the caudal vein using a final concentration of 10-20 Units heparin/ml of blood. Trypanosomes in blood were imaged immediately or blood was kept at 4°C overnight in siliconized tubes. The following morning, trypanosomes were enriched at the interface between the red blood cells and plasma, this buffy coat was recovered and centrifuged at 600xg for 8 minutes at room temperature. Trypanosomes were resuspended in RPMI without glutamine and phenol red (Lonza, Verviers, Belgium). To separate trypanosomes from red blood cells, the suspension was carefully loaded on top of a 100% Ficoll-Paque layer (GE Healthcare, Uppsala, Sweden) and centrifuged at room temperature for 20 min, at 800xg. Cells at the interphase were transferred to a new 50 ml tube and washed with RPMI. Trypanosomes were then resuspended at a density of 5×10^5 - $1 \times 10^6/\text{ml}$ and cultured in 75-165 cm² flasks in complete medium: 22.5% MEM with glutamine and phenol red (Gibco), 22.5% Leibovitz's L-15 medium without L-glutamine, with phenol red (Lonza, Verviers, Belgium), 45% Hanks' Balanced Salt solution (HBSS) with phenol red (Lonza, Verviers, Belgium), 10% sterile water, completed with 10% pooled carp serum, 2% 200 mM glutamine (Fisher Scientific) and 1% penicillin-streptomycin solution (10.000:10.000, Fisher Scientific). Cultures were incubated at 27°C without CO₂. Trypanosomes were kept at a density below $5 \times 10^6/\text{ml}$ and subcultured 1-3 times a week. Using this medium, *T. carassii* was kept in culture without losing infectivity for up to 2 months. For zebrafish infection, trypanosomes were cultured for 1 week and never longer than 3 weeks.

Zebrafish infection with *Trypanosoma carassii* and morbidity signs

Cultured trypanosomes were centrifuged at 800xg for 5 minutes and resuspended in 2% polyvinylpyrrolidone (PVP, Sigma-Aldrich) prior to injection. PVP was used to increase the viscosity of the medium and prevent trypanosomes from settling in the capillary, thus assuring a homogenous

trypanosome solution throughout the injection period. Trypanosomes number in 1-2 nl drop size varied depending on the intended dose and was monitored at the beginning and after 50 injections using a Bürker counting chamber. Prior to injection, 5 dpf (days post-fertilization) zebrafish larvae were anaesthetized with 0.017% Ethyl 3-aminobenzoate methanesulfonate (MS-222, Tricaine, Sigma-Aldrich) in egg water, and injected intravenously. Experimental groups received either *T. carassii* resuspended in PVP solution or PVP alone as a negative control. Injected larvae were directly transferred into pre-warmed egg water and kept in the incubator at 27°C. Viability was monitored daily. During the course of the optimization of the infection model, we noticed that some fish showed a lethargic behaviour and no escape reflex to a pipette and that such fish usually had a high parasitaemia leading to death. These clinical signs were therefore used to monitor morbidity and the progression of infection. When necessary, fish were removed from the experiment and euthanized with an overdose of anaesthetic (0.4% MS-222).

Real-time quantitative PCR

At various time point after infection, 3-6 zebrafish larvae were sacrificed by an overdose of anaesthetic, pooled and transferred to RNA later (Ambion). Total RNA isolation was performed with the Qiagen RNeasy Micro Kit (QIAGEN, Venlo, The Netherlands) according to manufacturer's protocol. Next, 250-500 ng of total RNA was used as template for cDNA synthesis using SuperScript III Reverse Transcriptase and random hexamers (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions with an additional DNase step using DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA). cDNA was then diluted 25 times to serve as template for real-time quantitative PCR (RT-qPCR) using Rotor-Gene 6000 (Corbett Research, QIAGEN), as previously described (Forlenza et al., 2012, 2008). Primers for zebrafish *elongation factor-1a* (*ef1a* Fw: 3'-CTGGAGGCCAGCTCAAACAT-5' and RV: 3'-ATCAAGAAGAGTAGTAGTACCG-5'; ZDB-GENE-990415-52) and *T. carassii* *heat-shock protein-70* (FW: 3'-CAGCCGGTGGAGCGCGT-5' 3'-AGTTCCTTGCCGCCGAAGA-5'; GeneBank-FJ970030.1) were obtained from Eurogentec (Liège, Belgium). Gene expression was normalized to the *ef1a* housekeeping gene and expressed relative to the time point PVP control.

High-speed light microscopy, image and video analysis

For imaging of *T. carassii* swimming behaviour *in vitro*, the high-speed camera was mounted on an automated DM6b upright digital microscope (Leica Microsystems), controlled by Leica LASX software (version 3.4.2.) and equipped with 100x oil (NA 1.32), 40x (NA 0.85, DIC) and 20x (NA 0.8, DIC) short distance objectives (Leica Microsystems). For high-speed light microscopy a three grey-scale (12 bits) Photron APX-RS High Speed Camera (Photron, resolution (128 x 16) to (1024 x 1024) pixels), with Leica HC 1x Microscope C-mount Adapter was used, controlled by Photron FASTCAM Viewer (PFV) software (version 3.5.1). Images were acquired at a resolution of 900 x 900 or 768 x 880 pixels depending on the C-mount adapter. Trypanosomes were transferred to non-coated microscopic slides (Superforst, Thermo Scientific), covered with a 24 x 50 mm coverslip and imaged immediately and for no longer than 10 minutes.

Prior to imaging of *T. carassii* swimming behaviour *in vivo*, the high-speed camera was mounted on a DMI8 inverted digital microscope (Leica Microsystems), controlled by Leica LASX software (version 3.4.2.) and equipped with 40x(NA 0.6) and 20x(NA 0.4) long distance objectives (Leica Microsystems). For high-speed light microscopy a three grey-scale (8 bits) Mikrotorn EoSens MC1362 (Mikrotron GmbH, resolution 1280 x 1024 pixels), with Leica HC 1x Microscope C-mount Camera Adapter, was used, controlled by XCAP-Std software (version 3.8, EPIX inc.). Images were acquired at a resolution of 900 x 900 or 640 x 640 pixels. Zebrafish larvae were anaesthetised with 0.017% MS-222 and embedded in UltraPure LMP Agarose (Invitrogen) on a microscope slide (1.4-1.6 mm) with a well depth of 0.5-0.8 mm (Electron Microscopy Sciences). Upon solidification of the agarose, the specimen was covered with 3-4 drops of egg water containing 0.017% MS-222, by a 24 x 50 mm coverslip and imaged immediately.

For all high-speed videography, image series were acquired at 240–500 frames per second (fps) and analysed using a PFV software (version 3.2.8.2) or MiDAS Player v5.0.0.3 (Xcite, USA); 240-250 fps were found optimal for imaging of trypanosome swimming behaviour *in vitro*, whereas 480-500 fps were used for *in vivo* imaging of infected zebrafish. Quantification of trypanosome length, swimming speed and directionality

was performed with ImageJ-Fijii (version 1.51n) using the MTrack plug-in. For livestream light microscopy (acquisitions at 20 fps) a DFC3000G camera (Leica Microsystems) was mounted on the DMI8 inverted digital microscope and controlled by the Leica LASX software. Images were acquired at a resolution of 720x576 or 1296 x 966 pixels.

For fluorescence microscopy of *Tg(fli1:egfp)^{v1}* casper lines, marking the zebrafish vasculature in green, the Zeiss LSM-510 confocal microscope, with a 20x long-distance objective was used with the following settings: laser excitation = 488nm with 73% transmission; HFT filter = 488nm; BP filter = 505-550; detection gain = 800; amplifier offset = -0.01; amplifier gain = 1.1; bright field channel was opened with Detection Gain = 130; frame size (pixels) = 2048 x 2048; pinhole = 300 (optical slice < 28.3µm, pinhole $\varnothing$ = 6.26 airy units). Videos were produced using CyberLink PowerDirector 16.

Results

Characterization of trypanosome swimming behaviour *in vitro*

Previous *in vitro* studies reported on the heterogeneity in swimming behaviour of African trypanosomes, and on how this was dependent on viscosity of the culture medium or host blood (Bargul et al., 2016; Engstler et al., 2007; Heddergott et al., 2012). To investigate the swimming behaviour of *T. carassii* in fish blood or culture medium, we used high spatio-temporal resolution microscopy. For the initial description of trypanosome swimming behaviour *in vitro*, we adopted the classification and quantification method described by Bargul et al (Bargul et al., 2016): *persistent swimmers*, i.e. trypanosomes exhibiting a directional movement, having an elongated and stretched cell body shape and covering several hundreds of micrometres; *tumbling swimmers*, trypanosomes exhibiting a non-directional movement, having a curled up shape and moving no further than their body length; and *intermediate swimmers*, trypanosomes alternating periods of directional and non-directional movement.

High spatio-temporal resolution analysis of the swimming behaviour of freshly isolated trypanosomes in carp blood, revealed that up to 96.4% of *T. carassii* could be classified as *tumbling swimmers* (**Figure 1A-B**, and **Video 1** (00:00-45:19 sec)) displaying the typical cellular waveform (Bargul et al., 2016). The remaining trypanosomes (3.5%) behaved as *intermediate swimmers* having a stretched and slim waveform when compared to tumblers (**Video 1**, 00:00-45:19 sec), and only 0.1% could be classified as *persistent swimmers* (**Figure 1A-B** and **Video 1**, 45:19-58:03 sec). Persistent swimmers showed an average speed of 32 $\mu\text{m}/\text{sec}$, whereas tumblers and intermediate swimmers both showed a lower average speed of 14 $\mu\text{m}/\text{sec}$ (**Figure 1B**). While tumblers would not move further than their body length ($\sim 23 \pm 2.4 \mu\text{m}$, **Figure 1C**), intermediate swimmers could cover a 'straight-line' distance of up to 240 μm in 20 seconds. Comparison of the movement of freshly isolated trypanosomes kept in either carp serum or culture medium revealed comparable swimming behaviour (**Video 1**, 58:03-1:15 min). Cultured in medium or serum for a period of up to 2 months did not alter morphology, the proportions of tumblers, intermediate or persistent swimmers (data not shown), suggesting that the swimming behaviour is an intrinsic property of parasites in blood.

Upon direct isolation from fish blood, trypanosome was observed that were anchored to red blood cells or to leukocytes. Attachment always occurred through their posterior end leaving the flagellum free to move (**Video 2**, 0-28:00 sec). Similarly, also when cultured, trypanosomes could attach to the flask or glass surface through their posterior end (**Video 2**, 28:00-55:00 sec). Whether the site of attachment coincides with the cell membrane, flagellum base, or neck of the flagellar pocket it was not readily visible with current image resolution. Finally, trypanosomes were also observed to crawl backwards without using the propelling motion of the flagellum (**Video 2**, 55:00 sec-1:15:00 min)).

Figure 1. Majority of freshly isolated parasites in carp blood are tumbling swimmers. Parasites were isolated from carp blood and immediately imaged using high resolution microscopy acquiring 240 frames per second (fps). **(A)** Relative percentage of tumbler, intermediate or persistent swimmers (defined as described in the text) was calculated over a total number of 944 parasites, isolated from 6 different carp infections and imaged over 60 independent acquisitions. **(B)** Representative tracks of a tumbler (green), intermediate (yellow) and persistent (red) swimmer. The diameter of the circles (23 μm) indicates the average cell-body size of a parasite as also shown in C. The inset table summarizes the straight-line distance covered by the parasite (between the first and last track point); the total track length, i.e. the path covered by the parasite in approximately 20 seconds of acquisition time indicated in matching colours; and the average speed ($\mu\text{m}/\text{sec}$) was calculated on a selection of the acquisitions used in A. **(C)** Detailed image of two parasites indicating the total body length including the flagellum (left) and the total cell-body length excluding the flagellum (right). Measurements were acquired on high resolution images of at least 10 freshly isolated parasites obtained from 4 independent infections, using more than 20 frames within the same acquisition. Quantification of parasite length, swimming speed and directionality was performed with ImageJ-Fijii using the MTrack plug-in. **Video 1** displays high-speed videos of the swimming behaviour of tumblers, intermediate and persistent swimmers in carp blood, or of parasites resuspended in serum or culture medium.

Video 1

Blood was drawn from infected carp and resuspended in carp serum.

The majority of the parasites are tumblers

real-time, 240 fps

Video 1. Swimming behaviour of freshly isolated parasites in carp blood, or diluted in either carp serum or culture medium, showing representative tumblers, intermediate and persistent swimmers.

Video 2

Upon isolation from fish blood, several parasites are attached to blood cells. Attachment always occurs through the posterior end leaving the flagellum free to move

real-time, 240 fps

Video 2. Parasites attach to cells or surfaces through their posterior end leaving the flagellum free to move. Parasites were also observed to crawl backwards without using the propelling motion of the flagellum.

Establishment of a *Trypanosoma carassii* infection model in zebrafish

To observe the swimming behaviour of *T. carassii* in a host, we developed an infection model in transparent zebrafish larvae. First the susceptibility and kinetics of parasitaemia were determined. Infection of 5 day-post-fertilization (dpf) zebrafish resulted in an acute infection associated with low survival (**Figure 2A**) and high parasitaemia (**Figure 2B**), independent of the infection dose. This confirms that zebrafish, similarly to other cyprinid fish, are susceptible to *T. carassii* infection.

Figure 2. Zebrafish larvae are susceptible to *T. carassii* infection. Zebrafish larvae (5 dpf) were injected with the indicated number of *T. carassii* per fish. PVP was used as injection control. **(A)** Kinetics of zebrafish larval survival. Fish (n=60/group) were observed daily for signs of infection and survival. **(B)** Kinetics of parasitaemia. Trypanosome levels were quantified by real-time quantitative-PCR using *T. carassii hsp70*-specific primers. RNA of n=5 fish was pooled at each time point. Expression was normalized relative to the host house-keeping gene *elongation factor-1 alpha* and expressed relative to fish injected with the corresponding trypanosome dose at time point zero.

The complex swimming behaviour of *T. carassii* in vivo in blood vessels

Having established that *T. carassii* can indeed infect zebrafish, we took advantage of the transparency of zebrafish larvae to characterize trypanosome swimming behaviour *in vivo*. Zebrafish larvae were infected at 5 dpf with 200 *T. carassii*/fish and imaged using either live stream imaging (20 fps) or high spatio-temporal resolution microscopy (500 fps) at various time points after infection and in differently-sized blood vessels. When parasitaemia was low, typically early during infection, trypanosomes were most readily detected in small to medium-sized blood vessels with reduced blood flow and a lower density of red blood cells, such as the tail tip loop or intersegmental capillaries (ISCs). In the tail tip loop, trypanosomes were passively dragged by the blood stream along with red blood cells (Figure 3A, Video 3, 00:00-42:23 sec) and were seen to either curl or stretch the cell body as well as occasionally propel their flagellum in the same or opposite direction as the blood flow (Video 3, 42:23-01:57:16 min) but were never seen swimming faster than the flow. ISCs are narrow with a diameter equivalent to a single cell. In ISCs, trypanosomes were elongated with their flagellum in the opposite direction to the blood flow (Figure 3B, Video 3, 1:57:16-02:49:06 min); the

diameter of the vessel, the speed of the flow and the presence of colliding red blood cells within the ISC, force the trypanosomes to passively move forward in the direction of the flow.

In larger diameter vessels with a strong blood flow and higher density of red blood cells, such as the cardinal caudal vein (**Video 3**, 02:49:06-04:11:17) or artery (**Video 3**, 04:11:17-04:37:05), detection and description of swimming behaviour was greatly aided by the use of high spatio-temporal resolution microscopy. In these vessels, trypanosomes are also passively dragged by the blood stream, curling among the densely packed red blood cells. In real time speed, only the occasional trypanosome was seen to slow down by rolling or bouncing against the vessel in the peripheral cell-free layer (**Figure 3C** and **Video 3**, 03:53:23-04:11:17). In general, the typical tumbling movement described *in vitro* was not observed in the fish, except for those locations where the blood flow was highly reduced or absent as in sharp turns of the tail tip (**Video 3**, 04:37:05-05:09:12 min), and in locations where leukocytes adhering to the endothelium would create a local disturbance of the flow rate allowing parasites to tumble (**Video 3**, 05:09:12-05:24:12 min).

Physiological changes in the blood flow or red blood cells density associated with the infection, allowed visualization of additional swimming behaviours in blood vessels. For example, in cases where the blood flow was (temporarily) interrupted, trypanosomes were able to swim directionally, repeatedly invert direction, or tumble (**Figure 4A**, and **Video 4**, 00:00:00-01:23:12). In cases where the blood flow continued but red blood cells were occluded, trypanosomes were able to persistently swim against the blood flow (**Figure 4B**, and **Video 4**, 01:23:12-02:13:20). We did not attempt to distinguish, *in vivo*, between intermediate and persistent swimmers because physical factors such as the presence of red blood cells, the length of a capillary or the stability of the blood flow could all interfere with the directionality of their movement. Instead, all trypanosomes observed to swim directionally in the blood, independent of the distance covered before changing direction, were considered swimmers.

Altogether, we observed that *T. carassii* can adopt different swimming behaviours all greatly influenced by the blood flow, size of the blood vessel

and presence of red blood cells. Most frequently, in blood vessels with an intact blood flow and high density of red blood cells, trypanosomes are passively dragged by the flow along with red blood cells, in a curling motion. Occasionally, when the blood flow or the number of red blood cells is reduced, trypanosomes can swim directionally and persistently (swimmers) in blood vessel. This indicates that, at least *in vivo*, it is not possible to assign a specific swimming behaviour to trypanosomes in blood vessels; on the contrary, trypanosomes rapidly adapt their swimming behaviour to changes in micro-environmental conditions.

Figure 3. Schematic representation of parasite swimming behaviour in blood vessels of various sizes. In general, in vessels with an intact blood flow (grey arrows) and a normal number of red blood cells (RBC), parasites are passively dragged by the flow along with RBC. Under these conditions, parasites were never seen swimming directionally against the flow or faster than RBC. **(A)** In medium-sized blood vessels with moderate flow, while being passively dragged by the flow, parasites can either curl or stretch their body, as well as occasionally propel their flagellum in the same or opposite direction of the blood flow. **(B)** In small-sized blood vessels of one-cell diameter, such as intersegmental capillaries (ISC), parasites are pushed forward by the blood flow and by colliding RBC. **(C)** In large-sized blood vessels the blood flow is very strong and the density of RBC high, making it more difficult to detect parasites without the aid of high-speed microscopy. Only the occasional parasite that would slow down by bouncing against the vessel wall would be visible in the cell-free layer. **Video 3** contains high-speed videos showing details of the parasite movements schematically depicted above.

Video 3

T. carassii movement in:
medium-size vessels (e.g. tail tip loop),
intersegmental capillaries (ISC),
larger vessels (e.g. caudal vein).

Video 3. Swimming behaviour of parasites in small, medium and large sized vessels with an intact blood flow. Under these conditions, parasites are passively dragged by the flow or pushed forward by colliding RBC.

A Vessel with reduced blood flow and fewer RBC

B Vessel with intact blood flow and temporary absence of RBC

Figure 4. Schematic representation of parasite swimming behaviour in blood vessels with altered blood flow or RBC number. **(A)** Blood vessels with a reduced blood flow (dashed arrows) and fewer RBC. **(B)** Blood vessels with an intact blood flow (grey arrows) but with a (temporarily) absence of RBC. In both cases, parasites can swim directionally (black straight arrows) by propelling the flagellum (distorted circular arrows) in the same or opposite direction of the flow or can tumble (circular arrows). **Video 4** contains high-speed videos showing parasite movement in medium-sized blood vessels and in capillaries as schematically depicted above.

Video 4

when the blood flow is temporarily interrupted
parasites are able to:

repeatedly invert direction,
swim directionally
or tumble

Video 4. Swimming behaviour of parasites in medium-sized blood vessels with interrupted blood flow, or in capillaries with intact blood flow and reduced number of red blood cells. Under these conditions, parasites can repeatedly invert direction, swim directionally or tumble.

Trypanosomes can attach *in vivo* by anchoring through their posterior end

Besides being passively dragged within blood vessels, trypanosomes could often be seen attached to the endothelium on the dorsal luminal side of the cardinal caudal vein (referred to as caudal vein, **Figure 5A**). Remarkably, despite the strong blood flow, attachment (anchoring) could last for several seconds (**Video 5**, 00:00:00-01:11:03) and involved a small area of the posterior end of the trypanosome, leaving the cell body and the flagellum free to move (**Video 5**, 01:11:03-01:59:03). Although anchoring could be observed already at 1 dpi, it was more easily detected at later stages of the infection. Anchoring was not the only mode of attachment, in fact, trypanosomes were also seen crawling on the vessel wall involving the entire cell body (**Figure 5B**, and **Video 5**, 01:59:03-02:38:05). Within blood vessels, anchoring occurred exclusively at the dorsal side of the caudal vein, whereas crawling could occur anywhere in the vein. No attachment or crawling was observed in arteries or capillaries, independently of the speed of the blood flow.

Altogether, our data show that *T. carassii* attaches to host cells through their posterior end, both *in vitro* (**Video 2**) and *in vivo*, leaving the flagellum free to move, and suggest that the posterior end acts as an anchoring site. Whether the exact anchoring site corresponds to the flagellum base or to the flagellar pocket and whether it may possibly favour extravasation, could not be confirmed under the current conditions and will be the focus of further investigation.

Figure 5. Parasite attachment to the blood vessel wall. (A) Parasite anchor themselves through their posterior end, leaving the entire parasite body and the flagellum free to move. Anchoring occurred only to the dorsal luminal side of the caudal vein and was not observed in other blood vessels. (B) Parasites crawl on the vessel wall of the caudal vein; the transparent square allows visualization of trypanosomes through the pack of RBC. Crawling occurred everywhere in the vein and involved the entire cell body. **Video 5** contains high-speed videos showing anchored and crawling parasites in the caudal vein as schematically depicted above.

Video 5. Parasites anchor to the dorsal luminal side of the caudal vein and crawl on the vessel wall of veins.

2

Movement in fluids outside the blood vessels and tissue

A characteristic of *T. carassii* infection in zebrafish is extravasation from blood vessels into surrounding tissues and tissue fluids. This occurred already at 1 dpi and allowed us to investigate the swimming behaviour in tissue fluids other than blood, including those of the peritoneal and heart cavity. In these locations, we could thus evaluate the swimming behaviour of trypanosomes in a natural fluidic environment, but now in the absence of red blood cells and of the hydrodynamic flow typical of blood vessels. Interestingly, in these environments, similarly to the swimming behaviour observed *in vitro* (**Video 1** and **Figure 1**), the majority of the trypanosomes were tumbling swimmers (**Figure 6A** and **Video 6**, 00:00:00-00:42:01). Indeed, in a view with more than 100 trypanosomes in the peritoneal cavity, only 3 persistent swimmers could be identified (**Video 6**, 00:42:01-01:35:03). Furthermore, trypanosomes were seen anchored to cells of the peritoneal membrane through their posterior end, in a manner similar to the one observed in the caudal vein (**Video 6**, 00:34:03-00:42:01).

Next, we analysed the swimming behaviour of *T. carassii* in tissues. Key locations included the tail tip, fins, muscle and interstitial space lining the blood vessels. Here, we observed no apparent consistency in swimming behaviour and trypanosomes alternated between directional and non-directional swimming, depending on the compactness of the tissue. For example, in the compact tissue of the fins, most were directional swimmers (**Figure 6B**) although their path can often be interrupted by an obstacle or hindered by the compactness of the tissue that does not allow the trypanosome to move further. Swimmers moved at an average speed of 47.5 $\mu\text{m}/\text{sec}$, covering up to 187 μm , before disappearing from the

view or hitting an obstacle that made them change direction (**Video 7**, 00:00-47:12). Similar to observations *in vitro* (**Video 2**), trypanosomes could invert their swimming direction by swimming backwards (**Figure 6B**, **Video 7**, 47:12-01:07:22). Backward swimming was exclusively observed in the fins.

In the interstitial space lining the cardinal blood vessels (artery and vein), trypanosomes swim directionally or tumble (**Video 8**, 00:00-23:02), but can also use the space between cells to pin themselves and effectively invert swimming direction in a 'whip-like' motion, a movement distinct from the more random tumbling movement. (**Video 8**, 00:23:00-01:17:04). Such 'whip-like' movement was also observed for trypanosomes swimming in ISC in which the blood flow is absent, and in more compact tissues such as the fin (**Figure 6C**, **Video 8**, 01:17:04-02:18:18). Furthermore, in compact tissues that do not allow the trypanosome to swim through, their persistent swimming translates into a drilling (auger) movement, which in some cases can lead to an enlargement of the space between somatic cells (**Figure 6D**, **Video 8**, 02:18:18-03:52:06).

Altogether, also in tissues and tissue fluids, *T. carassii* can adopt all swimming movements and can adhere through the posterior end to endothelial cells of the peritoneal cavity. Besides the previously described tumbling and directional (forward) swimming, trypanosomes were also able to invert direction through a 'whip-like' motion or by backward swimming.

Figure 6. Schematic representation of parasite swimming behaviour in tissue and tissue fluids outside the blood. **(A)** In the peritoneal or heart cavity, both environments without hydrodynamic flow and RBC, most of the parasites are tumbling swimmers; only occasionally a persistent swimmer could be observed. **(B)** In highly compact tissues such as the fins, most of the parasites are directional swimmers. Here, both forward and backward swimming could be observed. **(C)** In less compact tissues and in capillaries without blood flow, parasites can invert their swimming direction (arrows) in a whip-like motion by pinning themselves to the tissue wall (grey arrow head) and swiping their flagellum. **(D)** In tissues where parasites reach dead ends such as the interstitial space between vessels, persistent forward swimming translates into a drilling (auger) movement. **Video 6, Video 7** and **Video 8** contain high-speed videos showing all locations and swimming behaviours schematically depicted above.

Video 6

A typical feature of *T. carassii* infection is extravasation. Parasites can be found in the heart and peritoneal cavity. Here there is a natural fluidic environment without the stream typical of blood vessels.

The majority of the parasites are tumblers.

11 dpf Zebrafish, 6 dpi,
real time, 20 fps

Video 6. Swimming behaviour of parasites extravasated from blood vessels into tissue fluids that lack blood flow and RBC. These include the peritoneal and heart cavity.

Video 7

In the narrow spaces of the fins, the majority of the parasites are swimmers.

Of a selected number of parasites the tracks will be shown

11 dpf zebrafish, 6 dpi
real time, 500 fps

Video 7. Swimming behaviour of parasites in the narrow spaces of the fins, capturing persistent forward and backward swimmers.

Video 8

Parasites extravasate and can be seen in the tissue lining the blood vessel.

6 dpf zebrafish, 1 dpi,
real time, 500 fps

Video 8. Swimming behaviour of parasites in various tissues.

Video 9

Anaemia is a whole mark of *T. carassii* infection

Video 9. Anaemia is a whole mark of *T. carassii* infection.

Progression of infection and associated clinical signs

Physiological changes associated with the progression of the infection can affect the conditions within blood vessels or host tissues, and thus influence trypanosome behaviour. In addition to extravasation (**Video 6 to 8**) which occurred as early as 1 dpi we observed an onset of anaemia and vasodilation of blood vessels.

Non-infected larvae have a strong and steady blood flow, with all blood vessels packed with red blood cells (**Figure 7A**). In contrast, infected larvae progressively showed a decrease in the ratio between red blood cells and trypanosomes from 1 to 8 dpi (**Figure 7B-C**), until in some cases red blood cells disappeared completely (**Figure 7D** and **Video 9**). Anaemia therefore is a hallmark of later stage *T. carassii* infection in zebrafish larvae.

Highly infected fish that are anaemic also showed vasodilation, a clinical sign typical of advanced stages of infection (> 3dpi) with *T. carassii* (**Figure 8A-C**), most obviously observed in the caudal vein. The degree of vasodilation differed between individuals and in extreme cases the caudal vein was observed to enlarge up to three times that observed in control fish (**Figure 8D**). Vasodilation also occurs in the caudal artery but to a lesser extent. Interestingly, while the dilated blood vessels of larvae are packed with trypanosomes and have limited circulation, the number of extravasated parasites is very low (**Video 9**).

Figure 7. Onset of anaemia during *T. carassii* infection. Zebrafish larvae (5 dpf) were infected with 200 *T. carassii* or injected with PVP as non-infected control. Images are selected frames depicting the caudal artery, extracted from high-speed videos where parasites (white open-arrow heads) and red blood cells (RBC, black open-arrow heads) were identified and tracked. (A) Artery of a control, non-infected, fish. Only RBC are present. (B) Artery of an infected fish, 1 dpi, showing a high ratio of RBC:parasites. This frame corresponds to seconds 04:20-04:23 in **Video 3**, where the same parasites were tracked. (C) Artery of an infected fish, 6 dpi, showing a reduced ratio of RBC:parasites, indicating the onset of anaemia. (D) Artery of an infected fish suffering from severe anaemia, 8 dpi, where only parasites are present. The frame is extracted from the corresponding **Video 9**. Scale bars indicate 25 μm.

Figure 8. Advanced stages of *T. carassii* infection lead to vasodilation of the caudal vein. Wild type zebrafish larvae (5 dpf) were infected with 200 *T. carassii* or injected with PVP as non-infected control. Images are selected frames from high-speed videos. **(A-C)** Representative images of caudal artery and caudal vein (dashed lines) region at various time points after infection. Scale bars indicate 50 μm . **(D)** Maximum diameter of the caudal vein in non-infected (open symbols) and infected individuals (closed symbols) at 2-3 dpi (squares) and 6-8 dpi (triangles). Each value is the average of at least 3 measurements taken at different locations within the caudal vein of the same individual. Numbers indicate average and SD.

Discussion

In this study we describe a trypanosome infection model in zebrafish. By combining the transparency of zebrafish larvae with high spatio-temporal resolution microscopy, we were able to describe in detail the *in vivo* swimming behaviour of *T. carassii* in blood, tissues and tissue fluids of a vertebrate host. Besides the 'conventional' non-directional (tumbling) and directional forward swimming previously described *in vitro* (Bargul et al., 2016; Heddergott et al., 2012), we also provide evidence trypanosomes can reverse direction through a 'whip-like' motion or by swimming

backwards. Finally, we report a novel observation that the posterior end of the parasite, possibly coinciding with the flagellum base or flagellar pocket's neck, can act as an anchoring site. To our knowledge this is the first report of the swimming behaviour of trypanosomes *in vivo* in the vertebrate host environment.

Knowledge on trypanosome-host cell interaction, movement, and tropism in vertebrate hosts is important to understand trypanosome biology, pathology and ultimately improve current intervention strategies. To date, detailed analysis of parasite swimming behaviour and interaction with host cells has only been possible using isolated blood or conditions that best mimic those of the host blood or tissues. Although the current *in vitro* approaches have brought a wealth of information on the quantitative aspects of trypanosomes motility and potential immune evasion strategies, they could not fully reproduce the streaming nature of the blood, the heterogeneity of the blood stream between and within vessel, the different sizes of blood vessels between which trypanosomes regularly alternate, the different types of endothelium lining arteries and veins and finally, the changes that occur in the blood due to the infection itself.

In this study, we first investigated the motion of *T. carassii* freshly isolated from carp blood. Based on a previously proposed classification of the swimming behaviour of salivarian trypanosomes (Bargul et al., 2016a), clearly more than 90% of *T. carassii* parasites could be classified as tumblers, and this was independent of whether they were in serum or culture medium. These initial *in vitro* observations led to the suggestion that the tumbling behaviour might be an intrinsic property of parasites in the blood stream. Our subsequent *in vivo* observations however, showed that this applies only to trypanosomes swimming in fluids with highly reduced or absent flow, for example the peritoneal fluid or blood in vessels in which flow was slow or absent (**Figure 7A** and **Video 9**). In contrast, analysis of the swimming behaviour of *T. carassii* in the zebrafish blood stream revealed that it is not possible to assign a single or predominant swimming behaviour. In vessels with a normal blood flow and in the presence of red blood cells, trypanosomes were passively dragged by

the flow along with red blood cells. In the centre of large vessels, such as the portal artery or portal vein, the velocity of the flow does not allow either tumbling or swimming against or faster than the flow. Similarly, in narrow intersegmental capillaries (ISCs), trypanosomes are forced to move forward due to the streaming of the blood or to the presence of colliding red blood cells. Under these conditions, trypanosomes were never seen swimming faster than the passive movement of red blood cells (Heddergott et al., 2012; Langousis and Hill, 2014). Furthermore, since trypanosomes are carried along with red blood cells by the flow, it is difficult to envisage how the red blood cells could represent anything more than very occasional obstacles or surfaces for mechanical interactions that would favour forward swimming (Bargul et al., 2016; Heddergott et al., 2012). At the periphery of the portal vein, there is a cell-free layer where the number of red blood cells and the speed of the blood flow is reduced (Bagchi, 2007), here trypanosomes could clearly slow themselves down by crawling, rolling or by temporarily anchoring themselves to the dorsal endothelium (further discussed below). However, in arteries or small capillaries adherence to the epithelium was never observed, even when the flow was reduced or completely absent. This suggests that not only the velocity of the flow but also the type of endothelium influences trypanosome swimming behaviour.

Physiological changes occur as the infection progresses that modify conditions within blood vessels, the flow is strongly reduced or interrupted and the density of red blood cells is reduced due to obstruction of blood vessels or anaemia. In these conditions, directional swimmers were observed that swam faster than the flow and others that swam against the flow, or that repeatedly changed direction within blood vessels (**Video 4**). Taken together, our observations show that it is not possible to generalize the swimming behaviour of trypanosomes in blood, they can rapidly adopt different swimming behaviours: tumbling, directional swimming, anchoring, all largely influenced by the size and type of vessel, the speed of the flow, the presence, or not, of red blood cells as well as the highly dynamic microenvironment within a vessel, as for example the centre compared to the periphery, and the type of endothelium.

Outside blood, it has been suggested that the directionality of swimming is influenced by the density or compactness of the tissue (Bargul et al., 2016a). Our observation of swimming behaviour in various tissues revealed that it is the size of the interstitial space through which the trypanosome has to swim that largely determines whether it will swim directionally, tumble or both. For example, in the compact tissue of the fins, where the interstitial space between epithelial cells is very narrow and the epithelial cell membrane is not deformable, the vast majority of trypanosomes swim directionally. The space can be so restrictive that to invert direction the parasite is obliged to swim backwards (**Video 7**). Backward swimming was previously observed *in vitro* for *T. brucei* motility mutants or for wild type parasites swimming in high-density medium (Heddergott et al., 2012) (Baron et al., 2007; Branche, 2006; Engstler et al., 2007), and *ex vivo*, for procyclic and mesocyclic stages of *T. brucei* parasites swimming in confined spaces within the midgut of the tsetse fly (Schuster et al., 2017). The observation of *T. carassii* swimming in zebrafish tissues is the first report of backward swimming in a vertebrate host environment.

In the less compact areas of the fins or in the tissue lining the blood vessels, in addition to directional swimmers (forward or backwards) tumblers and trypanosomes that repeatedly inverted direction through a whip-like motion were observed (**Video 8**). When directional swimmers reached dead ends, the persistent forward swimming translated into either a drilling movement similar to the movement described for *T. brucei* in dead-end spaces within the midgut of the tsetse fly (Schuster et al., 2017), or backwards swimming as observed in the fins. Altogether, these observations indicate that trypanosomes can adopt several swimming behaviours and these are largely influenced by the compactness and confinement offered by the tissue.

Perhaps one of the most interesting observation is the discovery that trypanosomes can anchor themselves to the vein endothelium through their posterior end, leaving the flagellum and the entire cell body free to move. Anchoring was observed as soon as 30 min to 1h after parasite injection into zebrafish larvae, and by 1 dpi extravasation was observed. Whether this adhesion mechanism favours or is even required for extravasation, was

not possible to ascertain under the current conditions. So far, we have been unable to capture the exact moment of extravasation.

The anchoring site on the posterior of the trypanosome leaves the cell body and flagellum free to move (**Video 2** and **Video 5**) so it seems likely that the adhesion on the trypanosome occurs via the flagellum base or the neck of the flagellar pocket itself. The presence of specific adhesion molecules would at least partly explain how parasites can very rapidly anchor themselves, upon sudden collision with the endothelium, and remain in position for more than 15 seconds despite the very rapid blood flow and collisions with blood cells. Anchoring was only observed in the portal vein but trypanosomes were seen crawling on vein endothelium (**Video 5**) through dynamic interactions that involved the cell membrane, not just the flagellum membrane, suggesting that the molecules required for whole-cell adherence might be different from those required for anchoring through the posterior end. This is reminiscent of leukocyte rolling, which also occurs only in veins and not in arteries. Altogether, the possibility to observe *T. carassii* behaviour both *in vitro* and *in vivo*, in the presence or absence of a strong hydrodynamic flow, and at various locations within the vertebrate host, demonstrated how the environmental conditions, especially the presence or absence of a flow, strongly influence the ability of the parasite to attach and the duration of the attachment.

T. carassii anchors to zebrafish cells in a manner clearly distinct from that described for other trypanosomes. The major difference being the lack of extensive interaction between the parasite's flagellum membrane and the host cell or artificial surface. Stable interaction involving large portions of the flagellum membrane have been described among others for haptomonads stages (surface-attached) of *Paratrypanosoma confusum*, or *Leishmania* promastigotes (**Figure 9A**) (Skalický et al., 2017; Wakid and Bates, 2004). In these liberform parasites (flagellum not laterally attached to the cell), adhesion occurs through an attachment pad forming from the bulge at the base of the flagellum. At least *in vitro*, the formation of the pad takes approximately 1h, causing extensive remodelling of the flagellum itself and effectively anchors the parasites to the surface, and in the case of *P. confusum*, also favours parasite division (Skalický et al., 2017). Similarly, *T. brucei* epimastigotes divide while attached to the brush border of the

salivary gland epithelium through extensive outgrowths of the non-cell-attached anterior part of the flagellum membrane (**Figure 9B**) (Beattie and Gull, 1997; Langousis and Hill, 2014; Schuster et al., 2017). Such adhesion mechanism was only recently captured *ex vivo* through high-speed videography of dissected tsetse fly salivary glands; the exact moment of attachment and the time required to establish the stable interaction *in vivo* however, were not reported (Schuster et al., 2017). *T. congolense* was reported to adhere *in vitro* to bovine aorta endothelial (BAE) cell monolayers *via* extensive membrane protrusions (filopodia) of the membrane-attached flagellum (**Figure 9C**) (Beattie and Gull, 1997; Hemphill and Ross, 1995) an interaction that was shown to involve sialic acid residues on BAE cells (Hemphill et al., 1994). Although adhesion was observed already at 1h, the filopodia increased in size over a period of 24-48h. Whether such type of interaction occurs with similar kinetics also *in vivo*, in vessels with an intact blood flow, is yet to be confirmed.

Figure 9: Schematic drawing depicting the attachment of various trypanosome species. A) haptomonads stages of *Paratrypanosoma confusum*: adhesion occurs through an attachment pad forming from the bulge at the base of the flagellum involving extensive remodelling of the flagellum membrane (based on: Skalický et al., 2017). The square indicates the location of the flagellar pocket B) *T. brucei* epimastigotes attached through the flagellum to the brush border

of the salivary gland epithelium (based on (Beattie and Gull, 1997; Schuster et al., 2017; Vickerman and Tetley, 1990). C) *T. congolense* adhesion to bovine aorta endothelial cell line *via* extensive membrane protrusions (filopodia) of the membrane-attached flagellum (based on (Beattie and Gull, 1997; Hemphill and Ross, 1995). D) *T. carassii* attached through the posterior end, leaving the cell body and flagellum free to move, as also shown in figure 5.

All the above attachment mechanisms are clearly geared towards creating a very stable interaction with a surface to either establish a permanent infection in the salivary glands of the insect host, or to possibly adhere to the vertebrate host endothelium. They all involve extensive modifications of the flagellum membrane that occur over time to increase the contact area between the parasite and the surface. Most of these interactions however were observed *in vitro* for parasites cultivated on glass or plastic surfaces or endothelial cell monolayers and studied by means of scanning or transmission electron microscopy, as well as *in vitro* binding assays (Beattie and Gull, 1997; Hemphill and Ross, 1995; Hemphill et al., 1994; Skalický et al., 2017; Vickerman, 1969; Wakid and Bates, 2004). Therefore, to what extent the kinetics of interaction and the size of the contact area described also apply to the more dynamic *in vivo* environment is yet to be ascertained. The interactions between *T. carassii* and zebrafish cells was limited to the tip of the posterior end of the parasite body (**Figure 9D**), leaving the entire flagellum and parasite's cell body free to rapidly move (**Video 2**). Despite the small surface involved, anchoring occurred very rapidly, suggesting a very strong, yet dynamic, type of interaction, the duration of which was influenced by the presence or absence of a strong hydrodynamic flow as well as colliding red blood cells. Given the *in vivo* dynamic conditions within a blood vessel, it seems unlikely that *T. carassii* would establish stable interactions that involve large portions of the trypanosome cell surface, as for example described *in vitro* for *T. congolense*.

We were unable to determine whether the anchoring area of *T. carassii* corresponds to the flagellum base, flagellar pocket neck or to the cell body membrane. Nevertheless, since *T. carassii* adherence to red blood cells and to glass surfaces was also observed *in vitro*, it will be possible

to investigate the adhesion mechanism at high resolution in the future, for example by mean of surface labelling of the flagellar pocket. Similar to what was reported for *Leishmania donovani* upon surface labelling with the lipophilic dye PKH26 (Ghedini et al., 2001), we also observe that the flagellar pocket can be intensely stained and easily identified upon labelling with fluorescent Wheat Germ Agglutinin (WGA) or Cell Bright dyes (data not shown). The stability of the dyes and resistance to photobleaching however, is currently too low to allow *in vivo* visualization over the course of an infection.

Interestingly, similarly to the vast majority of trypanosomes (Kelly et al., 2017), *T. carassii* does not express VSGs, yet it can establish a long-term infection in its vertebrate (Overath et al., 1999). Given the strategic phylogenetic position of *T. carassii* with respect to Salivarian and Stercorarian trypanosomes, we recently took a genomic, transcriptomic and proteomic approach to characterize in detail this ancestral trypanosome (manuscript in preparation). This will open the possibility to generate transgenic trypanosome lines which, combined with the transparency of the zebrafish and the availability of existing mutant and transgenic zebrafish lines marking blood vessels and several immune cell types, will allow to address questions related to immune evasion strategies alternative to VSG-mediated antigenic variation. Most importantly it will be possible to do so *in vivo* in the natural environment of a vertebrate host. For example, it will be interesting to investigate the efficiency of antibody removal from the parasites' surface *in vivo*, during an ongoing infection. As previously demonstrated *in vitro* for *T. brucei*, besides plasma membrane recycling, directional forward swimming further facilitates antibody accumulation at the posterior end of the parasite owing to hydrodynamic drag-forces generated by the propelling motion of the flagellum (Engstler et al., 2007; Russo et al., 1993). Using our *in vivo* model, it will be possibly to evaluate to what extent directional swimming plays a role *in vivo*, especially for parasites residing in the blood stream where the vast majority of the parasites was shown to be passively dragged by the flow and where directional swimming was observed only in situation in which the blood flow was (temporarily) interrupted.

In conclusion, we described for the first time the swimming behaviour of a trypanosome *in vivo* in the natural environment of a vertebrate host. We reported on the complex and heterogeneous environment in which the parasites reside and how this highly influences the swimming behaviour of trypanosomes. We describe how it is not possible to assign specific behaviours to parasites swimming in any of the host compartments, as the parasites were extremely effective in rapidly adapting their motion to the highly dynamic host environment. We describe backward swimming and a whip-like movements that allowed the parasite to invert direction as well as identified the posterior end of the parasite as a novel anchoring site that allows the parasite to adhere to host cells in a manner different from those described to date for other trypanosomes. Altogether, the establishment of the *T. carassii* zebrafish infection model combined with the genetic tractability of the zebrafish and of the parasite, represent an unique possibilities to address questions related to: 1) parasite swimming behaviour *in vivo* in the natural vertebrate host environment, 2) host-pathogen interaction, 3) parasite biology, 4) the effect of specific immune factors on the progression of the infection, 5) the effect of drugs on both, the parasite and the host and 6) immune evasion strategies of parasites that do not present antigenic variation. For all these reasons, the *T. carassii*-zebrafish model holds the promise to become a valuable complementary model to those currently available, to study the complex biology of trypanosome and their interaction with the vertebrate host.

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3

CHAPTER

Differential response of macrophages and neutrophils to trypanosome infections in zebrafish: occurrence of foamy macrophages

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Abstract

Owing to its transparency and the availability of transgenic lines marking innate immune cells, zebrafish provides an excellent model to study host-pathogen interaction in a vertebrate host. We have previously reported on the establishment of a *Trypanosoma carassii* infection model of larval zebrafish. In the present work, we describe the preliminary characterization of the innate immune response of zebrafish to *T. carassii* infection. We developed a clinical scoring system based on multiple criteria that allowed us to consistently identify high and low infected individuals without having to sacrifice the zebrafish larvae. Next, we investigated the differential response of neutrophils and macrophages in these individuals. We observed that macrophages, and not neutrophils, strongly responded to the presence of trypanosomes independently of the infection level. Not only macrophage's number and distribution, but also morphology and activation state were different between high and low infected fish. Interestingly, exclusively in the cardinal caudal vein of high infected fish, large granular macrophages appeared. These cells were confirmed to be foamy macrophages, rich in lipid droplets and characterized by a strong pro-inflammatory profile, as assessed using *il1 β* and *tnfa* transgenic zebrafish lines. In contrast, low infected individuals displayed a lower inflammatory profile associated with survival of the infection. Altogether, our data provide a preliminary characterization of the differential response of innate immune cells to trypanosome infection and identify foamy macrophages as cells potentially associated with an exacerbated immune response. To our knowledge this is the first report of the development of foamy macrophages during an extracellular trypanosome infection.

Introduction

Trypanosomiasis comprises several diseases including Human Trypanosomiasis and Animal Trypanosomiasis caused by salivarian trypanosomes. These diseases are all caused by protozoan parasites of the genus *Trypanosoma* that live extracellularly in the bloodstream or tissue fluids of their host. For example, *T. vivax* can multiply rapidly and is evenly distributed throughout the cardiovascular system, *T. congolense* tends to aggregate in small blood vessels, whereas *T. brucei* especially can extravasate and multiply in interstitial tissues. Pathologically, anaemia appears to be a factor common to infections with trypanosomes although with different underlying causative mechanisms. These include erythrophagocytosis by macrophages, membrane damage caused by oxidative stress from free radicals, and increased sequestration of iron in macrophages (Constable et al., 2017). Immunologically, infections with trypanosomes are often associated with dysfunction and pathology related to exacerbated innate immune responses (Radwanska et al., 2018).

Trypanosoma carassii is an extracellular blood flagellate that infects several freshwater fish including goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) (Kent et al., 1993). *T. carassii* parasites are transmitted by blood sucking leaches and upon infection proliferate in the blood and interstitial fluids of the host. *T. carassii* (Simpson et al., 2006) shares morphological as well as genetic features of both salivarian and stercorarian trypanosomes (Chapter 4), placing it at a strategic phylogenetic position. Mortalities due to *T. carassii* infection can vary considerably; infection of goldfish can result in acute infection with mortalities between 60 and 100 per cent (Woo and Black, 1984), but infection of carp generally does not lead to mortality rather to the development of a long-lasting chronic infection (Joerink et al., 2007; Overath et al., 1999). In carp, infection with *T. carassii* seems to induce Th-17-like responses characterized by splenomegaly and neutrophilia in the spleen (Ribeiro et al., 2010) and to modulate macrophage activity (Joerink et al., 2006). Factors such as anaemia and immunopathology in fish and mice infected with trypanosomes (McAllister et al., 2019; Stijlemans et al., 2018) suggest a commonality in innate immune responses to trypanosomes across different vertebrates.

We first described the establishment of an experimental trypanosome infection of zebrafish (*Danio rerio*) based on *T. carassii* (Chapter 2). Zebrafish are a powerful model species owing to, among others, their genetic traceability, large number of transgenic lines expressing fluorescent proteins, knock-out mutant lines and transparency of developing embryos allowing high-resolution *in vivo* observation of cell behaviour. During the first 2-3 weeks of development they are mostly devoid of mature T and B lymphocytes and thus offer a window of opportunity to study innate immune responses (Torraca and Mostowy, 2018), including cell types such as neutrophils and macrophages. In the past years the number of pathogens studied in zebrafish has expanded enormously but, except for a single study (Akle et al., 2017), have not included trypanosomes. The response of macrophages and neutrophilic granulocytes towards pathogens has been thoroughly studied in zebrafish for many infections (García-Valtanen et al., 2017; Renshaw and Trede, 2012; Torraca and Mostowy, 2018), but never in the context of trypanosome infections. Here, we characterize innate immune responses of zebrafish to *T. carassii*. Based on a clinical scoring system we could consistently identify high and low infected individuals without having to sacrifice the larvae. We investigated the differential response of neutrophils and macrophages, making use of several transgenic lines marking neutrophils, macrophages as well as *il1b*- and *tnfa*-expressing cells. We observed that macrophages especially, strongly responded to the presence of trypanosomes, by increasing in number, redistributing towards the blood vessels and by exhibiting differential morphology and activation state depending on the infection level. Interestingly, exclusively in high infected individuals, we describe the presence of large, granular macrophages, reminiscent of foamy macrophages (Vallochi et al., 2018), in the cardinal caudal vein characterized by a strong inflammatory profile. Conversely, despite an increase in macrophages number, reduced inflammation was observed in low infected individuals. We discuss the nature of the differential responses of macrophages and neutrophilic granulocytes to this trypanosome infection and discuss the potential association of foamy macrophages with an exacerbated immune responses and susceptibility to *T. carassii* infections.

MATERIALS AND METHODS

Zebrafish lines and maintenance

Zebrafish were kept and handled according to the Zebrafish Book (zfin.org) and local animal welfare regulations of The Netherlands. Zebrafish embryo and larvae were raised in egg water (0.6 g/L sea salt, Sera Marin, Heinsberg, Germany) at 27°C with a 12:12 light-dark cycle. From 5 days post fertilisation (dpf) until 14 dpf larvae were fed once a day with Tetrahymena. From 10 dpf larvae were also fed daily with dry food ZM-100 (ZM systems, UK). The zebrafish lines used in this study included wild type AB, and transgenic *Tg(mpx:gfp)* (Renshaw et al., 2006), *Tg(mpeg1:mCherry-F)* (Nguyen-Chi et al., 2014), *Tg(il1b:eGFP-F)* (Nguyen-Chi et al., 2014), *Tg(tnfa:eGFP-F)* (Nguyen-Chi et al., 2015) or crosses thereof. The latter three transgenic zebrafish lines express a farnesylated (membrane-bound) mCherry (mCherry-F) or eGFP (eGFP-F) under the control of the *mpeg1*, *il1b* or *tnfa* promoter, respectively.

Trypanosoma carassii culture and infection of zebrafish larvae

Trypanosoma carassii were maintained and propagated through common carp and isolated from carp blood as described in Chapter 2. *T. carassii* cultures were kept at 27°C without CO₂ at a density below 5x10⁶/ml, and sub-cultured 1-3 times a week. In this way *T. carassii* could be kept in culture without losing infectivity for up to 2 months. For zebrafish infection, trypanosomes were cultured for 1 week and never longer than 3 weeks. Prior to injection into zebrafish larvae (5 dpf) *Trypanosoma carassii* parasites were centrifuged at 800xg for 5 minutes and resuspended in 2% Polyvinylpyrrolidone (PVP). Zebrafish larvae were anaesthetized in 0.017% Ethyl 3-aminobenzoate methanesulfonate (MS-222, Tricaine, Sigma-Aldrich) in egg water, and injected intravenously. Experimental groups received either n = 200 *T. carassii* or PVP alone as negative control. Parasite number in 1-2 nl drop size, was confirmed by counting in a Bürker chamber at the beginning and after 50 injections. Infected larvae were directly transferred into pre-warmed egg water and kept at 27°C.

Clinical scoring system of the severity of infection

Careful monitoring of the zebrafish swimming behaviour as well as *in vivo* observation of parasitaemia development in transparent larvae, led to the observation that fish could generally be segregated into high and low infected individual. To objectively assign fish to either one of these two groups, we developed a clinical scoring system. An infection score on a scale from 1 to 10 was based on counting parasite: blood cell ratios in 100 events passing through the intersegmental capillary (ISC) above the cloaca. The infection scores were assigned as follows: 1=no parasites observed, 2=1-10% parasite, 3=11-20% parasite, 4=21-30% parasite, 5=31-40% parasite, 6=41-55% parasite, 7=56-70% parasite, 8=71-85% parasite, 9=86-99%, 10=no blood cells observed. Larvae with infection scores between 1-3 were categorized as low infected while scores between 6-10 were categorized as high infected. Next to that, the swimming behaviour of larvae was observed and compared to the control group. Heartbeat of the larvae was monitored and noted if it was slower than the control. The diameter of the caudal vein in the trunk area after the cloaca region was measured in ImageJ 1.49o to quantify the degree of vasodilation. Eventual blockage of tail tip vessel-loop was also noted. Extravasation and the location of extravasated parasites (e.g. fins, muscle, intraperitoneal cavity, and interstitial space lining the blood vessels) was recorded.

Real-time quantitative PCR

Zebrafish were sacrificed by an overdose of MS-222 anaesthetic (50 mg/L). At each time point 3-6 zebrafish larvae were sacrificed and pooled. Pools were transferred to RNA later (Ambion), kept at 4°C overnight and then transferred to -20°C for further storage. Total RNA isolation was performed with the Qiagen RNeasy Micro Kit (QIAGEN, Venlo, The Netherlands) according to manufacturer's protocol. Next, 250-500 ng total RNA was used as template for cDNA synthesis using SuperScript III Reverse Transcriptase and random hexamers (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions with an additional DNase step using DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA). cDNA was then diluted 25 times to serve as template for real-time

quantitative PCR (RT-qPCR) using Rotor-Gene 6000 (Corbett Research, QIAgen), as previously described (Forlenza et al., 2012)(Fink et al., 2016). Primers (**Table 1**) were obtained from Eurogentec (Liège, Belgium). Gene expression was normalized to the expression of *elongation factor-1 alpha* (*ef1a*) housekeeping gene and expressed relative to the PVP control at the same time point or to 0 dpi time point.

Table 1. List of primers used in this study

Gene name	Fw primer sequence	RV primer sequence	Acc. number (zfin.org)
ef1a	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTAGTACCG	ZDB-GENE-990415-52
il-1 β	TTGTGGGAGACAGACAGTGC	GATTGGGGTTTGATGTGCTT	ZDB-GENE-040702-2
il10_1	ACTTGGAGACCATTCTGCC	CACCATATCCCGCTTGAGTT	ZDB-GENE-051111-1
tnfa_1	AAGTGCTTATGAGCCATGC	CTGTGCCAGTCTGTCTC	ZDB-GENE-050317-1
tnfb	AAACAACAAATCACCACACC	ACACAAAGTAAAGACCATCC	ZDB-GENE-050601-2
il6_1	ACTCCTCTCCTCAACCT	CATCTCTCCGTCTCTCAC	ZDB-GENE-120509-1
arg 2	GGTGGGAGCAGGACACAC	TGTCCCGCATGGAGAAGTACT	ZDB-GENE-030131-1334
igm	GAAGCCTCCAATTCTGTTGG	CCGGGCTAAACACATGAAG	ZDB-GENE-030925-46
lgz	CCGGGCTAAACACATGAAG	GCTTGGGGCAGTTACA	ZDB-GENE-040513-8
lck	CACCCAGCATTCTACAGCAA	CACCCAGCATTCTACAGCAA	ZDB-GENE-040617-1
<i>T. car.</i> hsp70	CAGCCGGTGGAGCGCGT	AGTTCCTTGCCGCCGAAGA	FJ970030.1 (GeneBank)

***In vivo* imaging and videography of zebrafish**

Prior to imaging, zebrafish larvae were anaesthetised with 0.017% MS-222 (Sigma-Aldrich). For total fluorescence acquisition, double transgenic *Tg(mpeg1:mCherryF;mpx:GFP)* (Nguyen-Chi et al., 2014)(Renshaw et al., 2006) were positioned on flat agar plates (1% agar in egg water with 0.017% MS-222) and imaged with Fluorescence Stereo Microscope (Leica M205 FA). The image acquisition settings were as following: Zoom: 2.0 - 2.2, Gain: 1, Exposure time (ms): 70 (BF)/700 (GFP)/1500 (mCherry), Intensity: 60 (BF)/700 (GFP)/700 (mCherry), Contrast: 255/255 (BF)/70/255 (GFP)/ 15/255 (mCherry).

Alternatively, anaesthetised larvae were embedded in UltraPure LMP Agarose (Invitrogen) and positioned on the coverglass of a of 35 mm petri dish, (14 mm microwell, coverglass No. 0 (0.085-0.13mm), MatTek corporation) prior to imaging. Roper Spinning Disk Confocal Microscope, equipped with a 40x 0.2 WD objective, was used with the following settings: GFP excitation: 488nm, emission: 496-560nm with 80% transmission,

gain: 1, digitizer: 200 MHz (12-bit); 561 BP excitation: 561nm; emission: 570-620 with 71% transmission, gain: 1, digitizer: 200 MHz (12-bit); BF gain: 1, digitizer: 200 MHz (12-bit). Z-stacks of 1 μ m. Andor Spinning Disk Confocal Microscope, with a 20x 1.0 WD objective was used with the following settings: Dual pass 523/561: GFP excitation: 488nm, emission: 496-560nm, EM gain: 300ms, digitizer: 1 MHz (16-bit); RFP excitation: 561nm; emission: 570-620, EM gain: 300ms, digitizer: 1MHz (16-bit); BF DIC EM gain: 300ms, digitizer: 1 MHz (16-bit). Z-stacks of 1 μ m. High-speed videography was performed as described in Chapter 2.

Fluorescence quantification

Quantification of total cell fluorescence in zebrafish larvae was performed in ImageJ 1.49o program using the free-form selection tool and by accurately selecting the larvae area. Area integrated intensity and mean grey values of each selected fish were measured by the software. To correct for the background, three consistent black areas were selected in each image. Analysis was performed using the following formula: corrected total cell fluorescence (CTCF) = Integrated density - (Area X Mean background value).

BODIPY injection

BODIPYTM FL pentanoic acid (BODIPY-FL5, Invitrogen) was diluted in DMSO to a 3 mM stock solution. Stock solution was diluted 100x (30 μ M) with PVP. Infected larvae 3dpi (8dfp) were injected i.p (heart cavity) with 1 nl of the solution and imaged 18-20 hours later.

Statistical analysis

Analysis of gene expression and total fluorescence data were performed in GraphPad PRISM 5. Statistical analysis of gene expression data was performed on Log(2) transformed values followed by One-way ANOVA and Dunnett's multiple comparisons test. Analysis of fluorescence quantification was performed using a Two-way ANOVA followed by a Bonferroni post-hoc test. In all cases, $p < 0.05$ was considered significant.

RESULTS

Immune response to *T. carassii* infection in zebrafish larvae

We recently reported the establishment of a trypanosome infection in zebrafish larvae using a natural parasite of fish, *Trypanosoma carassii* (Chapter 2). To further investigate the immune response to *T. carassii* infection, we first investigated the kinetics of susceptibility of zebrafish larvae as well as the kinetics of expression of various immune-related genes. Similar to what was reported in chapter 2, *T. carassii* infection of 5 dpf zebrafish larvae leads to approximately 10-20% survival by day 15 with the highest incidence of mortality between day 4-7 (**Figure 1A**). The onset of mortality coincided with the peak of parasitaemia as assessed by microscopical observation (see next section). Interestingly, we consistently observed between 10-20% survival in the infected group, suggesting that even in the absence of a fully developed adaptive immune system, some zebrafish larvae can control *T. carassii* infection. Although the expression of immune-related genes, such as *il6*, *il10* and *tnfa* was elevated (**Figure 1B**), only *il6* was significantly different. Analysis of the kinetics of expression of genes related to the development of adaptive immunity revealed that *lck* transcripts could be detected from time point 0 (5 dpf) onwards, but were not influenced by the infection, suggesting that developing T cells are not proliferating in response to the infection. *Igm* and *igz* transcripts were also detected throughout the sampling period and were significantly affected at 10 dpi (15 dpf).

Based on this preliminary gene expression analysis we could not point at a specific response triggered by the parasite. Analysis was performed on pools of whole larvae which might have obscured any tissue-specific response. Furthermore, we also noticed that, despite the fact that up to 5 larvae were pooled at each sampling point and up to 3 pools were analysed at each time point, a large variation in the response was always observed. This prompted us to investigate the kinetics of parasitaemia and development of (innate) immune responses at the individual level.

Figure 1. *T. carassii* infection of larval zebrafish. A) wild type AB zebrafish larvae (5 dpf) were infected intravenously with $n=200$ *T. carassii*/fish and survival was monitored over a period of 15 days. **B)** *Tg(mpeg1:mCherryF;mpx:gfp)* zebrafish (5 dpf) were injected intravenously with 150 *T. carassii* and sampled at various time points. At each time point 3-6 pools of 3-5 larvae were sampled for real-time quantitative PCR analysis. Bars indicate average and standard deviation (SD) on $n=3-6$ pools per time point. Bars without standard deviation, indicate genes that were low expressed and detected in only one pool. Relative fold change was normalised to the *ef1a* and expressed relative to the PVP-injected control.

Clinical signs of *T. carassii* infection and clinical scoring system

To characterize the response to *T. carassii* infection in individual zebrafish larvae, we developed a clinical scoring system to determine individual infection levels, enabling us to group together individual larvae based on severity of infection. From 4 dpi onwards, we could consistently sort larvae into groups of high or low infected individuals based on *in vivo* microscopical observations, without the need to sacrifice animals (**Video 1**). Infection levels were categorised using four criteria: 1) escape reflex (slow vs fast) upon contact with a pipette tip, 2) infection scores (1-10, see details in Materials and Methods), based on the ratio of blood cells and parasites passing through an intersegmental capillary (ISC) in 100 events (**Fig. 2B, 2C**) (**Video 1**, 00:06-00:39 sec), 3) extravasation, based on the presence of parasites outside blood vessels (**Fig 2D**) (**Video 1**, 00:40-1:20 sec) and 4) vasodilation, based on the diameter of the caudal vein (**Fig. 2E, 2F**). The first criterion defined all individuals with a minimal escape reflex (slow swimmers) as high-infected individuals: they were mostly located at the bottom of the tank and showed minimal reaction upon direct contact with a pipette. Larvae with a normal escape reflex (fast swimmers) however, were not exclusively low-infected individuals. Therefore, a second criterion was used based on parasite counting in ISC (**Video 1**, 00:06-00:39 sec). Individuals with an infection score between 1-3 were categorized as low infected and always survived the infection (see Materials and Methods). Individuals with an infection score between 6-10 were categorized as high infected and generally succumbed to the infection. Individuals with an intermediate score (4-5) could go both ways: they either showed a delayed parasitaemia and later developed high parasitaemia (common) or recovered from the infection (rare). The third criterion clearly identified high-infected individuals as those showing extensive extravasation at two or more of the following locations: peritoneal cavity (**Fig. 2D**) (**Video 1**, 00:40-00:59 sec), interstitial space lining the blood vessels, muscle tissue (**Video 1**, 01:00-01:07 sec) or fins (**Video 1**, 01:08-01:20 sec), in particular the anal fin. At these locations, in high-infected individuals, parasites could accumulate in high numbers filling up all available spaces. Extravasation however could also occur in low-infected individuals, but to a lesser extent. The fourth criterion,

vasodilation of the caudal vein associated with high numbers of parasites in the blood vessels, was a definitive sign of high infection level, and never occurred in low infected larvae. To validate our scoring system, expression of a *T. carassii*-specific gene was analysed in pools of larvae classified as high or low infected. As expected, in individuals categorized as high-infected, *T. carassii*-specific gene expression increased more than 60-fold whereas in low-infected individuals the increase was less than 20-fold (**Fig. 2G**). Altogether these data show that *T. carassii* infects zebrafish larvae, but that the infection can develop differently among individuals, leading to different outcomes. The clinical scoring system based on numerous criteria is suitable to reliably separate low and high infected larvae to further investigate individual immune responses.

Figure 2. Progression of *T. carassii* infection in zebrafish larvae. **A)** representative frame of *T. carassii* freshly isolated from carp blood and imaged using high-speed videography with Leica DM6 upright microscope, using 100x magnification and DIC lens. **B-C)** 5 dpf zebrafish were infected with $n=200$ parasites and imaged 2 dpi (B) or 5 dpi (C). Shown are representative images

of intersegmental capillaries (ISC) containing various number of *T. carassii* (red arrows). Frames are extracted from high-speed videos acquired with a Leica DMI8 inverted microscope at a 40x magnification. **D)** Zebrafish were infected and imaged as described in B-C. Images show extravasated *T. carassii* parasites (some indicated with red arrows) in the intraperitoneal cavity. **E-F)** 5 dpf zebrafish were infected with $n=200$ *T. carassii* and imaged 7 dpi with a Leica DMI8 inverted microscope at 40x magnification. Square brackets and numbers indicate the diameter of the caudal vein $\pm$ SD. **G)** *T. carassii* infection level measured by the expression of the *heat-shock protein-70* (*hsp70*). *Tg(mpeg1:mCherryF; mpx:GFP)* zebrafish larvae (5 dpf) were injected intravenously with $n=200$ *T. carassii* and sampled at the indicated time points. High and low infected fish were separated from 4 dpi onwards based on our clinical score sheet. At each time point 3 pools of 3-5 larvae were sampled for RNA isolation and cDNA synthesis followed by real-time quantitative gene expression analysis. Each data point represents the mean of 3 pools, except for the high infected group at 10 dpi where only one pool could be made due to high mortalities. Relative fold change of genes is normalised to the zebrafish *ef1a* housekeeping gene and expressed relative to the parasite injected group at time point 0h.

Video 1. Clinical signs of *T. carassii* infection in high and low infected zebrafish larvae. High-speed videos (500 frames/sec, fps) or real-time videos (20 fps) capturing trypanosomes *in vivo* in blood vessels or extravasated in tissues, as well as describing typical signs of anaemia and vasodilation.

***T. carassii* infection induces a strong macrophage response in zebrafish larvae**

After having established a method to determine infection levels in each larva, we next investigated whether a differential innate immune response would be mounted in high- and low-infected fish. To this end, using double-transgenic *Tg(mpeg:mCh;mpx:gfp)* zebrafish, we first analysed overall macrophage and neutrophil responses by quantifying total fluorescence in high- and low-infected individuals (**Fig. 3**). Total neutrophil responses (green fluorescence) was not significantly affected by the infection (**Fig. 3A, 3B**). In some cases, however, a redistribution of neutrophils could be observed in infected fish, where cells moved from lining the caudal vessels to a sparse distribution throughout the body (data not shown). Fish-to-fish variation however, obscured this observation and therefore no strong correlation between infection level and neutrophil activation could

be made. In clear contrast, total macrophage responses (red fluorescence) increased significantly in infected fish from 3 dpi onwards (**Fig. 3D, 3E**). The increase in fluorescence can be indicative of macrophage activation and/or proliferation. Interestingly, despite the strong macrophage response to the infection, there was no significant difference between high- or low-infected individuals, suggesting that macrophages respond to the presence of parasites but not to the total number of parasites. Gene expression levels of *mpeg* and *mpx* genes was also examined by real-time quantitative PCR confirming our microscopic observations (**Fig. 3C, 3F**). Up to 4-fold upregulation of *mpeg* was observed at 6 dpi in both the high- and low-infected group. Conversely, *mpx* expression was not found to be significantly upregulated throughout the experiment. For both genes, high expression was observed at 10 dpi, but this was measured in only one pool of three high infected larvae which later succumbed to the infection.

Figure 3. Macrophage activation in *T. carassii* infected zebrafish is independent of parasite number. A, D) *Tg(mpeg1:mCherryF;mpx:GFP)* were infected intravenously at 5 dpf with $n=200$ *T. carassii* and imaged at the indicated time points. Starting at 5 dpi larvae were separated in high and low infected groups. The images are representatives of $n=3-44$ larvae per group, depending on the number of high or low infected larvae categorized or surviving at each time point. Images were acquired with Leica M205 FA Fluorescence Stereo Microscope with 1.79x zoom. **B, E)** Corrected Total Cell Fluorescence (CTCF) quantification of infected and non-infected larvae of the same experiment as in A. Bars represent average and standard deviation of red and green fluorescence in $n=3-44$ whole larvae. * indicates significant differences ($P<0.05$) to the PVP control as assessed by Two-way ANOVA followed by Bonferroni post-hoc test. **C, F)** Relative fold change of *mpeg* and *mpx* genes during the infection. 5 dpf *Tg(mpeg1:mCherryF;mpx:GFP)* zebrafish larvae were injected intravenously with 150 *T. carassii* and sampled at various time points. High and low infected fish were separated from 4 dpi onwards based on our clinical score sheet. At each time point 3 pools of 3-5 larvae were sampled for RNA isolation and cDNA synthesis followed by real-time quantitative gene expression analysis. Each data point represents the mean of 3 pools, except for the *T. carassii* high infected group at 10 dpi where only one pool could be made due to high mortalities. Relative fold change of immune genes is normalised to *ef1a* housekeeping gene and expressed relative to the PVP injected control.

Differential response of neutrophils and macrophages in high and low infected zebrafish larvae

When analysing total macrophages and neutrophils fluorescence in infected individuals, we consistently observed increased fluorescence around the head kidney region and the cardinal vein, suggesting possible proliferation and/or cell redistribution to these locations. Proliferation of macrophages is partly supported by gene expression data in whole larvae (Fig. 3F) that showed a 3-fold increase in *mpeg* transcription around the peak of parasitaemia. However, proliferation of macrophages should be further investigated and confirmed (work in progress).

Next, we focused on the cardinal caudal vein region at 5 and 7 dpi (**Fig. 4A**), time points at which clear differences in parasitaemia were observed between high and low infected fish (**Fig. 2**). Neutrophils were generally numerous and located along the cardinal caudal vein in control, low and high infected fish. No apparent differences could be observed between the groups, although the total number of neutrophils appeared to be lower in infected fish at 7 dpi (**Fig. 4A, upper panels**). When looking at macrophages, increased cell number was indeed observed along the caudal vein of high and low infected larvae, especially at 5 dpi (**Fig. 4A, lower panels**).

When looking in more details to the immune cells present within and around the caudal vein, clear differences could be observed in macrophage morphology and location in high infected fish (**Fig. 4B**). In control fish, macrophages generally exhibited an elongated and dendritic morphology, were very rarely observed inside the vessel and were mostly located in the ventral fin, along the vessel or in the tissue between the cardinal vessels (**Figure 4B, left panel**). A similar morphology and distribution was observed also in low infected fish (not shown). Strikingly, in high infected larvae, we consistently observed large, dark, granular and round macrophages located almost exclusively inside the cardinal vein on the dorsal luminal side. These dark macrophages were clearly visible already in bright field images due to their size, colour and location, and could be present as single cells or as aggregates (**Fig 4B, right inset**). The occurrence of these large macrophages increased with the progression of the infection, and was exclusive to high infected individuals since they were never observed in low-infected or control fish.

The rounded morphology, the size and the dark appearance of these cells was reminiscent of that of foamy macrophages. Therefore, to further investigate the nature of these cells, the green fluorescent fatty acid BODIPY-FLC5 was used to track lipid accumulation in infected larvae (**Fig. 5**). Interestingly, administration of BODIPY-FL5 in high infected fish, 1 day prior to the expected appearance of the large macrophages, confirmed the accumulation of lipids in these cells (**Fig. 5**). This result suggests that the large rounded macrophages in the cardinal caudal vein are indeed foamy macrophages.

Figure 4. Macrophages are recruited to the cardinal vein of high infected zebrafish larvae. A) *Tg(mpeg1:mCherryF; mpx:GFP)* zebrafish larvae were infected intravenously at 5 dpf with $n=200$ *T. carassii* or PVP and imaged at 1-7 dpi with Andor Spinning Disk Confocal Microscope using 20x magnification with Bright Field, GFP or mCherry filters. Representative images of each time points are shown with the cardinal caudal vein outline indicated with dashed lines. **B)** *Tg(mpeg1:mCherryF; mpx:GFP)* zebrafish larvae were treated as in A, and imaged at 7 dpi using 40x magnification with Bright Field or mCherry filters. Representative images are shown with blue arrowheads pointing at macrophages outside the vessel and white arrowheads indicating large round macrophages inside the cardinal vein (dashed line).

Figure 5. The large macrophages inside the vessel of high infected zebrafish are foamy macrophages. Casper zebrafish larvae (5dpf) were infected with $n=200$ *T. carassii* per fish. 3-days post-infection, larvae received 1 nl of 30 μ M BODIPY-FLC5 and were imaged 24 hours later using a Roper spinning disc Confocal microscope with 20x magnification.

Foamy macrophages have a pro-inflammatory activation state

To further investigate the activation state of foamy macrophages, we made use of the *Tg(tnfa:gfp-F;mpeg1.4:mCherryF)* double transgenic zebrafish line, having macrophages in red and *tnfa*-expressing cells in green, and of the *Tg(il1b:gfp-F)* transgenic line having *il1b*-expressing cells in green (Fig. 6 and Fig. 7). We first focused on the time point at which the foamy macrophages were most clearly present in highly infected individuals. With respect to *tnfa* expression, our results clearly show that all large foamy macrophages, were strongly positive for *tnfa*, suggesting an inflammatory activation state (Fig. 6A, white asterisks). Interestingly, not only the large foamy macrophages within the vessel, but also dendritic or lobulated macrophages outside or lining the vessel showed various degrees of activation. It appeared that macrophages that

where still partly in the vessel (Fig. 6B, yellow arrow head) displayed higher *tnfa* expression than macrophages lining the outer endothelium of the vessel (white arrow heads). This could suggest that the presence of *T. carassii* components within the vessels might trigger macrophage activation.

At the time of the study homozygous *Tg(il1b:gfp-F;mpeg1.4:mCherryF)* double transgenic zebrafish were not available. Crosses between the two separate lines generally led to very faint GFP signal. For this reason, to analyse *il1b* expression in foamy macrophages we proceeded with homozygous *Tg(il1b:gfp-F)* and took advantage of the fact that foamy macrophages could be easily identified in the bright field due to their typical large and granular morphology. Similar to what was observed for *tnfa* expression, all foamy macrophages within the vessel were strongly positive for *il1b* (**Fig. 6C**), confirming their pro-inflammatory profile.

Figure 6. Foamy macrophages have an inflammatory profile. *Tg(tnfa:gfp-F; mpeg1.4:mCherryF)* **A-B**) and *Tg(il1b:gfp-F)* **C**) 5 dpf zebrafish larvae, were infected with $n=200$ *T. carassii*. At 3 dpi, high infected individuals were imaged with an Andor Spinning Disk Confocal Microscope. Foamy macrophages (asterisks) were easily identified within the cardinal caudal vessel (dashed lines) and were strongly positive for *tnfa* (**A**) and *il1b* (**C**) expression (GFP signal). **B**) same as in A, but a few stacks up focusing on the cells lining the endothelium. Macrophages that were partly inside and partly outside the vessel (yellow arrow head) were also strongly positive for *tnfa*, whereas macrophages lining the outer vessel wall had a lower *tnfa* expression (white arrowheads).

High infected fish have a strong inflammatory profile associated with susceptibility to infection

When comparing the overall activation state in low and high infected fish it was apparent that high infected individuals generally exhibited a higher pro-inflammatory response than low infected fish. Generally, a higher number of *il1b*- and *tnfa*-expressing cells was observed in high infected fish when compared to low infected and non-infected PVP controls (Fig. 7). In high infected fish, *il1b* and *tnfa* expression was observed not only in foamy macrophages, but also in other leukocytes. Although *tnfa* positive macrophages could be seen in low infected individuals, these were generally few. Altogether, these results suggest that an exacerbated pro-inflammatory response is detrimental for the host and leads to susceptibility to the infection since, as mentioned above, high infected individuals do not survive the infection. Low infected individuals however, with increased macrophage number and moderate cytokine response, will be able to recover from the infection.

3

Figure 7. High infected fish have a strong inflammatory profile. Zebrafish larvae (5 dpf), either *Tg(il1b:gfp-F)* (**A**) or *Tg(tnfa:gfp-F; mpeg1.4:mCherryF)* (**B**), were infected as described in figure 6. At 3 dpi, larvae were separated in high and low infected individuals and at 5 dpi imaged with a Roper Spinning Disk Confocal Microscope. **A**) *il1b-gfp* expression was generally low in non-infected PVP controls and in low infected individuals and was mostly localized in the endothelial cells lining the vessels endothelium. In low infected fish (middle panel), only a few *il1b*-expressing cells (yellow arrowhead) were present. In high infected individual however (right panel), foamy macrophages (asterisks) as well as other leukocytes (yellow arrowhead), were strongly positive for *il1b-gfp* expression. **B**) In non-infected PVP controls (left panel), several macrophages can be observed outside the vessel but none was positive for *tnfa*. In low infected individuals (middle panel) macrophages were present inside and outside the vessel. Except the occasional macrophage showing *tnfa-gfp* expression (white arrowhead), they generally did not exhibit strong GFP signal. In high infected individual however, foamy macrophages (asterisks) as well as other leukocytes (white arrow), were strongly positive for *tnfa-gfp* expression.

Discussion

In this study we report a preliminary characterization of the differential response of macrophages and neutrophils *in vivo* during a trypanosome infection. After having established a clinical scoring system of infected larvae, we were able to consistently differentiate high and low infected individuals. We observed that macrophages, and not neutrophils, strongly respond to the presence of the parasites, and that this response was independent of the parasitaemia level. Redistribution and recruitment of macrophages to the caudal vein was confirmed, while neutrophils did not seem to respond to the presence of the parasite in the vessel. Macrophages displayed different morphologies depending on their location, inside or around the vessel, and their activation state was different in high and low infected fish. Exclusively in high infected fish, we observed the occurrence of foamy macrophages in the cardinal caudal vein, and these were characterized by a strong inflammatory profile. To our knowledge this is the first report of the development of foamy macrophages during an extracellular trypanosome infection.

We recently established a *Trypanosoma carassii* infection in larval zebrafish (Chapter 2). After having investigated the swimming behaviour of the parasite, we wanted to investigate the immune response of the

zebrafish host to *T. carassii* infection. First, we observed that infected larvae could be divided into low and high infected individuals, where the latter generally succumbed to the infection. Next, we observed an increase in total red fluorescence in both high and low infected fish, indicative of a strong macrophage response. Although it cannot be excluded that the increase in fluorescence is due to upregulation of the *mpeg* promotor, the strong fluorescence signal observed especially in the head kidney (haematopoietic organ) and the more detailed image analysis reported in figure 4, are indicative that macrophage proliferation might occur. Experiments aiming at quantifying cell proliferation in live cells (EdU assay) or in fixed larvae (PCNA staining) are currently ongoing.

Interestingly, exclusively in high infected individuals, by 3 dpi, large, rounded, dark and granular cells were observed, already under the bright field view, in the lumen of the caudal vein. These cells were confirmed to be macrophages. Using BODIPY-FLC5 labelled fatty acid precursor we were able to confirm that these were foamy macrophages with high cytoplasmic lipid content. Foam cells, or foamy macrophages have been named after the lipid bodies accumulated in their cytoplasm leading to their typical morphological appearance (Dvorak et al., 1983), they are enlarged in size and contain diverse cytoplasmic organelles (Melo et al., 2003). They have been associated with several infectious diseases, including leishmaniasis, Chagas disease, experimental malaria, toxoplasmosis and tuberculosis, reviewed in (López-Muñoz et al., 2018; Vallochi et al., 2018). For example, during *T. cruzi* infection of rat, increased numbers of activated monocytes or macrophages were reported in the blood or heart (Melo and Machado, 2001) and interestingly, parasite uptake was shown to directly initiate lipid body formation in macrophages leading to the appearance of foamy macrophages (D'Avila et al., 2011). During human *Mycobacterium tuberculosis* infections, foamy macrophages play a role in sustaining the presence of bacteria and contribute to tissue cavitation enabling the microbes to spread around (Russell et al., 2009). Independently of the disease, it is clear that foamy macrophages are generally associated with inflammation, since their cytoplasmic lipid bodies are a source of strong mediators of inflammation, eicosanoids (Melo et al., 2006; Wymann and Schneider, 2008). In turn, inflammatory mediators such as Prostaglandin

E2, benefit parasite survival, as shown in *Trypanosoma*, *Leishmania*, *Plasmodium*, and *Toxoplasma* infections (reviewed in (Vallochi et al., 2018)). Our results are consistent with these reports as we show the occurrence of foamy macrophages exclusively in fish that developed high parasitaemia, a strong pro-inflammatory response and succumbed to the infection. Although we did not investigate in detail the kinetics of parasitaemia and occurrence of foamy macrophages, it will be interesting to investigate whether the presence of foamy macrophages precedes or follows the increase in parasite numbers. Despite the presence of foamy macrophages has been reported for intracellular trypanosomatids (*T. cruzi*, *Leishmania*), and arachidonic acid-derived lipids were reported to act as regulators of the host immune response and parasite burden during *T. brucei* infections (López-Muñoz et al., 2018), to our knowledge our study is the first to report the presence of foamy macrophages during an extracellular trypanosome infection.

The fact that we could detect the occurrence of large, granular cells already in the bright field and that using transgenic lines, we could confirm these to be macrophages, further emphasizes the power of the zebrafish model. This allowed us to visualize *in vivo*, in real time, the occurrence of cells in specific locations of the animal, that we otherwise could have missed if we for example were to bleed an animal, perform immunohistochemistry or gene expression analysis. In fact, as mentioned above, our gene expression analysis, even on pools of high and low infected larvae, failed to detect the clear differential activation state between the two groups. The possibility to separate high and low infected animals without the need to sacrifice them, allowed us to follow at the individual level the progression of the infection and ensuing differential immune response.

Previous work by our group suggested that *T. carassii* induces, *in vitro* and *in vivo*, an alternative activation of carp macrophages, characterized by increased arginase activity and upregulation of regulatory cytokines that *in vivo* leads to the establishment of a chronic type of infection. In contrast, another natural blood parasite of carp, *Trypanoplasma borreli*,

typically leads to an acute infection characterized by the development of classically activated macrophages marked by increased inducible Nitric Oxide Synthase (iNOS) activity and pro-inflammatory cytokines gene expression (Forlenza et al., 2008; Joerink et al., 2007, 2006). Given the fact that *T. carassii* induces an acute infection in zebrafish larvae but a chronic infection in carp, we looked at signature genes of both classically and alternatively activated macrophages in zebrafish. Using real-time quantitative PCR, we could not confirm upregulation of either the signature genes of classically activated macrophages (iNOS2a or iNOS2b), or the signature gene of alternatively activated macrophages (arginase2). Based on gene expression, our analysis could not confirm any particular macrophage polarization route. This could however have been due to the use of pooled whole larvae. In parallel, we also performed gene expression analysis on pools of either high or low infected larvae, but again, no significant differences were observed that could explain the different outcomes of the infections (data not shown). In the future it will therefore be interesting to analyse the transcription profiles of sorted macrophage populations from low and high infected larvae. Given the marked heterogeneity in macrophage activation observed especially within high infected individuals, single cell transcriptome analysis, of foamy macrophages in particular, may provide insights in the differential activation state of the various macrophage phenotypes. Furthermore, as recently reviewed (Fink et al., 2015), the zebrafish has already emerged as being a particularly useful animal model for the study of inflammation and infection *in vivo* and can be a powerful complementary model to investigate particularly the plasticity of macrophages to switch subtypes during an inflammation or infection response. As also seen in Chapter 2, zebrafish may help to examine macrophage polarization *in vivo* by truly reflecting the complex nature of *in vivo* environments during an ongoing infection.

It is important to realise however, that our study was performed in zebrafish larvae and therefore the response of macrophages could be different in the context of a fully developed adaptive immune response. It will be indeed interesting to investigate whether a similar response and the formation of foamy macrophages occurs in adult larvae. The

availability of transparent adult zebrafish lines, such as the casper line (White et al., 2008), may aid the *in vivo* analysis of macrophage activation in adult individuals.

During the course of the study we attempted to investigate the specific role of macrophages in protection or susceptibility to *T. carassii* infection. To this end we made use of a cross between the *Tg(mpeg1:Gal4FF)*^{g12} (Ellett et al., 2011) and the *Tg(UAS-E1b:Eco.NfsB-mCherry)*^{c26} (Davison et al., 2007) line that allows the timed metronidazole (MTZ)-mediated depletion of macrophages in zebrafish larvae. Unfortunately, *in vitro* analysis of the effect of MTZ on the parasite itself, revealed that trypanosomes are susceptible to MTZ rendering the *nfsB* line not suitable to investigate the role of macrophages during this particular type of infection. Alternatively, we attempted to administer clodronate particles as described previously (Travnickova et al., 2015). In our hands however, administration of 5 mg/ml clodronate (3 nl) to 5 dpf larvae, led to the rapid development of oedema.

Altogether, in this study we provide the preliminary characterization of the innate immune response of zebrafish larvae to *T. carassii* infection. Taking advantage of the transparency and availability of various transgenic zebrafish lines, we established a clinical scoring system that allowed us to segregate individuals into high and low infected fish, and were able to describe a differential response of neutrophils and macrophages in these two groups. Interestingly, for the first time in an extracellular trypanosome infection, we report the occurrence of foamy macrophages characterized by a strong inflammatory profile and associated with susceptibility to the infection. Our model paves the way to investigate which mediators of the parasite are responsible for the induction of such inflammatory response as well as study the condition that lead to the formation of foamy macrophages *in vivo*.

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4

CHAPTER

Transcriptome sequencing of the fish parasite *Trypanosoma carassii* reveals widespread loss during the evolution of the *Trypanosoma brucei* cell surface.

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Abstract

Trypanosoma brucei, the causal agent of African trypanosomiasis, is the best characterised kinetoplastid pathogen. It proliferates in the blood and tissue spaces of its vertebrate hosts and escapes elimination by the immune system through antigenic variation of the variant specific glycoprotein (VSG), the single immunodominant surface protein that forms a densely packed cell surface coat. Although the biology of antigenic variation in *T. brucei* is moderately well understood, little is known of how this VSG-based system of antigenic variation evolved. To provide insight into the changes that occurred, we determined the transcriptome and performed a proteomic analysis of the cell surface of the fish parasite *Trypanosoma carassii*, a sister species to the Stercorarian and Salivarian clades that comprise mammal-infective trypanosomes including *T. brucei*. Through comparative analysis we show that the evolution of *Trypanosoma brucei* antigenic variation occurred coincidentally with dramatic loss of gene families encoding putative cell surface proteins. In addition, we report the identification of two novel large families of GPI-anchored surface proteins that are unique to *T. carassii* and propose that these surface protein genes may act in immune evasion in these parasites.

Introduction

The trypanosomatida are a group of single-celled eukaryotes that encompass numerous important pathogens of crops, livestock and people. Though there is thought to have been a single evolution of parasitism within this monophyletic clade¹, there have been multiple independent adaptations to intracellular and extracellular lifestyles that have resulted in a varied array of immune evasion strategies²⁻⁴.

The best characterised trypanosomatid, *Trypanosoma brucei*, causes human and animal African trypanosomiasis. *T. brucei* is exclusively extracellular and within the human host the parasites can be found in both the bloodstream, lymphatic system and tissues. These locations mean that *T. brucei* is constantly exposed to attack from both innate and adaptive immune responses. In the mammalian host the *T. brucei* cell surface is covered in a densely packed coat of ~ 5 million molecules of a single GPI-anchored variant surface glycoprotein (VSG)⁵⁻⁸. Although this protein coat may shield some surface antigens from antibody recognition^{9,10}, its primary function is in mediating immune evasion via antigenic variation as a population survival strategy. In this process an individual *T. brucei* cell changes its expressed VSG gene, and thus presents a novel protein surface to the host immune system. It has been proposed that additional immune evasion is provided by clathrin-mediated endocytosis and degradation of antibody bound to surface proteins^{7,11,12}. This additional protection is thought to increase the survival time of stumpy form trypanosomes and thus increase transmission rate¹³.

Though the molecular and cell biology of antigenic variation have been characterised, and the evolution of antigenic diversity within the VSG repertoire of individual Salivarian trypanosome species is well-documented¹⁴, little is known of how this strategy of antigenic evolved. It is unclear what the ancestral cell surface looked like and, until recently, few cell invariant surface proteins had been identified. However novel insight into these proteins was provided by a detailed proteomic investigation of the *T. brucei* cell surface¹⁵. This study identified several novel proteins that localised to the flagellar pocket in addition to multiple other surface localised proteins of unknown function.

These included two main classes of proteins: receptor-like surface proteins predicted to be GPI- anchored and unique to *T. brucei*, and transporter-like surface proteins that are conserved in other species and are predicted to contain transmembrane helices¹⁵.

T. carassii is a kinetoplastid parasite of cyprinid fish such as carp (*Cyprinus carpio* L.), goldfish and some non-cyprinids¹⁶ that is transmitted between fish hosts by leeches. Like Salivarian trypanosomes, *T. carassii* lives extracellularly in the blood and tissue fluids of the fish. Both the innate and adaptive immune system of teleost fish (including carp) is highly similar to that present in mammals with the presence of immunoglobulins (IgD, IgM, IgT), the thymus gland and presence of (restricted) immune memory¹⁷⁻¹⁹. However, the mechanism by which *T. carassii* evades the immune system of carp is unknown²⁰. Infections occur in both wild and farmed fish populations where prevalence of the parasite is high but parasitaemia is often low²¹. Moreover, infection is rarely lethal to the common carp^{22,23}. There is no evidence that antigenic variation occurs in this parasite, and thus the mechanism by which this parasite is able to achieve chronic infections is unknown²⁴. Though the mechanisms of immune evasion are unknown some protein components of the *T. carassii* cell surface have been identified. These include a GPI-anchored mucin-like glycoprotein, a trans-sialidase-like protein and a member of the major surface protease (MSP, also known as GP63 and leishmanolysin)^{25,26}.

To provide insight into the evolution of the *T. brucei* cell surface we have sequenced the transcriptome and performed cell surface proteomics on bloodstream form *T. carassii*. We provide robust phylogenetic evidence that ancestor of *T. carassii* diverged from the trypanosomatid lineage prior to the divergence of the Stercorarian and Salivarian trypanosomes. Thus *T. carassii* is an outgroup for these two disparate lineages that enables inference about the ancestral gene content of the clade. We show that 80 families of putative surface protein genes previously thought to be unique to Stercorarian trypanosomatids were present in the common ancestor Stercorarian and Salivarian trypanosomes and thus lost from the *T. brucei* lineage concomitant with the evolution of antigenic variation. In contrast, only two surface protein families present in the last common ancestor of

the trypanosomes were lost in Stercorarian trypanosomatids. In addition, we identify two novel large families of GPI-anchored surface that are unique to *T. carassii* and propose that these act in immune evasion in these parasites.

Results

De novo transcriptome assembly and phylogenetic inference supports *T. carassii* as a sister species to both Salivarian and Stercorarian trypanosome clades

mRNA from bloodstream cells of *T. carassii* was extracted, sequenced and subject to *de novo* transcriptome assembly. The assembled transcriptome contained 13,960 transcripts that encoded open reading frames ≥ 100 amino acids. The predicted protein coding genes contained within these transcripts were subject to orthogroup inference using OrthoFinder²⁷ and the predicted proteomes of 15 other trypanosomes. Of these proteomes, 3 were from members of the Salivarian trypanosome clade (*T. brucei*, *T. vivax*, *T. congolense*) and 3 were from members of the Stercorarian trypanosome clade (*T. cruzi*, *T. rangeli* and *T. grayi*). Orthogroup inference resulted in identification of 17,608 orthogroups that were found in *T. carassii* or one or more of the Salivarian or Stercorarian trypanosomes (Supplementary Table S1). A phylogenetic tree was inferred from a 100 bootstrap replicate concatenated multiple sequence alignment of ubiquitously conserved single copy genes. Each alignment contained 63,842 phylogenetically informative un-gapped sites sampled equally from 466 single copy genes ubiquitously conserved in all 16 species. All trees provided support for *T. carassii* as a sister group to both Salivarian and Stercorarian trypanosomes (**Fig. 1**), having diverged from the trypanosome lineage prior to the divergence of the Salivarian and Stercorarian trypanosomes.

Figure 1. Bootstrapped maximum likelihood phylogenetic tree inferred from 466 ubiquitously conserved single copy genes. Support values indicated at internals expressed as fractions (i.e. 1.0 = 100%). Scale bar indicates 0.1 amino acid substitutions per site.

The evolution of antigenic variation is coincident with a loss of 27% of ancestral surface protein gene families.

Given the phylogenetic relationship of *T. carassii* to other trypanosomatids (Fig. 2A) the differential patterns of presence and absence of genes between *T. carassii*, Salivarian and Stercorarian trypanosomes can inform about patterns of gene loss in this clade. In total, *T. carassii* shared 834 orthogroups with Stercorarian trypanosomatids that were not found in any Salivarian trypanosome (Fig. 2B). In contrast *T. carassii* shared 42 orthogroups with Salivarian trypanosomes that are not found in any sequenced Stercorarian trypanosome (Fig. 2B). This differential presence of orthogroups indicates that a larger number of gene families lost were lost in the Salivarian lineage than in the Stercorarian lineage since the divergence from the last common ancestor.

Figure 2. A summary of the results of orthogroup clustering. **A)** Phylogenetic tree showing the relationship between the species used in this analysis. Clades are highlighted in different colours to facilitate easier interpretation of subsequent figure parts. **B)** Venn diagram showing the number of shared orthogroups between the *T. carassii* and the Salivarian and stercoarian trypanosomes. Numbers in parenthesis indicate the number of orthogroups that are found in more than one species in the Salivarian or stercoarian groups. **C)** As in B but for putative surface protein orthogroups. **D)** The predicted cell surface localisation method for the putative cell surface orthogroups shared between different species groups.

To determine which if any of these gene family loss events affected the putative surface proteins the full set of predicted protein sequences used for orthogroup clustering were searched for sequences encoding domains associated with cell surface localisation. These comprise the signal peptide (SP), transmembrane domains (TMD) and the glycoposphatidylinositol (GPI) anchor. While these domains (either SP and TMD, or SP and GPI) can facilitate cell surface localisation, their presence is not diagnostic of cell surface localisation. Moreover, proteins containing SP and TMD may reside in multiple internal compartments within the cell including the endoplasmic reticulum, Golgi apparatus, or other endomembranes.

Therefore, the presence of these domains is used as a bioinformatic screen to identify those proteins that, in the absence of other information, may potentially be localised to the cell surface.

This screen identified a set of 1384 orthogroups that were comprised of possible cell surface localised protein coding genes which we define here as “possible surface orthogroups” (PSO). The complete set of PSOs is provided in Supplementary Table S1. Of the 1384 PSOs the majority ($n = 1016$, 73%, Fig. 2C) were found to be unique to a single species. These are likely to represent gene families that have evolved following the last speciation events captured in this phylogeny (i.e during the time encompassed by the terminal branches in the tree). This is consistent with previous reports that state the predicted complement of surface proteins are largely species specific²⁸. Of the total set of PSOs, 294 ($n = 212 + 80 + 2$, 21%, Fig. 2C) had a species distribution consistent with presence in the last common ancestor of all species under consideration and represent a common core set of ancestral PSOs (Supplementary Table S1). Of this ancestral set, 212 were present in at least one species from both the Salivarian or Stercorarian trypanosome groups, and 114 were found in every species in this analysis. Of the 114 ubiquitously conserved PSOs the majority (103, 90%, Fig. 2D) had a signal peptide and transmembrane domain, 11 had both a signal peptide & GPI anchor. These observations are consistent with previous reports that GPI-anchored proteins are largely parasite- specific whereas transmembrane proteins are more broadly conserved across the trypanosomatids

¹⁵. The overlap between the predicted PSOs and those identified in previous studies^{15,28} are shown in Supplementary Table S1. Approximately half of the ubiquitously conserved PSOs (59, 52%) are retained as single copy genes in each species and thus the other half of these surface protein genes have been subject to gene duplication events and expansions in gene copy number since the last common ancestor. In contrast nearly all of the species specific surface orthogroups ($n = 618$, 94%) are represented by single copy genes. This suggests that the *de novo* evolution of surface localised genes is a common theme in these trypanosome lineages.

In total, 22 PSOs were found in multiple Stercorarian trypanosomes but not in *T. carassii* or in Salivarian trypanosomes and 24 were found in

multiple Salivarian trypanosomes but not in *T. carassii* or in Stercorarian trypanosomes (Supplementary Table S1 and Fig. 2C). These gene sets may represent candidate genes for adaptation to different transmission routes within the respective groups. Of the core set of 292 ancestral PSOs 80 are not found in any of the Salivarian trypanosomes (Fig. 2C). In contrast to this only 2 PSOs that were presented in the last common ancestor of these species appear to have been lost from the Stercorarian lineage (Fig. 2C). Thus 27% (80 out of 294) of the ancestral cohort of PSOs and were lost from the Salivarian lineage coincident with the evolution of antigenic variation. This extent of gene loss in PSOs is much higher than expected from an equivalent analysis of all genes encoded in the genome (12%, 834 lost out of 7101, Fig. 2B).

***T. carassii* transcriptome contains two large families of putative surface protein encoding genes not found in any other trypanosomatid.**

Analysis of the species distribution of all PSOs resulted in the identification of two large orthogroups that were unique to *T. carassii*. The first of these two families is PSO 2 (orthogroup 5, Supplementary Table S1, Fig. 3A), contains ~500 distinct members in the *de novo* assembled transcriptome. Of these, 99% of the complete predicted open reading frames (i.e. those that end in a stop codon) are predicted to encode a conserved GPI anchor sequence. Similarly 63% of those that begin with a start codon that is not located at the start of the assembled transcript are predicted to encode a signal peptide. A LOGO summarising the multiple sequence alignment of the conserved GPI anchor region is shown in Fig. 3A. For comparison a LOGO of the same region from VSG from *T. brucei* (Fig. 3B) and mucin from *T. cruzi* (Fig. 3C) are shown. To estimate the relative abundance of the mRNAs encoding the genes in this PSO the pooled mRNA abundance of the all genes in the group as compared to that of beta tubulin. This revealed that the combined mRNA abundance of this gene family was ~3.3 times that of beta tubulin (orthogroup cumulative abundance = 18070 TPM, beta tubulin abundance TryCa.3580.10_ntrunc = 5516 TPM). To put this value in context, the VSG mRNA in bloodstream form *T. brucei* is ~9.9 times more abundant than beta tubulin. Thus collectively the novel surface protein genes in *T. carassii* are approximately 1/3rd as abundant as VSG in *T. brucei*. However, given that a population of cells isolated from

a carp was sequenced it was not possible to determine whether individual cells in that population expressed a single surface coat gene (analogous to VSG in *T. brucei*) or whether individual cells expressed all or a subset of different genes from this orthogroup.

The other surface orthogroup unique to *T. carassii* is PSO 7 (orthogroup 21, Supplementary Table S1, Fig. 3D). This surface orthogroup contains fewer distinct transcripts ($n = 128$) of which 83% are predicted to encode a GPI anchor sequence and 65% are predicted to encode a signal peptide (Supplementary Table S1). The GPI-anchor signal is distinct from the other family of surface genes, VSGs and mucins (Fig. 3D). Similar to PSO 2 above, the cumulative mRNA abundance of this family of genes is ~ 2.9 times that of beta tubulin as above, again it is not possible to determine whether individual cells in the population expressed a single gene from this orthogroup or whether individual cells expressed a subset of different genes.

There is a large number of genes encoding MSPs in *T. carassii*. In total 157 MSP genes were detected that are distributed between two orthogroups (orthogroup 13 and orthogroup 31, Supplementary Table S1). The cumulative mRNA abundance of these genes is ~ 1.3 times that of beta tubulin in *T. carassii*. Thus taken together it appears from mRNA expression data that the surface of *T. carassii* is a complex mix of three distinct gene families (PSO 2, PSO 7, and MSP) that taken together are ~ 7.5 times as abundant as beta tubulin and thus similar in relative abundance to that of VSG in *T. brucei*.

Figure 3. LOGOs constructed from multiple sequence alignments of GPI anchor regions of putative novel surface proteins identified in this study. **A)** The GPI anchor region of the largest family of putative surface localised genes in *T. carassii*, surface orthogroup 2. **B)** The equivalent region from *T. brucei* VSGs. **C)** The equivalent region from *T. cruzi* mucins. **D)** The equivalent region from the second largest group of surface localised proteins in *T. carassii*, Surface orthogroup 7.

Cell surface biotinylation and isolation of the *T. carassii* surface proteome.

A proteomic investigation was conducted to identify cell surface proteins on *T. carassii* cells isolated from infected carp. Live cells were labelled chemically, biotinylated proteins were enriched by affinity purification and tandem mass spectrometry was performed on affinity purified fractions. The mass spectrometry data were searched against a database of *T. carassii* protein sequences inferred from the transcriptome.

Peptides corresponding to 22 proteins were identified in the affinity purified fraction whereas peptides corresponding to 1326 proteins were found in the unbound material. Of the 22 proteins detected in the

biotinylated fraction three were also detected in a non-biotinylated control and thus were discarded. Spectral index values were computed for the remaining 19 proteins and their relative abundance compared to those identified in the unbound material. In total 6 proteins were detected as being twofold or more abundant in the biotinylated fraction than in the unbound material Table 1. The protein that was most enriched by the cell surface enrichment procedure was a cell surface metallopeptidase gp63. The 2nd and 3rd most enriched proteins were encoded by genes that belonged to PSO 7. Peptides corresponding to genes from the other species specific gene family PSO 2 were not detected in the biotin affinity purified fraction. A further 3 hypothetical proteins of unknown function were also identified (Table 1), two of which are conserved in other trypanosomatids (orthogroup 3380 and 5295), and the third which has no similar genes in any other trypanosomatid genomes (TryCa.5636.10_full). These genes have no putative assigned functions and no PFAM domains from which clues to function could be obtained. Of these three genes, only the unique protein (TryCa.5636.10_full) contains a GPI-anchor sequence and thus it is possible that the other two proteins were contaminants.

Although cells were washed three times in TBS prior to biotinylation a number of carp proteins were detected in the in the proteomic dataset. The results of searching the mass spectra against a database of predicted carp proteins are also provided in Supplementary Table S1. The detection of transferrin variants, complement factors, and Alpha-1 antitrypsin is consistent with previous proteomic studies of carp blood plasma²⁹.

Table 1

Accession	Annotati-on	Conser-vation	OG	GPI/TM D	Elu-tion	Flow-through	Fold Change
TryCa.2544.10_ctrunc	GP63	Ancestral	SurfOG 11	GPI	1.1E-06	3.1E-09	343.1
TryCa.8020.10_nt-runc	NSP1	Unique	SurfOG 7	GPI	3.6E-07	3.1E-09	118.3
TryCa.5961.10_full	NSP1	Unique	SurfOG 7	GPI	3.6E-07	3.1E-09	116.7
TryCa.1036.10_nt-runc	hypothet-ical	Ancestral			1.3E-05	2.3E-07	57.2
TryCa.9345.10_full	hypothet-ical	Ancestral			3.0E-06	6.0E-08	48.8
TryCa.5636.10_full	hypothet-ical	Unique		GPI	6.8E-07	2.4E-07	2.8

Discussion

Here we present a *de novo* transcriptome assembly and comparative bioinformatic analysis of the predicted cell surface proteome of *T. carassii*, an extracellular parasite of cyprinid fishes. *T. carassii* is a sister species to both the Stercorarian and Salivarian trypanosomes that split from the trypanosomatid lineage prior to the divergence of *T. cruzi* and *T. brucei*. Thus comparison of the gene complement of *T. carassii* with *T. brucei* and *T. cruzi* has potential to inform about the gene loss events that occurred during the evolution of these two human pathogens.

This analysis revealed that there was a substantial loss of putative surface protein genes in the lineage that lead to Salivarian trypanosomes. This loss is concomitant with the evolution of antigenic variation. In total 27% of ancestral surface orthogroups, those orthogroups with genes present in *T. carassii* and at least one species of both the Stercorarian and the Salivarian clades, were lost in the lineage that lead to the Salivarian trypanosomes. In contrast only 1% of the ancestral surface proteome has a pattern of occurrence that indicated they have been lost in Stercorarian trypanosomes. This finding indicates that the cell surface proteome of Salivarian trypanosomes is likely to have contained a complex cohort of proteins prior to the advent of antigenic variation in this lineage. As none of these putative cell surface proteins have known functions it is impossible to speculate on the loss of function that these gene losses confer.

Analysis of the predicted surface protein genes in *T. carassii* also lead to the discovery of two large groups of putative surface proteins unique to *T. carassii*. Lack of detectable similarity by BLAST to known surface proteins such as trans-sialidase, mucin VSG etc. suggests that they are novel. A proteomic investigation provided support that members of one of these protein families were present on the cell surface of *T. carassii* when parasites were isolated from carp blood. However, the proteomic analysis detected fewer proteins than expected and proteomic evidence for the cell surface localisation of members of the other *T. carassii* specific gene family was not obtained. This discordance between the proteome and the transcriptome may be due to the differential presence of members of this highly diverse family in both the proteome and transcriptome

samples, as these were isolated from temporally separated populations of cells isolated from different fish. Thus the cohort of surface proteins expressed at the time of isolation of the transcriptome and the proteome may have been different. Alternatively, it may also be due to the high amino acid diversity exhibited by members of this gene family and the difficulty of unambiguously assigning spectra to peptides. In Summary, the cell surface of *T. carassii* is a complex mixture of at three large and diverse gene families, two of which are unique and distinct from those found in Salivarian and Stercorarian trypanosomes.

Materials and Methods

Isolation of *T. carassii* cells from carp

For all experiments used in this analysis *T. carassii* was grown in carp as described previously^{16,24}. For cell surface biotinylation parasites were harvested 4 weeks post infection. Here, carp blood was collected and left to settle overnight at 4°C. Live *T. carassii* cells were isolated from infected blood on a 1×12 cm ion-exchange chromatography using DEAE cellulose (DE-52; Whatman international) as described previously²² and grown for 14 days in culture (22.5% Minimum Essential Medium, 22.5% L-15 Medium (Leibovitz), 10% sterile water, 45% Hank's Balanced Salt Solution, 10% pooled carp serum, 2% L-Glutamine and 1% Pen/Strep) at 27°C without CO₂¹⁶. Parasites were further purified by DE52 column to remove dead cells and cells originating from the host prior to mRNA extraction and cell surface biotinylation.

De novo transcriptome assembly

Total RNA was extracted from these parasites and the mRNA fraction sequenced using Illumina paired-end sequencing technology by the Beijing Genomics Institute. Raw reads were deposited to NCBI SRA under the accession number SRR5120186. Reads were subject to *de novo* transcriptome assembly using the following pipeline. Raw reads were subject to quality filtering using trimmomatic³⁰. This was done to remove

low quality bases and read-pairs as well as contaminating adaptor sequences prior to assembly. Sequences were searched for all common Illumina adaptors (the default option) and the settings used for read processing by trimmomatic were LEADING:10 TRAILING:10 SLIDINGWINDOW:5:15 MINLEN:50. The trimmed and quality filtered paired-end reads were then filtered to remove reads coming from ribosomal RNA using SortMeRNA³¹ using the default settings and then subject to read error correction using BayesHammer³² using the default settings. Reads were then digitally normalised using khmer³³ with settings -C 20 -k 21 -M 8e9. Following normalisation, overlapping paired-end reads were joined together using ALLPATHS-LG³⁴ and all reads (joined and those that were unable to be joined) were subject to initial de novo assembly using SGA³⁵ using minimum overlap size of 80 nucleotides (with no mismatches). The assembled contigs were subject to 4 additional rounds of assembly using SGA, each round reducing the overlap size by 10 nucleotides such that the last round of assembly was performed with an overlap size of 40 (with no mismatches). The filtered, normalised joined reads were then mapped to this assembly using bowtie2³⁶. Reads that were absent from the assembly were identified and combined to the assembly file. The combined un-mapped reads and assembled contigs were subject to assembly using SGA with an overlap size of 70. This process of identifying unmapped reads and reassembling with SGA was repeated each time decreasing the overlap size by 10 nucleotides until the final round of assembly was performed with an overlap size of 40 nucleotides. Contigs were then subject to scaffolding using SSPACE³⁷ and the full set of corrected, normalised paired-end reads using the settings -k 10 -a 0.7 -n 50 -o 20. Scaffolds were subject to gap filling using the SGA gap filling function. Finally contigs are subject to assembly base-error correction using Pilon³⁸. The complete set of assembled contigs was deposited to NCBI TSA under the accession number SUB2183395.

Inference of orthogroups

The annotated proteins for *Trypanosoma brucei* TREU927, *Trypanosoma congolense* IL3000, *Trypanosoma cruzi* CL brenner Esmeraldo-like, *Trypanosoma grayi* ANR4, *Trypanosoma vivax* Y486, *Leishmania*

tarentolae ParrotTarII, *Leishmania major* Friedlin, *Leishmania infantum* JPCM5, *Leishmania mexicana* MHOMGT2001U1103, *Leishmania braziliensis* MHOMBR71973M2269 and *Crithidia fasciculata* CfCl were downloaded from TriTrypDb V8.0³⁹. The predicted protein sequences from *Trypanosoma rangeli* were downloaded from NCBI. The predicted proteins from *Phytomonas EM1* and *Phytomonas Hart1* were obtained from⁴⁰. The predicted proteins from *Phytomonas serpens* were obtained from NCBI. Orthogroups were inferred from these predicted protein coding genes using OrthoFinder²⁷ using default program parameters.

Phylogenetic inference

Phylogenetic inference was performed using only single copy genes present in all species were selected for inclusion in the phylogenetic analysis. The individual orthologous groups were aligned using MergeAlign⁴¹ and edited to remove all gap-containing columns and columns containing fewer than two character states. This resulted in a set of 716 alignments each containing ≥ 100 phylogenetically informative ungapped aligned positions. 100 bootstrapped concatenated multi-gene multiple sequence alignments were then constructed from this subset using an equi-sampling strategy. In this strategy each bootstrap replicate alignment was constructed by sampling (at random) 137 un-gapped phylogenetically informative columns from each multiple sequence alignment containing ≥ 137 ungapped aligned positions. This equi-sampling strategy was performed so that each gene contributed equally to the final phylogenetic tree thus preventing longer genes from biasing the result. Each bootstrap replicate contained 63,842 phylogenetically informative, un-gapped aligned positions spanning all species. Trees were inferred from each resampled multi-gene alignment using RAxML⁴² utilising the PROTGAMMAGTR and PROTGAMMAAUTO model of sequence evolution and using minimum evolution principle (with log corrected scores) implemented in FastME⁴³. Each method (GTR, AUTO and minimum evolution) gave the same topology with 100% support for all internal nodes.

Inference of cell surface orthogroups

The predicted protein sequences of each species used in this analysis were searched to identify protein features that are characteristic of

cell surface proteins; signal peptides, transmembrane domains and GPI anchors. Signal peptides were predicted using SignalP 3.0⁴⁴, GPI anchors were predicted using FragAnchor⁴⁵ and transmembrane helices predicted using TMHMM v2.0⁴⁶. Pseudogenes were discarded and not considered in this analysis. *T. carassii* proteins that were truncated at either the N or C terminus (determined from analysis of where the gene model finished in the transcript) were deemed ineligible for signal peptide or GPI anchor predictions respectively. Orthogroups were designated as surface orthogroups if $\geq 40\%$ of the genes in the orthogroup contained a signal peptide and at least one transmembrane helix, or a signal peptide and a GPI anchor. This cut-off of $\geq 40\%$ was employed on the basis of observed values for a gp63, a ubiquitously conserved cell surface protein that had 48.4% GPI and 46.4% signal peptide.

Biotinylation of surface proteins

Following isolation of cells from carp as described above, *T. carassii* cells were washed once in 10 ml ice cold trypanosome dilution buffer (TBD: 118 mM NaCl, 1.2 mM KH₂PO₄, 30 mM TES, 16 mM NaHPO₄, 5 mM NaHCO₃, 5 mM KCl, pH 8) and biotinylated by addition of EZ-link Sulfo-NHS-SS- Biotin (Thermo Scientific) to cells in trypanosome dilution buffer and incubated on ice for 10 minutes. The labelling reaction was quenched with ethanolamine and cells were washed 3 times with 1xTBS. Surface biotinylated cells were lysed with lysis buffer (8 M urea, 1% sodium deoxycholate, 0.2% Sodium dodecyl sulfate, 1 M NaCl in 1xTBS pH 7.5). Cell lysates were added to streptavidin Dynabeads and incubated at 4 °C for 1 hour with rotation. The beads were washed 3 times with lysis buffer and 3 times with bead wash buffer (50 mM Tris-Cl pH 7.5 and 1 sodium deoxycholate) to remove any residual SDS from the sample. Bound material was removed by reduction with 10 mM TCEP for 15 minutes at 37 °C. The eluate was alkylated with 20 mM iodoacetamide for 30 minutes in the dark, N-linked glycans removed with PNGase F and tryptic digestion in solution, using 1 mg trypsin at 37 °C with agitation.

Mass spectrometry

Tandem mass spectrometry was performed by the University of Oxford Central Proteomics facility (CPFP, <http://www.proteomics.ox.ac.uk/>). Samples were acidified with Trifluoroacetic acid to 0.5% to remove sodium deoxycholate. The supernatants were desalted on C18 and analysed by LC MS/MS using a QExactive Orbitrap mass spectrometer (Thermo Electron, Hemel Hempstead, UK) coupled to an Ultimate 3000 RSLCnano HPLC (Dionex, Camberley, UK) system run in direct injection mode. Samples were resolved on a 25 cm by 75 micron inner diameter home packed column with ProntoSIL 120-3 C18 Ace-EPS phase, 3 μ m bead (Bischoff Chromatography, Germany). The system was operated at a flow-rate of 300nL min⁻¹. A 100 minute gradient was used to separate the peptides with a linear gradient ranging from 5-50% buffer B. The mass spectrometer was operated in a "Top 20" data dependent acquisition mode with a scan range of 350-1650 m/z. Precursor scans were performed in the orbitrap at a resolving power of 70,000 from which the twenty most intense precursor ions were selected by the quadrupole and fragmented by higher energy collision dissociation at a normalised collision energy of 30%. The quadrupole isolation window was set at 3 m/z. Charge state +1 ions and undetermined charge state ions were rejected from selection for fragmentation. Dynamic exclusion was enabled for 40s. Data were converted from .raw file format to .mgf file format using ProteoWizard MSconvert⁴⁷. .mgf files were uploaded onto the Central Proteomics Facility Pipeline server⁴⁸. Data were searched against an in-house constructed database containing the predicted proteins encoded in the *T. carassii* transcriptome using the following search parameters: full tryptic cleavage; 2 missed cleavages; carbamidomethyl c fixed modification; oxidised (M) and addition of the reacted and reduced NHS-SS-biotin (C5H7O2NS) (K, N-terminal protein) variable modifications; precursor peptide tolerance 20ppm, fragment tolerance 0.1 Da and charge states =+1,+2,+3.

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5

CHAPTER

Successes and challenges in generating tools to study the biological activity of soluble and transmembrane zebrafish Tnf.

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Abstract

In mammals, TNFa is a pleiotropic pro-inflammatory cytokine produced by and having an effect on several cell types, including immune cells. TNFa is first produced as a transmembrane homotrimers that is then proteolytically cleaved by the TNFa converting enzyme (TACE) to release soluble TNFa (sTNFa). Both, transmembrane (tmTNFa) and sTNFa can bind to either TNFR1 or TNFR2 triggering a response in the receptor-bearing cell. Interestingly, it was reported that tmTNFa itself can also trigger an intracellular signalling pathway in the TNFa-bearing cell. A process referred to as reverse or bidirectional signalling. Our group previously reported on the biological characterization of two isoforms of TNFa in common carp, and reported for the first time in a non-mammalian species, a potential protective role of carp tmTnfa during trypanoplasma infections. In the current study, we generated tools to study the biological activity of soluble and transmembrane zebrafish Tnf. Similar to other fish species, also in zebrafish two *tnf* genes are present, referred to as *tnfa* and *tnfb*. Here we describe the identification of TACE cleavage sites on zebrafish Tnfa and Tnfb as well as the advantages and disadvantages of several tools generated to study the ligand-receptor preferences of soluble and transmembrane zebrafish Tnf. Finally, we describe our attempt to generate a zebrafish transgenic line constitutively expressing tmTnfa and discuss future approaches to study the biological activity of soluble and transmembrane zebrafish Tnf *in vivo*.

Introduction

The cytokine tumor necrosis factor- α (TNF α) has been thoroughly studied in mammals (Kolb and Granger, 1968)(Ruddle and Waksman, 1968). TNF α homologs with similar structure and transcriptional regulation have been described in many species of bony fish and several studies have addressed aspects of functional conservation in at least goldfish, rainbow trout (Laing et al., 2001)(Hong et al., 2013), carp (Saeij et al., 2003), Japanese flounder (Hirono et al., 2000), gilthead seabream(Garcia-Castillo et al., 2002), turbot (Ordas et al., 2007), catfish (Zou et al., 2003) and tilapia (Praveen et al., 2006). Mammalian TNF α is secreted primarily by monocytes and macrophages, but also by natural killer (NK) and endothelial cells (Chan et al., 2001)(Aggarwal et al., 2012)(Ming et al., 1987)(Gamble et al., 1985) and is a typical pleiotropic cytokine: TNF α can activate macrophages and neutrophils, regulate leukocyte recruitment (Mulligan et al., 1993)(Henninger et al., 1997), has pro-apoptotic activity (Tartaglia et al., 1993) and appears an important player in the pathophysiology of several diseases (Perez et al., 1990). Indeed, a tight regulation of TNF α expression during infection and disease appears essential, for example during infection of mice with *Trypanosoma brucei* (Magez et al., 1999), but also during infection of carp with *Trypanoplasma borreli* (Forlenza et al., 2009).

In mammals, TNF α is first expressed as a transmembrane monomer on the cell surface, forming a homotrimeric transmembrane TNF α (tmTNF α) which is proteolytically cleaved by the metalloprotease TNF α converting enzyme (TACE) to release soluble TNF α (sTNF α) (Schlondorff and Blobel, 1999) (Peschon et al., 1998). TACE is a multi-domain, type-I transmembrane protein belonging to the disintegrin and metalloprotease ADAM family (Black, 2002)(Solomon et al., 1999). The activity of TACE (also called ADAM17) is not limited to TNF α only, and is responsible for the shedding of multiple other cell surface proteins (Tartaglia et al., 1993)(Wajant et al., 2003)(Aggarwal, 2003). The trimeric TNF α can bind to two distinct cell-surface receptors: TNFR1 (p55), characterized by an intracellular death domain which activation leads to pro-inflammatory and apoptotic signalling, and TNFR2 (p75) which activation leads to induction of pro-

inflammatory and survival genes as well as angiogenesis and cell proliferation (REF) (Naude et al., 2011)(Cabal-Hierro and Lazo, 2012). While TNFR1 is expressed on most cell types, TNFR2 is expressed mostly on endothelial cells and immune cells (Grell et al., 1998). In fish, not much is known about Tnf receptors, however it has been previously identified in some species, for example, in Japanese flounder (Park et al., 2003), goldfish (Grayfer and Belosevic, 2009) and zebrafish (Bobe and Goetz, 2001). In contrast to the mammalian expression patterns of the receptors, fish TNF-R2 was the receptor found to be expressed on a broader level; it has been found in all tissues examined in goldfish (Grayfer and Belosevic, 2009).

Both tmTNFa (prior to cleavage by TACE) and sTNFa have the ability to bind to either of the two TNF receptors, at least in mammals, although sTNFa appears to preferably bind to TNFR1, tmTNFa shows binding preference to the soluble and transmembrane form of TNFR2 (Canault et al., 2006; Grell et al., 2002, 1995; Locksley et al., 2001). When tmTNFa binds to its receptor, signal transduction is induced not only in the TNFR-bearing cells but also in the tmTNFa-expressing cell; a phenomenon referred to as reverse or bidirectional signalling, reviewed in (Qu et al., 2017). Interestingly, overexpression of tmTnfa in carp had a crucial protective role during *T. borreli* parasite infection. Indeed, injection of a TACE inhibitor led to an overexpression of tmTnfa and resulted in 100% survival of carp and complete clearance from infection with *T. borreli* (Forlenza et al., 2009). The exact mechanism behind this protection and detailed knowledge of receptor preferences of sTnfa or tmTnfa in fish remains unknown.

In addition to TNFa receptor (TNFR)-dependent activation via cell surface molecules, typically leading to downstream pro-inflammatory and/or apoptotic or survival signals, mammalian TNFa can also exert receptor-independent lectin-like activities by directly binding to cell surfaces, for example to the variant specific surface glycoproteins (VSG) of African trypanosomes and thereby cause cell (here, parasite) lysis, a characteristic activity apparently conserved for fish Tnfa.

Ligand binding to the TNF receptors induces recruitment of accessory proteins and activation of an intracellular signalling cascade that leads to either cell survival via the activation of NF- κ B or AP-1; or cell death

via the activation of caspases. The often used double edge sword term for TNF α originated from the dual effect exerted via TNFR1 binding: on the one hand it mediates apoptosis while on the other it can trigger pro-inflammatory responses, cell proliferation and differentiation. On the contrary, binding to TNFR2 mainly leads to tissue repair and angiogenesis although apoptosis can be triggered but only when crosstalk between the two receptors occurs.

To study Tnf α -TnfR interaction in fish in more detail we chose a model organism complementary to carp: the zebrafish, for which we recently managed to establish a trypanosome or trypanoplasma infection model (this thesis) and for which genome editing tools exist that have revolutionized the generation of new transgenic zebrafish lines. Transcription activator-like effector nucleases (TALENs) are sequence-specific endonucleases consisting of two engineered arrays of TALE repeats separated by 10–20 base pairs and a non-specific *FokI* cleavage domain that introduces a double stranded break in the DNA (Auer and Del Bene, 2014), and have been used extensively for genome editing in zebrafish. Advantages of the TALEN system are the easy design with little restrictions regarding target site and rare off-target effects (Kim et al., 2013). A disadvantage of TALEN can be that the assembly of the TALE repeats can require laborious cloning steps. We anticipated that the possibilities for genome editing by TALENs could facilitate our studies into the biological function of tmTnf α in zebrafish.

We aimed to first investigate ligand-receptor interactions of zebrafish sTnf α and tmTnf α *in vitro* and then study the protective responses mediated by tmTnf α *in vivo*. To address ligand-receptor binding preferences of sTnf α and tmTnf α *in vitro* we designed multiple constructs for the overexpression of Tnf α receptors as well as of uncleavable zebrafish Tnf α . The latter constructs carried mutations and/or deletions in the predicted TACE cleavage sites of Tnf α and Tnf β . To validate the constructs, we used transfection of mammalian and fish cell lines stably transfected with a luciferase reporter gene under the control of a NF κ B promotor. To address the protective responses mediated by tmTnf α *in vivo* we used the sequence of few plasmid constructs selected from the *in vitro* work

and tried to generate a transgenic zebrafish line exclusively expressing uncleavable tmTnfa. Our strategy was to first knock-out the native form of Tnfa and then replace it with the mutated uncleavable version in a single knock-in step using the TALEN genome editing system. We discuss the methodology and apparent inefficiency and limitations of the TALEN gene editing system for knocking-in a whole gene and discuss alternative approaches for the future.

Materials and Methods

Eukaryotic expression constructs for zebrafish Tnfa and Tnf receptors (tnfr)

Zebrafish possesses 2 isoforms of *tnfa* gene located on 2 different chromosomes and named as *tnfaa* and *tnfab*; those will be referred to as *tnfa* and *tnfb* throughout this chapter.

Full-length zebrafish *tnfa* was kindly provided as pcDNA6-*tnfa*-V5-his by dr. Victor Mulero from the University of Murcia, Spain. This *tnfa* construct with V5 and 6xHistidin tags at the C-terminal end (tnfa-V5-His) was previously validated as leading to expression of soluble zfTnfa in cell culture supernatants (Roca et al., 2008). To avoid any possible interference of tags when assessing receptor binding of Tnfa, also new zebrafish *tnfa* constructs were generated, without tags. To this end, the pcDNA6V5-His construct was used as template for amplification of *tnfa* and the resulting PCR product cut with *NheI* and *EcoRI* to be cloned into pIRES-eGFP (Promega) for prokaryotic expression. In parallel, the same PCR product was also cloned in pGEM-T easy (Promega) from which it was excised using *EcoRI* for cloning into pcDNA3 (Thermo Fisher Scientific) for eukaryotic expression (pcDNA3-tnfa).

Zebrafish *tnfb* was obtained as cDNA IMAGE clone from Source BioScience (Nottingham, UK) and amplified using DNA polymerase with proofreading activity using the Expand High Fidelity^{PLUS} PCR System (Roche), using the cloning primers (Eurogentec) listed in **Table 1**. Full-length tnfb was amplified using primers designed to add a FLAG tag at the C-terminal end (see Table 1), and the product was first cloned into a pIRES-eGFP vector using the *NheI* and *EcoRI* restriction sites. In parallel, the same

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and resulting amino acid substitutions of all relevant residues into Alanine (A), except mutation of Lysine (K) to Glutamate (E), while (pcDNA3-) Tnfa_{D1-9K}, Tnfb_{D1-9K} and Tnfb_{D18-26} were designed to lead to deletion of the TACE cleavage site and additional amino acid substitution leading to mutation of Lysine (K) to Glutamate (E) (see further details in **Figure 1**).

ZfTNFb_ATG_Hind_FW	ATATAAGCTTAACATGGTGAGATACGAAACAACATTAG	cloning
ZfTNFb_His_BamHI_RV	ATATGGATCCTCACATCACCATCACCATCACCAACGCG AACACCCCGAAGAAT	cloning
ZfTnfr1_noSP_SfiI_FW	CTGGGGCCCGAGCCGGCCGGACTTGGTGGAAACAAGAC TAT	cloning
ZfTnfr1_Sall_RV	AGATGTCGACTTACGAAACGCTTGTGTTCTG	cloning
ZfTnfr2_NheI_FLAG_FW	ATATGCTAGCAACATGGATTACAAGGATGACGATGACA AGACGGTGGTGGCTCTTGGCGG	cloning
ZfTnfr2_HA_EcoRI_RV	ATATGAATTCTCAAGCATAATCTGGAACATCATATGGAT AGTAAGGACCGGGCCGCTCTCC	cloning
ssOligo_tnfa	TTGAGAGATCGCATTTACAGTCATGGCGTTTTAAACCTT <u>AATTAAGCTGTTGTAGAGGCAATTTCACTTCCAAGG</u>	donor for TALEN verification
ssOligo_tnfb *	AAGCAGATCGCTCAGAGGTCATGGCGTTTTAAACCTTAA <u>TTAAGCTGTTGTAGCACAAAGGCTGCCATTCACTTGC</u>	donor for TALEN verification
TNfa TALEN check_Fw	GGCGTTTTGGATGTTGAAG	TALEN verification by PCR
TNfa TALEN check_STOP_Rv	TAATTAAGGTTTAAACGCCATGAC	TALEN verification by PCR
TNfb TALEN check_Fw *	CAACAAATCACCACACCTTC	TALEN verification by PCR
TNfb TALEN check_STOP_Rv *	TAATTAAGGTTTAAACGCCATGAC	TALEN verification by PCR
TNfa_VLRK_FW	GGCAATCAACAAGATGGAAGTGIG	verification of uncleavable Tnf insertion
TNfa_VLRK_RV	TTTAGCGTACTGAGTGTGAGATGG	verification of uncleavable Tnf insertion
TNfb_PLKLLK_FW *	TTTTCTTCACCTGGCATGTGATGAA	verification of uncleavable Tnf insertion
TNfb_PLKLLK_RV *	TCTCCTTCTGAGCTCTAATAGGAAC	verification of uncleavable Tnf insertion
ZfTnfb_long_Bam_FW	atataGGATCCctaaacaacgaatcaccacacct	cloning construct for α Tnfb antibody
ZfTnfb_short_Bam_FW	atataGGATCCCTGAAGCAGATCGCTCAGAGCACA	cloning construct for α Tnfb antibody
ZfTnfb_Hind_RV	atataAAGCTTtcaCAACGCGAACACCCCGAAGAAT	cloning construct for α Tnfb antibody

Table 1. Cloning primers used in this study. Asterisk indicates primers that were designed for the current study but could not be used due to lack of time. Underlined nucleotides in the ssOligo sequences indicate the additional nucleotides not present in the Tnf gene including a stop codon (bold).

Anti-zfTnfb antibody production

Since two cleavage sites could be predicted for Tnfb, a long 532 bp, from amino acid 91 to aa 242 (23kDa), and a short 481bp, from aa 73 to aa 242 (21kDa) fragment, was amplified by PCR using pIRES-*tnfb* (*zftnfb*) as template, adding a *Bam*HI restriction site to the 5' end and *Hind*III site to the 3' end of the fragment. Both fragments were cloned into pET15b vector. JM109 *Escherichia coli* competent cells were heat shock transformed with the ligated products and protein was produced as previously described for carp Tnfa (Forlenza et al., 2009). Purified recombinant proteins were used for immunization of rabbit with a standard 2 months protocol (Naxo).

Cell culture

EPC (*Epithelioma Papulosum Cyprini*) cells (de Kinkelin et al., 1980) were cultured in RPMI (RPMI 1640, Lonza, Breda, Netherlands) supplemented with 10% foetal calf serum (FCS, Thermo Fisher Scientific), 1% Penicillin-Streptomycin Solution (10.000:10.000, Fisher Scientific) and 1% L-glutamine 200 mM (Fisher Scientific) at 27°C in the presence of CO₂. HEK293 (Human Embryonic Kidney cells) and HEK293-NFκB reporter cells (HEK293 cells stably transfected with pNifty2 plasmid containing luciferase gene under the control of *NFκB* promoter site (Fink et al., 2016)) were cultured in Advanced DMEM (Gibco, Life Technologies, Breda, The Netherlands) supplemented with 5% FCS, 1% Penicillin-Streptomycin Solution and 1% L-glutamine. Cells were kept at 37°C with 5% CO₂. PAC2 (Zebrafish fibroblast cells) and stable PAC2-NFκB cells (stable line made by us) were cultured in Leibovitz's L-15 medium (Gibco, Life Technologies) supplemented with 15% FCS, 1% Penicillin-Streptomycin Solution, and 1% L-glutamine. The cells were maintained at 27°C without CO₂. VERO cells (African green monkey kidney epithelial cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life Technologies) supplemented with 10% FCS, 1% Penicillin-Streptomycin Solution and 1% L-glutamine. The cells were grown at 37°C with 5% CO₂.

Cell transfections

EPC cells were seeded in a 6-well plate at a density of 700,000 cells/well and 24 hours later transiently transfected with 2 µg pcDNA3-tnfa, pcDNA3-tnfa_{VLRK}, pcDNA3-tnfa_{D1-9K} or pcDNA6-tnfa-V5-His using FuGENE® HD Transfection Reagent (Promega, USA) at a 1:3.5 plasmid:Fugene ratio according to the manufacturer's instructions. Supernatant was collected 48h or 72h after transfection, centrifuged at 14800xg for 5 min at 4°C, and stored at -20°C until use.

HEK293 cells were seeded in a 6-well plate at a density of 400,000 cells/well and 24 hours later transfected with 2 µg pDisplay-HA-tnfr1, pIRES-eGFP-FLAG-Tnfr2-HA, pIRES-eGFP-FLAG-ΔTnfr2, pBI-HA-ΔTnfr1 using jetPRIME™ DNA & isRNA transfection reagent (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions at a 1:3 ratio of plasmid:transfection reagent. Supernatant was collected 48h after transfection, centrifuged at 14800xg for 5 min at 4°C, and stored at -20°C until use.

VERO cells were seeded in a 6-well plate at a density of 300,000 cells/well and 24h later transfected with FuGENE® HD Transfection Reagent using 2 µg of plasmid DNA (pcDNA3, pcDNA3-tnfa, pcDNA6-Tnfa-V5-His, pcDNA3-Tnfa_{VLRK}). Six hours after transfection the medium was replaced with 1.5mL of DMEM with 2% FCS and supernatant was collected 48h later, centrifuged at 14800xg for 5 min at 4°C and stored at -20°C until use.

HEK293-NFκB cells were seeded in 96-well plates at a density of 50,000 cells/well and 24h later transfected with JetPRIME with 0.1 µg pDisplay-HA-tnfr1, pIRES-eGFP-FLAG-Tnfr2-HA, pIRES-eGFP-FLAG-ΔTnfr2, pBI-HA-ΔTnfr1. Stable HEK293-NFκB cell lines expressing Tnfr1 or ΔTnfr1 were created upon transfection of cells as described above and subsequent selection using 300 µg/ml Geneticin antibiotic.

In order to produce a new NFκB reporter cell line based on zebrafish cells, PAC2 cells were transfected with 3.5 µg pNiFty-Luc plasmid (InvivoGen) that encodes for a luciferase reporter gene under the control of the NFκB-inducible ELAM-1 promoter using jetPRIME™ DNA & isRNA transfection reagent (Polyplus Transfection, Illkirch, France) at a 1:3 ratio of plasmid:transfection reagent. Transfected cells were selected using 250 µg/ml Zeocin (Life technologies™).

Immunostaining

For immunostaining of surface proteins, EPC cells were stained 48 hours and HEK293 cells 24 hours after transfection. Cells were placed on ice and ice-cold FCS-free medium (supplemented with 0.01% NaN_3 and 1 % BSA) was added to the cells. After 5-10 minutes cells were collected, transferred to 5 ml tubes and kept on ice for 20 minutes, followed by centrifugation at $250 \times g$ for 5 minutes at 4°C . Cells were incubated with the primary antibody in 100 μl FCS-free medium for 30-45 minute, on ice. The following primary antibodies were used for staining of surface proteins: rabbit α -zfTnfa in 1:200 dilution (kindly provided by Prof. Victor Mulero, University of Murcia, Spain); mouse α -FLAG (Sigma) in 1:50 dilution; mouse α -HA (InvivoGen) in 1:200 dilution. Cells were then washed twice with 1 ml FCS-free medium. Secondary antibodies were added in 100 μl FCS-free medium and incubated for 30-45 minutes on ice. The following secondary antibodies were used: goat α -rabbit RPE (SouthernBiotech) in 1:200 dilution; goat α -mouse RPE (Dako) in 1:200 dilution. Cells were washed twice, re-suspended in 200 μl of FCS-free medium and 100 μl of each sample was centrifuged at $200 \times g$ for 8 minutes at 4°C on poly-L-lysine-coated glass slides. Vectashield (Vector Laboratories, Burlingame, USA) was directly added to each slide, which was covered with a cover slip and sealed. Slides were kept at 4°C in the dark. Cells were visualized using an EVOS Fluorescent Digital Inverted Microscope.

For immunostaining of intracellular proteins, HEK293 cells were stained on ice 24 hours after transfection. Cells were collected as described above, re-suspended in 200 μl cytofix/cytoperm buffer (BD Biosciences) and incubated on ice for 20-30 minutes. Cells were then washed with 100 μl 1x perm/wash buffer in PBS followed by a second wash with 200 μl of the same buffer. The following primary antibodies were used for staining of intracellular proteins: mouse α -HA (InvivoGen) in 1:200 dilution and mouse α -FLAG (Sigma) in 1:200 dilution, added in 100 μl 1x perm/wash and incubated for 30-45 minutes on ice or at 4°C . Cells were washed three times with 1 ml of FCS-free medium (supplemented with 0.01% NaN_3 and 1 % BSA). Goat α -mouse RPE (Dako) secondary antibody was added to each tube in 1:200 dilution in 100 μl 1x perm/wash and

incubated for 30-45 minutes on ice or at 4°C. Cells were then washed twice and re-suspended in 200 µl of FCS-free medium. For microscopy analysis 20 µl of the cells was transferred to poly-L-lysine-coated glass slides and fluorescence analysed using an EVOS Fluorescent Digital Inverted Microscope.

Western blot analysis

Prior to western blot, cell culture supernatant of pcDNA3-Tnfa transfected EPC cells was concentrated 10 times using 2mL Amicon Ultra Centrifugal Filter with 30,000 molecular weight cut off (Millipore Corporation, Bedford, MA, USA) and centrifuged for 7 min at 14800 x *g* at 4°C. Samples were run on a 12.5% SPS-PAGE gel and blotted onto a nitrocellulose membrane (Protran, Schleicher & Schuell BioScience) at 15V for 30min. Membranes were blocked with 5% non-fat dry milk (NFDM) (Elk skimmed milk powder, Friesland Campina) in 1x TBST (50mM Tris, 0.15M NaCl [pH 7.4], 0.2% [v/v] Tween-20) overnight at 4°C followed by incubation with either rabbit α-zfTnfa primary polyclonal antibody (kindly provided by dr. Victor Mulero) in 1:500 dilution; rabbit α-zebrafish Tnfb antibody (Anaspec, Anti-TNF-b (IN), Z-Fish™) in 1:500 dilution; or mouse α-FLAG antibody in 1:800 dilution in 5% NFDM TBST for 1 hour at room temperature. The tnfa-V5-His construct was used as a positive control for the α-zfTnfa antibody in this study since the same construct was used for the production of this antibody. Goat α-rabbit HRP (GAR-HRP, DAKO, Glostrup, Denmark) and goat α-mouse HRP (GAM-HRP, DAKO, Glostrup, Denmark) in 10% NFDM TBST were used as secondary antibody in 1:1000 dilution for 1 hour at room temperature. Chemiluminescence signal was detected using WesternBright ECL Enhanced Chemiluminescence Western Blotting detection kit (Advansta) and visualized on Lumni-film chemiluminescent Detection Film (Roche).

NFκB Reporter assay

HEK293-NFκB cells transfected with Tnfr1, ΔTnfr1, Tnfr2, or ΔTnfr2 plasmid or non-transfected HEK293-NFκB cells were seeded in 96-well culture plates at a density of

50,000 cells/well in 100µL Advanced DMEM supplemented with 2% FCS to be later stimulated with difference cell culture supernatants for the reporter assay.

PAC2-NFκB cells were seeded in 96-well plates at a density of 40,000 cells/well in 100µL L-15 medium supplemented with 2% FCS. After 24 hours, 50 µL of the PAC2-NFκB cell culture supernatant was removed and cells were stimulated with 50 µL of cell culture supernatants from VERO cells transfected with different zebrafish tn timeructs. Flagellin (recFLA-st, Recombinant Flagellin from Salmonella typhimurium Invivogen) in 0.1 µg/mL or LPS 10 µg/mL concentration was used as positive control for NFκB activation. Supernatant of cells transfected with empty plasmid was used as a negative control.

6h or 24h after transfection cells were lysed using Bright-Glo substrate (Promega, UK), lysate was transferred to a white 96-well opaque bottom plate (Corning) and luminescence was acquired with Filtermax F5 spectrophotometer (Molecular Devices) according to the manufacturer's instructions using Softmax Pro 6.2.2 software (Luminescence option, 1000 ms integration time).

TALEN design and assembling

TALEN restriction sites were identified using <http://zifit.partners.org>, choosing Zifit option and selecting TALE Nucleases (REAL and REAL-Fast) under the Design Genome Editing Nucleases/Nickases menu. The full nucleotide sequence of interest, obtained from Ensembl, was inserted and the nucleotide position of the desired break was indicated with brackets. The break point was chosen such that the two TALEN arms (given by the program based on the indicated break point) were overlapping with the mutated regions of the unclavable construct, to prevent the unclavable construct to be cut as well. TALEN arms were aimed to be located on the exon, if possible, which was not possible in the case of tn timeruct; the last 4 nucleotides of the right TALEN arm ended up on intron 3, see Figure 8). Position 38 on exon 3 for tn timeruct and position 40 on exon 3 for tn timeruct was selected as breaking point and the first tier sequence identified by the program was used. Customized graphical guide for TALEN assembly was obtained by clicking on the identified target site (**Supplementary Figure**

1).

Following the graphical guide, series of cloning steps were performed on plasmids encoding individual TALE repeats (Addgene, Joung Lab REAL Assembly TALEN kit). In each series of ligation, a fragment encoding the carboxy-terminal repeat (right side of the ligation pair) was ligated into the BamHI and BsaI sites of a DNA vector backbone encoding the amino-terminal repeat (left side of the ligation pair) using Quick Ligation Kit (New England Biolabs, cat. No. M2200L). Ligated constructs were transformed into XL1-Blue competent cells and plasmid was isolated using QIAprep Spin Miniprep Kit (Qiagen, cat. No. 27106). To validate the vectors, an additional digestion was performed in each round using XbaI and BamHI enzymes. Successful ligation resulted in a $[(M + N) \times 102] + 33$ bp fragment (M=numbers of TALE repeats encoded by the vector backbone, N=numbers of TALE repeats encoded by the fragment, used for ligation). The final TALE repeat sequence was then digested with *BbsI* and *BsaI* and subcloned into the *BsmBI* site of the nuclease vector indicated on the graphical guide. To validate the final vector, a digestion step was performed using *KpnI* and *BamHI* restriction enzymes. Successful ligation resulted in a $650 + [N \times \sim 102]$ bp fragment (N=number of TALE repeats encoded in the array).

Prior to injection into 1-cell stage zebrafish embryos, plasmid was linearized with PmeI and RNA was synthesized using mMACHINE™ T7 Transcription Kit (ThermoFisher Scientific) using lithium-chloride RNA recovery method.

Generation of eukaryotic expression constructs for zebrafish embryo injection

In order to provide sufficient DNA length for homologous recombination, a 370-620 bp long sequence was synthesized (see sequence in **Supplementary Figure 2**) identical to the UTR upstream and downstream of the relevant gene with restriction sites *HindIII* and *BamHI* in between. The selected *tnfaVLRK* or *tnfbPLKLKK* uncleavable gene was then subcloned into the synthesized sequence (*HindIII* and *BamHI* sites) followed by subcloning the construct into pBluescript II Ks+ (Addgene) between *KpnI* and *NotI* restriction sites providing the full donor plasmid for zebrafish embryo injection. Note that pBluescript II Ks+ was used because of availability at the time and thus constructs were in reverse orientation (normal orientation requires pBluescript II Sk+).

Zebrafish single cell injection

In order to test if the designed TALEN could generate a double-strand break, wild type AB zebrafish embryos were injected at a 1-cell stage with 1 nl of a solution containing 300 ng/ml tnfa TALEN left and right arms, 5% Tango buffer (ThermoFisher Scientific), 10% Phenol red (Sigma) and 300 ng/ml 1 mM ssOligo_tnfa; a short 24 bp additional sequence with a stop codon between homologous sequences on both ends to provide template for the repair machinery upon double-strand break (see sequence in Table 1). If TALEN are successful, Tnf knockout zebrafish can be obtained with a premature stop codon. To confirm the efficiency of TALENs, genomic DNA was isolated from pools of 10 embryos at 24 hours post-injection and PCR was performed using TNFa TALEN check_Fw primer to anneal on the native gene upstream of the TALEN cutting site, while TNFa TALEN check_STOP_Rv primer designed to anneal on the ssOligo introduced by injection. This combination of primers would allow verification of the insertion of the ssOligo. Amplified products were confirmed by sequencing.

In order to generate a transgenic zebrafish line expressing uncleavable Tnf, wild type AB zebrafish embryos were injected at a 1-cell stage with 1 nl of a solution containing 300 ng/ml tnfa TALEN left and right, 5% Tango buffer, 10% Phenol red and 300 ng/ml pBluescript II Ks+ containing the uncleavable tnfaVLRK, or tnfbPLKLKK, providing the donor sequence to be knocked-in while native tnfa/tnfb would be knocked-out. To confirm insertion of the sequence encoding for uncleavable TnfaVLRK only, 3-months-old injected fish were fin clipped and PCR performed on genomic DNA using TNFa_VLRK_FW and TNFa_VLRK_RV primers. The forward primer was designed to start on exon 3 and end on exon 4 in order to select for the transgenic individuals only, while the reverse primer was designed to anneal in the 3' UTR region outside the section included in the donor plasmid.

Statistics

Data of the reporter assays analysis were plotted and analysed by using GraphPad PRISM. One-way ANOVA followed by Dunnett's multiple comparisons test were used to compare data. Values of $p < 0.05$ at a confidence interval of 95% were considered significant.

RESULTS

Identification of putative TACE cleavage sites in zebrafish Tnfa

To generate uncleavable forms of Tnf and study receptor preferences *in vitro* we first predicted the recognition and cleavage sites of TACE in the sequence of both isoforms of zebrafish Tnfa, namely Tnfa and Tnfb. Site predictions were based on parameters and empirical analyses previously performed for several mammalian TACE targets (Caescu et al., 2009). These analyses predicted four recognition sites and one cleavage site at the hinge region of Tnfa, and six recognition sites and two cleavage sites at the longer hinge region of Tnfb (**Figure 1**). Amino acids in position (P) -5, 1 and 2, where cleavage occurs just before P1, have been reported to be important for TACE in substrate recognition and cleavage (Black et al., 2003). In humans the nonpolar, aliphatic valine is the most favoured amino acid by TACE in P1 position. Similarly, both zebrafish Tnfa and Tnfb sequences have a nonpolar and aliphatic yet other amino acid (leucine) at the predicted P1 site. Furthermore, also amino acids at the P-5 position show aliphatic properties in both human and zebrafish Tnfa, while P2 presents positively charged amino acids.

Our approach was to either introduce point mutations or delete part of the hinge region around the predicted TACE cleavage site. Both these approaches have been applied in mammals with various degree of success (Perez et al., 1990)(Caescu et al., 2009)(Tang et al., 1996a)(Tang et al., 1996b)(Decoster et al., 1995): mutation in the TNFa sequence of the primary cleavage site (P1) of TACE alone did not prevent cleavage (Decoster et al., 1995)(Migaki et al., 1995)(Mullberg et al., 1994) most likely due to the activity of proteases other than TACE; deletion of the region including both the recognition and cleavage sites prevented cleavage by TACE and other proteases, but also led to reduced TNFa bioactivity (Decoster et al., 1995). Taking all this into consideration, we designed several constructs using both approaches. *Tnfa*_{VL^{LRK}} and *Tnfb*_{PL^{KLKK}} were designed with point mutations resulting in substitutions of the amino acids V, L, and R to alanine (A) and K to glutamine (E) (**Figure 1**), while *Tnfa*_{D1-9K}, *Tnfb*_{D1-9K} and *Tnfb*_{D18-26} were designed to lead to complete deletions of the predicted TACE recognition and cleavage site and an additional point mutation at P13 in the *Tnfa*_{D1-9K} and P28 in the *Tnfb*_{D1-9K} construct. Owing to the availability of more tools to validate the Tnfa constructs, in the next paragraphs we first describe the characterization of Tnfa followed by the description of our preliminary characterization of the Tnfb constructs at the end of the chapter.

Figure 1. Schematic representation of the design of uncleavable *tnfa* and *tnfb* constructs. (A) P indicates position before or after the predicted TACE cleavage site indicated with red dashed line(s). Red asterisks indicate mutations of the corresponding amino acid and red line segments indicate deletions of the region. Within the *Tnfb* hinge region two cleavage sites were predicted and referred to as P1 and P18. **(B)** Sequence alignment of human TNFα, zebrafish *Tnfa* and *Tnfb*. Transmembrane region is highlighted in yellow, first amino acid after the predicted cleavage site (P1) is squared and amino acids that were mutated in the uncleavable constructs are highlighted in red.

Generation and validation of soluble and uncleavable zfTnfa

Surface expression of predicted uncleaved Tnfa proteins was assessed by immunohistochemistry, whereas the presence of soluble Tnfa in cell culture supernatant was assessed by Western blot analysis. To this end, VERO and EPC cells were transfected with plasmids encoding for either the native or the predicted uncleavable form of Tnfa. Cell culture supernatant was collected 48 hours after transfection and cells were stained with α -Tnf antibody. Western blot analysis of cell culture supernatants revealed a single band of expected molecular weight of approximately 28 kDa only when cells were transfected with *tnfa-V5-His* construct as the positive control (**Figure 2**). The native form of Tnfa was visible on Western blot only when supernatants were concentrated, suggesting that there is a difference in the secretion level or transfection efficiency of the two *tnfa* constructs. In the case of uncleavable Tnfa_{VL_{RK}} no soluble protein was expected, but the presence of a faint band around 25 kDa indicated that a very low percentage of Tnfa_{VL_{RK}} might still be cleaved.

EPC cells were stained with a polyclonal α -zfTnfa antibody 48 hours post-transfection. Constructs encoding for soluble Tnfa were used as negative control because proteolytically cleaved. As expected, image analysis revealed very few positive cells in wells transfected with the soluble Tnfa constructs (**Figure 3A and 3B**). In contrast, clear surface staining could be observed in wells transfected with the predicted uncleavable Tnfa constructs, indicating the presence of Tnfa on the cell surface. When comparing the fluorescence level of cells transfected with the Tnfa_{D1-9K} or Tnfa_{VL_{RK}} constructs, the number and fluorescence intensity of positive cells was higher in the Tnfa_{VL_{RK}} than in the Tnfa_{D1-9K} transfected cells. This indicated that the mutations introduced in the Tnfa_{VL_{RK}} construct were most efficient in preventing Tnfa cleavage. Given these results, for the subsequent *in vivo* experiments aimed at generating zebrafish expressing uncleavable form of Tnfa, we proceeded with the Tnfa_{VL_{RK}} sequence.

Figure 2. Validation of $Tnfa_{VLRK}$ construct by Western Blot. (A) Supernatant of VERO cells collected 48h after transfection with different Tnfa constructs or empty pcDNA3 (negative control), blotted on a 12.5% gel and visualized using rabbit α -zebrafish Tnfa polyclonal antibody. (B) 10x concentrated supernatant of VERO cells transfected with pcDNA3-Tnfa and detected as described in A.

Figure 3. Validation of uncleavable Tnfa by overexpression. EPC cells were transfected with the indicated constructs encoding soluble (A, B) and predicted uncleavable Tnfa (C, D), and 48 hours after transfection stained using rabbit α -zebrafish Tnfa followed by goat α -rabbit-RPE antibody (each at 1:200 dilution). Images were acquired with a LEICA M205 fluorescent microscope.

Validation of zebrafish TnfR expression constructs by overexpression

After having validated the constructs for the Tnf ligands we next validated the constructs for Tnf receptor (Tnfr) expression. HEK293 cells were transfected with expression constructs encoding for full-length HA-Tnfr1, FLAG-Tnfr2-HA, or truncated HA-ΔTnfr1 and FLAG-ΔTnfr2. Intracellular and extracellular immunostaining using α-tag antibodies was performed 24 hours post-transfection to assess receptor expression and localization. Extracellular staining (**Figure 4A-E**) using anti-HA for Tnfr1 and anti-FLAG for Tnfr2 confirmed the surface expression of all receptors although the expression levels differed between the full-length and truncated versions. Interestingly, for Tnfr2, we could detect a signal using the anti-FLAG antibody despite that the tag was placed before any potential leader peptide. The leader peptide indeed could not be identified using any of the available prediction softwares, possibly indicating that the leader peptide might not be cleaved during surface expression. Intracellular staining (**Figure 4F-H**) confirmed the expression of both, full-length and truncated receptors.

Figure 4. Validation of zebrafish Tnfr expression. HEK cells were transfected with the indicated constructs encoding either the full-length or truncated Tnfr. Labelling was performed 24 hours post-transfection. Proteins were detected using either mouse α-FLAG or mouse α-HA antibody. Images were acquired with a LEICA M205 fluorescent stereo microscope (A, C, D, F, G) and an EVOS Fluorescent Digital Inverted Microscope (B, E, H).

NFκB activation via Tnfr1 and Tnfr2

In order to investigate the bioactivity of Tnfa and of its receptors, Tnfr1 or Tnfr2, we made use of an NFκB reporter assay previously developed in our laboratory (Pietretti et al., 2014). HEK293-NFκB reporter cells were transiently transfected with the zebrafish Tnf receptors constructs and stimulated with cell supernatants containing Tnf ligands for 6 hours and 24 hours (data not shown due to lower responses). LPS was used as a positive control for NFκB activation in a Tnfr-independent manner, while supernatant of EPC cells transfected with empty pIRES-eGFP plasmid or with plasmids encoding for the uncleavable Tnfa were used as negative control. NFκB promoter activity was measured in luminescence and expressed as relative light unit (RLU). A schematic representation of the experiment design can be found in **Figure 5**.

Figure 5. Schematic representation of the reporter assay design used in this study. Blue cells indicate (EPC) cells transfected with either the native soluble, or the mutated uncleavable *tnf* constructs leading to the expression of transmembrane Tnf only. Supernatant of those cells were used to stimulate the HEK293-NFκB reporter cells (red cells) transiently transfected with full length or truncated zebrafish Tnf receptors. Only the cells expressing the full length receptors are able to induce intracellular signalling leading to NFκB and subsequent luciferase activation.

When HEK293-NFκB reporter cells not expressing zebrafish Tnf receptors were stimulated with any of the cell culture supernatants (negative controls), no significant change in NFκB promotor activity could be observed (**Figure 6**). Stimulation with LPS however (positive control), readily led to NFκB promotor activity and luciferase expression. When HEK293-NFκB reporter cells were transfected with Tnfr1, an elevated luciferase activity could be detected in all samples, independent of the stimulus, suggesting a constitutive NFκB activation induced by the overexpression of Tnfr1. Such constitutive activation did not occur when reporter cells were transfected with truncated Tnfr1 (ΔTnfr1). Further stimulation of HEK293-NFκB-Tnfr1 with cell culture supernatant containing soluble Tnfa did not trigger a further upregulation of luciferase activity when compared to cells stimulated with the (pIRES-eGFP) medium control. However, further stimulation of HEK293-NFκB-Tnfr1 with LPS did trigger a further upregulation of luciferase activity when compared to non-transfected cells or cells transfected with ΔTnfr1, suggesting a synergism in the intracellular pathways downstream of the Tnfr1 and LPS receptor(s).

Transfection of HEK293-NFκB reporter cells with constructs encoding the Tnfr2 did not lead to the constitutive NFκB promotor activity noticed for Tnfr1. Stimulation of these cells with supernatants containing Tnf did not lead to a further stimulation of NFκB activity. Stimulation of the same cells with LPS (positive control) did lead to higher luciferase activity than in non-transfected cells or cells transfected with ΔTnfr2.

Figure 6. Constitutive NFκB activation in HEK reporter cells expressing Tnfr1. HEK293-NFκB cells were transiently transfected with constructs coding for Tnfr1 and ΔTnfr1 (A) or Tnfr2 and ΔTnfr2 (B), and stimulated for 6 hours with supernatant from EPC cells transfected with constructs encoding soluble or predicted uncleavable Tnf (1:1 dilution). Supernatant from cells transfected with empty plasmids was used as negative control, LPS was used as positive control. RLU= Relative Light Unit. Each bar represents the mean + SD of n=3 experiments (different experiments using the same batch of supernatants for stimulation)

To investigate whether the constitutive NFκB activation observed in HEK293-NFκB reporter cells transiently transfected with Tnfr1 was due to the transient but high receptor expression, cell lines stably expressing (lower amounts of) zebrafish Tnfr1 were generated. Indeed, the overall constitutive activation of HEK293-NFκB-Tnfr1 was lower in stably- than in transiently-transfected cells (**Figure 6**).

While LPS induced a significant increase in luciferase activity in both Tnfr1- and ΔTnfr1-expressing HEK-NFκB cells, no significant change could be observed when these reporter cells were stimulated with different sources of Tnfa ligands or supernatant harvested from cells transfected with uncleavable Tnfa (**Figure 7**). Later however, it was shown that the stable reporter cell lines would gradually lose surface expression of the Tnf receptors while retaining antibiotic resistance, reducing their value after several passages.

Figure 7. Constitutive activation of NFκB in HEK293-NFκB reporter cells stably expressing Tnfr1. HEK-NFκB cells stably expressing the indicated Tnf receptors were stimulated for 24 hours with supernatant harvested from VERO cells transfected with different forms of recombinant zebrafish Tnfa in 1:1 dilution. Supernatant from VERO cells transfected with empty plasmids was used as negative control, LPS was used as positive control for NFκB activation. Data are shown as relative light units (each bar represents the mean of n=3 experiments + SD).

In an additional *in vitro* attempt, we generated a zebrafish fibroblast PAC2 reporter cell line stably transfected with the NFκB-luciferase reporter plasmid. PAC2 cells naturally express zebrafish Tnf receptors (Doctoral Thesis, Suning He, Leiden University, <https://openaccess.leidenuniv.nl/handle/1887/15555>) which means that constitutive activation of the NFκB pathway as reported above for HEK cells due to overexpression of TnfR can be avoided. PAC2-NFκB cells were stimulated with supernatant collected from VERO cells transfected with soluble or uncleavable Tnfa constructs (**Figure 8**). Supernatants from VERO cells transfected with empty plasmid were used as negative control, and flagellin rather than LPS was used as positive control as flagellin was previously shown to induce NFκB activation *in vitro* (Hayashi et al., 2001). Although the flagellin induced a positive luciferase signal, no clear signal was induced by any of the treatments with cell supernatants 24 hours after stimulation. Altogether, we conclude that overexpression of zebrafish Tnfr1 on the cell surface of mammalian and fish cell lines leads to constitutive expression of the luciferase reporter protein indicating a constitutive activation of the NFκB pathway. Such activation could not be further modulated by adding Tnf ligands which strongly reduces the value of these NFκB reporter cell lines for characterization of ligand-receptor preferences *in vitro*. It cannot be excluded that relatively low concentrations of Tnf ligands in the cell culture supernatants may have been insufficient to trigger a significant increase in activation of NFκB and subsequent luciferase reporter activity.

Figure 8. Tnfa is unable to induce NFκB activation in PAC2-NFκB cells. PAC2-NFκB were stimulated for 24 hours with supernatant from VERO cells transfected with the indicated forms of recombinant zebrafish Tnfa. Cell culture supernatant of VERO cells transfected with empty plasmid was used as negative control and flagellin was used as positive control (each bar represents the mean of n=3 experiments + SD).

Studies on the second isoform of zebrafish Tnf: Tnfb

In parallel, tools to characterize the bioactivity of both, soluble and uncleavable Tnfb, were also generated. CHO cells, typically easy to transfect with resulting high expression of recombinant proteins, were transfected with *tnfb-FLAG*. Supernatant was collected 72 hours after transfection and Western blot was performed in order to confirm the presence of soluble Tnfb. Unfortunately, both commercially-available antibodies (α -Tnfb and α -FLAG) failed to detect Tnfb on Western blot (**Figure 9**). Since our experiments could not exclude the presence of Tnfb in the supernatant, we continued characterizing the transfected cells rather than supernatants. Interestingly, when HEK293 cells transfected with *tnfb-FLAG* were labelled with α -FLAG antibody, a clear staining could be observed. The staining however, was observed at the surface of non-permeabilized cells (**Figure 10**), while it was expected that Tnfb would be cleaved off.

In another attempt to confirm soluble Tnfb in cell culture supernatant, the FLAG tag was replaced by an 6xHistidine tag. Detection of 6xHis-tagged protein however failed in supernatant, cell surface staining as well as in permeabilised cells.

Figure 9. (Lack of) detection of Tnfb in EPC cell culture supernatant.

Supernatants of EPC cells were collected 72h after transfection with pIRES-Tnfb-FLAG construct or with the empty plasmid control, and used for Western blot analysis. Rabbit α -zebrafish Tnfb (A) and mouse α -FLAG (B) antibody was used for protein detection.

Figure 10. Surface detection of Tnfb-FLAG on HEK293 cells.

HEK293 cells were transfected with pIRES-Tnfb-FLAG (top panel) or with empty pIRES (bottom panel) and 48 hours later upon fixation mouse α -FLAG antibody was used for protein detection. Images were acquired with an EVOS Fluorescent Digital Inverted Microscope.

At this point of the study, owing to the lack of suitable antibodies against zebrafish Tnfb, validation of the uncleavable Tnfb constructs was not possible, as these were designed without tags in order to retain the natural protein conformation. Therefore, we produced a new antibody against zebrafish Tnfb recognizing both, the short and long (uncleaved) version of Tnfb (**Figure 11**). The short and long form refer to the products that would potentially be obtained after cleavage, or not, at one of the two predicted TACE sites within the hinge region (**Figure 1A**). Unfortunately, due to lack of time, we did not test the ability of the rabbit polyclonal to recognize the uncleavable or soluble Tnfb proteins as described in figure 8 and 9.

Figure 11. Successful recognition of recombinant Tnfb-His by rabbit anti-zebrafish Tnfb polyclonal antibody. 2 μ g of bacterial recombinant Tnfb-His protein (either long or short form) was loaded in each lane and separated on a 12.5% gel. Proteins were visualized using the newly produced anti-zebrafish Tnfb polyclonal antibody in 1:2000 dilution.

Using an approach similar to the one used above for Tnfa, HEK293-NF κ B reporter cells transfected with full-length or truncated Tnfr1 and Tnfr2 were stimulated with EPC cell culture supernatant containing Tnfb (Tnfb treatment) or not containing Tnfb (Tnfb_{PLKLKK'}, Tnfb_{D1-9K'}, Tnfb_{D18-26}), (**Figure 12**). In a similar set-up, cell culture supernatants of EPC cells transfected with empty plasmid were used as negative control, and LPS was used as positive control. As expected, non-transfected HEK293-NF κ B reporter cells, when stimulated with cell culture supernatants from cells transfected with constructs encoding various Tnfb ligands, did not show increased luciferase activity. Stimulation with LPS did lead to a significant activation of luciferase activity. Stimulation of HEK293-NF κ B reporter cells expressing Tnfr1 with any of the supernatants resulted in increased luciferase activity, independent of the supernatant used. Stimulation with the supernatants originating from uncleavable Tnfb transfected cells were not expected to contain Tnf ligands, therefore no signal increase was expected either. The only stimulation that should have led to luciferase

increase was the Tnfb one. Constant and high light unit values between different stimulus could be the result of a constitutive NFkB activation induced by overexpression of Tnfr1. Indeed, when HEK293-NFkB cells were transfected with truncated Tnfr1 (Δ Tnfr1), constitutive NFkB activation was abolished. LPS stimulation of Tnfr1-expressing cells led to a further increase in NFkB promotor activity when compared to non-transfected cells, as previously observed in Figure 5, which was reduced in cells transfected with the truncated receptors. NFkB promotor activity was not observed when HEK293-NFkB cells expressing Tnfr2 were stimulated with Tnfb ligands. Luciferase activity was elevated, but not significantly different from non-transfected HEK293-NFkB cells or Δ Tnfr1-expressing cells.

Figure 12. Constitutive NFkB activation in HEK293 cells via Tnfr1. HEK293-NFkB cells were transiently transfected with the indicated receptors and were stimulated for 6 hours with supernatant from EPC cells transfected with constructs encoding soluble or predicted uncleavable Tnf (1:1 dilution). Supernatant of cells transfected with empty plasmid was used as negative control, LPS was used as positive control. Data are shown as relative light units (RLU) (each bar represents the mean of n=3 experiments + SD).

Generation of uncleavable Tnfa transgenic zebrafish using TALEN system

Given the technical difficulties of the *in vitro* system in evaluating Tnf ligand and receptor binding activities, we proceeded with an *in vivo* approach to characterize the biological function of uncleavable Tnfa. For this purpose, we attempted to produce a transgenic zebrafish line exclusively expressing uncleavable Tnfa by knocking out the native form of *tnfa* and at the same time introducing the mutated uncleavable *tnfa*_{VLRK} in a single homologous recombination step.

We first considered to use the CRISPR-Cas 9 system to knock out the native form of *tnfa* and insert uncleavable *tnfa*_{VLRK}. Even though this technique has been proven to be fast and cheap, we had a major concern: the guide RNA of CRISPR could potentially also anneal to the donor plasmid thereby leading to an undesirable cut in the donor plasmid. Therefore, our first approach was to design guide RNAs specifically targeting the sites of the native *tnfa* where the mutations are in our uncleavable construct. This however was not possible due to the fact that the specific sequence requirements of the CRISPR-Cas 9 system were not met within the relatively short exons of our target gene. As an alternative, Transcription activator-like effector nuclease (TALEN) technique was used. The TALEN system allows to design the two homology arms to target the TACE cleavage site of the native genomic *tnf*; the region which was mutated in our uncleavable construct. In this way our uncleavable construct would not be cleaved by the nucleases. Hence, we choose the TALEN system and designed two pairs of TALEN arms for each *tnf* gene. The arms were designed to anneal to the sequence encoding the recognition and cleavage site of TACE on the native *tnf* sequence, and therefore could not bind to the donor plasmid. TALEN arms were cloned according to the graphical guide in **Supplementary Figure 2** obtained from Zifit online targeter software.

As seen in the Supplementary figure 1, 2 and 3, the same strategy has been used for *Tnfb* as well, however due to limited time and considering that we could not confirm, *in vitro*, the uncleavable constructs only for *Tnfa*, we did not proceed with the generation and injection of the TALENs designed for *Tnfb*.

In order to validate the nuclease activity of the designed TALENs, 300 ng/ml TALEN_TNFa mRNA and 1 mM ssOligo were injected into 1-cell stage wild-type zebrafish embryos. The ssOligo consisted of a 35 bp sequence, including a stop codon and additional 17-23 bp long sequences up- and downstream of the stop codon that are homologous to the genome to provide template for homologous recombination occurring upon double stranded break (**Figure 13A**). Therefore, with optimally working TALEN, this step can lead to functional zebrafish knock-out due to the insertion of a premature stop codon in the *tnfa* coding sequence. To confirm the

insertion, pools of 10 embryos were collected 24 hours after injection with TALEN for subsequent PCR analysis. The forward primer of the primer set was designed to anneal upstream of the TALEN cleavage site (TNFa TALEN check_Fw) while the reverse primer was designed to anneal to the injected Oligo (TNFa TALEN check_STOP_Rv). The PCR results (**Figure 13B**) show bands of the correct size (463 bp) in all tested pools but not in the negative control (C-) (no Oligo injected) or the wild type (Wt) control (not treated) which confirmed the ability of the designed Tnfa TALENs to introduce double-stranded breaks in the genome. Injected F0 fish were raised and n=8 individuals were later confirmed by sequencing to carry the mutation.

Figure 13. Strategy and validation of *tnfa* TALEN. (A) The position of the TALEN left (tier 1) and right (tier 2) arms are underlined. Red nucleotides indicate the position of the mutations in the uncleavable constructs present on exon 3 of the donor plasmid (highlighted with red). Blue nucleotide in brackets indicate the position of the TALEN-induced double strand break. Red sequence of the ssOligo is an additional 35 bp that is not present in the gene and the underlined red sequence was the annealing region of the reverse primer TNFa TALEN check_STOP_Rv. Underlined black nucleotides of the ssOligo indicate the TALEN. (Acc: [ZDB-GENE-050317-1](https://www.ncbi.nlm.nih.gov/nuccore/ZDB-GENE-050317-1)) (B) Wild type zebrafish embryos were injected at 1 cell stage with the designed Tnfa TALEN left and right arms in combination with a short oligo sequence (ssOligo) and 24 hours post-injection groups of 10 embryo was sacrificed. PCR was performed using a FW primer annealing to the genomic region and a reverse primer annealing to the ssOligo sequence.

Upon successful *in vivo* validation of the nucleases, TALENs were injected in combination with pBluescript II Ks⁺-*tnfa*_{VLRR} donor plasmid containing the uncleavable *tnfa* to be inserted knocked-in at the same genomic level where the double stranded break would be induced (**Figure 14**). Injected embryos were raised up to 3-months-of-age and fin-clipped. To assess homologous recombination of the transgene into the genome, a PCR was performed using forward primer with the 5'-end annealing to exon 3 and the 3'-end to exon 4 thereby excluding the possibility to amplify the wild-type gene (see position in **Supplementary Figure 2**). The reverse primer was designed to anneal downstream of the gene in the 3' UTR region outside the region that was used in the donor plasmid. The combination of these primers should result in a band only when insertion of the transgene was successful. Unfortunately, no product could be amplified by PCR in none of the n=8 *Tnfa*^{-/-} fish which indicated that the homologous recombination was not successful.

Figure 14. Schematic representation of whole *tnf* gene replacement with the TALEN system. The position of the TALEN left (tier 1) and right (tier 2) arms are underlined. Red nucleotides indicate the position of the mutations in the uncleavable constructs present on exon 3 of the donor plasmid (red square). Blue nucleotide in brackets indicate the position of the TALEN-induced double strand break. Nucleotides in grey in the sequence of *tnfb* indicates intron localization. (*Tnfb* Acc: [ZDB-GENE-050601-2](#), *Tnfa* Acc: [ZDB-GENE-050317-1](#)). Yellow boxes: exons: Grey boxes: introns.

Overall conclusions

In this study we reported the generation of various uncleavable zebrafish Tnfa, and report on the preliminary work performed to generate uncleavable Tnfb. Several NFκB reporter cell lines were generated expressing the full-length or truncated forms of zebrafish Tnfr1 and Tnfr2. Unfortunately, the constitutive NFκB activation in the Tnfr1-expressing lines, and the lack of activation in the Tnfr2-expressing lines, did not allow us to further characterize *in vitro* the potential differences in biological activity between soluble and uncleavable Tnf. Furthermore, based on the validation of the uncleavable Tnfa constructs, we generated TALENs aimed at the insertion of a premature stop codon in the wild type *tnfa* gene and subsequent insertion of the sequence coding the validated uncleavable *tnfa*. In addition, several tools to study zebrafish Tnfa and Tnfb at the protein level were generated in this study. These can be used in future studies to improve the current knock-in strategy and ultimately study the activity of soluble and uncleavable Tnf *in vivo*.

Identification of putative TACE cleavage sites in zebrafish Tnfa

Critical to our study was the correct prediction of TACE recognition and cleavage sites in the hinge region of the two zebrafish Tnfa sequences. Previously, it has been shown that substrate recognition by TACE is not dependent on only one, but rather on multiple amino acids with specific properties and location within the TNF hinge region (Perez et al., 1990) (Creasey et al., 1987). Furthermore, the hinge region of TNF can be the target of other metalloproteases, including ADAM10, which was shown to cleave tmTNFa (Killar et al., 1999; Rosendahl et al., 1997). Thus, several mutations and/or deletion were required to obtain a transmembrane TNF that could not be cleaved by either TACE nor other proteases. Furthermore, deletion of amino acids between position 1-9 of the hinge region did lead to the generation of uncleavable TNF, but this time with reduced binding abilities to the TNFR (Decoster et al., 1995).

In our study, based on the characteristics of the relevant residues involved in TACE recognition and cleavage of mammalian TNF, four recognition and a single cleavage site was predicted for Tnfa, while six recognition and two

cleavage sites were predicted in the longer hinge region of Tnfb. Based on this information, and considering that deletion of large portion of the hinge region may affect Tnf bioactivity, we designed several constructs to introduce point mutations (*Tnfa*_{VLRK} and *Tnfb*_{PLKLKK}) or delete the complete recognition and cleavage sites as well as add an additional point mutation (*Tnfa*_{D1-9K}, *Tnfb*_{D1-9K} and *Tnfb*_{D18-26}) to achieve expression of uncleavable, tmTnfa on the cell surface. Indeed, surface expression of Tnfa, most evidently *Tnfa*_{VLRK}, was confirmed by immunohistochemical analysis of cell lines transfected with Tnfa constructs predicted to lead to uncleavable Tnfa. Considering that *Tnfa*_{VLRK} construct does not contain any deletions, and thus should not have a reduced binding efficiency to its receptors, it was selected for further *in vivo* studies. Surface expression of Tnfb could not be confirmed due to the lack of specific antibody. However, we have produced anti-Tnfb polyclonal antibodies that can be used in the future to finally validate the Tnfb constructs. Nevertheless, based on the results obtained for the various tmTnfa constructs, we could conclude that the *Tnfa*_{VLRK} mutant is the best candidate which successfully led to expression of uncleavable, transmembrane Tnfa and should be considered for follow up studies.

To date, most studies investigating the various functions of Tnf in zebrafish, mostly focused on the Tnfa paralogue (Clay et al., 2008; Espín-Palazón et al., 2016; Roca and Ramakrishnan, 2013). Therefore, very little is known about the function(s) of zebrafish Tnfb. With this in mind, we aimed to further study Tnfb, and we started by generating a FLAG-tagged cleavable Tnfb construct as source of soluble Tnfb. Unfortunately, both commercially-available antibodies (α -Tnfb from Anaspec and α -FLAG) failed to detect soluble Tnfb in cell culture supernatants by Western blot. Since our experiments could not confirm the presence of soluble Tnfb in the supernatant, we continued characterizing the transfected cells. Interestingly, when HEK293 cells transfected with *tnfb*-FLAG were labelled with α -FLAG antibody, a clear staining could be observed. The staining was observed on the surface of non-permeabilized cells, while it was expected that Tnfb would be cleaved off. This is surprising considering that the hinge region of Tnfb is longer than the one of Tnfa, and was

predicted to have two potential recognition and cleavage sites. Similar experiments, using the same conditions and cell type, were performed with the corresponding soluble Tnfa-V5 construct (Figure 3). Here, a very weak staining of the surface of non-permeabilized cells, and a stronger intracellular staining of permeabilized cells was observed, confirming that overexpression of cleavable Tnfa leads to release of the protein and no accumulation on the cell surface. It is therefore tempting to speculate that the two zebrafish paralogues may have intrinsic differences; they may exert different biological activities based on their post-translational processing, or might be cleaved at different efficiency. Tnfa might be preferentially present as soluble protein, whereas Tnfb might be, at least initially, present as transmembrane protein. Although Tnfb is most likely also present as soluble protein, it will be interesting to investigate under which circumstance the transmembrane or the soluble form are generated and to which receptor they preferentially bind to. Nevertheless, Tnfb is an understudied but important cytokine that should not be neglected.

Limitation of the *in vitro* system to study Tnf signalling

One of the initial aims of the study was to generate tools to study in details bidirectional signalling between tmTnf and Tnfr. To this end we generated several constructs encoding full-length or truncated forms of Tnfr1 or Tnfr2, tagged to facilitate protein detection and localization. Immunohistochemical staining confirmed surface expression of all receptors although the expression levels differed between full-length and truncated versions. Transfection of HEK-NFκB reporter cells with Tnfr1, but not truncated Tnfr1 (Δ Tnfr1), led to constitutive NFκB promoter activity and luciferase expression, which could not be further induced with cell culture supernatants containing Tnfa.

It has been well studied that binding of mammalian TNFα to TNFR1 leads to receptor trimerization (Jiang et al., 1999). Normally, aggregation of death domains from each cytosolic site of the TNFR1 monomer is ligand binding-dependent, leading to the recruitment of the TNFR1-associated death domain protein (TRADD) and further signaling through TNFR-associated factor (TRAF) (Brenner et al., 2015)(Verstrepen et al., 2008). When TNFR1 are overexpressed, due to for example transient transfection, it is possible that the death domains are aggregated in the cytoplasmic

site due to their physical proximity leading to recruitment of TRAF and downstream NF- κ B activation (Horie et al., 2002)(Jiang et al., 1999).

In our study we attempted to decrease the relative expression levels of full length Tnfr1 by co-transfecting HEK NF- κ B cells with Tnfr1 along with the Δ Tnfr1 (data not shown). We then expected that the truncated receptors would 'dilute out' the full-length receptors, providing the more physical space between the cytosolic domains of each receptor. Alternatively, we also generated stable lines constitutively expressing the full-length receptors, but onset of antibiotic resistance made the lines not suitable for further study.

In order to overcome the problem of constitutive NF- κ B activation it would be an interesting alternative to replace the intracellular part of the zebrafish Tnfr with for example the human TLR2 intracellular TIR domain creating a chimeric receptor. In a previous study it has been confirmed that over expression of human TLR2 does not lead to constitutive NF- κ B activation in the same HEK NF- κ B reporter cell line used in the current study (Fink et al., 2016). Therefore, human TLR2 TIR domain might be a suitable candidate to use for future studies.

Furthermore, to come back to our original approach and study the bi-directional signaling of transmembrane Tnfa/b, a different reporter cell line could be transfected with the validated uncleavable constructs and the cells could be mixed with a cell line expressing the Δ Tnfr1/2 truncated receptor. Therefore, luciferase activity could be detected only in the transmembrane Tnf-expressing cells upon ligand-receptor binding (**Figure 15B**). The hypothesis in this case would be that also zebrafish Tnf signals *via* an NF κ B-mediated pathway (Qu et al., 2017; Sun and Fink, 2007). Bidirectional signaling by mammalian TNFa has been shown to be mediated *via* a casein kinase I (CKI) binding site (S/TXXS/T) (Qu et al., 2017; Watts et al., 1999). The downstream signaling events are still not fully understood and seem to be highly dependent on the mTnf-expressing cell (Qu et al., 2017). Besides NF κ B-mediated signaling, NFAT has also been implicated downstream of CKI activation (Sun and Fink, 2007; Watts et al., 1999). Analysis of the cytoplasmic region of zebrafish Tnfa and Tnfb reveals the presence of one putative CKI site in each of the proteins, but in a position more proximal to the transmembrane region than the one observed for human TNFa (**Figure 15A**). The availability

of an NFkB reporter cell line and the possibility to generate an NFAT reporter line, together with the identification of a suitable construct for the generation of mTnfa, altogether would allow us to study whether bi-directional signaling via tmTnf exist in zebrafish.

A

zftnfa	MKLESRAFLDVEEGELPLPLVIVSRRKAGSSKSGVWRVFGIILAVGLCAA AVCFTL HKTQGNQQDG
zftnfb	MVRYETTLVDMEAGVG GVYQT TVAPVPISSRSNIWKTLAAVAFVGLCVVAAFFFTWHVMKPNEEGQ
hTNFα	-MSTESAIRDVELAEEALPKKTGGPQ--GSRRCFLSLFSFLIVAG-ATTLFCLLHFGVIGPQREE

B

Figure 15. Schematic representation of the *in vitro* characterization of receptor binding properties of soluble and transmembrane Tnfa/b. (A) protein sequence alignment of the first 60 amino acids of the N-Terminal region of zebrafish and human tnfa. Orange squares indicate the casein kinase I (CKI) binding site (S/TXXS/T) in the human sequence, and the putative site in each of the zebrafish Tnf sequences. (B) HEK NF-kB cells transfected with uncleavable Tnfa/b and mixed with cells transfected with ΔTnfr1/2 will induce intracellular signaling leading to luciferase expression in the transmembrane Tnfa/b expressing cells indicating bidirectional signaling.

Generation of uncleavable Tnfa transgenic zebrafish using the TALEN system

We aimed to produce a transgenic zebrafish line exclusively expressing uncleavable Tnfa by knocking out the native form of *tnfa* and introducing the mutated uncleavable *tnfa*_{VLK} in a single homologous recombination step. Upon successful *in vivo* validation of the nucleases, TALENs were injected in combination with plasmid containing the uncleavable *tnfa* to be knocked-in. Injected embryos were grown and fin-clipped at 3-months-of-age to confirm by PCR homologous recombination of the transgene into the genome. Unfortunately, no correct PCR product could be amplified

in none of the n=8 *Tnfa*^{-/-} fish which indicated that the homologous recombination was not successful.

We hypothesized that this may have occurred because the homology arms have been designed too far from the double stranded break. Therefore, to overcome that problem, we suggest to use knockout lines for background that are already available and insert the construct encoding the uncleavable *tnf* directly downstream of the start codon of the genomic *tnf*. This would require to introduce a point mutation in the start codon of the uncleavable construct that would still lead to the same amino acid but wouldn't allow the TALEN to cut the donor plasmid.

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SUPPLEMENTARY FIGURES

tnfa

Tnfa	ATGAAGCTTGAGAGTCGGGCGCTTTTGGATGTTGAAGAAGGAGAGTTGCCTTTACCGCTGGTGAT
TnfaVLRK	ATGAAGCTTGAGAGTCGGGCGCTTTTGGATGTTGAAGAAGGAGAGTTGCCTTTACCGCTGGTGAT
TnfaD1-9K	ATGAAGCTTGAGAGTCGGGCGCTTTTGGATGTTGAAGAAGGAGAGTTGCCTTTACCGCTGGTGAT
Tnfa	GGTGCTAGGAGGAAAGCTGGATCTTCAAAGTCGGGTGTATGGAGGGTGTTGGGATCATTTTGG
TnfaVLRK	GGTGCTAGGAGGAAAGCTGGATCTTCAAAGTCGGGTGTATGGAGGGTGTTGGGATCATTTTGG
TnfaD1-9K	GGTGCTAGGAGGAAAGCTGGATCTTCAAAGTCGGGTGTATGGAGGGTGTTGGGATCATTTTGG
Tnfa	CTGTGGGCCTTTGTGCCGCTGCTGCCGCTGCTTCACGCTCCATAAGACCCAGGGCAATCAACAA
TnfaVLRK	CTGTGGGCCTTTGTGCCGCTGCTGCCGCTGCTTCACGCTCCATAAGACCCAGGGCAATCAACAA
TnfaD1-9K	CTGTGGGCCTTTGTGCCGCTGCTGCCGCTGCTTCACGCTCCATAAGACCCAGGGCAATCAACAA
Tnfa	GATGGAAGTGTGCTGAGACTCACATTGAGAGATCGCATTTTACAAGGCAATTTCACTTCCAAGGC
TnfaVLRK	GATGGAAGTGTGCTGAGACTCACAGGAGATCGCATTTTACAAGGCAATTTCACTTCCAAGGC
TnfaD1-9K	GATGGAAGTGTGCTGAGACTCACAGGAGATCGCATTTTACAAGGCAATTTCACTTCCAAGGC
	V>A L>A,R>A K>E
Tnfa	TGCCATCCATTTTAACAGGTGGATACAACCTCTGAATCAAAGACCTTAGACTGGAGAGATGACCAGG
TnfaVLRK	TGCCATCCATTTTAACAGGTGGATACAACCTCTGAATCAAAGACCTTAGACTGGAGAGATGACCAGG
TnfaD1-9K	TGCCATCCATTTTAACAGGTGGATACAACCTCTGAATCAAAGACCTTAGACTGGAGAGATGACCAGG
Tnfa	ACCAGGCCCTTTTCTTCAGGTGGCTTGAAATTAGTAAACAGGGAGATTATCATTTCCCGATGATGGC
TnfaVLRK	ACCAGGCCCTTTTCTTCAGGTGGCTTGAAATTAGTAAACAGGGAGATTATCATTTCCCGATGATGGC
TnfaD1-9K	ACCAGGCCCTTTTCTTCAGGTGGCTTGAAATTAGTAAACAGGGAGATTATCATTTCCCGATGATGGC
Tnfa	ATTTATTTTGTCTACAGCCAGGTGTCTTTGCACATCAGCTGCACGTCTGAACTGACTGAGGAACA
TnfaVLRK	ATTTATTTTGTCTACAGCCAGGTGTCTTTGCACATCAGCTGCACGTCTGAACTGACTGAGGAACA
TnfaD1-9K	ATTTATTTTGTCTACAGCCAGGTGTCTTTGCACATCAGCTGCACGTCTGAACTGACTGAGGAACA
Tnfa	AGTGCTTATGAGCCATGCAGTGATGCGCTTTTCTGAATCCTACGGAGGCCAAAAAGCCACTTTTCA
TnfaVLRK	AGTGCTTATGAGCCATGCAGTGATGCGCTTTTCTGAATCCTACGGAGGCCAAAAAGCCACTTTTCA
TnfaD1-9K	AGTGCTTATGAGCCATGCAGTGATGCGCTTTTCTGAATCCTACGGAGGCCAAAAAGCCACTTTTCA
Tnfa	GTGCAATCCGCTCAATCTGCACGCAGGAGCCTGAATCTGAAAACTCTGGTACAACACTATTTTAC
TnfaVLRK	GTGCAATCCGCTCAATCTGCACGCAGGAGCCTGAATCTGAAAACTCTGGTACAACACTATTTTAC
TnfaD1-9K	GTGCAATCCGCTCAATCTGCACGCAGGAGCCTGAATCTGAAAACTCTGGTACAACACTATTTTAC
Tnfa	CTCGGCGCTGCCTTCCATTTACGAGAAGGAGACAGACTGGGCACAGACACGACCACAGCACTTCT
TnfaVLRK	CTCGGCGCTGCCTTCCATTTACGAGAAGGAGACAGACTGGGCACAGACACGACCACAGCACTTCT
TnfaD1-9K	CTCGGCGCTGCCTTCCATTTACGAGAAGGAGACAGACTGGGCACAGACACGACCACAGCACTTCT
Tnfa	ACCGATGGTTGAAAAATGATAACGGAAAGACCTTCTTTGGGGTGTTTGGTTTGTGA
TnfaVLRK	ACCGATGGTTGAAAAATGATAACGGAAAGACCTTCTTTGGGGTGTTTGGTTTGTGA
TnfaD1-9K	ACCGATGGTTGAAAAATGATAACGGAAAGACCTTCTTTGGGGTGTTTGGTTTGTGA

tnfb

Tnfb	ATGGTGAGATACGAAACAACATTAGTAGATATGGAAGCCGGTGTCGGGGGAGTTTATCAGACAAC
TnfbPLKLKK	ATGGTGAGATACGAAACAACATTAGTAGATATGGAAGCCGGTGTCGGGGGAGTTTATCAGACAAC
TnfbD1-9K	ATGGTGAGATACGAAACAACATTAGTAGATATGGAAGCCGGTGTCGGGGGAGTTTATCAGACAAC
TnfbD18-26	ATGGTGAGATACGAAACAACATTAGTAGATATGGAAGCCGGTGTCGGGGGAGTTTATCAGACAAC
Tnfb	CGTGGCACCTGTACCGGTTAATTCTTCAAGAAGCTGGATATGGAAGACACTCGCTGCAGTTGCTT
TnfbPLKLKK	CGTGGCACCTGTACCGGTTAATTCTTCAAGAAGCTGGATATGGAAGACACTCGCTGCAGTTGCTT
TnfbD1-9K	CGTGGCACCTGTACCGGTTAATTCTTCAAGAAGCTGGATATGGAAGACACTCGCTGCAGTTGCTT
TnfbD18-26	CGTGGCACCTGTACCGGTTAATTCTTCAAGAAGCTGGATATGGAAGACACTCGCTGCAGTTGCTT
Tnfb	TGTGTTGGCCTCTGCGTTGTGGCTGCATTTTCTTACCTGGCATGTGATGAAGCCAAACGAAGAA
TnfbPLKLKK	TGTGTTGGCCTCTGCGTTGTGGCTGCATTTTCTTACCTGGCATGTGATGAAGCCAAACGAAGAA
TnfbD1-9K	TGTGTTGGCCTCTGCGTTGTGGCTGCATTTTCTTACCTGGCATGTGATGAAGCCAAACGAAGAA

TnfbD18-26	TTGTTGCGCTCTGCGTTGTGGCTGCATTTTTCTTCACCTGGCATGTGATGAAGCCAAACGAAGAA
Tnfb	GGTCAGAAACCCAAACAGAGAACATCTAAAACAACAAATCACCACACCTTCAGCTTCCTCAGACCA
TnfbPLKLKK	GGTCAGAAA■CCAAACAGAGAACAT■CA■AACAACAAATCACCACACCTTCAGCTTCCTCAGACCA
TnfbD1-9K	GGTCAGAAACCCAAACAGAGAACAT-----TCAGCTTCCTCAGACCA
TnfbD18-26	GGTCAGAAACCCAAACAGAGAACATCTAAAACAACAAATCACCACACCTTCAGCTTCCTCAGACCA
	P>A L>A K>E
Tnfb	CGGAAAAGTGCTGAAGCAGATCGCTCAGAGCACAAAGGCTGCCATTCACTTGCATGGTGACCCTT
TnfbPLKLKK	CGGAAAAGTG■G■AGCAGATCGCTCAGAGCACA■AGGCTGCCATTCACTTGCATGGTGACCCTT
TnfbD1-9K	CGGAAAAGTGCTGAAGCAGATCGCTCAGAGCACA■AGGCTGCCATTCACTTGCATGGTGACCCTT
TnfbD18-26	CGGAAAAGTG-----GCTGCCATTCACTTGCATGGTGACCCTT
	L>A K>E K>E
Tnfb	CAGGACAATCGCTGAAATGGGTTGGCGGTGTGGATCAGGCATTCCAACAGGGCGGCTCAGGCTG
TnfbPLKLKK	CAGGACAATCGCTGAAATGGGTTGGCGGTGTGGATCAGGCATTCCAACAGGGCGGCTCAGGCTG
TnfbD1-9K	CAGGACAATCGCTGAAATGGGTTGGCGGTGTGGATCAGGCATTCCAACAGGGCGGCTCAGGCTG
TnfbD18-26	CAGGACAATCGCTGAAATGGGTTGGCGGTGTGGATCAGGCATTCCAACAGGGCGGCTCAGGCTG
Tnfb	GAAAACAACGAGATCATCATTCCTAAGGATGGTCTTTACTTTGTGTACAGTCAAGTGTGCTACGA
TnfbPLKLKK	GAAAACAACGAGATCATCATTCCTAAGGATGGTCTTTACTTTGTGTACAGTCAAGTGTGCTACGA
TnfbD1-9K	GAAAACAACGAGATCATCATTCCTAAGGATGGTCTTTACTTTGTGTACAGTCAAGTGTGCTACGA
TnfbD18-26	GAAAACAACGAGATCATCATTCCTAAGGATGGTCTTTACTTTGTGTACAGTCAAGTGTGCTACGA
Tnfb	AACCTTTGTGCGTTGAAGATGTTGAAGGAGATGGTCAAAAGTACCTGAGCCACACCATCAATCGAT
TnfbPLKLKK	AACCTTTGTGCGTTGAAGATGTTGAAGGAGATGGTCAAAAGTACCTGAGCCACACCATCAATCGAT
TnfbD1-9K	AACCTTTGTGCGTTGAAGATGTTGAAGGAGATGGTCAAAAGTACCTGAGCCACACCATCAATCGAT
TnfbD18-26	AACCTTTGTGCGTTGAAGATGTTGAAGGAGATGGTCAAAAGTACCTGAGCCACACCATCAATCGAT
Tnfb	ACACAGATGCGGTGAGGGAAAAGATGCCTCTGCAAAACTCGGCCAATTCGGTCTGCCAGTCTCTA
TnfbPLKLKK	ACACAGATGCGGTGAGGGAAAAGATGCCTCTGCAAAACTCGGCCAATTCGGTCTGCCAGTCTCTA
TnfbD1-9K	ACACAGATGCGGTGAGGGAAAAGATGCCTCTGCAAAACTCGGCCAATTCGGTCTGCCAGTCTCTA
TnfbD18-26	ACACAGATGCGGTGAGGGAAAAGATGCCTCTGCAAAACTCGGCCAATTCGGTCTGCCAGTCTCTA
Tnfb	GATGGAACAAACGTCCTACAGCACCATTACCTTGGAGCCGCTCTTTGATCTTTTTGGGGATGACAG
TnfbPLKLKK	GATGGAACAAACGTCCTACAGCACCATTACCTTGGAGCCGCTCTTTGATCTTTTTGGGGATGACAG
TnfbD1-9K	GATGGAACAAACGTCCTACAGCACCATTACCTTGGAGCCGCTCTTTGATCTTTTTGGGGATGACAG
TnfbD18-26	GATGGAACAAACGTCCTACAGCACCATTACCTTGGAGCCGCTCTTTGATCTTTTTGGGGATGACAG
Tnfb	GCTGTCGACCCACACCACTCGAGTGGGCGACATCGAGAACAACACTACGCCAAAACATTCTTCGGGG
TnfbPLKLKK	GCTGTCGACCCACACCACTCGAGTGGGCGACATCGAGAACAACACTACGCCAAAACATTCTTCGGGG
TnfbD1-9K	GCTGTCGACCCACACCACTCGAGTGGGCGACATCGAGAACAACACTACGCCAAAACATTCTTCGGGG
TnfbD18-26	GCTGTCGACCCACACCACTCGAGTGGGCGACATCGAGAACAACACTACGCCAAAACATTCTTCGGGG
Tnfb	TGTTTCGCGTTGTGA
TnfbPLKLKK	TGTTTCGCGTTGTGA
TnfbD1-9K	TGTTTCGCGTTGTGA
TnfbD18-26	TGTTTCGCGTTGTGA

Supplementary Figure 1. Sequence alignment of native and mutated uncleavable zebrafish *tnf*. Mutated nucleotides leading to change of amino acid are highlighted in red, mutations not leading to amino acid change, used to remove internal restriction sites, are highlighted in yellow.

Supplementary Figure 2. Graphical guide for TALEN assembly from the TALEN library.

tnfb

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cctccctgcatcgtgatgacttgttttatatgtaaaacaaagccgatgcagtgagagttgtgactgaataccatgtagtcagtcgaagagcact
ataacaagggcactacaattgtgtacttatcgcaagctaattgtgacgtttgattatctttagctatttcggtgtagaataagtagacttttatttta
tttttgctgttgattgatctatttataacttttacaccttttatattgtattgtttgtatttatctatttataatgactccatgctgtta**gcggccgg**
...(91 bp)...gttcctatttagagctcagaaaggaga 3'

Supplementary Figure 3. Donor plasmids and ssOligo sequence. Low cased nucleotides indicate the 5' upstream and 3' downstream UTR region of the gene that was synthesized. Blue nucleotides were not included in the donor plasmid but reverse primer was designed in that region. Nucleotides in capitals are of the uncleavable *tnfa*_{VLRK} or *tnfb*_{PLKLLK} where alternating black and grey regions indicate different exons. Restriction sites used for cloning are in boxes: KpnI, HindIII, BamHI and NotI (in order). Red nucleotides in the sequence of *tnfa*_{VLRK} or *tnfb*_{PLKLLK} indicate the mutations from wild type to uncleavable Tnf. Start and stop codons are bold and the underlined sequence was used as primers.

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6

CHAPTER

General Discussion

Novel mechanism of trypanosome attachment to host cells *in vivo*

In this thesis we have characterized the movements of *T. carassii* in zebrafish larvae, and described the major behavioural features during the course of infection (**Chapter 2**). Attachment of the trypanosomes to the blood vessel could be readily observed as early as 30 minutes to 1 hour after infection of zebrafish larvae. Remarkably, anchoring to the endothelium or other stationary cells on the luminal side of the vein, always occurred via the posterior end of the trypanosomes leaving the flagellum free to move, and was very rapid. Differently from what observed in our study, previous *in vitro* reports describe that the flagellum is actively involved in the adhesion of other trypanosomes, including *T. brucei* (Beattie and Gull, 1997; Langousis and Hill, 2014; Schuster et al., 2017), *T. congolense* (Beattie and Gull, 1997; Hemphill and Ross, 1995), and *Paratrypanosoma confusum* (Skalický et al., 2017), via extensive remodelling of the flagellar membrane ensuring a stable interaction. That process is not only extensive, but also time consuming. Based on our observation, *T. carassii* uses a different mechanism, since the entire flagellum is free upon anchoring and anchoring occurs very rapidly despite the strong hydrodynamic flow. In Chapter 2, we have hypothesized that the anchoring site of *T. carassii* coincides with the flagellar base, the flagellum pocket neck, or parasite cell membrane, however, it is yet to be determined. In order to identify the adhesion site of *T. carassii*, it is essential to locate and label the flagellum base or flagellar pocket neck region. To this end, parasites could be labelled with fluorescent surface dyes and accumulation of the label in the flagellar pocket could be observed shortly after staining. Accumulation of the dye in the flagellar pocket occurs rapidly, due to the membrane recycling and high endocytic activity of this region. For this purpose, experimental infection of zebrafish is not needed, as adherence of *T. carassii* to blood cells also occurred *in vitro* in the same manner (**Chapter 2, Video 2**). Therefore, these experiments can be easily performed *in vitro* on trypanosome culture with the addition of carp blood, or *ex vivo* upon isolation from carp blood. This method would not only be faster, but would also enables us to acquire high resolution images.

Despite that we were not able to capture the exact moment of extravasation, it occurred very early during the course of infection. However, the exact mechanism behind the process of extravasation has yet to be determined. In order to extravasate from the blood vessel, decreasing the speed and adherence of the parasite to the endothelial cells seems to be the first logical step. Therefore, in order to investigate whether or not adhesion of *T. carassii* to the endothelial cells is involved in the initiation process of extravasation, the use of our currently established zebrafish infection model would be greatly beneficial.

For example, by blocking sugar molecules on the surface of *T. carassii* that are involved in adhesion (e.g. lectins), we could investigate whether extravasation still occurs without adhesion. In addition to that, we can use the recently obtained genomic, transcriptomic and proteomic data (**Chapter 4**) to identify molecules that might be involved in adhesion. The availability of transcriptomic and genomic data will allow us to knock-out or genetically modify parasites to express fluorescently labelled surface molecule. This, combined with the already available transgenic zebrafish lines marking the vasculature or immune cells, would allow us to address the role of adhesion molecules in extravasation.

Capturing the swimming behaviour of biflagellate *Trypanoplasma borreli* in vivo in a vertebrate host

Unlike *T. carassii* and other trypanosomes, *Trypanoplasma borreli* has 2 flagella: an anterior and a posterior one (**Fig. 1**). While trypanosomes have the flagellar pocket on the posterior end, a recurrent flagellum running along the body ending free on the anterior end and determining the swimming direction, in *T. borreli* this is the other way around. Both flagella originate from a single flagellar pocket that, based on the swimming direction, is located at the anterior end of the parasite, at least in this case. The recurrent flagellum, which runs from the flagellar pocket along the cell body, ends free at the posterior end and does not seem to be involved in determining the swimming direction of *T. borreli* (**Video 1**). The second, non-cell attached, flagellum is free to move at the anterior end of the parasite (**Video 1**) making wide sweeping movements at the front as to patrol the environment. Among trypanoplasma species, the cellular

organization of *T. borreli* is not unique, the salmonid infectious *Cryptobia* (*Trypanoplasma*) *salmositica* presents very similar ultrastructural features and flagellar organization (Woo, 2003). However, detailed high-speed imaging of flagellar movements of any biflagellate parasite has not been reported so far.

Fig. 1: Simplified Schematic representation of the anatomy of *Trypanoplasma borreli*.

T. borreli has two flagella both emerging from the flagellar pocket located at the anterior pole of the cell. The posterior flagellum is attached to the cell body, ending free at the posterior end; while the anterior flagellum is non-cell attached and exits directly from the flagellar pocket.

After having performed detailed high-speed imaging of the swimming behaviour of the uniflagellate *T. carassii* (**Chapter 2**), we performed a preliminary analysis of the biflagellate *T. borreli* swimming behaviour *in vitro* and *in vivo*. Not only the number of flagella, but also the main cellular movements of *T. carassii* and *T. borreli* were different. The overall impression of the cellular movement of *T. borreli* is reminiscent of a swimming flatworm showing a wide sinusoidal-like waveform at the periphery of the cell (**Video 1**). We have observed that, while most trypanosomes' (anterior) flagellum is usually pointing towards the swimming direction, this is not the case for *T. borreli*; the anterior (attached) flagellum is free to move and it is either sweeping in front of the cell, or it is most often curled backward pointing towards the posterior end, being dragged during the forward movement. At least *in vitro*, a typical propelling movement of either of the two flagella is not observed and is also not required for forward movement. *T. borreli* rather uses the cell body to adhere to the surface and 'pull' itself forward. The flagellar

movement which occurs in parallel, especially of the recurrent flagellum, deforms the flexible cell body allowing the parasite to adhere to a different point of the surface and move forward. Thus, the waves created by the recurrent flagellum at the periphery of the cell body allow the parasite to greatly deform its cell shape alternating between stationary and forward movements.

In Chapter 2, to characterize the swimming behaviour of *T. carassii*, we adopted a previously described classification method by Bargul et al (Bargul et al., 2016), discriminating between tumblers, intermediate and persistent swimmers. Although for *T. borreli* quantification of speed and distance has yet to be performed, upon observation of freshly isolated *T. borreli* in carp blood, it was clear that the majority of the parasites were tumblers (**Video 2**). A higher percentage of intermediate swimmers was observed in *T. borreli* than in *T. carassii* cultures (data not shown), while persistent swimmers were never observed *in vitro*.

The presence of a second flagellum certainly creates a completely different situation from *Trypanosomes* and therefore the question rises how the motility of the biflagellate *Trypanoplasma* changes compared to trypanosomes, in the host.

First, we assessed susceptibility of zebrafish to *T. borreli* infections. Zebrafish larvae (5 dpf) were infected with various doses of *T. borreli*, survival was monitored and the kinetics of parasitaemia development followed using the same imaging setup as described in Chapter 2. Zebrafish were shown to be susceptible to *T. borreli* infection in a dose dependent manner (**Figure 2**), with the onset of mortality starting around 6 days post-infection (dpi) and leading to an overall 20% survival after two weeks (**Figure 2**).

Figure 2. Zebrafish is susceptible to infections with *T. borreli*. 5-days post fertilization (dpi) zebrafish larvae ($n=60/\text{group}$) were injected with $n=100$, 200 or 400 *T. borreli*, or with PVP as control. Zebrafish larvae were monitored daily for survival.

Second, using high spatio-temporal resolution microscopy we preliminary analysed the swimming behaviour of the biflagellate *in vivo* in the dynamic environments of a vertebrate host. This included the blood, and upon extravasation, tissues and tissue fluids of zebrafish.

When *T. borreli* was located in large vessels with an intact blood flow, they were mostly passively dragged by the flow (**Video 3**). Interestingly, in small vessels of one-cell diameter, such as the intersegmental capillaries (ISC), *T. borreli* was seldomly observed. In fact, as seen in **Video 3**, several parasites pass through the vein and artery, but none enter the capillary. This could be due to the strong flow, although *T. carassii* was often seen alternating between large and small vessels. It has to be considered however, that *T. borreli* is much larger than *T. carassii* (**Figure 3**) thus its size may also influence its ability to enter small vessels while being dragged in large vessels with a strong blood flow. In cases in which *T. borreli* was observed in capillaries it had an elongated body shape and was moving forward along with red blood cells (**Video 3**). Similar to what

observed for *T. carassii*, changes due to the progression of the infection influence the swimming behaviour of *T. borreli* in the blood. In fact, in capillaries or blood vessels with reduced or absent flow, or when red blood cells would not be present due to the onset of anaemia, *T. borreli* was seen tumbling on the vessel wall, repeatedly invert direction in capillaries or swimming against the blood flow (data not shown).

Figure 3. Details of *T. carassii* and *T. borreli*. 100x magnification images of freshly isolated parasites showing that *T. borreli* is larger than *T. carassii*. Scale bar indicates 10 μ m.

Similar to infection with *T. carassii*, a characteristic of *T. borreli* infections is **extravasation**. Extravasation of *T. borreli* was observed as early as 1 hour after infection (data not shown), in multiple locations including the intraperitoneal cavity, muscle tissue, fin, heart cavity and the head. Extravasation of the biflagellate allowed us to evaluate its swimming behaviour in fluids without hydrodynamic forces and blood cells. Interestingly, *T. borreli* was mostly adopting tumbling swimming behaviour (**Video 4**). A similar observation was made when trypanoplasma were found in the interstitial spaces lining the cardinal vein (**Video 4**). However, when looking at the fin tissue, we see something completely different. Here the biflagellate adopts an elongated wave form and swims directionally in the narrow spaces of the fin epithelium (**Video 4**). Similar to what observed with *T. carassii*, when the path of the parasite is interrupted, it

inverts direction by swimming backwards.

One of our main observations in Chapter 2 was the identification of the posterior end of *T. carassii* as anchoring site *in vivo*. Based on our preliminary *in vivo* observations, we see that also *T. borreli* can anchor itself to the vein endothelium (**Video 5**). With the current resolution and due to the presence of two flagella, it has not been possible to ascertain whether the anchoring side corresponds to the anterior or posterior end of *T. borreli*. Nevertheless, both flagella can move freely, indicating that similarly to *T. carassii*, the flagella do not serve as anchoring site. Besides anchoring, we very often observed that *T. borreli* can slow itself down by crawling or tumbling on the vessel wall, without an obvious anchoring point, despite the strong hydrodynamic blood flow (**Video 5**). Altogether, we describe also for *T. borreli* an anchoring mechanism, similar to the one observed for *T. carassii*, which is very rapid and does not require extensive reorganization of the cellular or flagellar membrane. This also suggests that such anchoring mechanism might not be specific to *T. carassii* or *T. borreli* only, but it might be a more general, yet overlooked, anchoring mechanisms of kinetoplastids that was revealed by the possibility to observe parasites *in vivo* in the natural environment of a vertebrate host. Thus, our observations certainly expand our knowledge on the adaptation of *trypanosomes* and *trypanoplasma* to the vertebrate host.

Preliminary analysis of innate immune responses against *T. borreli*.

After having established that *T. borreli* can infect zebrafish larvae and having preliminary evaluated the swimming behaviour of *T. borreli in vivo* in a vertebrate host, we next performed a preliminary characterization of the innate immune response of zebrafish against *T. borreli*. To this end, a *Tg(mpeg1:mCherryF;mpx:GFP)* double transgenic line was used and overall macrophage and neutrophil responses were analysed by total fluorescence quantification. We observed a clear response by both macrophages and neutrophils to the infection (data not shown). Individuals could be grouped in high and low macrophage responders; however, no correlation between macrophage and neutrophil responses,

nor between macrophage or neutrophil responses and parasitaemia could be made (data not shown). Preliminary data suggest that distribution of neither macrophages, nor neutrophils was affected by the presence of the parasite. Such response was clearly different from the one triggered by *T. carassii* where high macrophage responses and redistribution to and around blood vessels was observed (Chapter 3). Currently we are developing a new scoring system to better monitor *T. borreli* levels and are investigating the activation levels of macrophages with respect to their pro-inflammatory profile.

Do immune cells 'see' *T. carassii* and *T. borreli*?

As mentioned above, extravasation of both parasites occurred very often. Interestingly however, extravasated *T. carassii* were observed moving next to macrophages and neutrophils without any visible response (chemoattraction) from those immune cells (**Video 6**). Such 'lack of' attraction was observed in both, high and low infected individuals (data not shown). This suggests that despite the strong macrophage activation, there is no direct attraction of immune cells to the parasites. A similar observation was made during *T. borreli* infections (data not shown). Thus, two questions arise: (1) What activates macrophages? (2) What makes the parasites 'invisible' to immune cells?

To address the first question, we collected soluble factors produced by *T. carassii* in culture supernatants (conditioned medium) and injected them intravenously in non-infected *Tg(mpeg1:mCherryF;mpx:GFP)* double transgenic larvae. Few hours after injection (8h), we measured macrophage and neutrophil responses by total fluorescence quantification. Data showed an increase in total red (macrophage) fluorescence, however due to high fish to fish variation differences were not significant. Within the timeframe used, this approach provides information as to whether soluble mediators can (indirectly) activate the *mpeg* promotor, and thus mCherry expression. It does not however, provide information on whether macrophages proliferate in response to such mediators or are directly attracted or activated by it. In the future, local (e.g. intramuscular)

injection of conditioned medium into transgenic zebrafish larvae, followed by quantification of cell recruitment and activation state, could give insights on whether or not parasite-produced chemoattractant are involved in activation of macrophage responses. If soluble factors produced by the parasites are involved in the activation of macrophages, that would explain why macrophages are activated throughout the body; through the circulatory system, the chemoattractant is spread around the whole fish. We have also considered that the macrophages might respond to damage caused by extravasated parasites, however we could not observe any visible tissue damage while observing infected individuals. Consistent with the possibility that soluble mediators might be involved in macrophage activation, our group previously reported on the ability of supernatant from phosphatidylinositol-specific phospholipase C (PI-PLC)-treated *T. borreli* and *T. carassii* to activate macrophages in a TLR2-dependent manner (Ribeiro et al., 2010b, 2010a). Which specific GPI-anchored protein might be responsible for the observed activation is yet to be uncovered. The preliminary proteomic analysis performed in Chapter 4 (for *T. carassii* only) will certainly help in identifying surface virulence factors involved in macrophage activation.

The answer to the second question, what makes the parasites 'invisible' to immune cells, is certainly less straightforward. Focus has always been placed on the fact that trypanosomes reside in the blood of their host and have devised mechanisms to evade the attack by various host humoral factors (e.g. complement and antibodies) (CNOPS et al., 2015; Engstler et al., 2007; Forlenza et al., 2009; Radwanska et al., 2018; Stijlemans et al., 2017, 2016). Very little is known about how trypanosomes escape the attack of for example phagocytes, once in the tissue. The fact that extravasated *T. carassii* is not 'seen' by surrounding neutrophils (**Video 6**) raises several hypotheses, not all mutually exclusive: 1) parasites hide by binding host proteins on their surface, 2) parasites constantly secrete soluble factors while continuously moving in the host thereby 'confusing' phagocytes with respect to their exact location, or 3) parasites secrete or express molecules that inhibit macrophages activation upon binding or phagocytosis of less viable parasites. Noteworthy is the fact that for as

long as parasites are motile, phagocytosis of *T. carassii* or *T. borreli* by carp macrophages was never observed, neither *in vitro* nor *in vivo* (personal observation). Only in case complement and parasite-specific antibodies would be present, parasites would slow down and could be phagocytosed (Forlenza et al., 2009). This suggests that motility is certainly a strong escape mechanism. Yet, we observed parasites tumbling in the tissue (thus not moving further than their body length), not being targeted by innate immune cells. Differently from VSG-expressing trypanosomes, very little is known about the surface proteins and associated virulence factors of non-VSG-expressing trypanosomes. It has been however reported that *T. brucei* can secrete immunosuppressive molecules such as *T. brucei* Kinesin Heavy Chain 1 (TbKHC1), triggering the secretion of IL-10 and the activation of the arginase pathway, leading to the formation of alternatively (non-inflammatory) activated macrophages (De Muylder et al., 2013). Or, through a mechanism of altruistic death, phagocytosed *T. brucei* would upregulate the expression of transmembrane receptor-like adenylate cyclases (AdCs), called *T. brucei* Adenylate Cyclase (TbAdC) (Rolin et al., 1996). This in turn, triggers the increase of cytoplasmic cAMP within the phagocyte, activating protein kinase A and leading to the inhibition of the synthesis of the trypanolytic cytokine TNF and of other pro-inflammatory molecules (Lucas et al., 1994; Magez et al., 1997; Salmon et al., 2012). This will ultimately inactivate pro-inflammatory macrophages, effectively suppressing the host response. Whether one or more of the above depicted mechanisms is exploited by either of the fish trypanosomes is yet unknown. It is worth noticing that we observed two groups in our infected zebrafish population. High infected individuals showed a clear pro-inflammatory profile, inability to control the infection and ultimately succumbed to the infection. Low infected individuals on the contrary, showed a less inflammatory profile, the ability to control parasitaemia and ultimately survived the infection. It is therefore tempting to speculate that if trypanosomes succeed in manipulating the host response to prevent inflammation (as described above), thereby avoiding the exacerbated pro-inflammatory response observed in high infected individuals, this is advantageous for both the host and the trypanosome, as it guarantees survival and possibly propagation of both. Using our

newly established models, the possibility to directly visualize immune cell-trypanosome interaction *in vivo* will certainly shed light on (novel) mechanism of immune evasion by non-VSG-expressing trypanosomes.

Flagellar motility has been shown to be essential for not only parasite migration, replication and morphological adaptation to different microenvironments, but very importantly also to immune evasion (Krüger and Engstler, 2015; Stellamanns et al., 2014). *T. brucei* can clear VSG-bound antibodies from the surface using hydrodynamic drag forces generated by directional swimming. VSG-bound antibodies would then accumulate in the flagella pocket and be eliminated by endocytosis (Engstler et al., 2007; Russo et al., 1993). Additional *in vivo* studies addressing flagellar motility, however, showed somewhat contradicting results in that motility-mutant parasites remained infectious (Krüger and Engstler, 2015). In our study (**Chapter 2**), *T. carassii* in blood vessels swam directional only when the blood flow was greatly reduced or absent, or upon extravasation; in the presence of an intact flow, parasites were passively dragged by the flow in a curling motion, which might not be a favourable position to rapidly eliminate antibodies from the surface, if directional swimming would be required. In addition to that, it is unlikely that in adult fish such disruption of the flow or blockage of blood vessels would occur (discussed later), which suggests that directional swimming might not be required to remove surface bound antibodies.

Monoclonal antibody against carp IgM (Secombes et al., 1983) or against *T. carassii* (D5D5) and *T. borreli* (Joerink et al., 2007; Lischke et al., 2000) are available in our group which would allow us to investigate antibody clearance *in vivo*. For example, *T. carassii*-infected transparent zebrafish larvae could be injected with fluorescently-labelled D5D5 antibody and by using high spatio-temporal resolution fluorescent microscopy, binding and internalization of the antibody could be investigated *in vivo*. However, this system also has limitations; the fast flow combined with the limited sensitivity of the fluorescence channel during high-speed videography would not allow us to follow a single parasite over time. Nevertheless, we would certainly gain overall knowledge about antibody clearance *in vivo*.

The fine art of producing transgenic fluorescent parasites

As discussed in chapter 4 we carried out a transcriptome shotgun assembly project for *T. carassii*, which allowed the analysis of transcripts coding for abundant cell surface proteins, and can act as a comparator for understanding the evolution and pathogenicity of the Kinetoplastida. In addition, we carried out a similar transcriptome shotgun assembly project for *T. borreli* (Carrington et al., 2017). The added advantage of these two assemblies was the identification of intervening regions between *alpha* and *beta tubulin* genes, which could be cloned into recombination vectors for the generation of transgenic trypanosomes expressing fluorescent reporter proteins. The tandemly arranged intervening regions of the alpha-beta tubulin locus, could be targeted for integration of an expression cassette encoding enhanced green or red fluorescent protein (eGFP or mChFP) (**Figure 4**), carried by a plasmid that could be transfected by electroporation. Thereby the different parasites, *T. carassii* and *T. borreli*, could be marked by constitutive expression of either eGFP or mChFP from transgenes inserted in the tubulin locus. The strategy requires large numbers of trypanosomes because of typically low transfection rates and the need to rapidly grow high numbers of trypanosomes *in vitro* for subsequent selection purposes.

Figure 4. Design of recombination vectors and strategy for generation of fluorescent trypanosomes. The p3605-eGFP vector contains the GFP and antibiotic resistance gene flanked by the β-α tubulin and α-β tubulin intervening regions of *T. borelli* or *T. carassii*. Upon transfection, due to a random recombination event, the intervening regions will recombine with the corresponding regions in the genome of *T. borelli* or *T. carassii* leading to the excision of one α-tubulin gene and the insertion of the GFP+ resistance gene cassette. The α and β tubulin genes are present in several copies in the genome of the parasites and are constitutively expressed thereby ensuring a constitutive expression of the reporter gene.

We achieved a first success with *T. carassii* as we were able to generate transgenic parasites stably expressing the eGFP. However, fluorescence intensity was too weak to be detected under fluorescent microscope when parasites were injected *in vivo* in zebrafish larvae. Nevertheless, parasite fluorescence was readily detected by FACS analysis that allowed us to detect and sort GFP+ *T. carassii* (**Figure 5**). Currently we are exploring the possibility to replace the eGFP for a brighter variant and to drive the expression under a stronger promoter. Finally, the possibility to generate transgenic *T. carassii* expressing the luciferase gene is also considered.

Figure 5. Validation of transgenic GFP⁺ *T. carassii*. *T. carassii* parasites were electroporated with 20 μ g of the plasmid described in figure 3, using Amaxa Nucleofector II with human T-cell kit (VPA-1002 Lonza). Parasites were cultured for 14 days in the presence of 10 μ g/ml blasticidine. Selected parasites were injected into carp and isolated 3 weeks later. **A)** Total parasites where **B)** gated for GFP expression and sorted using a BD-Canto A FACS sorter. More than 90% of the isolated parasites were GFP⁺ confirming the generation of transgenic GFP⁺ *T. carassii*.

The use of transgenic fluorescent trypanosomes would be most appealing for microscopical analysis, especially when in combination with zebrafish transgenic lines. It could allow for easier visualization of the *in vivo* dynamics of parasitaemia development, parasite distribution as well as concomitant identification of trypanosomes and immune cells.

The generation of transgenic lines for *T. carassii* and for *T. borreli*, with different colours, would allow us to monitor by fluorescence microscopy double (mixed) infections *in vivo*. The fact that these two parasites have different morphological characteristics (most obvious: *T. carassii* has one flagellum and *T. borreli* has two flagella, *T. borreli* is larger than *T. carassii*), will allow for an easy visual confirmation of parasites differentiated by fluorescence. Mixed infections are not rare in nature, in fact, mixed trypanosome infections have been frequently reported but not studied as frequently. Given the complex nature of trypanosomes themselves and the associated complexity of the host-parasite interaction observed during mono-parasitic infections (this thesis; among others), one can only

imagine the scientific challenges of investigating mixed infections.

In this thesis, we report on the development of an infection model in a vertebrate host for *Trypanosoma carassii*. Meanwhile, we also developed a similar infection model for *Trypanoplasma borreli* (as discussed above in this discussion chapter). Both occur naturally in blood of cyprinid fish, as single species infections but also as naturally occurring mixed infections (Lom and Dyková, 1992). Previously, we established an experimental mixed infection model in common carp by injection of fixed numbers of these two species of parasites (Joerink et al., 2007). Interestingly, in mixed infections, peak parasitaemia levels of *T. borreli* were reduced, and to a lesser extent survival was increased compared to mono-parasitic infection with *T. borreli*. Cross-reactive antibodies increased earlier and to higher levels in mixed infected fish than in mono-parasitic infection with *T. borreli*. Further, carp that had survived a prior *T. carassii* infection showed increased resistance to infection with *T. borreli*. This study indicated a protective effect of co-infection with *T. carassii* on the resistance to *T. borreli*, possibly mediated via cross-reactive antibodies. Re-establishment of this mixed infection model in zebrafish could make use of the powerful time-lapse imaging of biological and disease processes driven by mixed trypanosome infections in a live vertebrate.

Most but not all African trypanosomes are transmitted by tsetse flies. Some, including *Trypanosoma evansi* and *Trypanosoma vivax* are also mechanically transmitted. Such multiple transmission modes, in combination with increased movement of infected livestock, may be important drivers for the occurrence of mixed trypanosome infections. Indeed, mixed infections with different species of trypanosomes have been reported for tsetse flies, humans, pigs and cattle (JAMONNEAU et al., 2004). It is not easy to predict whether mixed trypanosome infections will result in mutually cross-protective immune responses or lead to more severe infections, but surely these health questions are becoming increasingly important with the (increasing) occurrence of mixed infections, not only mixed trypanosome infections, but also co-infections with mycobacteria, viruses or fungi. There could be an evolutionary advantage for co-evolution of different parasites species, but surely this question may remain unanswered for some time. No matter what, the transparency and

availability of transgenic zebrafish to study *in vivo* dynamics of single and mixed infections with different trypanosome species provides a unique animal model to study complicated questions on mixed infections with parasites as well as other pathogens.

Beyond larvae: infecting juvenile and adult zebrafish with *T. carassii*

Recent years have seen a fast increase in the use of zebrafish as infection models and the enormous increase in available transgenic and knock-out lines will only stimulate this further and help answer questions on immune response development in real time and *in vivo*. So far, most studies have been carried out on embryonic and larval stages up to a few days post-hatch for reasons of transparency and because the presence of only innate immune cells during the early life period 'simplifies' the biological system. To date, an increasing number of studies also included the use of adult stages (Singleman and Holtzman, 2014). Preliminarily, we also performed experiments with *T. carassii* not only on larvae but also on juvenile and adult zebrafish, representing different life stages within a developing immune system.

Laboratory strains of zebrafish hatch around 3–4 days post fertilization (dpf) with the end of embryogenesis defined as the protruding mouth stage (~3 dpf at 28.5°C). After the embryonic period, larval development will last approximately 6 weeks, during which the fish greatly increase in length and progress through a series of morphological changes into juveniles around 45 dpf. Juvenile morphology closely resembles that of the adult, but sexual maturity takes until approximately 3 months dpf when zebrafish are considered adults (Singleman and Holtzman, 2014). Thus, at a temperature of approximately 28°C zebrafish develop from embryo into adult over a period of roughly 3 months. Typically, during zebrafish development, only innate immune cells will be present during the first period of 2–7 dpf of embryonic and early larval life, providing a time frame for studies on host-parasite interaction against an innate-immunity-only background. Subsequently, mature T cells will start to leave the thymus and move to peripheral organs, followed by B cell development such that around 3 weeks post hatch, both innate and adaptive immune cell types

are present in developing zebrafish (Page et al., 2013).

After having characterized the development of parasitaemia and progression of infection in larval zebrafish, we also examined the kinetics of parasitaemia and susceptibility to *T. carassii* of juvenile and adult zebrafish. Here, we define zebrafish of 4 weeks old as juveniles with a competent (but perhaps immature) adaptive immune system and zebrafish of 10-12 weeks old as adults, with a fully developed immune system. In juveniles, a clear dose-dependent survival could be observed with parasitaemia developing over time and persisting for a period of at least 30 days post-infection (**Figure 6A-B**). Differently, adult zebrafish showed 100% survival independent of the initial infection dose with a kinetics of parasitaemia that appeared chronic with cyclical peaks, up to a period of at least 50 dpi as detected by PCR (**Figure 6C-D**). It may be clear that development of adaptive immunity with increasing age contributed to the development of protective immune responses. In common carp, the development of antibodies together with the presence of complement factors has been shown crucial for protective immunity (Forlenza et al., 2009; Saeij et al., 2003). It could be of interest to infect adult Rag-/- zebrafish with *T. carassii* to evaluate the importance of T and B cells, no longer present in these KO zebrafish line, that also present an enhanced innate response (Tokunaga et al., 2017). No matter what, the correlation of results in carp and zebrafish underlines the value of the zebrafish model to study infection with *T. carassii* in the natural environment of a vertebrate host.

Figure 6. Progression of infection with *T. carassii* in juvenile and adult zebrafish. Juvenile (4 wpf) and adult (10 wpf) zebrafish (*Tg:mpeg:mCherry;mpx:gfp*) were infected with the indicated dose of *T. carassii* per fish. PVP was used as injection control. **A and C**) Fish (n=80) were observed daily for signs of infection as described in the methods section and survival was monitored over the indicated period. **B and D**) Parasitaemia was quantified by real-time quantitative-PCR using *T. carassii*-specific primers. For gene expression n=3-5 individuals were used at each time point. Gene expression was normalized relative to the host house-keeping gene *elongation factor-1 alpha* and expressed relative to fish injected with the corresponding parasite dose at time point zero. Note the logarithmic scales for parasitaemia.

Swimming behaviour of *T. carassii* and response of host immune cells is most easily studied in naturally transparent larval zebrafish (this thesis). Yet, the availability of so-called 'casper' pigment-depleted transgenic zebrafish allowed us to also study *in vivo* swimming behaviour and extravasation of *T. carassii* in juvenile and – to some extent – in adult zebrafish. At these life stages, parasite detection was easiest in the fins and in small capillaries. In juvenile zebrafish, as early as 3 days post-infection in the fins, several extravasated parasites could be detected, which would

typically be classified as tumbling swimmers (**Video 7**). With regard to number of parasites in juvenile fish; by 7 dpi individual differences in infection level were clear and consistent with fluctuations in parasitaemia detected by PCR (**Figure 6B**): in some fish no parasites could be detected, neither in the vessels nor in the fins, whereas in others the tail fin could sometimes be seen crowded with extravasated parasites (**Video 7**). In adult zebrafish, always when *T. carassii* parasites were indeed detected in fins, they behaved as tumbling swimmers. Extravasation (at least to the fin) was a rare event and occurred only during the early phase of infection up to 13 dpi. Altogether, our data confirm susceptibility of all life stages of zebrafish to infection with *T. carassii*.

In summary, *T. carassii* infection of zebrafish could revise our interpretation of trypanosome swimming behaviour and provide novel insights into human or animal trypanosomiasis. The infection model may reveal fundamental mechanistic insights into trypanosome persistence and may well reveal evolutionarily conserved components of anti-trypanosome immunity. Such results could have broad implications for the design of novel therapeutic strategies that can effectively limit disease spreading.

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SUMMARY

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Samenvatting

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Trypanosomes are pathogenic unicellular eukaryotic flagellates, with a broad host range, they can be found in many vertebrate species, causing diseases in cold-blooded as well as livestock and humans. Owing to their pathogenicity and unicellular eukaryotic nature trypanosomes have attracted the attention of researchers trying to unravel fundamental questions about cell biology and pathogenicity of these parasites.

The current thesis aimed to: 1) establish an *in vivo* trypanosome infection model in zebrafish; 2) investigate the behaviour of trypanosomes *in vivo*, in a natural host environment; 3) characterize the general (innate) immune response to the infection in young zebrafish, and 4) study the biological properties of transmembrane Tnfa, which was previously associated with protection against trypanosome infections.

In **Chapter 1**, I provide the general introduction to the framework of this thesis. First, I introduce various species of trypanosomes and the most common immune evasion strategies used by these parasites to establish a chronic infection. Then *Trypanosoma carassii* and *Trypanoplasma borreli*, two kinetoplastids affecting cyprinid fish, are introduced. These parasites have been extensively studied in our laboratory, and I specifically summarize our current knowledge on host-pathogen interaction and differential immune responses triggered in carp, one of their natural hosts. Flagellar motility is one of the most crucial elements of trypanosome pathogenesis but how trypanosome move *in vivo*, in the natural vertebrate host environment, has not been reported thus far. Therefore, in order to gain insights in the swimming behavior of trypanosomes *in vivo*, we turned to the zebrafish, a well-established animal model and most importantly, a cyprinid fish very closely related to the natural host of both *T. carassii* and *T. borreli*. Finally, based on previous research from our group, we introduce a potential role of transmembrane TNFa in the protection against trypanosome infection, and explain our approach to study both, *in vitro* and *in vivo*, the biological activity of soluble and transmembrane TNFa in zebrafish.

In **Chapter 2** the establishment of a *T. carassii* infection model in zebrafish is described. Using high-speed videography, we describe in detail the swimming behaviour of *T. carassii* *in vivo* in the blood, tissue and interstitial spaces of transparent zebrafish larvae. First, the swimming behavior of trypanosomes *ex vivo* in carp blood was described. The majority of

the parasites were found to be tumbling swimmers, suggesting that the tumbling behavior would be typical of trypanosomes residing in the blood. Upon observation of the swimming behavior of *T. carassii* *in vivo* however, it became clear that it was not possible to assign a specific swimming behavior to parasites in the blood, since, as long as the blood flow was intact, trypanosomes were passively dragged by the flow along with red blood cells. Thus, in the streaming blood, trypanosomes were never observed to tumble, swim directionally faster than the flow, or to bypass red blood cells. Outside the vessels however, or in conditions in which the blood flow would be reduced, or red blood cells would be absent, trypanosomes could adopt different swimming behaviors rapidly adapting to the environment. Furthermore, we report a new “whip-like” motion and *in vivo* backwards swimming that allow parasites to invert their swimming direction. Finally, we described a novel attachment mechanism for which trypanosome anchor to the vein endothelium or tissue epithelium through their posterior end leaving the flagellum free to move. All these findings, not only provide novel insight in parasite swimming behavior and host-pathogen interaction, but also show how the hereby established zebrafish trypanosome infection model can serve as a powerful complementary tool to further investigate trypanosome biology.

To date, it is still not known what mechanism the majority of trypanosomes use to evade the host immune response; most do not present antigenic variation, neither can hide intracellularly. They still however are capable of establishing long-lasting infections. Therefore, transcriptome and proteome analysis of *T. carassii* was performed in **Chapter 3** to identify potential surface proteins/protein families that might be involved in immune evasion by *T. carassii*. Our preliminary analysis revealed that, parallel to the development of antigenic variation in other trypanosomes, a loss of 27% of ancestral surface protein gene families took place. Furthermore, we show that the transcriptome of *T. carassii* presents two large families of two putative surface protein-encoding genes that are unique to *T. carassii* and haven't been found in other trypanosomes. Their further characterization might shed light on the ancestral mechanisms leading to a VSG-independent immune evasion.

Next, in **Chapter 4**, we investigated the innate immune response of zebrafish larvae to *T. carassii* infection. First, a clinical scoring system was estab-

lished that allowed us to consistently allocate individual larvae into high or low infected groups, without having to sacrifice the larvae. We found that macrophage, but not neutrophils, strongly response to the presence of parasites, and such response is not dependent on the infection level. Although the macrophage response (measured as total mpeg:mcherry fluorescence) was comparable in high and low infected individuals, macrophage morphology and pro-inflammatory activation state differed between the two groups. Large granular macrophages appeared and increased in number exclusively in high infected individuals, and were observed in the luminal side of the cardinal caudal vein. Interestingly, lipid staining confirmed that such macrophages were rich in lipid droplets and were defined as 'foamy' macrophages. Foamy macrophages displayed as strong pro-inflammatory profile as assessed using *tnfa* and *il1b* transgenic lines. In low infected individual, macrophages had a more dendritic morphology and displayed a more heterogeneous proinflammatory activation state depending on whether they were in the tissue or inside/outside a vessel. Generally the presence of pro-inflammatory foamy macrophages was found to be associated with susceptibility to the infection, possibly due to an exacerbated immune response.

Our group has previously shown that transmembrane (tm) Tnfa plays a crucial role in protection against a close relative of *T. carassii*, *Trypanoplasma borreli*. When carp was infected with *T. borreli*, and cleavage of transmembrane Tnfa was prevented by administration of a Tnfa Converting Enzyme (TACE) inhibitor, all fish recovered from the infection. In order to further investigate the biological properties and role of tmTnfa we have taken two approaches in **Chapter 5**; (1) we attempted to generate tools for the *in vitro* analysis of the biological activities of soluble and transmembrane zebrafish Tnfa and Tnfb. In mammals, it was previously shown that both forms of TNFa can bind to both TNF receptors (TNFR1 and TNFR2), but it was also reported that tmTNFa itself can trigger a signaling pathway in the TNFa-expressing cell (referred to as bi-directional signaling). In our study we first produced and validated several constructs to generate uncleavable Tnfa and Tnfb. In parallel, we generated NFκβ reporter fish cell lines transiently or stably expressing the zebrafish Tnfr1 or Tnfr2. Due to the unexpected constitutive NFκB activation resulting from the overexpression of the receptors, it has not been possible to investigate *in vitro* the receptor-binding preferences of soluble and tmTnf. Finally, upon identification of the constructs encoding for uncleavable Tnfa and Tnfb, we attempted

to generate transgenic zebrafish lines exclusively expressing tmTnfa. To this end, a TALEN approach was used to first knock out the native *tnfa* and concomitantly introduce the mutant version encoding for uncleavable Tnfa. Unfortunately, although *tnfa* knockout was successful, homologous recombination of the construct encoding uncleavable Tnf was not successful. Although the study did not lead to the characterization of the biological activities of tmTnf, several tools were generated that can be used in the future to pursue such type of investigations.

Finally, in **Chapter 6**, I discuss the most important findings of this thesis and potential future research directions. I elaborate on possible hypothesis about the adhesion site of *T. carassii* and propose some ideas on how to test it. Then I present some interesting additional studies that we have performed on another, closely related parasite to *T. carassii*; the unique biflagellate *T. borreli*. We have also established a *T. borreli* infection model in zebrafish and performed *in vivo* high-speed imaging of the biflagellate in order to characterize its peculiar movement. Potential immune evasion mechanisms of both parasites are discussed, then I describe the strategy that was used for the generation of transgenic fluorescent *T. carassii* and its potential use to study mixed trypanosome (or other) infections in zebrafish. Lastly, as an outlook for future studies, I describe our work on *T. carassii* infection of juvenile and adult zebrafish that lay the basis to study the full immune response against *T. carassii* in a transparent vertebrate host.

Samenvatting

Trypanosomen zijn pathogene eencellige eukaryotische flagellaten, met een breed gastheerbereik. Ze zijn te vinden in veel gewervelde soorten en veroorzaken ziektes bij zowel koudbloedigen als vee en mensen. Vanwege hun pathogeniteit en eencellige eukaryote aard hebben trypanosomen de aandacht getrokken van onderzoekers die fundamentele vragen over celbiologie en pathogeniciteit van deze parasieten proberen te ontrafelen. Het huidige proefschrift richtte zich op: 1) het vestigen van een *in vivo* trypanosoom-infectiemodel in de zebravis; 2) onderzoeken van het gedrag van trypanosomen *in vivo*, in een natuurlijke gastheeromgeving; 3) karakteriseren van de algemene (aangeboren) immunrespons op de infectie bij juvenile zebravissen, en 4) bestuderen de biologische eigenschappen van transmembraan Tnfa, die eerder werd geassocieerd met bescherming tegen trypanosoom infecties. In hoofdstuk 1 geef ik de algemene inleiding tot de opzet van dit proefschrift. Eerst introduceer ik verschillende soorten trypanosomen en de meest voorkomende immuunontwikkelingsstrategieën die door deze parasieten worden gebruikt om een chronische infectie tot stand te brengen. Vervolgens worden *Trypanosoma carassii* en *Trypanoplasma borreli* geïntroduceerd, twee kinetoplastiden die karperachtigen infecteren. Deze parasieten zijn uitgebreid bestudeerd in ons laboratorium en ik vat met name onze huidige kennis samen over gastheer-pathogeen-interactie en differentiële immunresponsen veroorzaakt in karpers, een van hun natuurlijke gastheren. De beweeglijkheid van de flagella is een van de meest cruciale elementen van de pathogenese van trypanosomen, maar hoe de trypanosomen *in vivo* bewegen, in de natuurlijke vertebrate gastheeromgeving, is tot nu toe nog niet gerapporteerd. Daarom hebben we, om inzicht te krijgen in het zwemgedrag van trypanosomen *in vivo*, ons gewend tot de zebravis, een beproefd diermodel en bovenal een karperachtige die nauw verwant is aan de natuurlijke gastheer van zowel *T. carassii* en *T. borreli*. Tot slot, gebaseerd op eerder onderzoek van onze groep, introduceren we een mogelijke rol van transmembraan TNFα in bescherming tegen trypanosoom infectie, en verklaren we onze benadering om zowel *in vitro* als *in vivo* de biologische activiteit van transmembraan en niet-membraan-gebonden (oplosbaar) TNFα in de zebravis te bestuderen.

In hoofdstuk 2 wordt het tot stand brengen van een *T. carassii* infectie-

model in de zebravis beschreven. Met behulp van high-speed videografie beschrijven we in detail het zwemgedrag van *T. carassii* *in vivo* in het bloed, weefsel en interstitiële ruimten van transparante zebravislarven. Eerst werd het zwemgedrag van trypanosomen *ex vivo* in karpr bloed beschreven. De meerderheid van de parasieten bleken tuimelende zwemmers te zijn, wat suggereert dat het tuimelgedrag typisch zou zijn voor trypanosomen die in het bloed leven. Bij observatie van het zwemgedrag van *T. carassii* *in vivo* werd echter duidelijk dat het niet mogelijk was om een specifiek zwemgedrag toe te kennen aan parasieten in het bloed, aangezien, zolang de bloedstroom intact was, trypanosomen passief werden meegesleurd door de stroom samen met rode bloedcellen. Dus, in het stromende bloed, werden nooit trypanosomen waargenomen die tuimelenden, directioneel sneller zwommen dan de bloedstroom, of rode bloedcellen deden omzeilen. Buiten de bloedvaten echter, of in omstandigheden waarin de bloedstroom zou was verminderd, of rode bloedcellen afwezig waren, konden trypanosomen verschillende zwemgedragingen aannemen en zich snel aanpassen aan de omgeving. Verder vermelden we een nieuwe “zweepachtige” beweging en *in vivo* achterwaarts zwemmen, waardoor parasieten hun zwemrichting kunnen omkeren. Ten slotte beschreven we een nieuw aanhechtingmechanisme waarvoor trypanosoom zich via het achteruiteinde aan het bloedvat of weefselepitheel hecht en het flagellum vrij blijft om te bewegen. Al deze bevindingen bieden niet alleen nieuw inzicht in het zwemgedrag van parasieten en de interactie tussen gastheer en pathogeen, maar laten ook zien hoe het hierbij gevestigde trypanosoom-infectiemodel in zebravis kan dienen als een krachtig aanvullend hulpmiddel om trypanosoom biologie verder te onderzoeken.

Tot op heden is nog steeds niet bekend welk mechanisme de meerderheid van de trypanosomen gebruikt om de immuunrespons van de gastheer te omzeilen; de meeste vertonen geen antigene variatie, noch kunnen ze zich intracellulair verbergen. Ze zijn echter nog steeds in staat om langdurige infecties te veroorzaken. Daarom werd transcriptome en proteoom analyse van *T. carassii* uitgevoerd in Hoofdstuk 3 om mogelijke oppervlakte-eiwitten / eiwitfamilies te identificeren die mogelijk betrokken zijn bij immuun ontwijking door *T. carassii*. Onze voorlopige analyse onthulde dat, parallel aan de ontwikkeling van antigene variatie in andere trypanosomen, er een verlies van 27% van de voorouderlijke oppervlakte-eiwitgen families plaatsvond. Verder laten we zien dat het transcrip-

toom van *T. carassii* twee grote families presenteert van twee vermeende oppervlakte-eiwit-coderende genen die uniek zijn voor *T. carassii* en niet zijn gevonden in andere trypanosomen. Hun verdere karakterisering zou licht kunnen werpen op de voorouderlijke mechanismen die leiden tot een VSG-onafhankelijke immuun ontduiking.

Vervolgens hebben we in **hoofdstuk 4** de aangeboren immuunrespons van zebravislarven tegen *T. carassii*-infectie onderzocht. Eerst werd een klinisch scoresysteem opgezet dat ons in staat stelde consequent individuele larven toe te wijzen aan hoge of lage geïnfecteerde groepen, zonder de larven op te offeren. We ontdekten dat macrofagen, maar geen neutrofielen, sterk reageren op de aanwezigheid van parasieten, en een dergelijke respons is niet afhankelijk van het infectieniveau. Hoewel de macrofaagreactie (gemeten als totale mpeg: mcherry-fluorescentie) vergelijkbaar was bij hoog- en laag geïnfecteerde individuen, verschilden de morfologie van de macrofagen en de pro-inflammatoire activeringsstaat tussen de twee groepen. Grote granulaire macrofagen verschenen en namen toe in aantal, uitsluitend in hoog geïnfecteerde individuen en werden waargenomen in de lumenale zijde van de kardinale caudale ader. Interessant genoeg bevestigde lipide kleuring dat dergelijke macrofagen rijk waren aan lipide druppels en werden gedefinieerd als 'foamy' macrofagen. Foamy macrofagen lieten een sterk pro-inflammatoir profiel zien, zoals beoordeeld met behulp van *tnfa* en *il1b* transgene lijnen. Bij een laag geïnfecteerde individuen hadden macrofagen een meer dendritische morfologie en vertoonden ze een meer heterogene pro-inflammatoire activeringstoestand afhankelijk van of ze zich in het weefsel bevonden of binnen / buiten een vat. Over het algemeen bleek de aanwezigheid van pro-inflammatoire foamy macrofagen geassocieerd te zijn met gevoeligheid voor de infectie, mogelijk als gevolg van een verergerde immuunrespons.

Onze groep heeft eerder aangetoond dat transmembrane (tm) *Tnfa* een cruciale rol speelt bij de bescherming tegen een naaste verwant van *T. carassii*, *Trypanoplasma borreli*. Wanneer karpers werden geïnfecteerd met *T. borreli*, en afsplitsing van transmembrane werd *Tnfa* voorkomen door toediening van een *Tnfa* converterende enzym (TACE) -remmer, alle vissen herstelden van de infectie. Om de biologische eigenschappen en de rol van tm*Tnfa* verder te onderzoeken, hebben we in **Hoofdstuk 5** twee benaderingen gevolgd; (1) we hebben geprobeerd hulpmiddelen te genereren voor de *in vitro* analyse van de biologische activiteiten van zebravis transmembraan en oplosbaar *Tnfa* en *Tnfb*. Bij zoogdieren werd

eerder aangetoond dat beide vormen van TNFa kunnen binden aan beide TNF-receptoren (TNFR1 en TNFR2), maar er werd ook gemeld dat tmTNFa zelf een signaalroute in de TNFa tot expressie brengende cel kan triggeren (aangeduid als bidirectionele signalering). In onze studie hebben we eerst verschillende constructen geproduceerd en gevalideerd om niet-afsplitsbare Tnfa en Tnfb te genereren. Parallel hieraan genereerden we NFKB-reporter viscellijnen die kortstondig of stabiel de zebravis Tnfr1 of Tnfr2 tot expressie brengen. Vanwege de onverwachte constitutieve NFKB-activering als gevolg van de overexpressie van de receptoren, was het niet mogelijk om *in vitro* de receptorbindingsvoorkeuren van oplosbaar en tmTnf te onderzoeken. Uiteindelijk trachtten we na identificatie van de constructen die coderen voor niet-afsplitsbare Tnfa en Tnfb, transgene zebravislijnen te produceren die exclusief tmTnfa tot expressie brachten. Hiertoe werd een TALEN-benadering gebruikt om eerst de native *tnfa* uit te schakelen en tegelijkertijd de mutante versie codering voor niet-afsplitsbare Tnfa te introduceren. Helaas, hoewel *tnfa* knockout succesvol was, de homologe recombinatie van het construct dat codeert voor niet-afsplitsbare Tnf was niet succesvol. Hoewel de studie niet leidde tot de karakterisering van de biologische activiteiten van tmTnf, werden verschillende instrumenten gegenereerd die in de toekomst kunnen worden gebruikt om dergelijke onderzoeken uit te voeren.

Ten slotte bespreek ik in **hoofdstuk 6** de belangrijkste bevindingen van dit proefschrift en mogelijke toekomstige onderzoeksrichtingen. Ik bespreek de mogelijke hypothese over de adhesieplaats van *T. carassii* en stel een aantal ideeën voor over hoe het te testen. Vervolgens presenteer ik enkele interessante aanvullende onderzoeken die we hebben uitgevoerd op een andere, nauw verwante parasiet van *T. carassii*; de unieke biflagellate *T. borreli*. We hebben ook een *T. borreli*-infectiemodel in de zebravis vastgesteld en *in vivo* high-speed imaging van het biflagellaat uitgevoerd om zijn eigenaardige beweging te karakteriseren. Mogelijke immuun ontwikkelingsmechanismen van beide parasieten worden besproken, daarna beschrijf ik de strategie die werd gebruikt voor het genereren van transgene fluorescerende *T. carassii* en het potentiële gebruik ervan om gemengde trypanosoom (of andere) infecties bij de zebravis te bestuderen. Ten slotte beschrijf ik als een vooruitblik op toekomstige studies ons werk aan *T. carassii*-infectie van juveniele en volwassen zebravis die de basis leggen voor het bestuderen van de volledige immuunrespons tegen *T. carassii* in een transparante gewervelde gastheer.

ABOUT THE AUTHOR

Acknowledgements
Curriculum Vitae
List of Publications
Training Activities

Acknowledgement

I consider myself a very lucky person for having been around many wonderful, kind, smart and encouraging people during my time in Wageningen, and somehow all of them contributed to the creation of this thesis. Even though I won't be able to list all of you, I give it a try.

First of all, I would like to give a huge thank to my promotor and daily supervisor **Maria Forlenza**, who was the biggest help in the past years. Your dedication, knowledge and enthusiasm helped me through the toughest times. You were never tired to discuss anything; I will never forget our marathon brainstorming nights when we got kicked out of the building at midnight by the security team. Thank you for all your support, your hospitality at your home and the time you've invested in me.

My dear other promotor, **Geert Wiegertjes**, you've been the boss I've never had, with a weird sense of humor that not everyone understands but I always found it hilarious. Thank you for making things look easy and lighting up my days.

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Of course I am very grateful for my paranympths as well. **Sem**, I've had the pleasure to watch you growing from my student into a strong and independent PhD candidate. Thank you for your work, the many hours spend behind microscopes and I wish you a very smooth last few months of your PhD with lots of great results. **Reni**, my other paranympth and most importantly my sister, the only person from my family who actually knows what I was doing. I would like to thank you for all the support throughout my PhD, despite the distance you could always encourage me with your faith and strength.

Végül de nem utolsó sorban szeretném megköszönni a családomnak elsősorban hogy vagytok nekem, hogy mindig támogattok és felvidítotok, hogy veletek nem unalmas az élet. Köszí **Ati** és **Bence**, hogy nálatok mindig megszállhatok, és hogy mellé még teológiai eszmefuttatásokat is kapok. Köszönöm **Adri**, hogy helyettem is gondoskodsz a megjelenésemről, hogy a kreativitásod nem csak külsőségekre korlátozódik, de pl. ezt a művészi borítót is neked köszönhetem. Köszönöm **Reni, Balázs, Olivér** és **Lizike** a sok szeretetet és örömet amit kapok tőletek szerencsére most már nap mint nap. Köszönöm **apa** és **anya**, hogy kemény munkával minden anyagi, lelki és szellemi dolgot megadtatok nekünk, amire valaha szükségünk lehet, hogy az egész életeteket ránk áldoztátok.

És végül köszönöm **Neki**, akinek mindent köszönhetek.

Éva

Curriculum Vitae

Éva Dóró was born on January 30th, 1986 in Békéscsaba, Hungary and raised in a big family with 4 younger siblings. She obtained her high school diploma at the Leőwey Klára Gimnázium, Pécs in 2004 and started her university studies in 2005 at the University of Pécs, Faculty of Sciences in Hungary. During her studies she has spent a 6 months long internship at the University of Birmingham, UK where she has been working on the optimisation of embryonic and larval ECG measurement in zebrafish for quantifying the effect of QT prolonging drugs with

Prof. Attila Sík and Prof. Ferenc Müller. After she obtained her Biologist MSc degree in 2011 she received an EU COST travel fellowship (European Cooperation in Science and Technology) for Short Term Scientific Mission to continue and publish her thesis project. Following that she came back to Pécs and started working as a researcher at Biotecont Ltd., a company she was collaborating with from Birmingham.

In 2012, she joined the Cell Biology and Immunology Group of Wageningen University as a PhD candidate under the supervision of Maria Forlenza and Prof. Geert Wiegertjes. Her PhD project was part of an European Training Network called FishForPharma: Training Network on Zebrafish Infection Models for Pharmaceutical Screens; and funded by the Marie Skłodowska-Curie Initial Training Network 7th Framework People Programme of the European Commission. Her role in the network was to establish and characterize a trypanosome infection model in zebrafish. During her PhD she has visited and collaborated with many of the ITN partners (such as Universidad de Murcia, Spain, University of Sheffield, UK) and completed several trainings.

As of November 2017 she is a research advisor of the newly established Institute of Transdisciplinary Discoveries in the Medical School of University of Pécs. Furthermore, she is conducting research in the NEURAM – Visualizing Genetics H2020 European Union Project that aims to develop Stimulated Raman Spectroscopy (SRS)-based technologies to visualize endogenous transcription at single cell and single gene level.

List of publications

Published

Carrington M, **Dóró E**, Forlenza M, Wiegertjes GF, Kelly S. 2017. Transcriptome Sequence of the Bloodstream Form of *Trypanoplasma borreli*, a Hematozoic Parasite of Fish Transmitted by Leeches. *Genome Announc* 5:e01712-16. doi:10.1128/genomeA.01712-16

Dhillon SS, **Dóró É**, Magyary I, Egginton S, Sík A, Müller F. 2013. Optimisation of Embryonic and Larval ECG Measurement in Zebrafish for Quantifying the Effect of QT Prolonging Drugs. *PLoS One* 8. doi:10.1371/journal.pone.0060552

Manuscript submitted

Éva Dóró, Sem H. Jacobs, Ffion R. Hammond, Henk Schipper, Remco P.M. Pieters, Mark Carrington, Geert F. Wiegertjes, Maria Forlenza 2019. Visualising trypanosomes in a vertebrate host reveals novel swimming behaviours, attachment mechanisms and adaptation strategies.

Eleanor Jaskowska, **Éva Dóró**, Maria Forlenza, Geert F. Wiegertjes, Mark Carrington, Steven Kelly. Transcriptome sequencing of the fish parasite *Trypanosoma carassii* reveals widespread loss during the evolution of the *Trypanosoma brucei* cell surface.

Manuscript in preparation

Éva Dóró, Sem H. Jacobs, Ffion R. Hammond, Georges Lutfalla, Sylvia Brugman, Geert F. Wiegertjes, Maria Forlenza. Differential response of macrophages and neutrophils to trypanosome infections in zebrafish: occurrence of foamy macrophages.

Training Activities

Scientific conferences

Zebrafish Disease Models 6. Workshop, Murcia, Spain	2013
The 6th EMBO meeting, Birmingham	2015
Zebrafish Disease Models 8. Workshop, Boston, USA	2015

Presentations

(Zebra)Fish Immunology Workshop (poster)	2013
The 6th EMBO meeting, Birmingham (oral)	2015
Zebrafish Disease Models 7, Boston (poster)	2015
(Zebra)Fish Immunology Workshop, Wageningen (oral)	2016
WIAS Science Day, Wageningen (oral)	2016

Courses, workshops and trainings

WIAS Introduction Course	2012
Course on philosophy of science and/or ethics	2012
Human and Veterinary Immunology Course	2012
Zebrafish husbandry & facility management workshop, Paris	2012
Zebrafish infection & imaging workshop, Paris	2012
Fish Immunology Workshop, Wageningen	2012
Personal Development Planning workshop, Paris	2012
Drug discovery workshop, Wageningen	2013
Scientific Publishing and Communication workshop, Wageningen	2013
Fish Immunology Workshop, Wageningen	2013
Career Development 'Building a Successful Career in Science and How to Apply and Interview for Scientific Positions', Sheffield	2014
Career Development 'Self Organisation Skills including Effective Time and Resource Management', Sheffield	2014
Intellectual Property Rights Workshop, Leiden	2014
Fish Immunology/Vaccination Workshop, Wageningen	2014
Use of Laboratory Animals, Wageningen	2014
External training period, Murcia, Spain	2014
Genomics and Bioinformatics Workshop, Leiden	2014
Course Advanced Immunology, Utrecht	2015
Career Development 'Building a Successful Career in Science and How to Apply and Interview for Scientific Positions'	2015
WIAS Course The Final Touch: Writing the General Introduction and Discussion	2016

Teaching and other activities

Helping with HVI practicals	2013
Helping with HVI practicals	2014
Helping with HVI practicals	2015
Supervise 2 MSc major thesis	
Supervision of an intern	
Organizing Fish Immunology Workshop, Wageningen	2014
Chair member of "Early Stage Researchers" FishforPharma network	2012-13
Member of WIAS Science Day 2014 committee	2014

Total ECTS: 48,6

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