Macrophage polarisation:
immune responses of carp against parasites

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Maaike Joerink

Stellingen

1. Alternatieve activering is de positieve benaming voor de-activering.
   Dit proefschrift

2. Een dubbele infectie veroorzaakt niet per definitie dubbele ellende.
   Dit proefschrift

3. De grootste valkuil binnen de vergelijkende immunologie is de verwachting dat de immuunsystemen van verschillende diersoorten vergelijkbaar zijn.

4. In theorie is er geen verschil tussen theorie en praktijk, maar in de praktijk is dat verschil er wel.
   Naar J.L.A. van de Snepscheut 1953-1994

5. De geneeskunde doet zichzelf tekort door alternatieve geneeswijzen niet als serieus alternatief te erkennen.

6. Gebruik maken van je rechten is nooit een verplichting.

7. Alle topsport zou zonder scheidsrechter beoefend moeten worden.

Stellingen behorend bij het proefschrift
Macrophage polarisation: immune responses of carp against parasites
Maaike Joerink, Wageningen, dinsdag 12 september 2006

Contents

Chapter 1

General introduction

1. Comparative immunology

In 1905 Ilya (Elie) Metchnikoff described for the first time the existence of an immune response (Metchnikoff, 1905). Now, after a century of research our knowledge about this complicated system has greatly increased. In general, the immune system can be divided into two arms: the innate arm (incorporating the rapid and phylogenetically more primitive responses to infections, based on pattern recognition by germline encoded receptors) and the adaptive arm (involving more slowly developing, long-lived, and highly evolved antigen-specific protective responses, based on the use of rearranging gene fragments to generate epitope specific receptors). The adaptive immune system is based on the random generation of a massive array of recognition receptors coupled with the ability to select and propagate 'effective' receptors and their source cells (Magor and Magor, 2001). Although the adaptive immune system seems more complicated, it is well recognised that the innate immune responses are at least as important for protection as are the adaptive responses (Tosi, 2005). Since the discovery of the antifungal role of Toll in Drosophila (Lemaitre *et al.*, 1996) and Toll-like receptors (TLRs) in humans (Medzhitov *et al.*, 1997) and other vertebrates, research on the innate arm of the immune response has gained more attention.
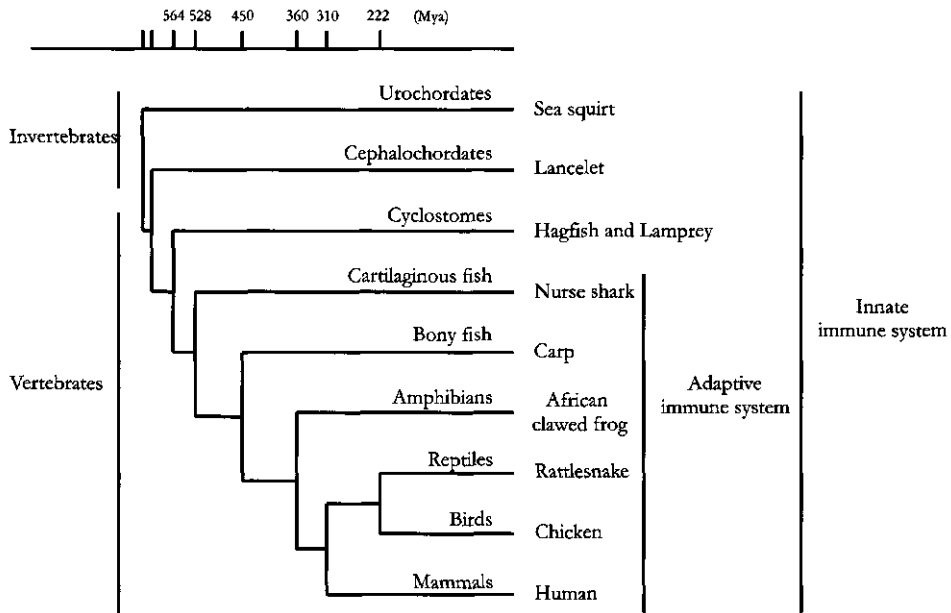

Fig. 1.1 Simplified phylogenetic tree of chordates.

Furthermore, also the field of comparative immunology, the study of the immune system of animals other than man, to shed light onto the evolution of the immune system, has greatly increased. Since (cartilaginous) fish are the first organisms with an adaptive immune system (Fig. 1.1), they represent a highly interesting group to study evolutionary / comparative immunology (Van Muiswinkel, 1995).

There are approximately 53,000 vertebrate species of which over 25,000 are fishes. Since these different fish species inhabit highly different environments they are challenged by all kinds of pathogens. While their immune system evolved under constant pressure from infectious micro-organisms such as viruses, bacteria and larger parasites, it is fascinating to see how the immune system of such an evolutionary successful group of animals evolved under so many different environments. Comparative research may not only give an idea of the evolution of the immune system, but may also distinguish what has been conserved from what has varied, which will shed light onto what is essential and what is accessory to the human immune system (Paul, 1999).

Within the fish species, teleosts (modern bony fish) are the most species-rich and diverse group and constitute about 95% of all fish species. Many components known from both the innate and adaptive arm of the mammalian immune system, have also been described for teleosts (Plouffe *et al.*, 2005; Secombes *et al.*, 2005). However, although the general aspects of the teleost immune system are indeed very similar to the mammalian immune system there are also some important differences. Two of the most striking differences are that teleost lack bone marrow and have no lymphatic system.

The teleost head kidney is considered to be the functional and structural homologue of the mammalian bone marrow, and the organ where hematopoiesis takes place (Zapata, 1981). Teleost macrophages originating from the head kidney perform similar functions as mammalian macrophages (Belosevic *et al.*, 2006). Phagocytosis, antigen processing and presentation, production of cytokines and radicals have all been observed. Furthermore, surface markers such as different TLRs, MH(C)-I and II and members of the Ig superfamily are described for teleosts (Plouffe *et al.*, 2005).

An organ or structure homologous to the mammalian lymph nodes, which is important for draining certain areas and bringing together antigen, macrophages, T cells and B cells, has never been described for teleosts. Although melano-macrophage centres in spleen and kidney have been suggested to represent equivalents of mammalian germinal centres (Vigliano *et al.*, 2006), it is still debated where exactly in teleosts antigen presentation could take place.

Teleosts do have a memory B cell response which is faster and leads to a higher production of antibodies upon exposure to the same antigen for a second time. Furthermore, teleosts are capable of producing different Ig isotypes; IgM, IgD, and IgT/Z (Danilova *et al.*, 2005; Hansen *et al.*, 2005; Hordvik *et al.*, 1999; Savan *et al.*, 2005). Class switching between different isotypes is initiated by activation-induced cytidine deaminase, this enzyme is present in teleosts (Barreto *et al.*, 2005). Functional Ig class switching has, however, never been demonstrated and only minor affinity maturation has been shown (Kaattari *et al.*, 2002). Similar to the T cells of mammalian species, teleost T cells are generated and most probably mature in the thymus. Furthermore, expression of recombination activating gene 1 and 2 was shown in these T cells, indicating the possibility for T cell receptor gene rearrangement (Huttenhuis *et al.*, 2005). Additional to thymic T cells, intraepithelial lymphocytes (IELs) have been described for several teleosts. These have, contrary to mammalian IELs, a highly diverse T cell receptor-β repertoire, which might be due to the lack of Peyer's patches and mesenteric lymph nodes in teleosts, and are suggested to fulfil a different function (Bernard *et al.*, 2006b). Until recently, research on the different T cell subtypes (e.g. T helper cells, cytotoxic T cells and T regulatory cells) has been severely hampered by the lack of appropriate T cells markers. To date, however, the sequences of various T cell markers and costimulatory receptor homologues have been described for teleosts (Bernard *et al.*, 2006a; Dijkstra *et al.*, 2006). Furthermore, the list of cytokines and chemokines, determining the type of immune response, is ever increasing (DeVries *et al.*, 2006; Huising *et al.*, 2004; Kaiser *et al.*, 2004). Taken together, this information will certainly lead to new insights in the function and differentiation of teleost T cells and related type I and type II immune responses.

2. Macrophage polarisation in mammals
2.1 Macrophage polarisation
Macrophages perform their function at the interface of the innate and adaptive immune system, and are one of the first cells to encounter entering pathogens. Macrophages are maintained in the different organs by local division and by migration from the blood, where they circulate as monocytes. Macrophages are able to phagocytose large (numbers of) pathogens but also participate in chemotaxis, cytokine-mediated inter-cellular communication and regulation of coagulation, of the complement cascade and of acute phase protein production. As such, macrophages perform a key function at the onset and later development of the immune response, and can form a bridge between the innate and adaptive arm of the immune system.

Upon activation, macrophages can polarise into different subsets with classically activated macrophages (caMF) and alternatively activated macrophages (aaMF) representing two extremes (Gordon, 2003; Mantovani *et al.*, 2002; Mills *et al.*, 2000). The alternatively activated macrophages can be further subdivided into three subtypes: M2a, M2b, and M2c. M2a are the truly alternatively activated macrophages, M2b are type II activated and M2c are deactivated macrophages (Mantovani *et al.*, 2004). Innate activation is an activation state functionally close to classical activation, although induced differently. Innate activation is triggered by a single microbial stimulation, while classical activation is induced by the combination of a microbial stimulation and interferon (IFN)γ (Gordon, 2003; Gordon and Taylor, 2005).

A typical difference between classically activated versus alternatively activated macrophages is that they metabolise L-arginine by two different pathways involving either the enzyme inducible nitric oxide synthase (iNOS) or arginase, respectively. The enzyme iNOS converts L-arginine into nitric oxide (NO) and L-citruline via N$^\omega$-hydroxy-L-arginine. The enzyme arginase converts L-arginine into urea and L-ornithine, the latter being a precursor for polyamines and proline (Fig. 1.2).
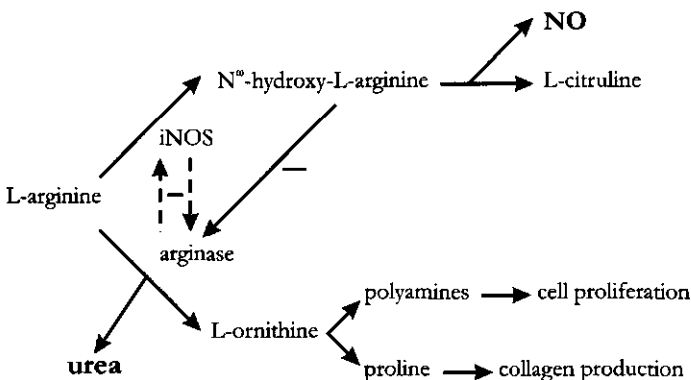
Fig. 1.2 L-arginine metabolism in macrophages.
L-arginine can be converted by the enzymes iNOS and arginase, which by means of substrate competition inhibit each others function (- - →). Furthermore N$^\omega$-hydroxy-L-arginine, the intermediate in the conversion to L-citruline and NO, can inhibit arginase activity.

By the production of NO and reactive oxygen species (ROS) classically activated macrophages play a role in the defence against intracellular pathogens. NO is effective against pathogens through its reactivity with DNA, proteins, thiols and the iron at the active site of many enzymes. Arginase activity by the alternatively activated macrophages does not only take away the substrate for iNOS, to

downregulate the type I immune response, but also takes care of the production of polyamines and proline. The latter two are important for cell proliferation and collagen production in wound healing. The balance between classically and alternatively activated macrophages is under influence of a competitive regulation by T helper $(T_H)1$ and T_H2 cells via their secreted cytokines. Cytokines produced by respective T_H1 and T_H2 lymphocyte subsets cross-inhibit each others development and function. This concept provides a widely applicable, molecular-based rationale for the understanding of the polarisation of immune responses observed in many infectious diseases.

Classically activated macrophages occur in type I immune responses, generally directed against intracellular pathogens. Type I immune responses are particularly associated with interleukin (IL)-1, tumour necrosis factor (TNF)α and IFNγ and are implicated in inflammation. Alternatively activated macrophages occur in type II immune responses, generally directed against extracellular pathogens. Type II immune responses are associated with the production of IL-4, IL-10, IL-13, and transforming growth factor (TGF)β which are essential for down regulation of the inflammation and the production of antibodies by B cells. caMF possess cytotoxic, antimicrobial and antiproliferative functions based on their ability to secrete ROS and NO, their signature mediator, and play a defensive role in several diseases (Bogdan *et al.*, 2000). However, the same caMF also secrete inflammatory mediators (TNFα, IL-1β, IL-6, and NO) that are involved in the induction of immunopathologies (Liew, 1993). In contrast, aaMF play an important role in type II immune responses against extracellular pathogens and secrete anti-inflammatory molecules (IL-10 and TGFβ) that down-regulate inflammatory processes and counteract NO synthesis by expressing arginase (Mills, 2001). Furthermore, aaMF increase their production of factors involved in tissue remodelling and wound repair (Shearer *et al.*, 1997).

2.2 Polarisation in response to parasites

During many parasitic infections macrophage polarisation (into caMF or aaMF) has been described. Currently there are no (molecular) properties described of the parasites that can predict the type of macrophage activation and the subsequent immune response. In the majority of the parasitic infections the early phase is characterised by the presence of caMF and the production of the antimicrobial compound NO, while the later phase is characterised by the presence of aaMF and related arginase activity (reviewed by Noël *et al.*, 2004). Alternatively, in some parasitic infections one type of macrophage activation prevails during the whole infection period.

For example, during infections with the helminth *Brugia malayi*, macrophages are preferentially alternatively activated (Noël *et al.*, 2004). On the other hand, during *Leishmania major* infections, parasites are cleared by caMF only (von Stebut and Udey, 2004).

More information on the parasite molecules responsible for the stimulation of aaMF, during a *Trypanosoma cruzi* infection, has become available. Cruzipain (syn. cruzain), a highly immunogenic glycoprotein of about 52-58 KDa with a highly mannose glycosylated C-terminal domain is a major *T. cruzi* antigen found in every developmental stage of the parasite. Giordanengo *et al.* found an increase of urea associated with a decrease in nitrate levels after injection of cruzipain, suggesting that this cysteine protease preferentially up-regulates the arginase pathway. Macrophages from immune mice cultured with cruzipain showed high urea levels but no increased nitrite levels. Cruzipain was found to directly activate macrophages to increase their arginase activity, possibly interacting through the mannose receptor (Giordanengo *et al.*, 2002). Interestingly, this mannose receptor is upregulated in aaMF.

In conclusion, parasitic infections are informative models to study the general immune response of a host and in particular macrophage polarisation. Therefore, parasitic infections could also be informative for studies on macrophage polarisation in teleosts.

3. Macrophage polarisation in teleosts

3.1 Macrophage polarisation

In teleosts, macrophages but also monocytes and granulocytes originate from the head kidney. Functions similar to those of macrophages of mammalian species have been described, including phagocytosis, radical production, antigen presentation and the production of cytokines. Polarisation into caMF and aaMF has, however, not been described for teleost macrophages.

In mammals, the balance between caMF and aaMF is under influence of the competitive regulation by T_H1 and T_H2 cells. Since IFNγ, TNFα, IL-1β, IL-6 and NO characterise the type I response, and IL-4, IL-10, IL-13 and TGFβ characterise the type II response, it is worthwhile to consider these molecules in teleosts. After several descriptions of functional evidence for the existence of IFNγ (Graham and Secombes, 1988; Neumann *et al.*, 1995; Verburg-van Kemenade *et al.*, 1996), and the cloning and sequencing of IFN-induced genes in teleosts (Collet and Secombes, 2002) the existence of IFNγ was recently confirmed by cloning and sequencing of the gene for pufferfish (*Takifugu*

rubripes) and rainbow trout (*Oncorhynchus mykiss*) (Zou *et al.*, 2005; Zou *et al.*, 2004). In addition, IL-6 has been identified in pufferfish through synteny cloning (Bird *et al.*, 2005), yet has not been described for other teleosts. IL-1β, TNFα, and iNOS have been identified in several teleost species (reviewed by Secombes *et al.*, 2001). No data on the existence of IL-4, and IL-13 in teleost have been reported. IL-10 has been identified in several teleost species (Inoue *et al.*, 2005; Savan *et al.*, 2003; Zhang *et al.*, 2005; Zou *et al.*, 2003) and several TGFβ homologues have been cloned in teleosts (Hardie *et al.*, 1998; Harms *et al.*, 2000; Laing *et al.*, 2000; Zhan and Jimmy, 2000). Although additional functional studies have to be performed, expression studies in common carp (*Cyprinus carpio* L.) indicate an inhibitory function for IL-10 and TGFβ (M. Forlenza, personal communication). Furthermore, studies with recombinant mammalian TGFβ1 demonstrated enhancement or inhibition of trout macrophage functions at low or high concentrations, respectively (Jang *et al.*, 1994).

In conclusion, the cytokines involved in type I immune responses are well described for teleosts, while not all the cytokines of 'true' type II responses (IL-4 and IL-13) have been described as yet. Therefore, it is not possible to use cytokine profiles to fully distinguish T_H1 and T_H2 cells as indicators of type I and type II immune responses, respectively. The levels of iNOS and arginase activity in macrophages may, however, reflect the type I and type II immune response designation. For teleosts, this allocation would allow for typing immune responses as type I or type II, based on the production of NO or arginase activity, respectively.

3.3 Polarisation in response to parasites

Common carp (*Cyprinus carpio* L.) is the natural host of two kinetoplastid parasites: *Trypanoplasma borreli* and *Trypanosoma carassii* (Fig. 1.3), that diverged more than 500 million years ago (Fernandes *et al.*, 1993). Infections with these trypanosomes could present informative models to study carp macrophage polarisation *in vivo*. Blood-sucking leeches (*Piscicola geometra* or *Hemiclepsis marginata*) act as vectors for transmitting kinetoplastid parasites between cyprinid fish and many carp will in fact carry mixed populations of *T. borreli* and *T. carassii* (syn. *T. danilewskyi*) (Lom and Dyková, 1992). Both protozoan parasites are kinetoplastids, with *T. borreli* belonging to the family of parabodonida, whereas *T. carassii* is classified in the family of trypanosomatida (Fig. 1.4). *T. carassii* is a member of the "aquatic clade" (Figueroa *et al.*, 1999), a group of trypanosomes all transmitted by leeches, that appeared early in the evolution of the genus Trypanosoma (Stevens *et al.*, 2001).
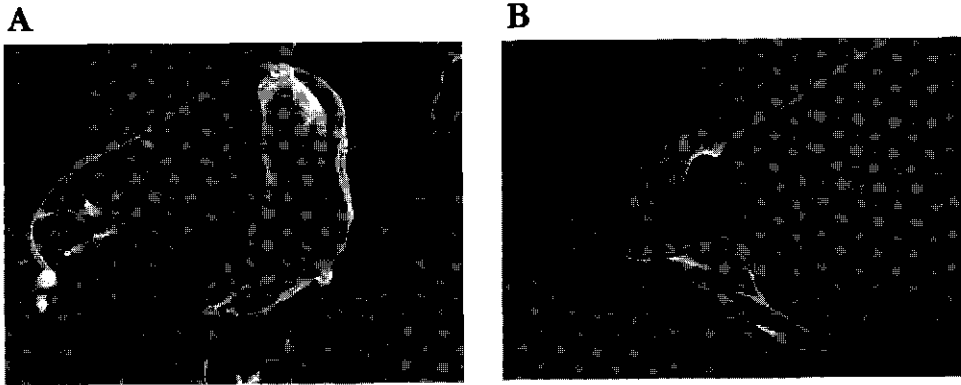
Fig. 1.3 SEM photographs of *Trypanoplasma borreli* (A) and *Trypanosoma carassii* (B)

T. borreli and *T. carassii* share the features typical of kinetoplastid flagellate parasites such as a kinetoplastid organelle containing the mitochondrial DNA, a glycosome compartmentalising glycolysis and a mini-exon; a highly conserved short RNA leader sequence trans-spliced onto every messenger RNA. Trypanosomatids (and thus also *T. carassii* but not *T. borreli*) have a single flagellum (Fig. 1.3B) and all genera are parasitic, either in vertebrates, invertebrates, ciliates or in flowering plants. The family of trypanosomatida is well-studied, because it contains the important mammalian parasites *T. cruzi*, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* and *Leishmania* spp a.o.. Bodonids (and thus also *T. borreli*, but not *T. carassii*) have two flagella (Fig. 1.3A) and most species are free-living inhabitants of aqueous environments, but some are vertebrate ecto- or endoparasites of fish (e.g. of the skin, gills, alimentary tract, reproductive organs or blood) or parasites of invertebrates (Stevens *et al.*, 2001). Bodonids represent an ecologically and economically important group of organisms, as they are present in all major aquatic ecosystems. They are also crucial components of sewage cleaning units and the causative agents of some fish diseases in aquaculture.

In analogy to the salivarian trypanosomes both *T. carassii* and *T. borreli* are believed to live exclusively extracellular in the blood and tissue fluids of their fish hosts. Differently, they are believed to lack antigenic variation, since fish that recovered from one infection are resistant to infection with other parasite lines or infection with parasites from a chronic phase (Overath *et al.*, 2001; Overath *et al.*, 1999).

Analogous to the stercorarian trypanosomes, the surface coat of *T. carassii* shows a clear resemblance with the carbohydrate-dominated surface coat of *T. cruzi* (Paulin *et al.*, 1980). Highly glycosylated mucin-like surface proteins are abundant and anchored in the plasma membrane by glycosylphophatidylinositol (GPI) residues (Lischke *et al.*, 2000). Although less data is available on the surface coat of *T. borreli*, electron microscopy suggests a much more massive surface coat than found for *T. carassii* (Lom and Nohýnková, 1977).
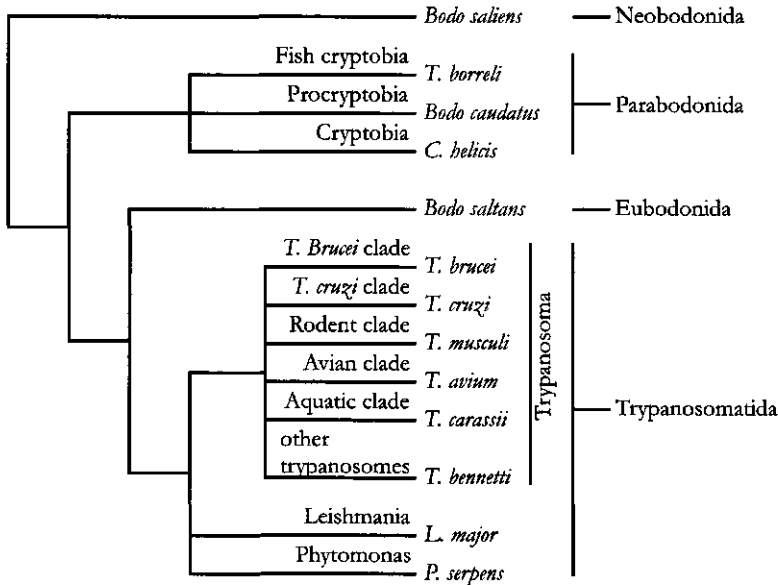
Fig. 1.4 Simplified phylogenetic tree of kinetoplastida

Possibly, *T. borreli* and *T. carassii* have different modes of adaptation allowing these parasites to infect and persist in carp. More importantly, it seems that the immune response of carp against these two parasites is fundamentally different. During *T. borreli* infection of carp high production of NO have been found (Saeij *et al.*, 2002b). Since *T. borreli* seems to induce a type I immune response (Saeij *et al.*, 2003a) we expect classically/innate activated macrophages to be involved. Interestingly, infections in carp with *T. carassii* do not seem to be associated with high NO levels (Saeij *et al.*, 2002b) and do not lead to an excessive inflammatory response (Dyková and Lom, 1979). Probably, divergent GPIs have evolved to the advantage of the various parasitic protozoa to activate or down-regulate the endogenous signalling pathways of the host (Tachado *et al.*, 1999). In theory, it is very well possible that *T. carassii* preferentially induces alternatively activated-

rather than classically/innate activated-macrophages in carp.

Assuming that teleost macrophages can indeed show polarisation, infections with fish kinetoplastid parasites that have evolved with this aspect of the innate immune system in place will certainly bring new insights into the evolution of host-parasite relationships and the polarisation of the immune system of teleosts.

Aim and outline of this thesis

In order to gain more insight in the evolution of the immune system and the presence of macrophage polarisation in teleosts, carp macrophages and their activation in response to parasites were investigated. We hypothesise that macrophage polarisation into classically activated and alternatively activated macrophages is present in teleosts. Furthermore, we hypothesise that *T. borreli* infections induce a classical activation of macrophages leading to type I immune responses, while *T. carassii* infections induce an alternative activation of macrophages leading to type II immune responses.

The principles of the teleost immune system and the host-parasite model (carp-*T. borreli* / *T. carassii*) are introduced (this chapter). The possibility to use iNOS and arginase activity as markers for type I (caMF) and type II (aaMF) immune response, respectively, is discussed (chapter 2). The arginase 1 and 2 genes are cloned and characterised for carp (chapter 3). A macrophage culture system is set-up, characterised and used for *in vitro* polarisation (chapter 4). *T. borreli* and *T. carassii* infections are performed to study macrophage polarisation *in vivo* (chapter 5). A mixed infection with *T. borreli* and *T. carassii* is performed in order to assess whether these parasites can influence each other in a mixed infection (chapter 6). *T. borreli* and *T. carassii* expression libraries are constructed and screened in order to identify antigenic proteins (chapter 7). Finally, the observations described in this thesis are discussed (chapter 8).

Chapter 2

Macrophage polarisation in the immune response to parasites

Geert F. Wiegertjes and Maaike Joerink

Introduction

Macrophages perform a key function in the immune response against parasites. No matter where a parasite enters the body it will encounter resident but highly active macrophages that can recognise pathogen-associated molecular patterns (PAMPs). These macrophages may not only be able to phagocytose parasites but participate in complement activation, chemotaxis, cytokine-mediated inter-cellular communication, regulation of acute-phase protein production and antigen presentation, among others. In general, it is the innate system that controls the initiation of the adaptive immune response by regulating the expression of costimulatory activity on professional antigen-presenting cells such as macrophages. The costimulation instructs the adaptive part of the immune system to develop a particular effector response by releasing cytokines. When indeed the immune system needs to recruit T helper cells to fight a parasite, it does so by instructing precursor T lymphocytes, which have not yet encountered a foreign antigen, to mature into either of two kinds of helper cells. These two, T helper 1 (T_H1) and T helper 2 (T_H2), typically differ by the cytokine profiles they produce and the functions they perform. For example, these cytokines, produced by the T_H cells will influence the activity of the macrophages attracted to the site of infection.

Macrophages have long been recognised as a heterogeneous cell population, probably reflecting the plasticity of these cells in response to micro-environmental signals, such as cytokines and PAMPs. Much of the understanding of the 'classical' activation of macrophages originates from studies on mice infected with typically intracellular parasites such as *Leishmania* spp., resulting in enhanced antimicrobial activities as part of a type I response. More recently, it has been recognised that particular cytokines from T_H2 cells can induce an 'alternative' activation of macrophages that induces distinct functional activities, integrating these macrophages in the type II response (Gordon, 2003; Mantovani *et al.*, 2002; Mills, 2001). It is important to realise that an effective immune response against a particular parasite requires a balanced differentiation between T_H1 lymphocytes/ classically activated macrophages (type I response) and T_H2 lymphocytes/ alternatively activated macrophages (type II response).

IFNγ, TNFα
IL-1

Classically activated

L-arginine

NO

NOHA → L-citruline

IL-1β, IL-6

IL-10, TGFβ

polyamines ⟶ cell proliferation

L-ornithine

L-arginine / Urea

proline ⟶ collagen production

Alternatively activated

IL-4
IL-10, IL-13
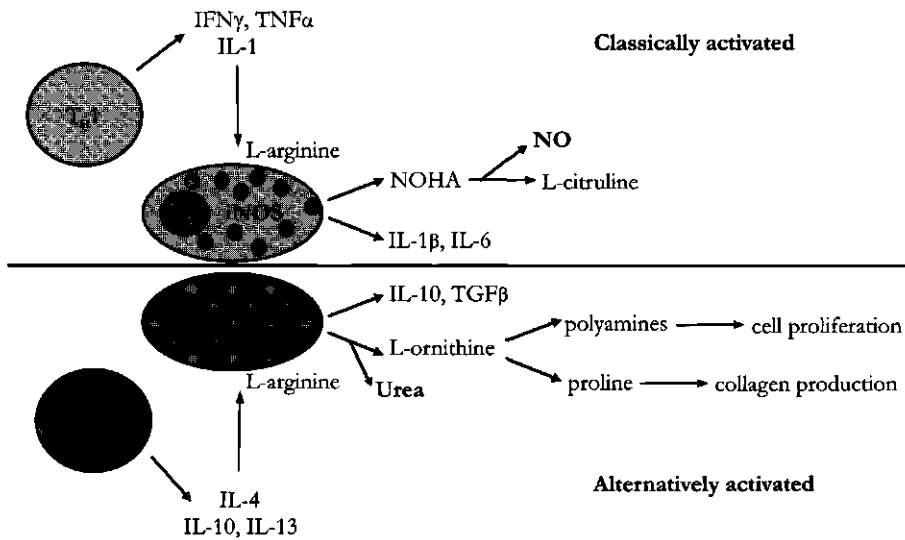
Fig. 2.1 Macrophages exhibit distinct functional activities in type I and type II immune responses. The classically activated macrophages are associated with the T_H1 cells and their cytokines, they have a higher inducible nitric oxide synthese (iNOS) expression and produce more nitric oxide (NO). The alternatively activated macrophages, associated with the T_H2 cells and cytokines, upregulate arginase activity important for cell proliferation and wound healing. (Adjusted from Sandor *et al.*, 2003.)

Macrophage polarisation

Classically-activated macrophages (caMF) possess cytotoxic, antimicrobial and antiproliferative functions based on their ability to secrete nitric oxide (NO) and reactive oxygen species (ROS) and in this way play a defensive role especially against intracellular parasites. Alternatively-activated macrophages (aaMF) play a regulatory role in humoral immunity (production of antibodies) against extracellular parasites, often helminths, and allergy and participate in the process of wound healing.

What has become clear during the last few years is the central role of the amino acid L-arginine in the polarisation of macrophages as it is the common substrate to both caMF and aaMF. In caMF L-arginine serves as a substrate for NO production by inducible nitric oxide synthase (iNOS). iNOS oxidizes L-arginine in two steps: L-arginine is first hydroxylated to N^ω-hydroxy-L-arginine (NOHA), which is further oxidized to L-citruline and NO. NOHA is a competitive inhibitor of arginase and in this way suppresses the alternative activation of macrophages (see Fig. 2.1). In aaMF L-arginine is hydrolysed to L-ornithine and urea by arginase. L-ornithine is a precursor for the synthesis of L-glutamine, polyamines

and proline, the latter two being important for cell replication and wound healing, respectively (Vincendeau *et al.*, 2003).

A reciprocal change in arginine metabolism is proposed to be extremely important for wound healing. Expression of iNOS creates a cytotoxic environment and favors vasodilatation, important in the early phase of wound healing. Increased arginase activity favors fibroblast replication and collagen production, required for tissue repair in the late phase of wound healing (Vincendeau *et al.*, 2003). An unregulated classical activation of macrophages may result in immunopathology due to high concentrations of (pro)-inflammatory mediators such as tumour necrosis factor (TNF)α, interleukin (IL)-1β, IL-6 and due to high concentrations of NO. An unregulated alternative activation of macrophages may lead to immunosuppression induced by anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)β. Thus, an effective and healing immune response against a particular parasite requires a balanced differentiation between caMF/iNOS and aaMF/arginase. The balance between caMF and aaMF is under influence of a competitive regulation by T_H1 and T_H2 cells via their secreted cytokine profiles. caMF develop as part of a type I response with TNFα and interferon (IFN)γ as main stimulating signals. In contrast, aaMF develop in a type II response and are particularly associated with the production of IL-4, IL-10 and IL-13 (see Fig. 2.1). The cytokines produced in the type I or II reaction cross-inhibit each others development (Fiorentino *et al.*, 1989; Mills, 2001).

Polarisation of the fish immune response

To date, there is no clear molecular nor functional evidence for the existence of T helper lymphocytes in fish, let alone a polarization into T_H1 and T_H2 cells. However, from Table 2.1 it is clear that an assignation of fish immune responses as type I or type II, although still in its infancy, could tentatively be based on the molecular evidence for those mediators that have been described for fish, even without directly implicating the cellular source. So far ignored, but evidently present are iNOS and arginase activity as potential markers for a type I or type II immune response, respectively, in fish.

An in vitro-derived carp head kidney macrophage culture system

Nitric oxide production by macrophages in response to lipopolysaccharide (LPS) has been demonstrated in several fish species, although the role of NO in fish parasite infections and its effect on parasite viability has remained largely uninvestigated. To our knowledge, no arginase activity has been reported for fish

macrophages. To further elucidate the role of fish macrophages and their ability to develop a type I (iNOS) or type II (arginase) response we established an *in vitro*-derived carp head kidney macrophage culture system, based on previous work by others on goldfish kidney macrophages (Neumann *et al.*, 1998; Neumann *et al.*, 1995). Flow cytometric analyses of the *in vitro*-derived carp head kidney cultures showed an enrichment for myeloid cells when cultured for a prolonged period. NO production by these cells could be determined with a Griess reaction measuring the amount of nitrite accumulating in the supernatant (Green *et al.* ,1982) after stimulation with LPS. Arginase activity by the carp myeloid cells could be determined by a micromethod measuring the amount of urea formed in one hour in the presence of excess L-arginine (Corraliza *et al.*, 1994) after stimulation with dibutyryl cyclic AMP or dexamethasone in combination with LPS (Morris *et al.*, 1998). Both activities could be detected as early as 18 h after stimulation, indicating the 'primed' state of the myeloid carp cells in culture.

There are three NOS genes, but only one is inducible and associated with macrophages. The availability of the full coding sequence for carp iNOS allowed us to perform expression studies after stimulation with LPS (Saeij *et al.*, 2000), or with parasites (Saeij *et al.*, 2003a).

There are two arginase genes and both seem to be activated in (murine) macrophages (Mori *et al.*, 2000; Morris *et al.*, 1998). Coding sequences for both arginase isoforms have been reported for fish (see Table 2.1) and we are in the process of identifying the arginase genes in carp. In murine cells, arginase 1 is located in the cell cytosol (together with ornithine decarboxylase facilitating polyamine synthesis), while arginase 2 is located in the mitochondria (together with ornithine aminotransferase enhancing L-proline and L-glutamate production). Quantitative expression studies on iNOS and arginases 1 and 2 will give us more insight into the polarisation of carp myeloid cells in response to parasite antigens. Expression studies on arginase will be of added value because of the possibility to distinguish between the two arginase isoforms.

Table 2.1. Type I and type II cytokines and mediators presently described for fish. The table is not complete and shows a selection of cytokines and mediators. Information is provided for a limited number of fish species (carp, rainbow trout, zebrafish and pufferfish).

Type I response		Type II response	
	Accession number		Accession number
IFNγ	-	IL-4	-
TNFα	Carp (**AJ311800**) Rainbow trout (**AJ278085**)	IL-10	Carp (**AB110780**) Pufferfish (**AJ539537**)
IL-1β	Carp (**AB010701**) Rainbow trout (**AJ278242**) Zebrafish (**AY340959**)	TGFβ	Carp (**AF136947**) Rainbow trout (**X99303**) Zebrafish (**AY178450**)
IL-6	-	IL-13	-
iNOS	Carp (**AJ242906**) Rainbow trout (**AJ295230**)	Arginase 1	Rainbow trout (**AY310733**) Zebrafish (ENSDART00000000974)* Pufferfish (SINFRUT00000148803)*
		Arginase 2	Rainbow trout (**AY056477**) Zebrafish (ENSDART00000023658)* Pufferfish (SINFRUT00000148943)*

* Not yet annotated but found in the ensembl database (www.ensembl.org)
– Not described for fish

Parasite infections

As mentioned above, an effective immune response against a particular parasite requires a balanced differentiation between a type I and a type II response. For example, in murine macrophages infected with *Leishmania major*, an intracellular parasite, a type I response induces NO synthesis and parasite killing, whereas

a type II response favours parasite growth (Vincendeau *et al.*, 2003). Likewise, resistance to the extracellular parasite *Trypanosoma brucei* is associated with the ability to produce a caMF response in the early phase of an infection, followed by an aaMF response in the late/chronic phase of the infection (Baetselier *et al.*, 2001).

With the use of our *in vitro*-derived head kidney myeloid culture it should become possible to examine putative type I or type II polarisation responses to fish parasites based on the balance between caMF/aaMF activities. We previously found that the NO induced by the extracellular parasite *Trypanoplasma borreli* could have a trypanostatic effect *in vitro* (Saeij *et al.*, 2002b). Carp highly susceptible to the parasite produce immunosuppressive amounts of NO, suggestive of an unbalanced immune response. In the late phase, resistant carp produce lytic antibodies (Saeij *et al.*, 2003a) and overcome the infection. We now can investigate whether, similar to what is observed for *T. brucei* infections, carp can and need to switch from a caMF response in the early phase to an aaMF response in the late phase of infection, enabling the production of protective antibodies.

Conclusion

Since evidence for the existence of type I and type II immune responses to fish parasites, based on polarised cytokine profiles secreted by T_H1 and T_H2 cells, is lacking, we propose to use iNOS and arginase activity as markers for polarised immune responses in fish. Carp *in vitro*-derived head kidney myeloid cells readily express iNOS and arginase activity upon specific stimulation and can be of true importance for providing new insights in the immune response against fish parasites. The role of L-arginine during infection deserves more attention, not only because parasites such as *T. borreli* possess arginase to convert L-arginine for their own growth but also because, unlike mammals, fish cannot synthesise L-arginine. Fish macrophages can be activated in a classical and/or alternative manner and, most likely, the balance between these caMF and aaMF will critically determine the outcome of the immune response to fish parasites.

Chapter 3

Evolutionary conservation of alternative
activation of macrophages:
Structural and functional characterisation of
arginase 1 and 2 in carp (*Cyprinus carpio* L.)

Maaike Joerink, Huub F.J. Savelkoul, Geert F. Wiegertjes

Abstract

Classically activated macrophages (caMF) play an important role in type I immune responses and alternatively activated macrophages (aaMF) function in type II immune responses. While the classical activation of fish macrophages has been well described, the existence of aaMF has not yet been described for teleosts. Arginase is the characteristic enzyme in aaMF and two isoforms have been described for mammals. To study the presence of aaMF in a primitive vertebrate species we cloned arginase 1 and 2 cDNA of common carp.

Carp arginase 1 is a 340 aa protein with 63% aa sequence identity to human arginase 1. Carp arginase 2 is a 347 aa protein with 63% aa sequence identity to human arginase 2. Three highly homologous arginase 2 genes were found, each showing only single non-synonymous substitutions. Basal arginase 1 expression is mainly found in carp mid kidney. In contrast, arginase 2 was expressed in all organs examined, with the highest basal gene expression in liver. Cultured carp head kidney-derived macrophages were used to study aaMF *in vitro*. Carp macrophages showed significant arginase activity which could be induced by dibutyryl cyclic adenosine mono phosphate (cAMP) and specifically inhibited by N^w-hydroxy-L-arginine (NOHA). At the gene level, arginase 2 gene expression was upregulated by cAMP stimulation, while arginase 1 gene expression was not influenced. LPS stimulation did not alter the arginase 1 or 2 expression, inducible nitric oxide synthase (iNOS) expression was however upregulated. This expression of iNOS was used as a measure of classical activation of carp macrophages. Thus, in contrast to mammals, fish arginase 2 and not arginase 1 is differentially regulated and likely involved in the alternative activation of fish macrophages. Our data suggest there may be an evolutionary conservation of the presence of aaMF down to teleost fish.

Introduction

Macrophages are described to occur in a resting, primed or activated state. Their activation can be subdivided in a classical and alternative state (Gordon, 2003; Mantovani *et al.*, 2004; Mantovani *et al.*, 2002; Mills *et al.*, 2000; Noël *et al.*, 2004; Sandor *et al.*, 2003). Classically activated macrophages (caMF) have been widely studied (Eisenstein *et al.*, 1994; Nathan, 1992) and find their role in type I immune responses against intracellular pathogens by the production of reactive oxygen species (ROS) and nitric oxide (NO). More recently, there is a growing interest in the role and functioning of alternatively activated macrophages (aaMF). Macrophages in this state of activation, via arginase activity, play an important role in type II immune responses against extracellular pathogens by showing increased phagocytic activity and enhanced gene expression of MHC class II. Besides this role aaMF promote proliferation and antibody class switching and function in allergic reactions and wound healing processes. Arginase is the characteristic enzyme in aaMF, and arginase activity plays a key role in the actions of aaMF (Gordon, 2003; Mantovani *et al.*, 2004; Mantovani *et al.*, 2002; Mills *et al.*, 2000; Mori and Gotoh, 2000; Noël *et al.*, 2004; Sandor *et al.*, 2003).

Arginase (L-arginine aminohydrolase, E.C. 3.5.3.1) is a binuclear manganese metalloenzyme that catalyses the hydrolysis of L-arginine to L-ornithine and urea. Two distinct isoforms encoded by two separate genes have been described in mammals (Jenkinson *et al.*, 1996; Morris *et al.*, 1998). In ureotelic animals (like mammals) arginase 1 takes part in the ornithine-urea cycle aimed at ammonia detoxification, is located in the cell cytosol and mainly expressed in the liver. In contrast, arginase 2 is found in mitochondria and expressed in almost all organs. Although in mice the highest gene expression can be found in kidney (Mori and Gotoh, 2000; Morris *et al.*, 1997), the organ distribution of arginase 2 gene expression is not the same for all mammals (Mori and Gotoh, 2000; Morris *et al.*, 1997; Salimuddin *et al.*, 1999).

There are a number of important functions of arginase that relate this enzyme to the immune system, often specifically to the function of macrophages. For example, arginase activity can deplete the extracellular environment of L-arginine, necessary for proliferation and survival of both pathogen and host cells (Currie *et al.*, 1979; da Silva *et al.*, 2002; Vincendeau *et al.*, 2003; Yu *et al.*, 2001). Also, arginase can deplete intracellular L-arginine, thereby removing the substrate of inducible nitric oxide synthase (iNOS) (Vincendeau *et al.*, 2003). For example, not only are iNOS and arginase 2 co-expressed in LPS-stimulated macrophages (Gotoh *et al.*, 1996; Mori and Gotoh, 2000; Morris *et al.*, 1998;

Wang *et al.*, 1995) but NO production is also enhanced when arginase gene expression is inhibited (Chang *et al.*, 1998). In this way arginase can modulate the production of NO by caMF (da Silva *et al.*, 2002; Nathan, 1995; Yu *et al.*, 2001). Further, arginase is involved in the production of polyamines and proline. Polyamines are important for cell proliferation and differentiation, while the production of proline is important for collagen production and wound healing (Albina *et al.*, 1990; Vincendeau *et al.*, 2003).

Teleost fish, such as the common carp (*Cyprinus carpio* L.), are one of the evolutionary oldest vertebrates to have both an innate and acquired immune system and can be considered a useful model to study the development of important functions of the immune system (Magor and Magor, 2001). The macrophage is considered an ancient cell type within the immune system (Beck and Habicht, 1996) and its function is most likely well-conserved throughout evolution. Indeed, caMF have been well characterised in teleost fish (Laing *et al.*, 1996; Laing *et al.*, 1999; Neumann *et al.*, 1995; Saeij *et al.*, 2000; Saeij *et al.*, 2002b). The presence of aaMF in teleost fish, however, has to the best of our knowledge, not been recognised so far.

To study the role of arginase in fish macrophages and hereby, an indication for the presence of aaMF in teleost fish, we first identified the carp arginase 1 and 2 cDNAs. Subsequently, we examined the gene expression of both isoforms in different organs. Arginase 1 expression was found mainly in the mid kidney which in teleosts consists of both haematopoietic and excretory tissue (Zapata, 1979). In clear contrast, arginase 2 was found to be expressed in all organs examined, with the highest gene expression in liver. Cultured carp head kidney-derived macrophages were used to study aaMF *in vitro*. Teleost fish do not posses bona fide bone marrow; instead, the head kidney is considered to be the functional and structural homologue (Zapata, 1981). Cultured head kidney-derived macrophages could be differentially stimulated to increase either arginase activity or NO production. Like in mice (Munder *et al.*, 1999) arginase activity could be induced by dibutyryl cyclic adenosine mono phosphate (cAMP) stimulation. This induced arginase activity could specifically be inhibited by N^{ω}-hydroxy-L-arginine (NOHA), which is the intermediate in the conversion of L-arginine into L-citruline and NO, and inhibits arginase by specifically interacting with the manganese-cluster of the active site of the enzyme. NO production could be induced by LPS stimulation. This induced NO production could be specifically inhibited by the L-arginine analogue N^{ω}-monomethyl-L-arginine acetate (L-NMMA), as was shown before (Saeij *et al.*, 2000). To distinguish between arginase 1 and arginase 2 -induced

activity in carp head kidney-derived macrophages we studied the expression of both genes by real-time quantitative PCR. The results clearly show a low gene expression of arginase 1 which could not be upregulated while arginase 2 gene expression was higher and could be significantly upregulated by cAMP. In addition, LPS could induce iNOS gene expression and NO production in carp macrophages, but neither gene expression of arginase 1 nor 2. Collectively our data show that, in contrast to mammals, fish arginase 2 but not arginase 1 is differentially regulated and likely involved in the alternative activation of fish macrophages.

Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility 'De Haar-Vissen' at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. R3 x R8 heterozygous carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). E5 carp are a gynogenetic homozygous carp strain (Komen *et al.*, 1991). The homozygous carp were used to distinguish between mutiple genes and allelic polymorphisms, for all other experiments heterozygous R3 x R8 carp were used.

Amplification of carp arginase 1 and 2 cDNA

Oligonucleotide primers for both arginase 1 and 2 (Table 3.1) were designed based on known partial fish arginase sequences. Aliquots (300 ng) of a λZAP cDNA library, made from phorbol 12-myristate 13-acetate (PMA)-stimulated carp head kidney phagocytes (Saeij *et al.*, 2000) were used in a PCR or anchored PCR with λZAP specific primers SK and T7. Based on partial carp sequences, new primers were designed to obtain extended (and for arginase 2, full-length) sequences.

(Anchored) PCR reactions were performed in Taq buffer, using 1 U Taq polymerase (Goldstar/Eurogentec S.A.) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 μM) and primers (400 nM each) in a total volume of 25 μl. PCR conditions included one cycle of 4 min at 94°C; followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C; and final extension for 7 min at 72°C, using a GeneAmp PCR system 9700 (PE Applied Biosystems).

5' and 3' Rapid amplification of cDNA ends assay

A full-length sequence of arginase 1 could not be found in the carp head kidney phagocyte cDNA library. The 5' and 3' ends of this gene were amplified by 5'and 3' rapid amplification of cDNA ends (RACE) (Frohman, 1993) using the GeneRacer™ RACE Ready cDNA Kit (Invitrogen) according to the manufacturer's protocol. Briefly, the GeneRacer™ RNA Oligo was ligated onto the 5'end of RNA from freshly isolated carp head kidney phagocytes. From this RNA cDNA was reverse transcribed using the oligo dT primer. Gene-specific primers (Table 3.1) were used in combination with the GeneRacer primers to amplify the first strands of cDNA. A second round with nested primers (Table 3.1) was performed to obtain a specific product. PCR and nested PCR were carried out under the following conditions: one cycle of 3 min at 94°C; followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 2 min at 72°C; and final extension for 7 min at 72°C.

Amplification of multiple carp arginase cDNAs

Multiple arginase sequences were found in heterozygous carp. To distinguish between mutiple genes and allelic polymorphisms, both arginase 1 and 2 genes were sequenced using organ material from carp, fully homozygous. If multiple sequences are detected in homozygous carp this indicates the presence of multiple genes and not alleles. Forward and reverse primer pairs were based on the sequences obtained from heterozygous carp (Table 3.1). RT-PCR was performed on RNA isolated from the head- or mid-kidney. For RT-PCR the SuperScript One-Step RT-PCR system (Invitrogen) was used. Briefly, 0.1 μg of total RNA, forward and reverse primers (200 nM each), 12.5 μl reaction buffer (2x), 20 U RNase inhibitor and 100 U Superscript II RT/*Taq* mix were mixed and diethyl pyrocarbonate (DEPC)-treated water was added to a final volume of 25 μl. RT-PCR was carried out under the following conditions: cDNA synthesis for 30 min at 50°C, followed by one cycle of 2 min at 95°C; and 40 cycles (arginase 1) or 30 cycles (arginase 2) of 30 sec at 94°C, 30 sec at 53 °C, and 1 min at 72°C; and final extension for 7 min at 72°C.

Cloning and sequencing

Products amplified by PCR, anchored PCR, RT-PCR or RACE-PCR were ligated and cloned in JM-109 cells using the pGEM®-Teasy kit (Promega) according to the manufacturer's protocol. Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's protocol. From each product both strands of four to eight clones were sequenced, using

the ABI Prism™ Bigdye™ Terminator Cycle Sequencing Ready Reaction kit, and analyzed using an ABI 377 automatic sequencer.

Analysis of sequences

Searches for similar sequences within the database were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) and the Swissprot, EMBL and Genbank databases using SRS 7.1 from the EBI (European Bioinformatics Institute) site (www.ebi.ac.uk). Comparisons between sequences were performed using the CLUSTALW 1.7 software (Thompson *et al.*, 1994) on the BioASP site, with minor optimisations made by hand. The PROSITE profile library (Falquet *et al.*, 2002) was used to identify family signatures and the MitoProt prediction tool (Claros and Vincens, 1996) was used to determine the presence of an N-terminal mitochondrial targeting sequence (MTS). Phylogenetic analysis was performed using MEGA version 2.1 software (Kumar *et al.*, 2001) using both the neighbour joining method and the minimal evolution method on the basis of amino acid differences (p-distance) and complete deletion of gaps (Saitou and Nei, 1987). Reliability of the trees was assessed by bootstrapping using 1000 bootstrap replications.

In vitro *culture of head- and mid-kidney-derived macrophages*

In teleost fish the head kidney is considered to be the functional and structural homologue of the bone marrow, the mid kidney consists of both haematopoietic and excretory tissue (Zapata, 1979; Zapata, 1981). To obtain a pure macrophage population carp head- or mid-kidney-derived macrophages were cultured essentially as described previously (Joerink *et al.*, 2004; MacKenzie *et al.*, 2003; Neumann *et al.*, 1998). Briefly, fish were euthanised with 0.25 g/l tricaine methane sulfonate (Crescent Research Chemicals) buffered with 0.38 g/l $NaHCO_3$, bled by syringe from the caudal vein and head- and mid-kidneys were aseptically removed. Organs were gently passed through a 100-µm sterile nylon mesh and rinsed with homogenisation buffer [incomplete-NMGFL-15 medium containing 50 mg/ml gentamicin, 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 U/ml heparin (Leo Pharma BV)] (Neumann *et al.*, 1998). Cell suspensions were layered on 51% (1.071 g.cm^{-3}) Percoll (Amersham Pharmacia Biotech AB) and centrifuged at 450 x *g* for 25 min at 4°C. Cells at the medium/Percoll interface were removed and washed twice. Cell cultures were initiated by seeding 1.75×10^7 head- or mid-kidney leukocytes in a 75 cm^2 culture flask containing 20 ml of complete-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 5% heat inactivated pooled carp

serum and 10% bovine calf serum (Invitrogen)] with 50 U/ml of penicillin and 50 µg/ml streptomycin. Cells were incubated at 27°C and cultured head- or mid-kidney-derived macrophages harvested after 6 days.

Flow cytometric analyses

Freshly isolated head- or mid-kidney leukocytes and cultured head- or mid-kidney-derived macrophages were analysed using a flow cytometer (Beckman Coulter, Epics XL-MCL). Forward scatter (FSC-H, reflecting cell size) sideward scatter characteristics (SSC-H, reflecting cell complexity) and fluorescence were recorded for 10^4 events per sample, propidium iodide (1 µg/ml) was added to each sample to identify necrotic cells. Cell populations were identified according to van Kemenade *et al.* (Verburg-van Kemenade *et al.*, 1994) and MacKenzie *et al.* (MacKenzie *et al.*, 2003).

Macrophage arginase activity and nitrite production

Head- or mid-kidney-derived macrophages (5×10^5) were seeded in 100 µl rich-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 2.5% heat inactivated pooled carp serum and 5% bovine calf serum (Invitrogen)] in 96-well flat-bottomed culture plates. Macrophages were stimulated with the cAMP analogue dibutyryl cAMP (0.5 mg/ml, dibutyryl cyclic adenosine mono phosphate, Sigma D0672) or with LPS (50 µg/ml; E. coli, Sigma L2880), inhibited with N^ω-hydroxy-L-arginine (NOHA 1 mM , Sigma H-7278) or with N^ω-monomethyl-L-arginine acetate (L-NMMA 500 uM, Sigma M7033), or left untreated, and incubated for 18 h at 27°C. cAMP has been shown to upregulate both arginase gene expression as well as activity (Gotoh and Mori, 1999; Morris *et al.*, 1998). LPS has been shown to upregulate iNOS gene expression and activity (Mori and Gotoh, 2000; Morris *et al.*, 1998; Salimuddin *et al.*, 1999). NOHA has been shown to inhibit arginase activity (Boucher *et al.*, 1994; Daghigh *et al.*, 1994) while L-NMMA has been shown to inhibit NO production (Saeij *et al.*, 2000).

Arginase activity was measured essentially as described by Corraliza *et al.* (Corraliza *et al.*, 1994): macrophages were lysed in 50 µl 0.1% Triton X-100 containing 5 µg pepstatin, 5 µg aprotinin and 5 µg antipain at room temperature for 30 min. Fifty µl of 10 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.5 was added and the mixture was incubated for 10 min at 55°C. To 50 µl of this activated lysate 50 µl of 0.5 M L-arginine, pH 9.7 was added and incubated for 1 h at 37°C. Reaction was stopped by adding 400 µl acid mixture containing H_2SO_4, H_3PO_4 and H_2O (1:3:7), then to each reaction 25 µl 9% α-isonitrosopropiophenone

(in 100% ethanol) was added and incubated for 45 min at 100°C. After 10 min cooling in the dark the absorbance was read at 540 nm and arginase activity (mU per million cells, mU= nmol urea formed per min) was calculated by comparison with a urea standard curve.

Nitrite production was measured essentially as described by Green *et al.* (Green *et al.*, 1982): to 75 µl cell culture supernatant 100 µl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentrations (µM) were calculated by comparison with a sodium nitrite standard curve.

Arginase and iNOS gene expression in cultured carp macrophages
For *in vitro* induction of gene expression 5×10^5 cultured head kidney-derived macrophages in 100 µl rich-NMGFL-15 medium were seeded in 96-well flat-bottom culture plates and stimulated with cAMP (0.5 mg/ml), with LPS (50 µg/ml) or left untreated as control. Optimal concentrations were determined by titration (data not shown). Head kidney-derived macrophages were incubated at 27°C and at 0, 3, 6, 9 and 18 h RNA was isolated. For this 2×10^6 cells (4 wells) per time point and per treatment were lysed and pooled before RNA was isolated.

RNA isolation, DNase treatment and first strand cDNA synthesis
RNA was isolated from nine organs (head- and mid-kidney, liver, brain, gills, spleen, thymus, skin and gut) of four carp using Trizol (Gibco BRL). RNA was also isolated from peripheral blood leukocytes (PBL) isolated on Ficoll (Amersham Pharmacia Biotech AB) and cultured head kidney-derived macrophages using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentrations were measured by spectrophotometry (Genequant, Amersham Pharmacia Biotech AB) and 1 µl was analysed on a 1% agarose gel to check the integrity. RNA was stored at -80°C until further use. For RNA isolated from cultured head kidney-derived macrophages DNaseI treatment was included on the columns of the RNeasy Mini Kit, using the accompanying RNase-Free DNase Set according to the manufacturer's protocol (Qiagen). For RNA isolated from organs and PBL DNaseI treatment was performed just before cDNA synthesis. To 1 µg of total RNA 1 µl of DNaseI reaction buffer (10x), 1 µl of DNaseI (Invitrogen) and RNase free water up to 10 µl was added. The mixture was incubated at room temperature for 15 min and DNaseI was

inactivated with 1 µl of 25 mM EDTA at 65°C, 10 min. For each sample a non-RT was included to which no reverse transcriptase was added. To each sample of 1 µg DNaseI treated total RNA, random primers (300 ng Invitrogen), 1 µl dNTP mix (10 mM), 4 µl First Strand buffer (5x), 2 µl DTT (0.1 M) and 1 µl RNase inhibitor (40 U/µl Invitrogen) were added and the mix was incubated at room temperature for 10 min. To each positive sample 200 U Superscript RNase H⁻ Reverse Transcriptase II (Invitrogen) was added and all samples were incubated at 37°C for 50 min. Reactions were stopped by adding MilliQ water up to 100 µl and cDNA was stored at -20°C until further use.

Real-time quantitative PCR

Specific real-time quantitative PCR primers (Table 3.1) were designed with the Primer Express software (Applied Biosystems). Because the three arginase 2 sequences each showed only a single non-synonymous substitution, real-time quantative PCR primers were designed to amplify all arginase 2 genes. To 5 µl of 10 times diluted cDNA, 12.5 µl Sybr Green Master Mix (Qiagen), forward and reverse primer (300 nM each) and MilliQ water up to 25 µl was added. Quantitative PCR was performed in a 72-well Rotor-Gene™ centrifugal real-time thermal cycler (Rotor-Gene 2000 Corbett Research). Following cycling conditions were used: one incubation step of 15 min at 95°C; followed by 45 cycles of 20 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C; an incubation for 1 min at 60°C was followed by a melting curve from 60°C to 90°C in steps of 1°C with 5 sec waiting. At the end of each cycle and during the waiting steps in the melting curve fluorescence intensities were measured. For each gene a standard dilution curve was run in triplicate to determine the threshold, efficiency and melting temperature (Table 3.1). Efficiencies were determined according to Rasmussen (Rasmussen, 2001). Raw data were analysed using Rotor-Gene Analysis Software V5.0 with 'dynamic tube' and 'slope correct' correction. Data were further analysed using the Pfaffl method (Pfaffl, 2001), the gene expression of 40S and β-actin in each sample were used to normalise the data. To be able to compare the gene expression of arginase 1 and 2 in the different organs data were analysed by using one threshold for both genes of interest and both house-keeping genes, reaction efficiencies at this threshold were determined for all genes. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked at least once by sequencing.

Table 3.1
Primer sequences, real-time quantitative PCR threshold, efficiencies (Eff.) and melting
temperatures (Melt. temp).

Primer	Sequence	Threshold	Eff.	Melt. temp
Arginase1fw1	CCAAATTTCTCATGGCTAAAGCC			
Arginase1rv1	ACTTTTGAAAACATGTGATCAC			
Arginase2fw1	GGAGCTCCGTTTTCCAAAGGA			
Arginase2rv1	TGGATCCACATCTCTAAGACCAATG			
SK	CGGCCGCTCTAGAACTAGTGGATC			
T7	TAATACGACTCACTATAGGG			
ForwardArginase1	AAGCACCTTGGAATCAAAACG			
NestedFwArginase1	GTCGATCGGCTTGGAATAGC			
ReverseArginase1	ACTTTTGAAAACATGTGATCAC			
Nested RvArginase1	GCTATTCCAAGCCGATCGAC			
Arginase1fw2	TACTGCTGYTGGATRAATAATG			
Arginase1rv2	CCTAAATAATGATGCCAGTAAG			
Arginase2fw2	CGGGCGACGGACTATTCG			
Arginase2rv2	CTAAAGTCGCAGTTCGGTGTC			
RQ arginase1fw	TGAGGAGCTTCAGCGGATTAC	0.0518	1.95	78.5
RQ arginase1rv	CCTATTATTCCCACGCAGTGATG			
RQ arginase2fw	GGAGACCTGGCCTTCAAGCATCT	0.006	1.98	81.0
RQ arginase2rv	CTGATTGGCACGTCCAACT			
RQ iNOSfw	AACAGGTCTGAAAGGGAATCCA	0.0148	1.96	87.7
RQ iNOSrv	CATTATCTCTCATGTCCAGAGTCTCTTCT			
RQ actinfw	GCTATGTGGCTCTTGACTTCGA	0.012	2.05	85.0
RQ actinrv	CCGTCAGGCAGCTCATAGCT			
RQ 40Sfw	CCGTGGGTGACATCGTTACA	0.0108	2.15	79.7
RQ 40Srv	TCAGGACATTGAACCTCACTGTCT			

Statistics

Gene expression data were analysed by a repeated measurement model (PROC
GLM, SAS, version 8.02, SAS Inst., Inc.) (SAS, 1995), with time as the repeating
factor. The treatments were tested for significance against the interaction of
treatment and individual. Arginase activity and nitrite production were tested
for significance by Student's *t*-test. $P \leq 0.05$ was accepted as significant.

Results

Carp possess a single arginase 1 and multiple copies of arginase 2

Primers based on partial fish arginase 1 and 2 sequences were used to obtain partial sequences from a carp phagocyte cDNA library. These sequences showed a high similarity with human arginase 1 and 2 sequences. Subsequent anchored and 5' and 3' RACE PCR resulted in full-length coding sequences of 340 and 347 aa for carp arginase 1 and 2, respectively. Several polymorphisms were found for both arginase isoforms in heterozygous carp. To distinquish between allelic and gene differences both arginase 1 and 2 were sequenced from fully homozygous carp. The complete sequences were amplified in single fragments. In homozygous carp, one unique arginase 1 sequence was found, suggesting arginase 1 exists as a single gene in carp. Although three sequences for arginase 2 were obtained (A-C), each was characterised by a single amino acid substitution only, suggesting these arginase 2 sequences were highly-homologous.

Carp arginase proteins were aligned with human, xenopus and chicken proteins (Fig 3.1). Carp arginase 1 and 2 are both 63% identical to the corresponding human protein. Carp arginase 1 and 2 proteins are 53% identical to each other. Analysis of the proteins with MitoProt predicted a 42 aa N-terminal mitochondrial targeting sequence for arginase 1 (probability 0.92) and a 41 aa N-terminal mitochondrial targeting sequence for arginase 2 (probability 0.99). To study the relationship of the newly found carp arginase genes with known sequences, a phylogenetic tree was constructed using the neighbour joining method (Fig. 3.2) and the minimal evolution method (data not shown). The overall topology for both methods was the same and showed two main clusters dividing the arginase 1 and 2 sequences. Within the two main clusters three sub-clusters could be distinguished representing fish, amphibian and mammalian taxons. All branching nodes were supported by high bootstrap values. The sequences of arginase 1 and 2A-C are deposited in the EMBL database under accession numbers **AJ871264** and **AJ618955**, **AJ871265**, **AJ871266**, respectively.

A

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CycaArg1    1 MTVMRSFSGLRAAFHIFTRDLHHHHCVGIIGAPFSKGQQRDGVQRGPDLIRAAGLVQKLKGQGCVVKDYGNLTFEDIPND
XelaArg1    1 ------------------MAKER.S..V.........P.R..EE..KYL.E...IE..REF.ND.R.C.D.D.P.V...
HosaArg1    1 ------------------MSAKSRTI...........P.G..EE..TVL.K...LE...E.E.D.....D.P.A.....

                                                    Ψ              ¶ + Ψ              Π
CycaArg1   81 EPIGRLKTPRAVGRANELLAGAVQKIKSDGNTCVMLGGDHSLAIGSISGHAASRHELSVLWVDAHADINTPLTTPTGNIH
XelaArg1   81 T.FNNV.N..T..K.T.I..N..TAV.KADK..QSI..,...V.T.A...VHPN.C.V..,.,..S.S.C..LI
HosaArg1   81 S.FQIV.N..S..K.S.Q...K.AEV.KN.RISLV..,.,........RVHPD.G.I..,T.......TS..LI

CycaArg1  161 GQPLSYLIHELHSKIPIIPNFSWLKPCVAAKDIVYIGLRDVDPEEHYILKHLGIKTFSMTEVDRLGIAKVMEQTCDHMFS
XelaArg1  161 .....F.MK..KA.M.AV.G.E.V...LRS..........G......T....YL..I...Y.KDD....E.LEYLVG
HosaArg1  161 ...V.F.LK..KG...DV.G...VT..IS..........G......T....Y...........G....E.LSYLLG

                     ¶ +                     Π
CycaArg1  241 KVKKPIHLSFDIDALDPSVSPATGTPVAGGLTYREGIYITENICQTGLLSAVD-MVPVNPKQGKTEDEIKSTVNAAVDLL
XelaArg1  241 .H.R.....,.....G....IA......CP..R.....RILHEQLHK.....G..TIWMESTSR.E.KRDVEV..KT.L.MT
HosaArg1  241 RK.R....,..V.G....FT.......V..,.....L...E.YK....GL.-IM,...SL...PE.VTR....T..AIT

CycaArg1  301 LGCFGRVREGSHEPDYQIPNP-
XelaArg1  301 .S...KA...F.AST-------
HosaArg1  301 .A...LA...N.K.IDYLNP.K
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B

```
CycaArg2    1 ---------------------------------------MALRGPLSRLLRSTMSSCQQNRSQSVAILGAPFSKGQKRRGVE
XelaArg2    1 ---------------------------------------MSIRSNFVRLLKKQVSIIKL.KKC.H...VI...........,..
GagaArg2    1 MPGTKGSPPQPACLLGSRGPHPLGSERVLLPKPEEHQA.NSACSHNGRILSSDEIQNFLEK.SGLCL.EVLAYLSFSFLH
HosaArg2    1 ---------------------------------------S..GSL....QTRVH.ILKKSVH...VI.....Q....K,L..

                                                                            Ψ
CycaArg2   81 HGPKAIRDAGLVERLSNLDYAVHDFGDLAFKHLEKDEHFMH-VPFPRTVGRANQLLSGAVSGAVGAGHTCIMLGGD,---
XelaArg2   81 ...A...S...I......GCN.C.....H.SQVPN..LYNSI.KH.....L.CKV.AEE..K........VT,...,---
GagaArg2   81 LSFLRAENWLTAVTQHAKRCQLY.....N.TQVPN..LYNNLIYY..S..L.S.V.AD...R..A...S.VTI...,RLC
HosaArg2   81 ...A...E...MK...S.GCHLK.....S.TPVP..DLYNNLIVN..S..L...E.AEV..R..SD.YS.VT,...,---

                  ¶ + Ψ              Π
CycaArg2  161 ----SLAIGSVEGHAQQCPDLCLIWVDAHABINTPLTSPSGNLGGQSVAFLLKDLQNKMPEVPGFSWMKPFLSARDLVYI
XelaArg2  161 ----...F..IT,........V...,....I.....T....,.P.S...RE..D.V.PI....A..C..KS.I...
GagaArg2  161 YCTC...L...S,..R...H.GV,....,....I.....TQ....,.PLS...RE..D.V.QL....L..C...S.I...
HosaArg2  161 ----....TIS,..RH....VV..,....I.....TS....,.P.S...RE..D.V.QL.....I..CI.SASI...

                                                          ¶ +
CycaArg2  241 GLRDVDPGEHIILKTLGIQYFSMRDIDRMGIQRVMEVTLDHLLARKQRPIHLSEDIDAFDPSLAPATGTPVNGGLTYREG
XelaArg2  241 ....L..A.QF...NYD.S.Y...H..C...KK...K.F.Q..G.RD.....,.,...A.........I..,....
GagaArg2  241 ....A.YY...NYD.....V..L...K...R.FEQ.MG.R.....,,........L..,......
HosaArg2  241 .......P..F.....NYD.....M.....L...K...R.F.L.IGKR......,,.......T.........V..,......

                  Π
CycaArg2  321 IYVTEEIHNTGLLSVMDVVEVNPTLGATPEAVEATTSLAVDVISSALGQTREGAHIAFPKIPEPKEDTELRL--------
XelaArg2  321 V.I.......M..AV.L,....V.A..S.E.K..AN......A.CFG.......TRADT.IDVLPTPSTSYESDNEEQV
GagaArg2  321 M.IA.......M..AV.M,....L...SQ.E.N..A.......ATCF.......T..DEL.T.SSPD.SDSEQQVRI--
HosaArg2  321 M.IA..........AL.L,...Q.ATSE.EAKT.AN......A.SF......G..VYDQL.T.SSPD.SENQARVRI--

CycaArg2  401 --
XelaArg2  401 RI
GagaArg2  401 --
HosaArg2  401 --
```

Fig 3.1 Amino acid sequences of carp arginase 1 (A) and arginase 2 (B) aligned with other arginase 1 and 2 proteins. Sequences were aligned by Clustal W1.7 programme. Dots indicate identity to the carp arginase 1 (A) or carp arginase 2A (B) protein, dashes denote gaps. I_{192} is V in carp arginase 2B and S_{364} is A in carp arginase 2C. The arginase family signature (boxes), histidines, aspartic acids and glycine involved in binding of the two manganese atoms: $Mn^{2+}A$ (+), $Mn^{2+}B$ (Ψ) or both $Mn^{2+}A$ and B (¶) and histidine and glutamic acid involved in L-arginine binding (Π), are indicated. I indicates the end of the predicted mitochondrial targeting sequence.

Proteins were taken from the Swissprot database with the following accession numbers: human HosaArg1 **P05089**, HosaArg2 **P78540**, African clawed frog XelaArg1 **P30759**, XelaArg2 **Q91553**, chicken GagaArg2 **XP421191**. Common carp CycaArg1 and CycaArg2 A-C are deposited in the EMBL database under accession numbers **AJ871264**, **AJ618955**, **AJ871265** and **AJ871266**, respectively.
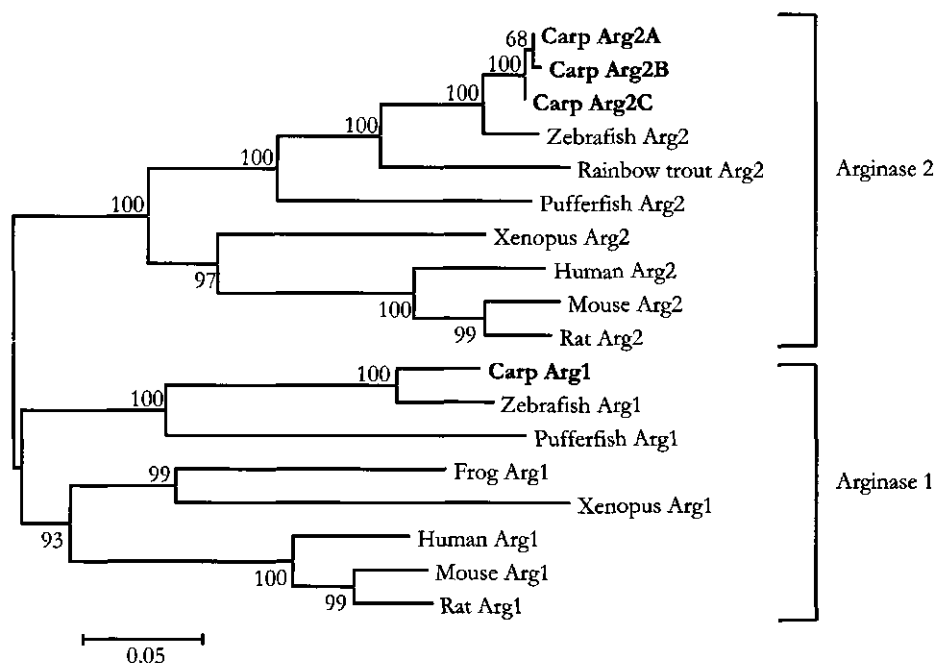
Fig 3.2 Neighbour joining tree of arginase 1 and arginase 2 proteins. Tree was based on p-distance calculations and positions with insertions/deletions were excluded. Numbers at branch nodes represent bootstrap confidence levels of 1000 bootstrap replications.
Zebrafish Arg1 **Q7T151**, zebrafish Arg2 **Q6PH54**, rainbow trout Arg2 **BK001400**, frog Arg1 **P49900**, xenopus Arg1 **P30759**, xenopus Arg2 **Q91553**, human Arg1 **P05089**, human Arg2 **P78540**, mouse Arg1 **Q61176**, mouse Arg2 **O08691**, rat Arg1 **P07824**, rat Arg2 **O08701**, pufferffish Arg1 SINFRUT00000148803 and pufferfish Arg2 SINFRUT00000148943 are gene scan peptide sequences from the ensembl database.

Carp arginase 1 and 2 are differentially expressed

To determine the basal gene expression of arginase 1 and 2 in different (immune) organs and in leukocytes (PBL and cultured head kidney-derived macrophages) total RNA was isolated and transcribed into cDNA. From this cDNA, arginase 1 and 2 gene expression was determined by means of real-time quantitative PCR (Fig. 3.3). Expression of both genes is shown relative to the gene expression of 40S. When β-actin was used as reference gene similar results were observed (data not shown). Real-time quantitative PCR allowed us to distinguish between arginase 1 and 2 gene expression despite their sequence similarity (63% nucleotide identity) and generally low basal gene expression levels. In carp, arginase 1 gene expression was low and found mainly in the mid kidney. Arginase 2 gene expression was more widely distributed.

The major sites of gene expression were liver and brain while lower gene expression levels were detected in gills and kidney. In cultured head kidney-derived macrophages arginase 2 basal gene expression was 5-6 fold higher than in the head kidney organ.

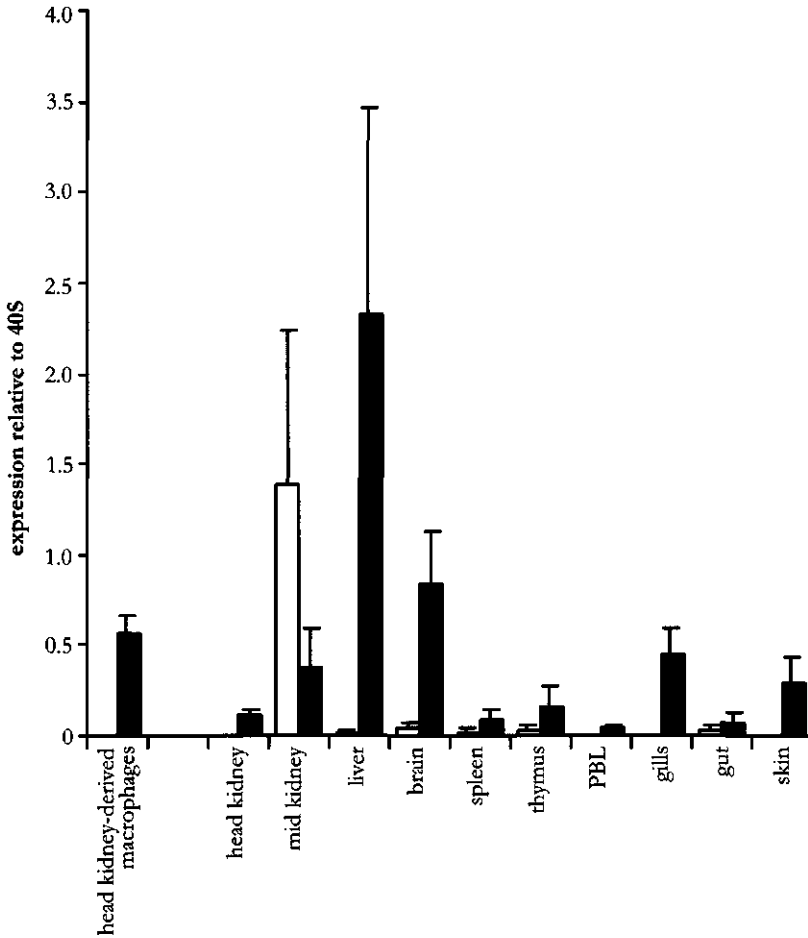Fig 3.3 Basal gene expression of arginase 1 (open bars) and arginase 2 (solid bars) in carp organs and leukocytes. Gene expression was measured by means of real-time quantitative PCR and shown relative to the gene expression of 40S. Threshold was set at 0.006 and efficiencies at this threshold were 1.94, 1.98, and 2.14 for arginase 1, arginase 2 and 40S, respectively. Averages and SD of n=4 fish are given.

Arginase activity and nitrite production can be stimulated in head kidney-derived macrophages

Head- and mid-kidney leukocytes were cultured for 6 days to obtain macrophages. The freshly isolated head kidney leukocytes (Fig. 3.4A) and cultured head kidney-derived macrophages (Fig. 3.4B) differ in their morphology. Flow cytometric analyses of these cells showed three distinct populations (R1–R3) of cells for freshly isolated head kidney leukocytes (Fig. 3.4C). R1 are small lymphocytes (71%), R2 are monocytes and lymphoblasts (8%) and R3 are granulocytes (21%). After 6 days of culturing head kidney leukocytes differentiated into major population (79%) of macrophages (R2'). The remainder of the leukocytes (R1) are myeloid precursors and some lymphocytes (Fig. 3.4D). Freshly isolated mid kidney leukocytes and cultured mid kidney-derived macrophages showed flow cytometric profiles and overall morphology similar to the ones shown for the head kidney and are not shown here.

A　　　　　　　　　　**B**

C　　　　　　　　　　**D**
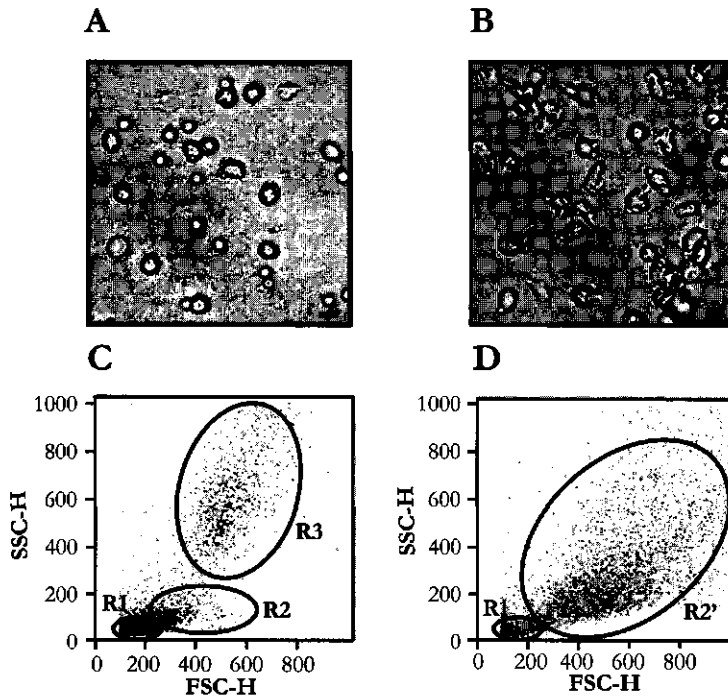
Fig 3.4 Light microscopy photographs (bar is 10 μm) and flow cytometric profiles of freshly isolated head kidney leukocytes (A and C) and cultured head kidney-derived macrophages (B and D). Per sample 10^4 events were recorded and PI positive events were subtracted afterwards. R1 are small lymphocytes (71%), R2 are monocytes and lymphoblasts (8%) and R3 are granulocytes (21%). After 6 days, cultures typically consist of 79% macrophages (R2') the remainder (R1) are myeloid precursors and some lymphocytes.

Freshly isolated head- or mid-kidney leukocytes and cultured macrophages were stimulated with cAMP or LPS, and arginase activity and NO production were measured. Freshly isolated head kidney leukocytes showed a low basal arginase activity, which could not be increased by stimulation with cAMP. After culture, head kidney-derived macrophages had an increased basal arginase activity, which could be further stimulated by cAMP. In contrast, freshly isolated mid kidney leukocytes showed a very high basal arginase activity, which could not be stimulated further. Cultured mid kidney-derived macrophages had a low basal arginase activity and could not be stimulated by cAMP (Fig. 3.5A).
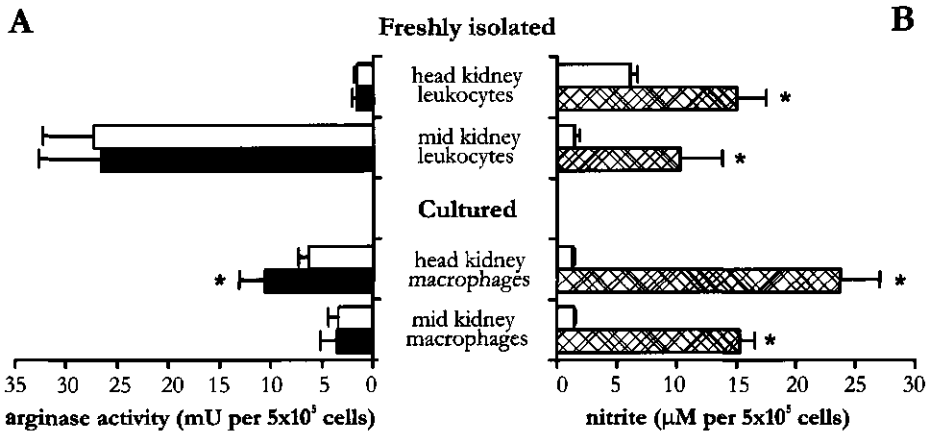

Fig 3.5 Arginase activity (A) and nitrite production (B) of freshly isolated head- or mid- kidney leukocytes and cultured head- or mid- kidney-derived macrophages. Cells, 5×10^5 per well, were stimulated with cAMP (solid bars) (0.5 mg/ml), LPS (hatched bars) (50 µg/ml) or left untreated as control (open bars), for 18 h at 27°C. Data are from a representative experiment out of n=3 fish, average and SD of triplicate measurements are given. * $P\leq0.05$ Student's t-test, treatment compared to the corresponding control.

Freshly isolated head kidney leukocytes produced low basal levels of nitrite which could be 3-fold stimulated with LPS. After culture, head kidney-derived macrophages could readily be stimulated to produce up to 18-fold higher levels of nitrite. Freshly isolated mid kidney leukocytes produced very low basal levels of nitrite which could be 7-fold stimulated with LPS. Mid kidney-derived macrophages could be stimulated to produce up to 10-fold higher levels of nitrite (Fig. 3.5B). LPS was never able to stimulate arginase activity nor was cAMP able to induce nitrite production (data not shown). Because of their active state further experiments were performed with macrophage cultures derived from head kidney.

To further assess the specificity of the stimulations with LPS and cAMP, cultured head kidney-derived macrophages were stimulated with LPS or cAMP and simultaneously inhibited with NOHA or L-NMMA. Again both arginase activity as well as nitrite production were measured after 18 h of stimulation and inhibition. cAMP specifically induced significant arginase activity up to 4.3 mU per 5×10^5 cells but did not induce nitrite production (Fig. 3.6A-B). Nitrite production was even significantly lower after stimulation with cAMP probably due to competition of the two enzymes for the same substrate, L-arginine. LPS specifically induced significant nitrite production up to 12.8 µM per 5×10^5 cells but did not induce arginase activity. Furthermore, arginase activity could be inhibited by NOHA, while NOHA did not influence nitrite production. Nitrite production could be inhibited by L-NMMA, while L-NMMA did not influence arginase activity.

A **Cultured head kidney macrophages** **B**
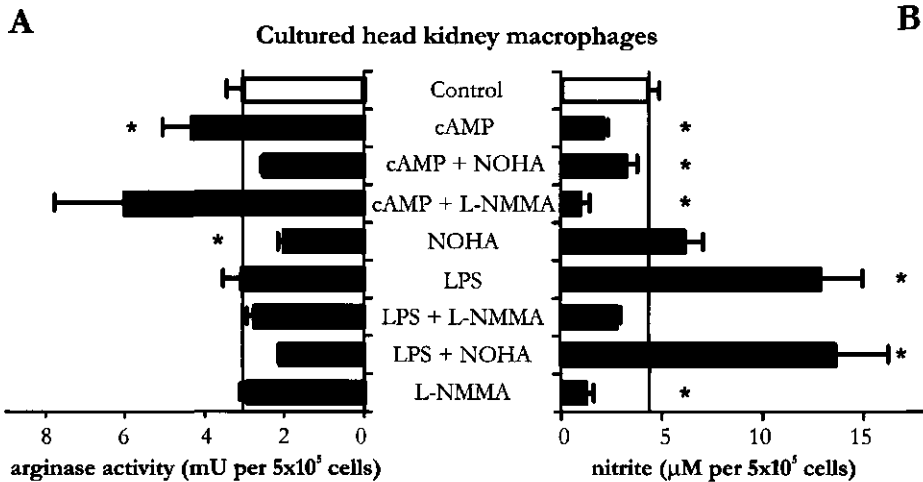
Fig 3.6 Arginase activity (A) and nitrite production (B) of differentially stimulated and inhibited cultured head kidney-derived macrophages. Cells, 5×10^5 per well, were stimulated (solid bars) with cAMP (0.5 mg/ml), LPS (50 µg/ml) inhibited with NOHA (1 mM) or L-NMMA (500 µM) or left untreated as control (open bars), for 18 h at 27°C. Data are from a representative experiment out of n=3 fish, average and SD of triplicate measurements are given. * $P\leq0.05$ Student's *t*-test, treatment compared to the control.

Arginase 2 and iNOS gene expression can be induced in carp macrophages

To determine whether arginase 1 or arginase 2 is responsible for the arginase activity in carp macrophages, we measured the gene expression in cultured head kidney-derived macrophages after stimulation with cAMP. As a measure of classical activation we also stimulated with LPS and measured iNOS

gene expression. Stimulation with cAMP significantly induced arginase 2 gene expression compared to the controls with a maximum increase at 6 h of approximately 14-fold (Fig. 3.7B). cAMP did not induce gene expression of arginase 1 nor iNOS (Fig. 3.7A,C). Stimulation with LPS induced gene expression of iNOS up to a maximum increase at 9 h of approximately 670-fold (Fig. 3.7C) but did not induce gene expression of arginase 1 nor arginase 2 (Fig. 3.7A-B). This suggests that arginase 2 is responsible for the increased arginase activity in cAMP stimulated carp macrophages.
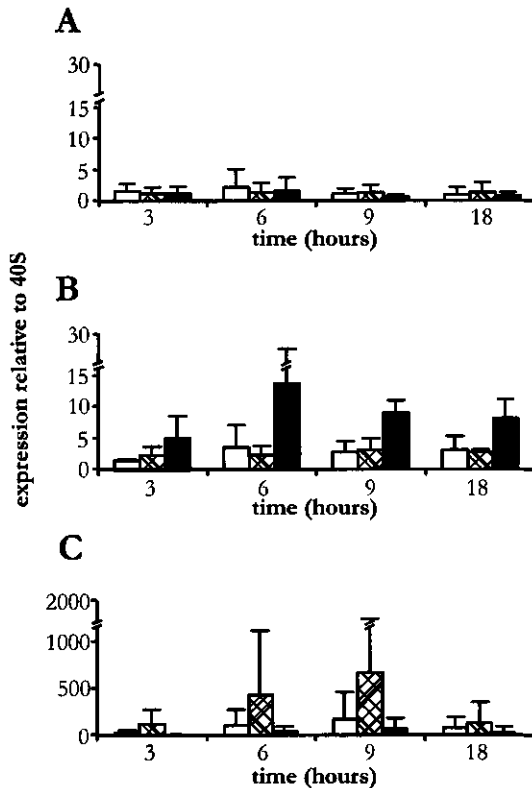
Fig 3.7 Gene expression of arginase 1 (A), arginase 2 (B) and iNOS (C) in carp head kidney-derived macrophages. Macrophages, 5×10^5 per well, were stimulated with cAMP (solid bars) (0.5 mg/ml), LPS (hatched bars) (50 μg/ml) or left untreated as control (open bars). After 0, 3, 6, 9 and 18 h at 27°C RNA was isolated from 2×10^6 cells. Gene expression was measured by means of real-time quantitative PCR and is shown relative to the gene expression of 40S. Averages and SD of n=3 fish are given.

Discussion

The classical activation route of macrophages has been well described, also for teleost fish (Eisenstein *et al.*, 1994; Laing *et al.*, 1996; Laing *et al.*, 1999; Nathan, 1992; Neumann *et al.*, 1995; Saeij *et al.*, 2000; Saeij *et al.*, 2002b). Far less is known about the regulation of aaMF and the role of arginase, especially in evolutionary older vertebrates like teleost fish. Alternatively activated macrophages are important for type II immune responses, including the defence against extracellular pathogens, allergic reactions and wound healing (Gordon, 2003; Mantovani *et al.*, 2004; Mantovani *et al.*, 2002; Mills *et al.*, 2000; Noël *et al.*, 2004; Sandor *et al.*, 2003). To better understand the evolution of aaMF we identified the arginase 1 and 2 cDNA from common carp, a primitive vertebrate species.

A single arginase 1 and multiple arginase 2 genes were found in fully homozygous carp. The latter is not uncommon, since the common carp is considered to be tetraploid because of their chromosome number (2n=100) and high DNA content (David *et al.*, 2003). Also in the rainbow trout, a species with a tetraploid ancestor, multiple arginase 2 sequences have been described (Wright *et al.*, 2004). For both fish species the differences between the arginase 2 proteins are only minor, however, suggesting that their enzymatic activity (conversion from L-arginine to L-ornithine and urea) might not be different. Arginase 1 and 2 sequences grouped in two separate clusters and within each of these two main clusters three sub-clusters could be distinguished representing fish, amphibian and mammalian representatives.

The carp arginase 2 sequences contain a mitochondrial targeting sequence, similar to all other arginase 2 sequences identified to date. Different, however, fish arginase 1 sequences, including the carp arginase 1 sequence, also contain a mitochondrial targeting sequence. This is in contrast to the arginase 1 of mammals, amphibians, and lungfish that all have a cytosolic arginase, an ammonia-dependent carbamoyl phosphate synthase (CSP III) and a mitochondrial transporter specific for ornithine (Mommsen and Walsh, 1989). In the carp mid kidney, high basal gene expression of arginase 1, but not arginase 2 was observed. Furthermore, freshly isolated mid kidney leukocytes showed a high basal arginase activity which could not be increased by cAMP. This was not seen in the carp head kidney. The major difference between the head- and mid-kidney in carp is the additional excretory function of the mid kidney (Zapata, 1979). We propose that the high arginase activity in freshly isolated mid kidney leukocytes could be ascribed to arginase 1 and not arginase 2 gene expression. The reason for this is the fact that mid kidney arginase activity could not

be increased by stimulation with cAMP, while also cAMP was shown unable to up-regulate arginase 1 gene expression (in cultured head kidney-derived macrophages). The functional relevance of this high arginase 1 activity might be linked to the excretory rather than to the haematopoietic functions of the mid kidney, although this requires further investigations. In ureotelic animals arginase 1 is mainly expressed in the liver, where it takes part in the ornithine-urea cycle aimed at ammonia detoxification. Fish, however, can directly secrete ammonia in their aquatic environment and have no need for the ornithine-urea cycle. This may explain the relatively low gene expression of arginase 1 in carp liver. Our data suggest that arginase 1 might have a function in fish different from ureotelic animals.

Basal gene expression of carp arginase 2 was widely distributed, similar to what has been observed for human, mouse and rat (Mori and Gotoh, 2000; Morris *et al.*, 1997). High gene expression was found in liver and brain. In brain, arginase forms ornithine, a precursor for glutamate, which can be transformed into the neurotransmitter GABA (γ-amino butyric acid) (Yu *et al.*, 2001). In liver, arginase activity can be expected to be involved in protein synthesis. Inside the cell, arginase 2 is located in the mitochondria together with aminotransferase both necessary for the production of proline and glutamate which are important for protein synthesis (Jenkinson *et al.*, 1996; Vincendeau *et al.*, 2003). Furthermore, arginase 2, via ornithine decarboxylase, is involved in the synthesis of polyamines. Polyamines are believed to be important for cell proliferation and growth (Jenkinson *et al.*, 1996; Vincendeau *et al.*, 2003). This might explain the relatively high basal gene expression in kidneys, haematopoietic tissues and skin, where cell proliferation can be expected.

To further investigate the function of arginase in the carp immune system, cultured head kidney-derived macrophages were used. To induce a classical activation, macrophages were stimulated with LPS. Cultured macrophages were shown to be 'primed' in that a significant NO production could be measured as early as 18 h following LPS stimulation. This is a very early time-point to measure a significant NO production in carp compared to previous measurements at 72-96 h in freshly isolated cells (Saeij *et al.*, 2000). This activity could be specifically inhibited by L-NMMA. LPS induced iNOS gene expression up to 670-fold but could not induce arginase gene expression nor arginase activity in carp macrophages. Although iNOS gene expression could clearly be induced by LPS, differences were not statistically significant, because of high variation between individual fish. This phenomenon is quite common for carp and has frequently been observed both at gene expression

and functional level (Saeij, 2002a).

cAMP stimulation induced arginase activity in carp macrophages. Studies in mice have shown that arginase gene expression and activity can be induced by IL-13 via the increase of intracellular cAMP and tyrosine kinase phosphorylation. Exogenous cAMP also increased arginase activity and enhanced the effect of IL-13 on arginase induction (Chang *et al.*, 2000). The cytokine IL-13 has not yet been identified in fish. Exogenous cAMP, however, clearly induced arginase activity but not NO production in carp macrophages. Apparently, the function of arginase in the immune system is well conserved and thus provides a unique opportunity to study alternatively activated macrophages in a primitive vertebrate species.

Arginase is the characteristic enzyme of aaMF. Freshly isolated carp macrophages could not be stimulated to increase arginase activity, while cultured (primed) head kidney-derived macrophages could easily be stimulated to express a high level of arginase activity. This phenomenon has also been observed for rat and mouse macrophages (Stickings *et al.*, 2002; Jakway *et al.*, 1980). The induced arginase activity could be specifically inhibited by NOHA, which is the intermediate in the conversion of L-arginine into L-citruline and NO. The gene expression of arginase 1 and 2 in carp macrophages is clearly differently regulated from that observed in mammalian macrophages. In carp, stimulation with cAMP specifically induced arginase 2 gene expression (up to 14-fold) but not arginase 1 gene expression. Stimulation with LPS induced gene expression of iNOS, but not arginase 1 nor arginase 2. In rats, LPS induces arginase 1 gene expression together with iNOS. In mice, LPS induces an early arginase 2 and iNOS gene expression but a delayed arginase 1 gene expression (Gotoh *et al.*, 1996; Salimuddin *et al.*, 1999; Wang *et al.*, 1995). Mouse macrophages stimulated with type II cytokines or schistosoma egg antigens upregulate arginase 1 while keeping arginase 2 gene expression constant (Hesse *et al.*, 2001; Munder *et al.*, 1999; Pauleau *et al.*, 2004; Stickings *et al.*, 2002).

Our observations that carp head kidney-derived macrophages can be stimulated by cAMP to specifically induce gene expression of arginase 2, leading to increased arginase activity, without inducing iNOS expression or NO production, suggest that fish macrophages may have the ability to differentiate into alternatively activated macrophages.

Acknowledgements

Chapter 4

Head kidney-derived macrophages of
common carp (*Cyprinus carpio* L.) show
plasticity and functional polarisation
upon differential stimulation

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Trudi Hermsen, Huub F.J. Savelkoul, Geert F. Wiegertjes

Abstract

Cells from the myeloid lineage are pluripotent. To investigate the potential of myeloid cell polarisation in a primitive vertebrate species, we phenotypically and functionally characterised myeloid cells of common carp (*Cyprinus carpio* L.) during culture. Flow cytometric analysis, antibody labeling of cell surface markers and light microscopy showed the presence of a major population of heterogeneous macrophages after culture. These head kidney-derived macrophages can be considered the fish equivalent of bone marrow-derived macrophages and show the ability to phagocytose, produce radicals and polarise into innate activated or alternatively activated macrophages. Macrophage polarisation was based on differential activity of iNOS and arginase for innate and alternative activation, respectively. Correspondingly, gene expression profiling after stimulation with LPS or cAMP showed differential expression for most of the immune genes presently described for carp.

The recently described novel immunoglobulin-like transcript 1 (NILT1) and the CXCR1 and CXCR2 chemokine receptors were upregulated after stimulation with cAMP, an inducer of alternative activation in carp macrophages. Upregulation of NILT1 was also seen during the later phase of a *Trypanosoma carassii* infection, where macrophages are primarily alternatively activated. NILT1 could however not be upregulated during a *Trypanoplasma borreli* infection, a model for innate activation. Our data suggest that NILT1, CXCR1 and CXCR2 could be considered markers for alternatively activated macrophages in fish.

Introduction

Macrophages play a role in both the innate and the adaptive immune system. In the innate immune system they act as phagocytic cells, phagocytosing pathogens and producing oxygen and nitrogen radicals. In the adaptive immune system macrophages act as professional antigen presenting cells. As such, macrophages can provide a bridge between the innate and adaptive immune response. Altogether, macrophage differentiation exhibits a wide array of functional and phenotypic heterogeneity (Rutherford *et al.*, 1993). Within this functional heterogeneity, classically and alternatively activated macrophages (caMF and aaMF, respectively) are proposed to represent the extremes of a continuum (Gordon, 2003; Mantovani *et al.*, 2004; Mills *et al.*, 2000). In literature a further distinction has been made between classical and innate activation of macrophages (Gordon, 2003). Classical activation is induced by stimulation with both IFNγ and a microbial trigger such as LPS, while innate activation is induced by stimulation with LPS (or other microbial triggers) alone (Gordon, 2003). Although classically and innate activated macrophages are induced by different stimuli, their functions overlap. Innate activated macrophages have microbicidal activity and produce pro-inflammatory cytokines, reactive oxygen species (ROS) and NO (Gordon and Taylor, 2005). caMF find their role in type I immune responses against intracellular pathogens by the production of ROS and NO. aaMF are active in type II immune responses against extracellular pathogens by showing increased phagocytic activity and enhanced gene expression of MHC class II. Furthermore aaMF increase their production of factors involved in tissue remodeling and repair and are able to inhibit type I inflammations (Noël *et al.*, 2004). Macrophage activation in fish has been well studied with regard to NO production (Laing *et al.*, 1996; Laing *et al.*, 1999; Neumann *et al.*, 1995; Saeij *et al.*, 2000; Saeij *et al.*, 2002b) and ROS (Chung and Secombes, 1988; Jørgensen and Robertsen, 1995; Norum *et al.*, 2005; Sveinbjørnsson and Seljelid, 1994). Recently, we described carp arginase gene expression and activity, which can be used as markers for alternatively activated macrophages, and we proposed an evolutionary conservation of alternatively activated macrophages down to teleost fish (Joerink *et al.*, 2006d).

The aim of the present study was to investigate the macrophage polarisation in teleosts, using functional assays and gene expression profiling. Hence we developed a primary cell culture system of carp head kidney-derived macrophages. In fish, only few such culture models exist for goldfish, rainbow trout and brook trout (MacKenzie *et al.*, 2003; Neumann *et al.*, 1998; Stafford *et al.*, 2001). We studied morphological changes by flow cytometry and light

microscopy. We investigated functional changes by determining phagocytic ability, the ability to produce radicals (both ROS and NO) and by measuring arginase activity. A well-accepted way to study polarisation is by determining gene expression profiles after differential stimulation. We quantified gene expression, by real-time quantitative PCR, in head kidney-derived macrophages stimulated with LPS or cAMP. Gene expression of carp CXCR1 and CXCR2 were upregulated after cAMP stimulation.

In addition to the CXCR1 and 2, head kidney-derived macrophages expressed the recently identified novel immunoglobulin-like transcripts (NILT)-1 and NILT2 (Stet *et al.*, 2005) at different levels following stimulation. NILT1 and NILT2 are polymorphic receptors belonging to the immunoglobulin superfamily (IgSF) which, in general, recognise pathogen-associated molecular patterns. These type of receptors frequently exist in pairs with antagonistic signaling functions, are co-expressed on the same cell and bind similar, if not identical, ligands (Taylor *et al.*, 2000). The cytoplasmic region of NILT1 contains a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) and of NILT2 an immunoreceptor tyrosine-based inhibition motif (ITIM). The use of NILT1, CXCR1 and CXCR2 as possible surface markers for alternatively activated macrophages in fish is discussed.

Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility 'De Haar-Vissen' at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. R3 x R8 heterozygous carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). Carp were between 9 and 11 months old. All studies were performed with approval from the animal experimental committee of Wageningen University.

Cell culture for macrophages

Carp head kidney leukocytes (HKL) were cultured essentially as described before for goldfish and trout (MacKenzie *et al.*, 2003; Neumann *et al.*, 1998; Stafford *et al.*, 2001). Briefly, fish were euthanized with 0.25 g/l tricaine methane sulfonate (Crescent Research Chemicals) buffered with 0.38 g/l $NaHCO_3$, bled by syringe from the caudal vein and head kidneys were aseptically removed. Head kidneys were gently passed through a 100-μm sterile nylon mesh and rinsed with homogenisation buffer [incomplete-NMGFL-15 medium containing

50 U/ml penicillin, 50 µg/ml streptomycin, and 20 U/ml heparin (Leo Pharma BV)] (Neumann *et al.*, 1998). Cell suspensions were layered on 51% (1.071 g .cm⁻³) Percoll (Amersham Pharmacia Biotech AB) and centrifuged at 450 x *g* for 25 min at 4°C without brakes. Cells at the medium/Percoll interface were removed and washed twice. Cell cultures were initiated by seeding 1.75×10^7 HKL in a 75 cm² culture flask containing 20 ml of complete-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 5% heat-inactivated pooled carp serum and 10% bovine calf serum (Invitrogen)] with 50 U/ml of penicillin and 50 µg/ml streptomycin. Cells were incubated at 27°C and head kidney-derived macrophages were harvested after 6 days by placing the flasks on ice for 10 min and gentle scraping.

Flow cytometry
Flow cytometry was used to follow the development of the cultures, forward scatter (FSC-H, reflecting cell size) and sideward scatter characteristics (SSC-H, reflecting internal cell complexity) were recorded for 10^4 events per sample, using a flow cytometer (Beckman Coulter, Epics XL-MCL). For all cytometric measurements the same settings were used: FS 350 volt, gain 2; SS 700 volt, gain 10; FL-1 800 volt, gain 1; FL-2 870 volt, gain 1; FL-3 675 volt, gain 1 and FL-4 950 volt, gain 1. The baseline offset was on and the discriminator at the FS at 20. For the phagocytosis assay a gate was set to measure cells only and not the (smaller) bacteria. Propidium iodide (PI 0.1 µg/ml) was added to each sample to detect and gate out PI⁺ cells, and 100 µl of a standard diluted bead solution (Fluoresbrite® YG Carboxylate Microspheres 10 µm, Polysciences Inc) was added to determine the amount of cells in culture. Cell populations were identified by FSC-H/SSC-H as described by van Kemenade *et al.* (Verburg-van Kemenade *et al.*, 1994) for freshly isolated head kidney leukocytes (HKL) and by MacKenzie *et al.* (MacKenzie *et al.*, 2003) for head kidney-derived macrophages.

Cell surface markers (WCI12, TCL-BE8 and WCL15)
To further characterise the cell culture we determined the number of WCI12, TCL-BE8 and WCL15 positive cells at day 0, 2, 4 and 6 of culture. WCI12 binds to carp Ig, staining Ig-immunopositive B lymphocytes (Secombes *et al.*, 1983). TCL-BE8 mainly binds to neutrophilic granulocytes but also has cross reactivity with monocytes (Nakayasu *et al.*, 1998). WCL15 has been shown to bind to macrophages and monocytes and to a lesser extend to basophilic granulocytes in head kidney cell suspensions (Weyts *et al.*, 1997).

Cells (2.5×10^5 per well of a 96-well round-bottom plate) were incubated with diluted primary antibody in predetermined optimal dilutions (WCI12 1:200; TCL-BE8 1:1000 and WCL15 1:100) for 30 min on ice and washed with flow cytometer medium (incomplete medium supplemented with 1% bovine serum albumin (BSA) and 0.01% Na-azide). Subsequently, cells were incubated with a 1:50 dilution of a secondary antibody (rabbit-anti-mouse R-Phycoerythrin (RPE) and goat-anti-mouse fluorescein isothiocyanate (FITC) Dako) for 30 min on ice, washed, and resuspended in 200 µl flow cytometer medium containing PI (0.1 µg/ml) to detect and gate out PI^+ cells. Per sample 10^4 events were measured by flow cytometer. A control sample incubated with the secondary antibody only, was included in each experiment and consistently found to be negative.

Light microscopy
For light microscopy, cell suspensions of freshly isolated HKL and head kidney-derived macrophages were pelleted (10 min 450 x *g*). Cell pellets were fixed in 1% (w/v) $K_2Cr_2O_7$, 2% (v/v) gluteraldehyde and 1% (w/v) OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at 0°C, and subsequently washed in double-distilled water, dehydrated in alcohol and propylene oxide and embedded in Epon 812 (Electron Microscopy Science). Semi-thin sections were cut on a Reichert Ultracut E (Leica), stained with 1% (w/v) toluidine blue O, 1% (w/v) borax on a hot plate for 1 min, rinsed with tap water and embedded in depex (Serva).

Phagocytosis, radical production, arginase activity and nitrite production
Freshly isolated HKL or head kidney-derived macrophages (5×10^5) were seeded in 100 µl rich-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% bovine calf serum (Invitrogen)] in wells of a 96-well flat-bottom culture plate.
For measurement of phagocytosis, cells were stimulated with LPS (50 µg/ml; *Escherichia coli*, Sigma L2880), or left untreated to measure basal phagocytosis, and incubated for 18 h at 27°C. Stimulated cells were resuspended by pipetting, transferred to flow cytometry tubes and incubated with FITC-stained bacteria (5×10^6) for an additional hour at 27°C. Bacteria (*Staphylococcus aureus*) (Pansorbin, EMD Biosciences) were stained by overnight incubation with FITC (5 µg/ml) at room temperature and continuous rotation. Bacteria were washed, at least three times, with incomplete medium to remove all unbound FITC.

Phagocytosis was stopped by placing the tubes on ice and adding 1-2 ml ice-cold PBS. PI (0.1 µg/ml) was added to each sample to detect and gate out PI$^+$ cells, fluorescence of non-phagocytosed bacteria was quenched by adding trypan blue (130 µg/ml). A total number of 10^4 events in the cell gate were measured by flow cytometer, the cell gate excluded the free bacteria from the measurements.

For measurement of radical production cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (0.1 µg/ml) or left untreated as controls. At the same time dihydrorhodamine 123 (DHR) (10 µg/ml Sigma D1054, 28.8 µM)) was added to all samples, and samples were incubated for 1 h at 27°C. Cells were resuspended by pipetting, transferred to flow cytometry tubes and PI (0.1 µg/ml) was added to each sample to detect and gate out PI$^+$ cells, 10^4 events were measured by flow cytometer.

For measurement of arginase activity cells were stimulated with the cAMP analogue dibutyryl cAMP (0.5 mg/ml, dibutyryl cyclic adenosine mono phosphate, Sigma D0672), or left untreated, and incubated for 18 h at 27°C. Arginase activity was measured essentially as described by Corraliza *et al.* (Corraliza *et al.*, 1994): cells were lysed in 50 µl 0.1% Triton X-100 containing 5 µg pepstatin, 5 µg aprotinin and 5 µg antipain at room temperature for 30 min. Fifty µl of 10 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.5 was added and the mixture was incubated for 10 min at 55°C. To 50 µl of this activated lysate 50 µl of 0.5 M L-arginine, pH 9.7 was added and incubated for 1 h at 37°C. Reaction was stopped by adding 400 µl acid mixture containing H_2SO_4, H_3PO_4 and H_2O (1:3:7), then to each reaction 25 µl 9% α-isonitrosopropiophenone (in 100% ethanol) was added and incubated for 45 min at 100°C. After 10 min cooling in the dark the absorbance was read at 540 nm and arginase activity (mU per million cells, mU= nmol urea formed per min) was calculated by comparison with a urea standard curve.

For measurement of nitrite production, cells were stimulated with LPS (50 µg/ml), or left untreated, and incubated for 18 h at 27°C. Nitrite production was measured essentially as described by Green *et al.* (Green *et al.*, 1982): to 75 µl cell culture supernatant 100 µl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentrations (µM) were calculated by comparison with a sodium nitrite standard curve.

Gene expression profiling of LPS and cAMP stimulated head kidney-derived macrophages

To analyse the functional heterogeneity of head kidney-derived macrophages, gene expression levels of an array of genes (TNFα, IL-11, IL-1β, IL-1R, IL-10, IL-12P35, IL-12P40.1, IL-12P40.2, IL-12P40.3, iNOS, arginase 1, arginase 2, CXCa, CXCb, CXCR1, CXCR2, MHC-II, NILT1 and NILT2) were measured after LPS or cAMP stimulation. Head kidney-derived macrophages were stimulated with LPS (50 μg/ml) or cAMP (0.5 mg/ml) or left untreated as control, and incubated for 6 h at 27°C. To study NILT gene expression in more detail, samples were taken after 0, 3, 6, 9 and 18 h of incubation at 27°C. From all samples RNA was isolated, cDNA synthesised and gene expression levels were determined by means of real-time quantitative PCR (see later sections).

Gene expression during T. carassii *and* T. borreli *infections*

Trypanosoma carassii was cloned and characterised by Overath *et al.* (Overath *et al.*, 1998). *Trypanoplasma borreli* was cloned and characterised by Steinhagen *et al.* (Steinhagen *et al.*, 1989). Parasites were maintained by syringe passage through carp. Thirty-two carp were i.p. injected with 10,000 *Trypanosoma carassii* per fish and four were left untreated as control. In a separate experiment twenty carp were i.p. injected with 10,000 *Trypanoplasma borreli* per fish and five were left untreated as control. At every time point four fish for the *T. carassii* and five fish for the *T. borreli* experiment were sacrificed and their head kidneys removed. Head kidneys were snap frozen in liquid nitrogen and stored at -80°C. From all head kidneys RNA was isolated, cDNA synthesised and gene expression levels were determined by means of real-time quantitative PCR (see later sections).

RNA isolation, DNase treatment and first strand cDNA synthesis

RNA was isolated from a 27 mm^3 head kidney (organ) or $2x10^6$-$5x10^6$ cells per treatment using the RNeasy Mini Kit (Qiagen) including the accompanying DNase I treatment on the columns, according to the manufacturer's protocol. Final elution was performed with 25 μl diethyl pyrocarbonat (DEPC)-treated water. RNA concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech) and 1 μl was analysed on a 1% agarose gel to check the integrity. Routinely, 10 μg of RNA approximately was isolated from 27 mm^3 head kidney (organ) and 15 μg of RNA from 2-5x10^6 cells. RNA was stored at -80°C until further use. For each cDNA synthesis a negative sample (non-RT), to which no reverse transcriptase was added and a positive sample containing

the reverse transcriptase were included. After DNAse treatment, 1 µg of total RNA was combined with, random primers (300 ng Invitrogen), 1 µl dNTP mix (10 mM), 4 µl First Strand buffer (5x), 2 µl DTT (0.1 M) and 1 µl RNase inhibitor (40 U/µl Invitrogen) and the mix was incubated at room temperature for 10 min. To each positive sample, 1 µl Superscript RNase H⁻ Reverse Transcriptase II (200 U/µl Invitrogen) was added. To each negative sample, 1 µl DEPC-treated water was added. All samples were incubated at 37°C for 50 min. Reactions were stopped by adding MilliQ water up to 100 µl and cDNA stored at -20°C until use.

Real-time quantitative PCR

Specific real-time quantitative PCR primers (Table 4.1) were designed with the Primer Express software (Applied Biosystems). To 5 µl of 10 times-diluted cDNA, 7 µl Sybr Green Master Mix (Stratagene), forward and reverse primer (300 nM each) and MilliQ water up to 14 µl was added. Quantitative PCR was performed in a 72-well Rotor-Gene™ centrifugal real-time thermal cycler (Rotor-Gene 2000 Corbett Research). Following cycling conditions were used: one holding step of 10 min at 95°C; followed by 40 cycles of 20 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C; an incubation for 1 min at 60°C was followed by a melting curve from 60°C to 99°C in steps of 1°C with 5 sec waiting. At the end of each cycle and during the waiting steps in the melting curve, fluorescence intensities were measured. Raw data were analysed using the comparative quantitation of the Rotor-Gene Analysis Software V5.0. Data were further analysed using the Pfaffl method (Pfaffl, 2001), average efficiencies per run per gene were used. Gene expression of 40S in each sample was highly constant and used to normalise the data. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked at least once by sequencing.

Table 4.1
Primer sequences corresponding accession numbers, real-time quantitative PCR melting temperatures (Melt. temp) and efficiencies (Eff.).

Gene	Acc. No.	Sense (5'-3') Antisense (5'-3')	Melt. temp	Eff.
TNFα	**AJ311800-01**	GCTGTCTGCTTCACGCTCAA		
		CCTTGGAAGTGACATTTGCTTTT	78.0	1.72
IL-1β	**AJ245635**	AAGGAGGCCAGTGGCTCTGT		
		CCTGAAGAAGAGGAGGCTGTCA	78.8	1.74
IL-1R	**AJ843873**	ACGCCACCAAGAGCCTTTTA		
		GCAGCCCATATTTGGTCAGA	76.7	1.75
IL-10	**AB110780**	CGCCAGCATAAAGAACTCGT		
		TGCCAAATACTGCTCGATGT	74.5	1.86
IL-11	**AJ632159**	CAGCAGCACAGCTCAGTACCA		
		AGCCTCTGCTCGGGTCATCT	78.8	1.74
IL-12P35	**AJ580354**	TGCTTCTCTGTCTCTGTGATGGA		
		CACAGCTGCAGTCGTTCTTGA	77.3	1.86
IL-12P40.1	**AJ621425**	GAGCGCATCAACCTGACCAT		
		AGGATCGTGGATATGTGACCTCTAC	77.0	1.79
IL12P40.2	**AJ628699**	TCTTGCACCGCAAGAAACTATG		
		TGCAGTTGATGAGACTAGAGTTTCG	76.7	1.41
IL12P40.3	**AJ628700**	TGGTTGATAAGGTTCACCCTTCTC		
		TATCTGTTCTACAGGTCAGGGTAACG	77.3	1.85
iNOS	**AJ242906**	AACAGGTCTGAAAGGGAATCCA		
		CATTATCTCTCATGTCCAGAGTCTCTTCT	77.7	1.69
Arginase 1	**AJ871264**	TGAGGAGCTTCAGCGGATTAC		
		CCTATTATTCCCACGCAGTGATG	77.3	1.76
Arginase 2	&	GGAGACCTGGCCTTCAAGCATCT		
		CTGATTGGCACGTCCAACT	80.3	1.73
CXCa	**AJ421443**	CTGGGATTCCTGACCATTGGT		
		GTTGGCTCTCTGTTTCAATGCA	78.8	1.78
CXCb	**AB082985**	GGGCAGGTGTTTTTGTGTTGA		
		AAGAGCGACTTGCGGGTATG	77.3	1.76
CXCR1	**AB010468**	GCAAATTGGTTAGCCTGGTGA		
		AGGCGACTCCACTGCACAA	80.8	1.75
CXCR2	**AB010713**	TATGTGCAAACTGATTTCAGGCTTAC		
		GCACACACTATACCAACCAGATGG	80.5	1.82
MHC-II DAB1-2	**Z47731-32**	ACAGCTCCCGTGATTTCAGT		
		CTCTGCGTTATATACTCCAAGTGC	77.0	1.85
MHC-II DAB3-4	**Z47733/X95435**	GCGTTTCAGGCGGACTCTT		
		ACACCATATCACTGTAATACACT	77.3	1.60
NILT1	**AJ811996**	CATACTCTGATTTCTGGACACAGA		
		CTGTTTTACCAGCAACAAAATCTC	77.8	1.69
NILT2	**AJ811997**	GGTGCTCCAGTTAAAGTCACAGGA		
		CTGTTTAACCAGCAACACAATCTC	79.5	1.74

Table 4.1 continued

Gene	Acc. No.	Sense (5'-3') Antisense (5'-3')	Melt. temp	Eff.
40S	**AB012087**	CCGTGGGTGACATCGTTACA		
		TCAGGACATTGAACCTCACTGTCT	78.5	1.77
β-actin	**M24113**	GCTATGTGGCTCTTGACTTCGA		
		CCGTCAGGCAGCTCATAGCT	81.8	1.73

& there are three arginase 2 isoforms known **AJ618955**/**AJ871265-66** this primer set amplifies all three.

Statistics

Phagocytosis, radical production, arginase activity, nitrite production and gene expression differences between the differential stimulated head kidney-derived macrophages and during the infections were tested for significance by Student's *t*-test. $P \leq 0.05$ was accepted as significant.

NILT1 and NILT2 gene expression in the head kidney-derived macrophages over time, were analysed by a repeated measurement model (PROC GLM, SAS, version 8.02, SAS Inst., Inc., Cary NC)(SAS, 1995), with time as the repeating factor. The treatments were tested for significance against the interaction of treatment and individual. $P \leq 0.05$ was accepted as significant.

Results

Macrophage cell culture: characterisation by cell number, surface marker staining and morphology

Carp head kidney leukocytes (HKL) were separated on a 51% Percoll layer and cultured *in vitro* for several days. A pilot experiment where cells were cultured up to 8 days and analysed by flowcytometer indicated an expanding cell population until day 7. To characterise these *in vitro* cell cultures we followed their development by flow cytometry, determining cell number, size and internal complexity (forward scatter (FSC-H) and sideward scatter (SSC-H), respectively) and expression of cell surface markers up to day 6 in culture.

During the first 2 days of culture, total cell numbers decreased drastically, while after 4 days of culture cell numbers increased again, indicating proliferation (Fig. 4.1A). At the start of the culture (day 0), three cell populations could be identified based on FSC-H/SSC-H dot plots (Fig. 4.1B). First, a population of lymphocytes and precursor cells was characterised by a low FSC-H/SSC-H (lower left hand corner). Most of the WCI12$^+$ cells (B lymphocytes) could be found in this population.

Over time, during the first two days of culture especially, this lymphocyte population decreased dramatically (Figs 4.1B and 4.1C). Second, a population of monocytes was characterised by a medium FSC-H/SSC-H (spreading out to the middle). A low percentage of WCL15$^+$ cells (macrophages, monocytes, basophilic granulocytes) could be found primarily in this population. Over time, the relative percentage of WCL15$^+$ cells increased steadily (Fig. 4.1C). Third, a population of neutrophilic granulocytes was characterised by a high FSC-H/SSC-H (upper right hand corner). Almost all of these cells were TCL-BE8$^+$ (neutrophilic granulocytes, monocytes). During the first two days of culture the relative percentage of TCL-BE8$^+$ cells remained constant (Fig. 4.1C). However, since the total cell number decreased rapidly (Fig. 4.1A), the absolute number of TCL-BE8$^+$ cells also decreased. From day 4 onwards, the percentage of TCL-BE8$^+$ cells increased again.

After four days, cell cultures consisted of two populations (Fig. 4.1B). One major population of head kidney-derived macrophages with a high FSC-H/SSC-H that were TCL-BE8$^+$, WCL15$^+$, or TCL-BE8$^+$WCL15$^+$, as confirmed by fluorescence microscopy (data not shown). The second population was smaller in number and consisted of precursor cells with a low FSC-H/SSC-H that were negative for all three cell surface markers used. During the last two days of the culture especially, the head kidney-derived macrophages further increased in number, both relative and absolute. Changes in cell number were also reflected by the changes in the percentage of PI$^+$ cells. The percentage of PI$^+$ cells increased from 5% at day 0 to 10% at day 2, but decreased again to 7% at day 6.
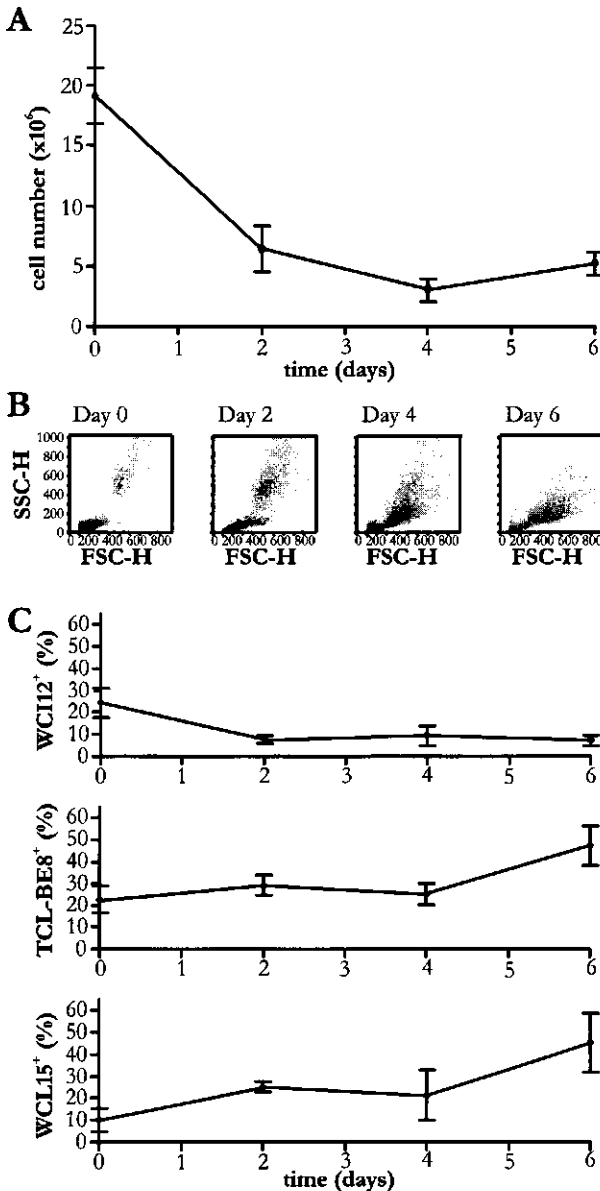
A

B

C

Fig. 4.1 Flow cytometric analyses of carp head kidney leukocyte cultures. Head kidney leukocytes were cultured in complete NMGFL-15 medium. At day 0, 2, 4 and 6 cells were collected to determine: cell numbers per culture (A), cell size and internal complexity (B) and surface marker staining (C). To determine cell numbers 100 μl of a standard bead solution was added and cells with beads were analysed by flow cytometry. Averages and SD of n=3 fish are given. To determine cell morphology, dot plot profiles for forward scatter (FSC-H, reflecting size) and sideward scatter (SSC-H, reflecting internal complexity) were measured. Data from a representative experiment out of n=8 fish are shown. For cell surface marker staining the monoclonals WCI12 (1:200), TCL-BE8 (1:1000), and WCL15 (1:100) were used. Averages and SD of n=4 fish are given. For all flow cytometric measurements PI (0.1 μg/ml) was included to detect and gate out PI^+ cells.

Light microscopy (Fig. 4.2) supported the decrease in lymphocytes and granulocytes and the increase in myeloid cells as detected by the antibody labeling. Within this myeloid cell population there is still a degree of heterogeneity visible.
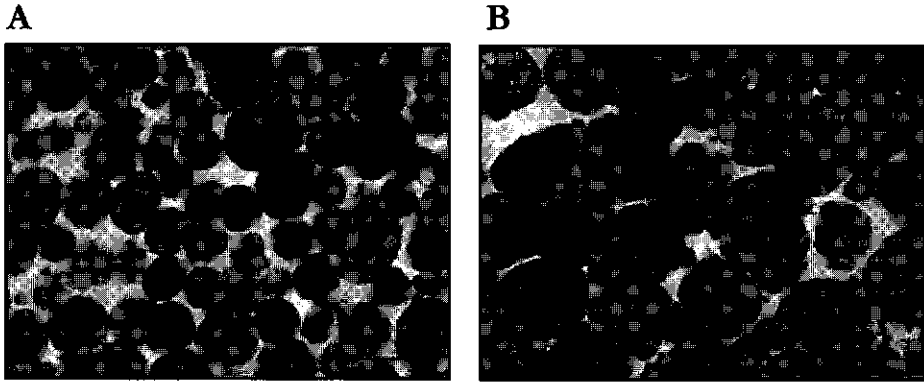
A   **B**

Fig. 4.2 Light microscopic photographs (bar is 10 μm) of freshly isolated head kidney leukocytes (A) and head kidney-derived macrophages (B). Cells were pelleted and fixed, semi-thin sections were cut and stained with toluidine blue and borax.

Phagocytic ability, (oxygen) radical production, arginase activity and nitrite production are high in head kidney-derived macrophages

Carp HKL were cultured *in vitro* for 6 days to obtain head kidney-derived macrophages, which were assessed for cell function by measuring phagocytosis and (oxygen) radical production by flow cytometry and arginase activity and nitrite production by a calorimetric method.

The phagocytic ability, the number of cells capable of phagocytosis, was measured both in freshly isolated HKL (day 0) and in head kidney-derived macrophages (day 6). In a pilot experiment, a time curve showed a plateau phase of phagocytosis after 1 h incubation at 27°C, which was chosen as the time of incubation in the following experiments. Head kidney-derived macrophages showed significantly higher basal phagocytic ability (19%) of FITC-labeled bacteria, as compared to freshly isolated HKL (8%). Pre-stimulation with LPS for 18 h further increased the phagocytic ability both in head kidney-derived macrophages (23%) and in freshly isolated HKL (13%) (Fig. 4.3A).

Radical production was measured by flow cytometry following incubating with DHR. Head kidney-derived macrophages showed a very high (77%) basal activity, as compared to freshly isolated HKL (22%) (Fig. 4.3B). While radical production in freshly isolated HKL could be significantly increased by PMA

stimulation (51%), radical production by head kidney-derived macrophages could not be further increased (79%).

Arginase activity was measured after 18 h of incubation with or without cAMP stimulation. Head kidney-derived macrophages showed a significantly higher arginase activity, as compared to freshly isolated HKL (Fig. 4.3C). This was true both for basal activity (2 vs. 6 mU) as well as for cAMP-induced activity (2 vs. 14 mU). cAMP stimulation increased the arginase activity of head kidney-derived macrophages significantly.

Nitrite production was measured after 18 h of incubation with or without LPS stimulation. Head kidney-derived macrophages showed a significantly higher ability to produce nitric oxide, measured as nitrite in a Griess reaction, as compared to freshly isolated HKL (Fig. 4.3D). This was true for both basal activity (2 vs. 15 μM) and for LPS-induced activity (3 vs. 36 μM). LPS stimulation increased the NO production of head kidney-derived macrophages significantly. Basal nitric oxide levels were high in head kidney-derived macrophages, considering a stimulation with LPS of 18 h only, already showed a considerable nitrite production of 15 μM.
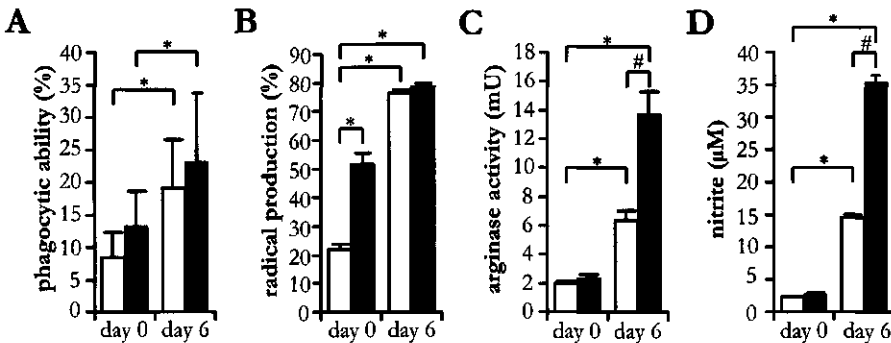
Fig. 4.3 Phagocytosis (A), radical production ($^{\cdot}$OH, $CO_3^{\cdot-}$, NO_3^-, H_2O_2 and $NO_2^{\cdot}$) (B), arginase activity (C) and nitrite production (D) by freshly isolated head kidney leukocytes (day 0) and head kidney-derived macrophages (day 6). Cells (5×10^5 per 100 μl) were stimulated (closed bars) or left untreated to measure basal activity (open bars). Cells were stimulated with LPS (50 μg/ml) for phagocytosis (A) and nitrite production (D), with PMA (0.1 μg/ml) for radical production (B) or with cAMP (0.5 mg/ml) for arginase activity (C). Averages and SD of n=4 fish are given for phagocytosis and radical production. Averages and SD or triplicate measurements of a representative experiment out of n=7 are given for arginase activity and nitrite production. * $P\leq0.05$, by Student's t-test, compared to day 0 control. # $P\leq0.05$ Student's t-test, compared to corresponding time point control.

Gene expression profiles of LPS- or cAMP-stimulated head kidney-derived macrophages are different

RNA was isolated from head kidney-derived macrophages (6 day culture) stimulated for 6 h with LPS or cAMP or from unstimulated cells, to assess differences in gene expression as a measure of macrophage polarisation. Gene expression levels of an array of immune-relevant genes currently described for carp were studied by real-time quantitative PCR, using 40S and β-actin gene expression as house keeping gene references. Results with 40S and β-actin were comparable; only the results relative to 40S are shown (Table 4.2).
Gene expression levels of cells stimulated with LPS or cAMP were compared to unstimulated cells, as shown in Table 4.2. In addition, gene expression levels of LPS-stimulated head kidney-derived macrophages were compared with gene expression levels in cAMP-stimulated head kidney-derived macrophages.

Table 4.2 Gene expression of head kidney-derived macrophages
after 6 h stimulation with LPS or cAMP relative to unstimulated cells,
take off values of unstimulated cells are given.

Gene	Take off value[&]	LPS	cAMP
TNFα[#]	21.2	1.14*	0.74
IL-1β	24.9	6.62*	5.24*
IL-1R	22.8	1.33	1.10
IL-10[#]	21.4	1.01	0.60*
IL-11	27.5	1.72	2.33
IL-12P35	27.8	2.04*	1.93
IL-12P40.1	29.2	1.27	0.75
IL-12P40.2	27.3	1.27	1.19
IL-12P40.3[#]	22.7	1.53	3.04*
iNOS[#]	25.5	8.08*	0.74
arginase 1	30.2	1.31	0.79
arginase 2[#]	21.4	1.33	3.08*
CXCa[#]	24.0	1.56*	0.91
CXCb	29.5	1.17	1.00
CXCR1[#]	27.3	0.73	7.18*
CXCR2[#]	25.8	1.26	3.46*
MHC-II DAB1-2	18.1	0.94	0.91*
MHC-II-DAB3-4	28.2	1.50	1.32
NILT1	31.8	1.07	2.05*
NILT2[#]	22.3	1.22*	0.81*

& Take off value is the point 80% before the peak of the second
    derivative of the raw data (Rotor-Gene Analysis Software V5.0).
Significant difference between the LPS and cAMP stimulated cells.
* Significant difference compared to unstimulated cells.

From the differentially expressed genes, TNFα, IL-10, iNOS, CXCa, and NILT2 were significantly higher expressed in LPS-stimulated head kidney-derived macrophages, while IL12P40.3, arginase 2, CXCR1, and CXCR2 were significantly higher expressed in cAMP-stimulated head kidney-derived macrophages.
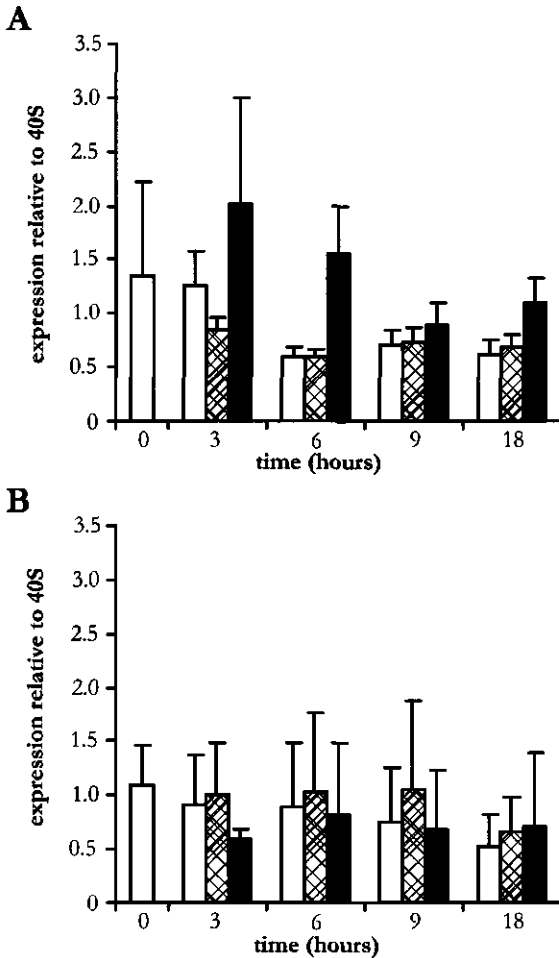
Fig. 4.4 Gene expression of NILT1 (A) and NILT2 (B) in head kidney-derived macrophages. Head kidney-derived macrophages, 5×10^5 per well, were stimulated with LPS (50 μg/ml) (hatched bars), cAMP (0.5 mg/ml) (solid bars) or left untreated as control (open bars). Gene expression was measured by means of real-time quantitative PCR and is shown relative to the gene expression of 40S. Averages and SD of n=3 fish are given. Statistical differences were calculated by a repeated measurement model with time as a repeating factor. NILT1 gene expression in the cAMP stimulated head kidney-derived macrophages is significantly different from both the LPS stimulated and unstimulated head kidney-derived macrophages. NILT2 gene expression in the cAMP stimulated head kidney-derived macrophages is significantly different from the LPS stimulated head kidney-derived macrophages.

NILT1 gene expression is upregulated in cAMP-stimulated head kidney-derived macrophages and in T. carassii-*infected carp but not in* T. borreli-*infected carp*

From the genes differentially expressed in LPS- or cAMP-stimulated head kidney-derived macrophages we selected the NILT genes for a more detailed study because of the unknown function of these novel receptors in fish. To further characterise the gene expression of NILT1 and NILT2, head kidney-derived macrophages were stimulated with LPS, with cAMP, or left untreated as control and gene expression relative to 40S was followed over time. NILT1 gene expression was significantly upregulated after stimulation with cAMP. In contrast, LPS did not influence NILT1 expression (Fig. 4.4A). NILT2 gene expression was consistently upregulated after LPS stimulation. cAMP stimulation showed a consistent downregulation of NILT2 (Fig. 4.4B).

In addition, we measured the *in vivo* gene expression of both NILT1 and NILT2 in head kidney (whole organ) of carp infected with either *Trypanosoma carasssii* or *Trypanoplasma borreli*. Both NILT1 and NILT2 gene expression were downregulated during the early phase of infection with *T. carassii* but upregulated during later time points in the *T. carassii infection* (Figs 4.5A and 4.5C). Observed effects were stronger for NILT1 than for NILT2.

The *T. borreli* infection was more severe and lasted shorter than the *T. carassii* infection. During *T. borreli* infection, NILT gene expression was different. NILT1 gene expression was significantly upregulated only at week 4 (Fig. 4.5B), while NILT2 gene expression was not significantly regulated (Fig. 4.5D).
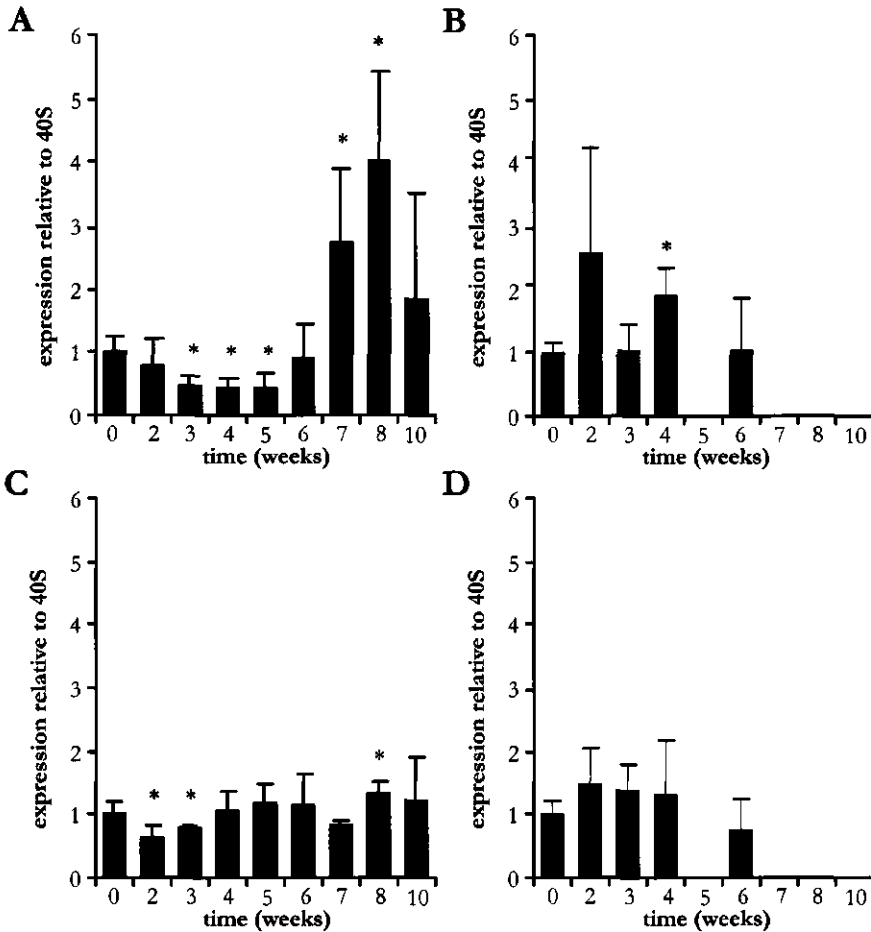
Fig. 4.5 Gene expression of NILT1 (A and B) and NILT2 (C and D) in head kidney (organ) of *Trypanosoma carassii*-infected carp (A and C), or *Trypanoplasma borreli*-infected carp (B and D). A number of n=32 carp were i.p. infected with 10,000 *T. carassii* per fish and a number of n=20 carp were i.p. infected with 10,000 *T. borreli* per fish. At week 0, the week of infection, n=4-5 carp were sacrificed as uninfected control fish. At every sample point n=4 fish from the *T. carassii* and n=5 fish from the *T. borreli* infection were sacrificed, head kidneys were removed, snap frozen in liquid nitrogen and stored at -80°C. Gene expression was measured by means of real-time quantitative PCR and is shown relative to the gene expression of 40S. Averages and SD of n=4-5 fish are given. * P≤0.05, by Student's *t*-test, compared to uninfected control.

Discussion

Our experiments show that carp head kidney-derived macrophages are heterogeneous and upon *in vitro* stimulation can give rise to functionally different polarisation states. According to FSC-H/SSC-H profiles the cell cultures consisted of two populations. The majority of the cells (>80%) with a high FSC-H/SSC-H profile are mature macrophages, as discussed in detail later. The remainder of the cells with a low FSC-H/SSC-H profile most likely are precursor cells of the myeloid lineage, as was shown for goldfish (Neumann *et al.*, 2000). The functional heterogeneity gives rise to macrophage populations that can be activated innate or alternatively. Gene expression profiling suggested that NILT1, CXCR1 and CXCR2 might be used as surface markers for alternatively activated macrophages. The use of NILT1 as putative cell surface maker for aaMF in fish was confirmed in an *in vivo* experiment.

Until present, carp macrophages have been studied using head kidney-derived leukocytes isolated by Percoll density centrifugation, followed by an adherence step in RPMI medium with adjusted molarity. This method leads to slightly-enriched fractions of macrophages (Verburg-van Kemenade *et al.*, 1994). To study macrophage polarisation and functional heterogeneity of a primitive vertebrate in more detail we here describe a method to culture carp macrophages, based on a procedure previously developed for goldfish and trout (MacKenzie *et al.*, 2003; Neumann *et al.*, 1998; Stafford *et al.*, 2001). Head kidney leukocytes (HKL) were isolated on a 51% Percoll layer, seeded in NMGFL-15 medium (Neumann *et al.*, 1998) and cultured for six days.

NMGFL-15 medium enhances myeloid but not lymphoid cell growth of goldfish and trout (Neumann *et al.*, 1998; Stafford *et al.*, 2001). Indeed, also for carp the NMGFL-15 medium leads to an early reduction in the number of lymphocytes (WCI12$^+$, low FSC-H/SSC-H (Verburg-van Kemenade *et al.*, 1994; Secombes *et al.*, 1983)) and supported a subsequent increase in the number of macrophages (WCL15$^+$TCL-BE8$^+$ (Nakayasu *et al.*, 1998; Weyts *et al.*, 1997), high FSC-H/SSC-H; (MacKenzie *et al.*, 2003)). Light microscopy supported head kidney-derived cell cultures to be primarily macrophages with phenotypic heterogeneity.

Head kidney-derived macrophages present at day 6 of culture were functional macrophages as could be seen from their apparent capacity to phagocytose fluorescent bacteria and increased radical production. To measure the ROS production cells were incubated with DHR. Free radicals and some other molecules ($^{\bullet}OH$, $CO_3^{\bullet-}$, NO_3^-, H_2O_2 and $NO_2^{\bullet}$) can oxidise the nonfluorescent DHR into the highly green fluorescent rhodamine 123 which localises in the

mitochondria (Wrona *et al.*, 2005). DHR is not oxidised by $O_2^{\cdot-}$ (Jourd'heuil *et al.*, 2001; Royall and Ischiropoulos, 1993). The radical $O_2^{\cdot-}$ can, however, react with other molecules to form radicals that can be measured by DHR (Jourd'heuil *et al.*, 2001). The low radical production by freshly isolated HKL could be ascribed, based on FSC-H/SSC-H, primarily to neutrophilic granulocytes and to a lesser extend to monocytes. In contrast, we observed a very high spontaneous radical production in carp head kidney-derived macrophages. Also, when radical production was measured using reduction of nitro blue tetrazolium (NBT), similar results were observed. In fact, for freshly isolated HKL, 90 minutes incubation was needed to obtain a substantial amount of NBT reduction, while head kidney-derived macrophages accounted for two times higher NBT reduction within 30 minutes only (unpublished data). Most likely, this high basal activity is caused by dead cells and/or cytokines also present in the cell cultures. In conclusion, cultured carp head kidney-derived macrophages provide us with an excellent model to investigate the polarisation and functional heterogeneity of fish macrophages.

There is a growing interest in the role and functioning of alternatively activated macrophages. Recently, we described the possible conservation of alternative macrophage activation down to teleost fish (Joerink *et al.*, 2006d). In the present study we used cAMP to stimulate arginase activity, as a measure of alternative activation. Both NO production and arginase activity were higher in head kidney-derived macrophages than in freshly isolated HKL. Furthermore, we observed a clear increase of NO production or arginase activity after stimulation with LPS, or cAMP, respectively. This increase in activity suggested that our culture system leads to enrichment for macrophages which are still able to polarise into either innate activated macrophages or alternatively activated macrophages.

For fish macrophages, it may be difficult to distinguish between innate and classically activated macrophages, since LPS sensitivity and subsequent intracellular signalling are recognised to be different from the mammalian situation (Iliev *et al.*, 2005). Fish cells not only require high amounts of LPS for stimulation they also seem to lack several proteins involved in the LPS signalling pathway (Iliev *et al.*, 2005). In fish, LPS is believed to signal via beta-2 integrins and not through toll-like receptor 4 (Iliev *et al.*, 2005). True classical activation of macrophages would also require stimulation with IFNγ. Previous experiments in our laboratory, using macrophage-activating factor (supernatants from mitogen-stimulated cells, believed to contain IFNγ or IFNγ-like proteins) in addition to LPS, did not result in an additional stimulation

of nitrite production, neither in cultured nor in freshly isolated macrophages. We therefore believe the present NO response is due to innate rather than classical activation. Recently, the first fish IFNγ sequences were identified and characterised (Zou *et al.*, 2005; Zou *et al.*, 2004) and, in time, production of recombinant fish IFNγ might allow for a further distinction between innate and classical activation of fish macrophages.

To further assess the polarisation of head kidney-derived macrophages we measured the gene expression of an array of immune-relevant genes after differential stimulation. Gene expression profiles of caMF and aaMF in both human and mouse are relative well studied and extensively reviewed by Mantovani *et al.* (Mantovani *et al.*, 2004; Mantovani *et al.*, 2002). From the differentially expressed genes in carp, TNFα, IL-10, iNOS, CXCa, and NILT2 were significantly higher expressed in LPS-stimulated head kidney-derived macrophages. IL12P40.3, arginase 2, CXCR1 and CXCR2 were significantly higher expressed in cAMP-stimulated head kidney-derived macrophages. The upregulation of TNFα and iNOS after LPS stimulation (innate activation) is similar to the mammalian situation. It is more difficult to compare the CXC gene expression with the mammalian situation, since phylogenetic analyses could not assign carp CXC chemokines according to the human CXC nomenclature (Huising *et al.*, 2003). CXCL8-11 and CXCL-16 are typically upregulated in mammalian caMF, similarly, CXCa was upregulated in the LPS-stimulated head kidney-derived macrophages. The, in comparison, higher gene expression of IL-10 in LPS-stimulated macrophages could be primarily ascribed to a downregulation of IL-10 in cAMP-stimulated macrophages. This is different from the mammalian situation. The expression of IL-10 in fish certainly requires further study (Savan *et al.*, 2003). The expression of IL-12 is difficult to compare between different species since expression and translation of IL-12P35 and IL-12P40 are tightly, but differently, regulated even between human and mouse, as reviewed by Trinchieri (Trinchieri, 2003). Expression can be regulated by both classical activators such as IFNγ, but also by alternative activators like IL-4 and IL-13. Here, we describe an upregulation of IL-12P40.3 gene expression after stimulation with cAMP, an alternative activator of fish macrophages. The upregulation of arginase, CXCR1 and CXCR2 after cAMP stimulation (alternative activation) is comparable to the mammalian situation where CXCR1 and CXCR2 gene expression are upregulated in alternatively activated macrophages (Mantovani *et al.*, 2004). Increased arginase expression in alternatively activated macrophages confirms our previous findings (Joerink *et al.*, 2006d), the increased CXCR1 and CXCR2 gene expression is, however, a

new finding. We suggest that CXCR1 and CXCR2 might be useful new surface markers for alternative macrophage activation in fish.

This is the first report on a differential expression of NILT genes. The recently described NILT genes show significant similarity to the human triggering receptors expressed on myeloid cells (TREM), to CMRF35 and to the natural cytotoxicity receptor NKp44. The location of zebrafish NILT homologues on chromosome 1 at 7 Mb downstream of the MHC class I cluster, suggests that the carp NILT genes are related to either the NKp44 or the TREM genes (Stet *et al.*, 2005). Expression of NKp44 is restricted to NK cells (Vitale *et al.*, 1998), while TREMs are expressed on different cells of the myeloid lineage (Bouchon *et al.*, 2000; Bouchon *et al.*, 2001; Schmid *et al.*, 2002; Washington *et al.*, 2002). Preliminary studies of NILT expression on carp peripheral blood lymphocytes were inconclusive as to ascribe a function to these genes (Stet *et al.*, 2005). Here, we show both NILT1 and NILT2 expression in head kidney-derived macrophages, cells of the myeloid lineage, suggesting a close relationship to TREM.

The TREM gene family comprises at least six members of which TREM-1 and -2 are best studied. TREM-1 expression is upregulated in myeloid cells after LPS stimulation (Bouchon *et al.*, 2000), or during sepsis (Gibot *et al.*, 2005). TREM-2 expression is associated with regulating myeloid lineage development (Cella *et al.*, 2003). TREM-2 is also believed to play a role in chronic inflammation and may stimulate production of constitutive rather than inflammatory chemokines and cytokines (Bouchon *et al.*, 2000; Bouchon *et al.*, 2001). Typically, aaMF are associated with chronic inflammation (Nair *et al.*, 2003). In parallel, TREM-2 was shown to be upregulated during chronic infections of mice with African trypanosomes (Hassanzadeh Ghassabeh *et al.*, 2006), together with other markers for alternative activation (Raes *et al.*, 2005; Raes *et al.*, 2002).

We have shown an upregulation of NILT1 in head kidney-derived macrophages stimulated with cAMP, an inducer of alternative activation in carp macrophages (Joerink *et al.*, 2006d). Furthermore, NILT1 gene expression was upregulated during the later time points of infection with *Trypanosoma carassii*. Recent *in vivo* experiments suggest that, during infection with *T. carassii*, macrophages are predominantly alternatively activated during the late phase of infection (Joerink *et al.*, 2006a). In addition, NILT1 gene expression was hardly upregulated during an infection with *Trypanoplasma borreli*, where macrophages are primarily innate activated (Saeij *et al.*, 2000; Saeij *et al.*, 2002b). Currently we are developing specific antibodies against the NILT and CXCR proteins to

unequivocally assign these proteins as surface markers of fish macrophages. We suggest that the NILTs might be TREM homologues and that NILT1, together with CXCR1 and CXCR2, could likely be used as novel surface markers for alternatively activated macrophages in fish.

Acknowledgements

Chapter 5

Differential macrophage polarisation
during parasitic infections in
common carp (*Cyprinus carpio* L.)

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Fish Shellfish Immunol. (accepted)

Abstract

In many parasitic infections both classically activated macrophages (caMF) and alternatively activated macrophages (aaMF) play a pivotal role. To investigate if both types of macrophages also play an important role during parasitic infections in fish, we infected carp with either *Trypanoplasma borreli* or *Trypanosoma carassii* and determined the activation state of the head kidney leukocytes (HKL). Nitrite production was used as read-out for caMF and arginase activity as read-out for aaMF. Basal nitrite production and arginase activity of HKL were moderately different between the two infections. Differences were observed, however, after *ex vivo* re-stimulation of HKL. Re-stimulation with LPS and *T. borreli* lysates increased nitrite production by HKL of *T. borreli*-infected fish. Re-stimulation with cAMP increased arginase activity in HKL of *T. carassii*-infected fish. Our results indicate that *T. borreli*-infected carp are more prone to increase nitrite production by caMF while *T. carassii*-infected fish are more prone to increase arginase activity by aaMF.

Introduction

Macrophages are one of the most pleiotropic cell types within the immune system. Depending on the (cytokine) environment macrophages can differentiate into a continuum of different activation states with classical activation and alternative activation representing two extremes (Gordon, 2003; Mantovani *et al.*, 2004; Mantovani *et al.*, 2002; Mills *et al.*, 2000; Noël *et al.*, 2004; Sandor *et al.*, 2003). Classically activated macrophages (caMF) play an important role in type I immune responses against intracellular pathogens by the production of reactive oxygen species (ROS) and nitric oxide (NO), and have been well characterised also in teleost fish (Laing *et al.*, 1996; Laing *et al.*, 1999; Neumann *et al.*, 1995; Saeij *et al.*, 2000; Saeij *et al.*, 2002b). Alternatively activated macrophages (aaMF) play an important role in type II immune responses against extracellular pathogens by showing increased phagocytic activity and enhanced gene expression of MHC class II genes (Gordon, 2003; Mantovani *et al.*, 2004; Mantovani *et al.*, 2002; Mills *et al.*, 2000; Noël *et al.*, 2004; Sandor *et al.*, 2003). Recently, we described the existence of aaMF in carp (*Cyprinus carpio* L.) (Joerink *et al.*, 2006d). In this study, we identified the genes for both arginase 1 and 2 and we used inducible nitric oxide synthase (iNOS) activity and arginase activity as measures for the classical and alternative activation of head kidney-derived macrophages, respectively. We demonstrated a classical activation of macrophages after LPS stimulation and an alternative activation of macrophages after cAMP stimulation. To continue this initial *in vitro* study we now examined the role of caMF and aaMF *in vivo* during infection with two related parasites.

During a parasitic infection, macrophages are among the first host immune cells to encounter the infecting parasites and their derived products. The subsequent activation state of these macrophages plays a major role in the further development of the immune response and infection. In many parasitic infections the early phase is characterised by the presence of caMF and the production of the antimicrobial compound NO. Often, the later phase is characterised by the presence of aaMF and related arginase activity (reviewed by Noël *et al.*, 2004). Alternatively, in some parasitic infections one type of macrophage prevails, such as during infections with the helminth *Brugia malayi* where macrophages are preferentially alternatively activated (Noël *et al.*, 2004), or during *Leishmania major* infections where parasites are cleared by caMF (von Stebut and Udey, 2004).

So far, it remains unclear whether the induction of aaMF is beneficial to host or parasite. On one hand, the induction of aaMF downregulates the initial activation

of caMF characterised by high iNOS activity, subsequent NO production and accompanying inflammation, thereby minimising immune-related pathology. On the other hand, the induction of aaMF can be beneficial for parasite development, enabling chronic infections instead of parasite clearance. For example, intracellular (*Leishmania*) as well as extracellular (*Trypanosoma brucei*) parasites can benefit from the induction of arginase in aaMF (Gobert *et al.*, 2000; Iniesta *et al.*, 2005). Arginase activity depletes cells of L-arginine, while L-arginine is also the substrate of iNOS. In addition, arginase activity results in the production of L-ornithine which is a precursor of the polyamines involved in DNA and trypanothione synthesis. Parasite trypanothione and its related enzymes are involved in the defence against damage by oxidants (Fairlamb and Cerami, 1992).

Trypanoplasma borreli and *Trypanosoma carassii* (Syn. *T. danilewskyi*, (Lom and Dyková, 1992)), the parasite species used in the present study, are extracellular kinetoplastid protozoan parasites that are transmitted by blood-sucking leeches. The common carp is one of their natural hosts and while *T. borreli* and *T. carassii* infections are widespread in nature they cause mortality only in intensive aquaculture (Lom, 1979). Experimental infections with *T. borreli* result in mortalities varying between 0 and 100%, depending on the carp strain used (Jones *et al.*, 1993; Wiegertjes *et al.*, 1995). Experimental infections with T. carassii cause mortalities varying between 60 and 100% in goldfish (Lom, 1979). The high but practically unnoticed prevalence in nature suggests a balanced evolution of these parasites and their fish hosts. In the laboratory, parasite dose and route can be tightly controlled by injection, which allows us to study the host-parasite balance in more detail (Wiegertjes *et al.*, 2005).

Previous studies showed different responses of carp against the two parasite species with regard to nitrite production (Saeij *et al.*, 2002b; Scharsack *et al.*, 2003). *T. borreli* was shown to induce a high amount of nitrite production by head kidney leukocytes, both *in vitro* and *in vivo*. In contrast, *T. carassii* did not induce nitrite production by head kidney leukocytes *in vitro* nor did serum nitrite levels increase in *T. carassii*-infected carp (Saeij *et al.*, 2002b). Additionally, in general, *T. borreli* infections are more severe than infections of carp with *T. carassii*. The observation that the induction of caMF with accompanying nitrite production is different between both infections, suggests that the presence of aaMF with accompanying arginase activity, could also be different between both infections. To test this hypothesis we infected carp with either *T. borreli* or *T. carassii* and followed the activation state of their head kidney leukocytes *ex vivo*.

Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility 'De Haar-Vissen' at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. R3 x R8 heterozygous carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). Carp were 9 months old at the start of the experiment with an average weight of 120 g. All studies were performed with approval from the animal experimental committee of Wageningen University.

Parasites

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (Steinhagen *et al.*, 1989). *Trypanosoma carassii* was cloned and characterised by Overath *et al.* (Overath *et al.*, 1998). Both parasites were maintained by syringe passage through carp, with intervals of three weeks. Parasitaemia was monitored using a Bürker counting chamber. The minimum detection limit by this method was 10^4 parasites/ml. Parasites were cultured as described by Steinhagen *et al.* (Steinhagen *et al.*, 2000) for *T. borreli* and by Overath *et al.* (Overath *et al.*, 1998) for *T. carassii*. Parasite lysates were made by washing cultured parasites once in sterile PBS, resuspending them ($5x10^7$ parasites/ml) in RPMI 1640 (Cambrex) and lysing them by sonication (3 min). The lysates were aliquoted and stored at -80°C until use.

Arginase activity and nitrite production

Arginase activity was measured essentially as described by Corraliza *et al.* (Corraliza *et al.*, 1994): cells were lysed in 50 µl 0.1% Triton X-100 containing 5 µg pepstatin, 5 µg aprotinin and 5 µg antipain at room temperature for 30 min. Fifty µl of 10 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.5 was added and the mixture was incubated for 10 min at 55°C. To 50 µl of this activated lysate 50 µl of 0.5 M L-arginine, pH 9.7 was added and incubated for 1 h at 37°C. Reaction was stopped by adding 400 µl acid mixture containing H_2SO_4, H_3PO_4 and H_2O (1:3:7). Then, to each reaction 25 µl 9% α-isonitrosopropiophenone (in 100% ethanol) was added and incubated for 45 min at 100°C. After 10 min cooling in the dark the absorbance was read at 540 nm and arginase activity (mU per million cells, mU= nmol urea formed per min) was calculated by comparison with a urea standard curve.

Nitrite production was measured essentially as described by Green *et al.* (Green *et al.*, 1982): to 75 µl cell culture supernatant 100 µl 1% (w/v) sulphanilamide

in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentrations (µM) were calculated by comparison with a sodium nitrite standard curve.

Experimental set up

At least three weeks before the start of the experiment fish were moved to the infection quarantine facilities and acclimatised to 20 ±2°C. At the day of infection 2 to 4 fish were kept separate and sampled as uninfected controls. All other carp were intraperitoneally (i.p.) injected with 100 µl RPMI 1640 (Cambrex) containing 10,000 parasites (*T. borreli* or *T. carassii*) per fish. Parasites for this infection were obtained from a three weeks earlier infected carp. At different time points after the start of infection 2 to 4 fish were sampled. Both *T. borreli* and *T. carassii* infections were repeated at least once and data are shown for 2 to 8 fishes per time point.

At time of sampling, carp were euthanized with 0.25 g/l tricaine methane sulfonate (Crescent Research Chemicals) buffered with 0.38 g/l NaHCO$_3$, bled from the caudal vein by a syringe containing heparinised (50 U/ml Leo Pharma BV) RPMI 1640 (Cambrex), and head kidneys were aseptically removed. Blood was used to determine the parasitaemia using a Bürker counting chamber. A small part of the head kidney was cut off, snap frozen in liquid nitrogen and stored at -80°C for RNA isolation (see next section). The remainder of the head kidney was used to isolate head kidney leukocytes (HKL). Head kidneys were gently passed through a 100-µm sterile nylon mesh and rinsed with homogenisation buffer [incomplete-NMGFL-15 medium containing 50 mg/ml gentamicin, 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 U/ml heparin] (Neumann *et al.*, 1998). Cell suspensions were layered on 51% (1.071 g·cm^{-3}) Percoll (Amersham Pharmacia Biotech AB) and centrifuged at 450 x *g* for 25 min at 4°C. Cells, HKL at the medium/Percoll interface were collected and washed twice.

Freshly isolated HKL (5x10^5) were seeded in 100 µl rich-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% bovine calf serum (Invitrogen)] in wells of a 96-well flat-bottom culture plate. Cells were either left untreated to determine the basal *ex vivo* arginase activity and nitrite production or stimulated with cAMP (0.5 mg/ml), LPS (50 µg/ml), or 5 µl parasite lysate (equivalent of 2.5x10^5 parasites per well) and incubated at 27°C. Arginase activity was measured (as

described above) after 18 h of incubation, nitrite production was measured (as described above) after 72 h of incubation.

RNA isolation, DNase treatment, and first strand cDNA synthesis
RNA was isolated from four head kidneys per time point, of the first *T. carassii* infection and from five head kidneys per time point, of a separate *T. borreli* infection. RNA was isolated from approximately 27 mm^3 per organ using the RNeasy Mini Kit (Qiagen, Leusden) including the accompanying DNase I treatment on the columns, according to the manufacturer's protocol. End elution was performed with 40 µl diethyl pyrocarbonate (DEPC)-treated water. RNA concentrations were measured by spectrophotometry (Genequant, Amersham Pharmacia Biotech) and 1 µl was analysed on a 1% agarose gel to check the integrity. RNA was stored at -80°C until further use. For each cDNA synthesis a negative sample (non-RT), to which no reverse transcriptase was added and a positive sample containing the reverse transcriptase were included. After DNAse treatment, 1 µg of total RNA was combined with, random primers (300 ng Invitrogen) and 1 µl dNTP mix (10 mM) and the mix was incubated at 65°C for 6 min and for 1 min on ice. A volume of 4 µl First Strand buffer (5x), 1 µl DTT (0.1 M) and 1 µl RNase inhibitor (40 U/µl Invitrogen) were added. To each positive sample, 1 µl Superscript RNase H⁻ Reverse Transcriptase III (200 U/µl Invitrogen) was added. To each negative sample 1 µl DEPC-treated water was added. All samples were incubated at 25°C for 5 min followed by 1 h at 50°C. Reactions were inactivated by incubating the samples at 70°C for 15 min. MilliQ water up to 100 µl was added to each sample and cDNA stored at -20°C until further use.

Real-time quantitative PCR
Specific real-time quantitative PCR primers (Table 5.1) were designed with Primer Express software (Applied Biosystems). To 5 µl of 10 times-diluted cDNA, 7 µl Sybr Green Master Mix (Stratagene), forward and reverse primer (300 nM each) and MilliQ water up to 14 µl was added. Quantitative PCR was performed in a 72-well Rotor-Gene™ centrifugal real-time thermal cycler (Rotor-Gene 2000, Corbett Research). Following cycling conditions were used: one holding step of 10 min at 95°C; followed by 40 cycles of 20 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C; an incubation for 1 min at 60°C was followed by a melting curve from 60°C to 99°C in steps of 1°C with 5 sec waiting. At the end of each cycle and during the waiting steps in the melting curve fluorescence intensities were measured. Raw data were analysed using

the comparative quantitation of the Rotor-Gene Analysis Software V5.0. Data were further analysed using the Pfaffl method (Pfaffl, 2001), using average efficiencies per run per gene. Gene expression of 40S in each sample was used to normalise the data. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked at least once by sequencing.

Table 5.1
Primer sequences corresponding accession numbers (Acc. No.), real-time quantitative PCR melting temperatures and efficiencies (Eff.).

Gene	Acc. No.	Sense (5'-3')		
		Antisense (5'-3')	Melt temp	Eff.
iNOS	**AJ242906**	AACAGGTCTGAAAGGGAATCCA		
		CATTATCTCTCATGTCCAGAGTCTCTTCT	77.7	1.69
Arginase 1	**AJ871264**	TGAGGAGCTTCAGCGGATTAC		
		CCTATTATTCCCACGCAGTGATG	77.3	1.76
Arginase 2	&	GGAGACCTGGCCTTCAAGCATCT		
		CTGATTGGCACGTCCAACT	80.3	1.73
40S	**AB012087**	CCGTGGGTGACATCGTTACA		
		TCAGGACATTGAACCTCACTGTCT	78.5	1.77

& there are three arginase 2 isoforms known **AJ618955**/**AJ871265**/**AJ871266** this primer set amplifies all three.

Statistics

Arginase activity and nitrite production data was log transformed and tested for significant effect of week by using analysis of variance (ANOVA). Differences between weeks were considered significant when ANOVA's F-test was less than 5%. Subsequently, significance of the contrasts between means per week and the control were assessed by pair wise comparisons using Student's t-test with a Dunnett correction for multiple comparisons. $P \leq 0.05$ was accepted as significant. In case of re-stimulation data from uninfected and 1 week after infection were combined. Statistical analysis was carried out using SAS software (version 9.1). Expression differences were tested for significance by Student's t-test. $P \leq 0.05$ was accepted as significant.

Results

Basal nitrite production and arginase activity are different between T. borreli- *and* T. carassii-*infected fish*

In this set of experiments parasite levels during the peak of parasitaemia were comparable in number between *T. borreli* and *T. carassii* infection, development of infection was however different for each parasite species (Fig. 5.1A). Parasitaemia of *T. borreli*-infected fish peaked at week 3-4 post-infection, and parasites were undetectable 8 weeks post-infection. During *T. carassii* infection, parasites could be detected from week 2 until at least week 8 post-infection, without a clear peak response, parasites were undetectable 10 weeks post-infection.
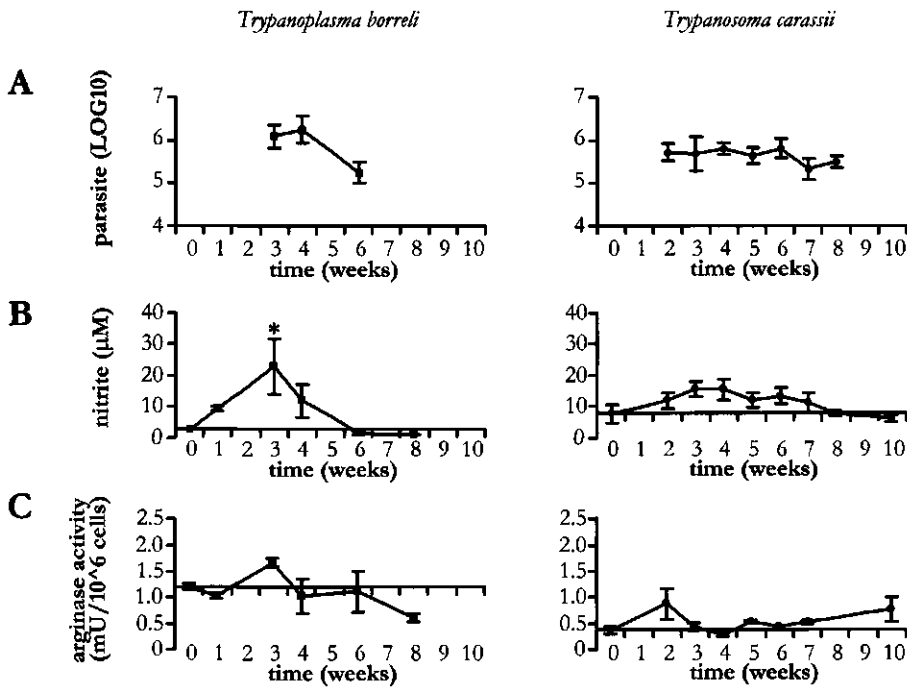
Fig. 5.1 Parasitaemia (A), basal *ex vivo* nitrite production (B), and basal *ex vivo* arginase activity (C) of *Trypanoplasma borreli* (■) or *Trypanosoma carassii* (•) infected fish. Carp were i.p. infected with 10,000 parasites per fish or left untreated as control (week 0). At indicated time points n=2-8 fish were sacrificed, blood samples were collected and head kidneys removed. Parasitaemia was determined with a Bürker counting chamber. Head kidney leukocytes were isolated and incubated at 27°C. After 18 h of incubation arginase activity was determined and after 72 h nitrite production was measured. Data points represent average ± SEM of n=2-8 fish per time point. *T. borreli* infection: W0 n=2; W1 n=2; W3 n=4; W4 n=3; W6 n=4 and W8 n=2. *T. carassii* infection: W0 n=4; W2 n=8; W3 n=7; W4 n=8; W5 n=4 W6 n=4, W7 n=4; W8 n=4 and W10 n=4. * $P \leq 0.05$ Student's *t*-test compared to uninfected controls.

To assess the activation state of head kidney leukocytes, basal nitrite production and arginase activity were measured from uninfected, and *T. borreli*- or *T. carassii*-infected fish (Fig. 5.1B and C). During *T. borreli* infection, nitrite production increased with a significant difference at week 3. Nitrite production significantly correlated with increasing parasitaemia (df $_{1,10}$ F=13.53 P=0.004 R^2=0.58). No significant correlation was found between *T. borreli* parasitaemia and arginase activity (P=0.45) or between *T. carassii* parasitaemia and nitrite production (P=0.59) or arginase activity (P=0.87). During *T. carassii* infection, basal nitrite production was slightly higher but never significantly different from the nitrite levels in uninfected control fish.

During *T. borreli* and *T. carassii* infection, arginase activity did not significantly change compared to basal levels in uninfected control fish. Variation between individuals was sometimes high.

Gene expression of iNOS, arginase 1 and arginase 2 confirm the basal nitrite production and arginase activity in the T. borreli- *and* T. carassii-*infected fish*

During infection experiments a small part of the head kidney was snap-frozen in liquid nitrogen and stored at -80°C. RNA was isolated from this material, and cDNA was synthesised which was used as template for real-time quantitative PCR. Gene expression was determined for iNOS, arginase 1 and arginase 2 and 40S as a house keeping gene.

Expression of iNOS was significantly upregulated during the third week of infection with *T. borreli*. Expression was still high in the fourth week although, owing to high individual variation, not significantly different from week 0 (25 times control level, P=0.12). During infection with *T. carassii* no significant regulation of iNOS was detected (Fig. 5.2A). The expression of both arginase 1 and 2 were moderately regulated during both parasite infections. Arginase 1 gene expression (Fig. 5.2B) was upregulated at some time points, while arginase 2 (Fig. 5.2C) gene expression was downregulated especially during the early weeks of infection with *T. carassii*.

Trypanoplasma borreli   *Trypanosoma carassii*
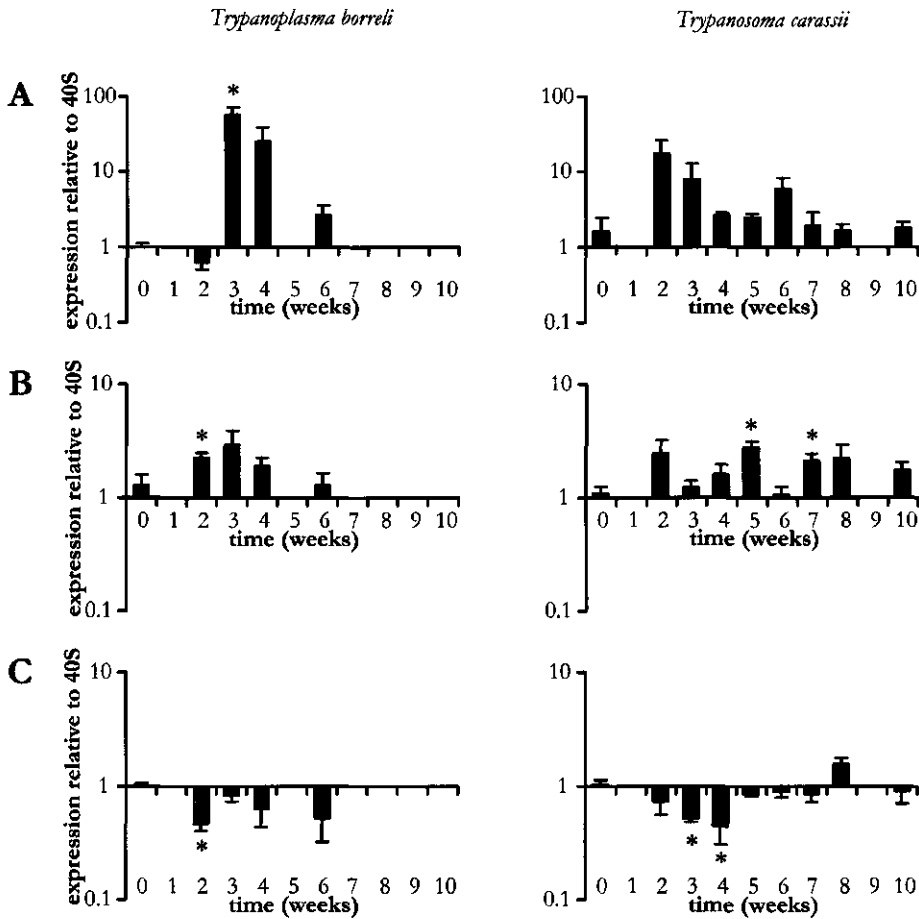
Fig. 5.2 Gene expression of iNOS (A), arginase 1 (B), and arginase 2 (C) in head kidney (whole organ) of *Trypanoplasma borreli*- or *Trypanosoma carassii*-infected carp. Carp were i.p. infected with 10,000 parasites per fish or left untreated as control (week 0). At indicated time points n=5 (*T. borreli*) or n=4 fish (*T. carassii*) were sacrificed, head kidneys were removed and snap frozen in liquid nitrogen. cDNA was synthesised from 1 μg RNA per sample. Gene expression was measured by means of real-time quantitative PCR and is shown relative to the gene expression of 40S, on a logarithmic scale. Averages ± SEM of n=4-5 fish are given. * $P \leq 0.05$ Student's *t*-test compared to uninfected controls.
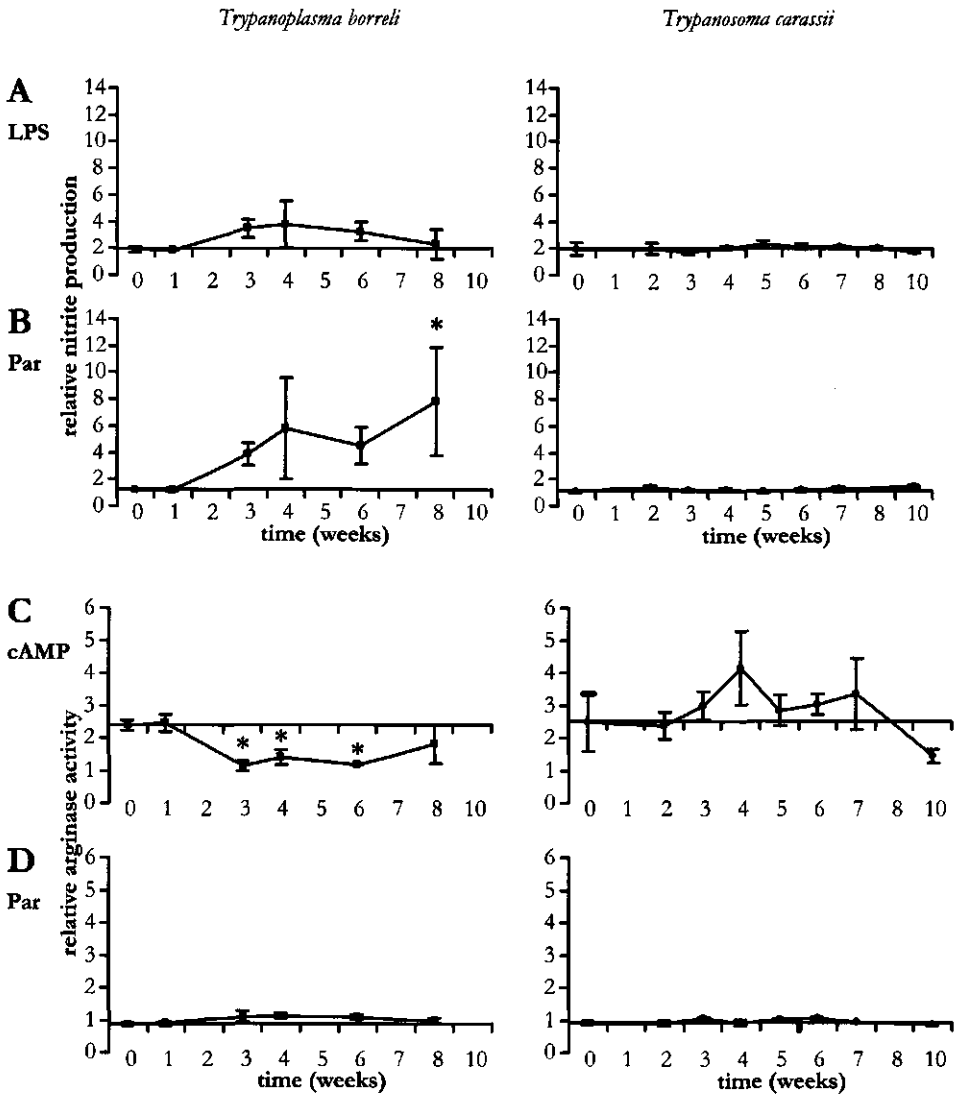
Fig. 5.3 Nitrite production (A and B), and arginase activity (C and D) of head kidney leukocytes of *Trypanoplasma borreli*- (■) or *Trypanosoma carassii*-infected (•) carp, re-stimulated with LPS (50 μg/ml), cAMP (0.5 mg/ml) or homologues parasite lysate (equivalent of 2.5×10^5 parasites per well). Carp were i.p. infected with 10,000 parasites per fish or left untreated as control (week 0). At indicated time points n=2-8 fish were sacrificed and head kidneys removed. Head kidney leukocytes were isolated, re-stimulated and incubated at 27°C. After 18 h of incubation arginase activity was determined and after 72 h nitrite production was measured. Data are expressed relative to unstimulated head kidney leukocytes. Data points represent average ± SEM of n=2-8 fish per time point. *T. borreli* infection: W0 n=2; W1 n=2; W3 n=4; W4 n=3; W6 n=4 and W8 n=2. *T. carassii* infection: W0 n=4; W2 n=8; W3 n=7; W4 n=8; W5 n=4 W6 n=4, W7 n=4; W8 n=4 and W10 n=4. * $P\leq0.05$ Student's *t*-test compared to uninfected controls.

Ex vivo *re-stimulation emphasises the* in vivo *polarisation effects*

Head kidney leukocytes from infected fish were re-stimulated with LPS, cAMP or parasite lysates to assess the potential to increase nitrite production or arginase activity. Increased nitrite production could be measured after re-stimulation with LPS, but also after re-stimulation with the corresponding parasite lysate. LPS re-stimulation (Fig. 5.3A) doubled the nitrite production both in uninfected fish and in *T. carassii*-infected fish. In *T. borreli*-infected fish, LPS re-stimulation increased nitrite production up to 4 times, although not significantly because of individual differences. Re-stimulation of HKL of *T. borreli*-infected fish with *T. borreli* lysates significantly induced more nitrite at week 8 post-infection (*P* values at week 4 and 6, 0.09). Re-stimulation of HKL of *T. carassii*-infected fish with *T. carassii* lysates, did not increase nitrite production (Fig. 5.3B).

Arginase activity was measured after re-stimulation with cAMP or corresponding parasite lysate. cAMP re-stimulation increased arginase activity 2-3-fold in HKL from uninfected control fish. Arginase activity in response to cAMP re-stimulation could not be elevated during *T. borreli* infection (Fig. 5.3C). This unresponsiveness was significantly different from the effect on uninfected fish. At week 8 post-infection, when *T. borreli* could no longer be detected, the ability of cAMP to increase arginase activity was restored. In contrast, HKL from *T. carassii*-infected fish were responsive to cAMP. In HKL from these fish, arginase activity could be increased three to four times compared to the unstimulated cells. At week 10 of the *T. carassii* infection, when no parasites could be detected anymore, cAMP could hardly increase arginase activity. Parasite lysates were never able to induce any arginase activity, either in the uninfected or in the infected fish (Fig. 5.3D).

Discussion

Here we show that carp macrophages can differentially polarise upon infection with parasites. During a *Trypanoplasma borreli* infection nitrite production was elevated and increased after re-stimulation with LPS or *T. borreli* lysates, indicating the presence of caMF. During a *Trypanosoma carassii* infection re-stimulation with cAMP could elevate the arginase activity, indicating the presence of aaMF.

Macrophages can be activated via different pathways, leading to either a classical or an alternative state of activation (Gordon, 2003; Mills *et al.*, 2000). These two activation states are considered to be the extremes of a continuum (Mantovani *et al.*, 2004). caMF are relatively well studied also in teleost fish and

can be characterised by their nitrite production (Laing *et al.*, 1996; Laing *et al.*, 1999; Neumann *et al.*, 1995; Saeij *et al.*, 2000; Saeij *et al.*, 2002b). Lately, attention focuses on aaMF and their role in parasitic infections (reviewed by Noël *et al.*, 2004). The best described features of aaMF are their enhanced endocytosis and phagocytosis capacity, their increased production of factors involved in tissue remodelling and repair and their ability to inhibit type I inflammation (Noël *et al.*, 2004). We recently showed that carp macrophages increased their arginase activity, a measure of alternative activation, in response to cAMP (Joerink *et al.*, 2006d). In addition, we have found that not only arginase 2 gene expression, but also gene expression of IL-1β, IL12-P40.3, CXCR1, CXCR2 and NILT1 could be upregulated in cAMP stimulated / alternatively activated head kidney-derived macrophages (Joerink *et al.*, 2006c). To assess the relevance of this alternative activation *in vivo* we infected carp with either *T. borreli* or *T. carassii* and followed nitrite production and arginase activity of the head kidney leukocytes (HKL) *ex vivo*.

Previous experiments in our laboratory by Seaij *et al.* (Saeij *et al.*, 2002b) showed differences in the immune response of carp to *T. borreli* and *T. carassii* with regard to their ability to induce nitrite production. It was shown that HKL stimulated *in vitro* with *T. borreli* lysates produced high amounts of nitrite. *In vivo*, serum nitrite levels of *T. borreli*-infected fish were found to be high. *T. carassii* lysates, however, were not able to induce nitrite production. Serum nitrite levels of *T. carassii*-infected fish remained low during infection as well. The initial finding that the presence of caMF was different between both infections let us to hypothesise that the presence of aaMF could also be different between both infections. Hence our choice for *in vivo* experiments with *T. borreli* and *T. carassii* as infectious agents.

Basal *ex vivo* nitrite production and arginase activity of HKL of infected animals showed a first indication of differences between *T. borreli*- and *T. carassii*-infected carp (Fig. 5.1). The most striking difference between the arginase activity of the two infections was a decreased arginase activity at the end of *T. borreli* infection, at which time point there was an increased arginase activity during *T. carassii* infection. Although the differences were small, this suggested a higher number of aaMF in *T. carassii*-infected fish compared to *T. borreli*-infected fish.

Real-time quantitative gene expression data (Fig. 5.2) confirmed basal *ex vivo* nitrite production and arginase activity. iNOS expression was upregulated at week 3 and 4 of *T. borreli* infection, at the same time point the peak of nitrite production was observed. In contrast, during *T. carassii* infection, iNOS

expression and thus nitrite production were not significantly induced. We studied the expression of both the arginase 1 and arginase 2 gene. Arginase activity is the additive result of arginase 1 and 2 gene expression, making interpretation more difficult. Arginase 1 gene expression was low (take off value between 30 and 33) while arginase 2 gene expression was intermediate (take off value between 21 and 24). Their regulation was only modest. During the first weeks of both the *T. borreli* and *T. carassii* infection we observed an increased basal arginase activity (Fig. 5.1C). This early activity was most likely caused by arginase 1, since gene expression of arginase 1 remained unchanged or was upregulated during the first 2-4 weeks of both infections while arginase 2 gene expression was downregulated (Fig. 5.2). At week 6 of the *T. borreli* infection, when parasites were still present, both arginase 1 and 2 gene expression were equal to the expression in uninfected fish. During the later time points of the *T. carassii* infection, preferentially, arginase 1 gene expression was upregulated, while arginase 2 gene expression returned to basal values. This indicated that elevated basal *ex vivo* arginase activities, measured during infections, primarily could be ascribed to arginase 1 gene expression. This seems in contrast with previous observations where we found arginase 2 to be preferentially upregulated in cAMP-stimulated head kidney-derived macrophages. The stimuli used in these studies are, however, different. Most likely, arginase 1 and 2 gene expression are preferentially induced by different stimuli. Currently we are re-evaluating the role of arginase 1 and 2 gene expression and related arginase activity in aaMF of carp. In mice, *in vivo* inhibition of arginase activity during *Leishmania major* infection showed a major role for arginase 1 in promoting parasite growth (Kropf *et al.*, 2005). A similar study in carp could shed more light on the relation between gene expression and arginase activity in fish.

The most clear differences between the two infections were observed when analysing nitrite production and arginase activity after re-stimulation of HKL with LPS, cAMP or corresponding parasite lysates (Fig. 5.3). Only in *T. borreli*-infected fish, LPS re-stimulation increased nitrite production up to 3-4-fold the levels in unstimulated cells. When HKL of infected fish were re-stimulated with the respective parasite lysates, differences were more pronounced. Lysates of *T. borreli* induced up to 8 times more nitrite. This indicated that HKL of *T. borreli*-infected fish were more prone to produce nitrite, suggesting the presence of caMF.

Differences in arginase activity after re-stimulation of HKL by cAMP were observed as well. In *T. borreli*-infected animals cAMP-induced arginase activity

was severely and significantly hampered during infection, but returned to basal values at week 8 when parasites were undetectable again. In contrast, in *T. carassii*-infected animals, cAMP re-stimulation could still induce arginase activity up to four times the levels in unstimulated cells. This indicated that HKL of *T. carassii*-infected fish were more prone to increase arginase activity, suggesting the presence of aaMF. The higher number of aaMF in *T. carassii*-infected fish, downregulating the inflammation, is in line with our observation that *T. carassii* infections are less severe than *T. borreli* infections. Increased endocytosis and phagocytosis capacity of aaMF together with the increased expression of MHC class II molecules could also have a positive effect on the production of antibodies, which are important for host survival (Jones *et al.*, 1993; Saeij *et al.*, 2003a).

Interestingly, parasite lysates were never able to re-stimulate arginase activity, while nitric oxide production could be induced. A first explanation might be that live and dead parasites induce different responses. This was also shown for the helminth *Brugia malayi* where dead parasites preferentially induced caMF, and excretory products of living parasites preferentially induced aaMF (Allen and Loke, 2001). A second explanation could be a lack of sensitivity of freshly isolated HKL to respond to parasite lysates. Previous observations showed that cultured head kidney-derived macrophages are more responsive towards cAMP stimulation than freshly isolated HKL (Joerink *et al.*, 2006d). Culturing the cells would however have reduced the direct effects of the infection.

When isolating the HKL, granulocytes and lymphocytes are co-purified with the monocytes and macrophages. It could be that the granulocytes, which are expected to have iNOS activity (Scharsack *et al.*, 2003), affect the NO production or arginase activity measured.

In our experiments it was easier to measure a caMF response than an aaMF response. It is not clear exactly which receptors are involved in the polarisation of macrophages. Depending on the cytokine environment, however, macrophages can differentiate into a continuum of activation states. Inducers of caMF, like IL-1β and TNFα can be produced by macrophages as well as by T_H1 cells. Inducers of aaMF such as IL-4 an IL-13 are mainly produced by T_H2 cells and not macrophages. In our HKL, T cells numbers are low and alternative activation by cytokines, especially, could therefore be hampered.

Nevertheless, the *in vivo* *T. borreli* infection as well as the *ex vivo* re-stimulation of HKL with *T. borreli* lysates are suitable models to study caMF. Infection with *T. carassii* seems a less appropriate model to study aaMF. Possibly, another infection model such as that of carp infected with *Sanguinicola inermis* could give

further information on the functioning of aaMF in carp. During infections with this particular parasite granulomas are formed around the eggs trapped in immune organs (Richards *et al.*, 1994). Similar granulomas are induced by *Schistosoma mansoni*. For this latter parasite, it has been shown that aaMF are essential for host survival (Herbert *et al.*, 2004).

In conclusion, our results indicate that *T. borreli*-infected carp are more prone to increase nitrite production by caMF while *T. carassii*-infected fish are more prone to increase arginase activity by aaMF.

Acknowledgements

Chapter 6

Mixed infection with *Trypanoplasma borreli* and *Trypanosoma carassii* induces protection: involvement of cross-reactive antibodies

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Submitted

Abstract

Mixed infections of *Trypanoplasma borreli* and *Trypanosoma carassii* in common carp (*Cyprinus carpio* L.) are commonly found in nature. So far, only mono-parasitic infections have been examined in more detail and indicated that infections with *T. borreli* are more severe than infections with *T. carassii*. We studied the influence of mixed rather than mono-parasitic infections on the protective immune response in this naturally-occurring host-parasite combination. Mixed infections were obtained by i.p. injection of fixed numbers of both parasite species and confirmed by species-specific antibody staining. Species-specific parasitaemia was determined by morphological differences and by real-time quantitative PCR. *T. carassii* parasitaemia always developed prior to *T. borreli*. In mixed infections, peak parasitaemia levels and to a lesser extend mortality were reduced compared to *T. borreli* mono-parasitic infections. Cross-reactive antibodies increased earlier and to higher levels in mixed infected fish than in *T. borreli* mono-parasitic infections. Carp surviving a *T. carassii* infection showed reduced susceptibility to re-infection with *T. borreli*. Our data indicate a protective effect of co-infection with *T. carassii* on the resistance to *T. borreli*, possibly mediated via cross-reactive antibodies. We suggest an evolutionary advantage for a co-evolution of *T. borreli* and *T. carassii* in carp.

Introduction

Owing to the movement of infected livestock, the geographical areas where different human African trypanosomes prevail are expanding, and it is very likely that in coming years mixed infections will occur in humans (Picozzi *et al.*, 2005). It is unclear whether such mixed infections might lead to more severe health problems or result in cross-protective immune responses. So far, experimental research has concentrated on mixed infections with different strains of the same trypanosoma species (Anez *et al.*, 2004; Cheesman *et al.*, 2006; Franco *et al.*, 2003; Marcet *et al.*, 2006), rather than on mixed infections with different kinetoplastid species. Mixed infections of different kinetoplastid species have, however, been demonstrated in flies (Jamonneau *et al.*, 2004; Kubi *et al.*, 2005), pigs (Jamonneau *et al.*, 2004), cattle (Van den Bossche *et al.*, 2004a) and fish (Lom and Dyková, 1992). Common carp (*Cyprinus carpio* L.) is the natural host of *Trypanoplasma borreli* and *Trypanosoma carassii* (syn. *Trypanosoma danilewskyi* (Lom and Dyková, 1992)), two kinetoplastid parasites that diverged more than 500 million years ago (Fernandes *et al.*, 1993). *T. borreli* belongs to the family of parabodonida and *T. carassii* to the family trypanosomatida (Simpson *et al.*, 2006). Blood-sucking leeches *Piscicola geometra* or *Hemiclepsis marginata* act as vectors for transmitting kinetoplastid parasites between cyprinid fish and many carp will in fact carry mixed populations of these two parasite species (Lom and Dyková, 1992).

Mono-parasitic infections with *T. borreli* and *T. carassii* have been shown to induce different immune responses of carp against these two parasites. While *T. borreli* preferentially induces the production of NO and the development of classically-activated macrophages, *T. carassii* does not induce the production of NO but rather stimulates an increased arginase activity involved in the alternative activation of macrophages (Joerink *et al.*, 2006a; Saeij *et al.*, 2002b). So far, only the mono-parasitic infections have been examined in more detail. To study the influence of mixed rather than mono-parasitic infections on the induced immune responses, we infected carp with a mixture of *T. borreli* and *T. carassii* and followed the parasitaemia of these fish over time. For a period up to ten weeks, blood samples were taken weekly to determine parasitaemia and antibody titers. Fish receiving a mixed infection had significantly lower peak parasitaemia levels than fish with a mono-parasitic *T. borreli* infection. Cross-protective antibodies were detected in serum by ELISA and Western blotting. Carp surviving *T. carassii* infection showed reduced susceptibility to re-infection with *T. borreli*, suggesting a long-lasting protective effect of the cross-reactive antibodies.

Our data indicate a protective effect of co-infection with *T. carassii* on the resistance to *T. borreli*, possibly mediated via cross-reactive antibodies. We suggest an evolutionary advantage for a co-evolution of *T. borreli* and *T. carassii* in carp. This would be beneficial to the carp host because mixed infections resulted in lower peak parasitaemia levels and increased survival. It would also be beneficial to *T. borreli* because mixed-infected fish have a better chance of survival than mono-parasitic *T. borreli*-infected fish, increasing the chances for transmission.

Materials en Methods

Animals

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility 'De Haar-Vissen' at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. Carp (R3 x R8) (Irnazarow, 1995) were 9 months old at the start of the experiment with an average weight of 120 g. All studies were performed with approval from the animal experimental committee of Wageningen University.

Parasites

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (Steinhagen *et al.*, 1989). *Trypanosoma carassii* was cloned and characterised by Overath *et al.* (Overath *et al.*, 1998). Both parasites were maintained by syringe passage through carp, with intervals of three weeks. Parasitaemia levels were monitored using a Bürker counting chamber. The minimum detection limit by this method was 10^4 parasites/ml. Parasite lysates, for coating of ELISA plates, were made by washing parasites once in sterile PBS, resuspending them (5×10^7 parasites/ml) in RPMI 1640 (Cambrex) and lysing by sonication (3 min). The lysates were aliquoted and stored at -80°C until use.

In vivo infection of carp with T. borreli and T. carassii

Three weeks before the start of the experiment fish were moved to the infection quarantine facilities and acclimatised to 20 ±2°C. All fish were individually numbered by tattoo for recognition during the experiment. Fish were randomly divided over the experimental groups and equally divided over four replicate tanks. At the day of infection carp were i.p. injected with PBS as a negative control or with a mixed or mono-parasitic infection with *T. borreli* or *T. carassii*. Injection volume was 100 µl for all different groups.

From week 2 onwards a weekly sample of 200 μl blood was taken from the caudal vein of each fish with a syringe containing 200 μl of EDTA (2 mM) RPMI 1640 (Cambrex).

Fish were checked daily and removed from the experiment when lethargic to prevent unnecessary suffering. Number of fish removed was recorded and used to calculate the survival.

Re-infection with heterologous parasite species

Thirteen weeks after the initial infection n=14 carp, which had recovered from *T. carassii* infection, were re-infected with 5,000 *T. borreli* per fish. Three naïve fish served as controls. Parasitaemia levels were monitored using a Bürker counting chamber from week 15 until week 19 (week 2 until week 6 of the re-infection).

Antibody staining

To confirm that fish receiving a mixed infection developed a mixed parasitaemia, blood smears were made and stained with *T. borreli-* and *T. carassii*-specific antibodies. *T. borreli*-specific antibodies were raised against *T. borreli* grown for 3 days in serum-free culture medium and purified by anion-exchange (Overath *et al.*, 1998). A number of $9x10^7$ parasites were emulsified 1:1 with Specol and injected into a rabbit. Two weeks after a booster injection with $5x10^7$ parasites, immune serum was collected. Rabbit antiserum was preabsorbed against carp red blood cells and against *T. carasssii*. Serum was used at a 1:750 dilution. Culture supernatant of a rat hybridoma cell line D5D5 producing a monoclonal antibody against *T. carassii* (Lischke *et al.*, 2000) was used at a 1:1 dilution.

Blood smears where made with 25 μl of blood on duplicate poly-L-lysine coated slides, slides were air dried for 15 min and fixed in ice cold acetone for 7 min. Incubations with antibodies were performed at room temperature for 1 h, with two PBS-T (PBS with 0.1% triton X-100) washing steps in between. Secondary antibodies used were a goat anti-rat (1:200) conjugated with horseradish peroxidase (HRP, Dako) and goat anti-rabbit (1:200) conjugated with alkaline phophatase (AP, Dako). Substrates used were diaminobenzidine tetrahydrochloride (DAB) giving a brown/red colouration and Nitro-Blue Tetrazolium Chloride/ 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (NBT/BCIP) giving a blue colouration.

Counting

Parasitaemia levels were monitored using a Bürker counting chamber. The minimum detection limit by this method was 10^4 parasites/mL. To determine the species-specific parasitaemia of the two parasite species separately in fish receiving a mixed infection two different methods were used: (i) distinction was made during counting of live parasites with the Bürker counting chamber: *T. borreli* are bigger, move slower and have two flagella while *T. carassii* are smaller, move faster and have a single flagellum, (ii) distinction was made by real-time quantitative PCR using parasite-specific primers for a gene sequence known in both *T. borreli* and *T. carassii* (heat shock protein (HSP)70).

For real-time quantitative PCR, DNA was released by the HotSHOT method (Truett *et al.*, 2000). Briefly, blood samples were 10 times-diluted in 1 mM EDTA RPMI 1640 (Cambrex). To 10 µl 10 times diluted blood 40 µl of 31.25 mM NaOH was added and heated for 20 min at 95°C. Reaction mixture was cooled down to 4°C and neutralised by adding 50 µl of 40 mM Tris. Samples were stored at -20°C until further use. Parasite-specific primers for HSP70 were designed by hand based on the known sequences of *T. borreli* accession number AY288515, and of *T. carassii* tca-10f15.b1 (Aguero *et al.*, 2002). Primers were checked for specificity by PCR on parasite and carp cDNA libraries, parasite lysates, and parasite and carp genomic DNA. Primer sequences were: Borreli. fw2 5'-CATGGCCAACATCACCCGT-3' Borreli.rv2 5'-ATCCACCCAC TAGCACAACA-3' Carassii.fw 5'-CAGCCGGTGGAGCGCGT-3' Carassii.rv 5'-AGTT CCTTGCCGCCGAAGA-3'. For the real-time quantitative PCR 0.5 µl of DNA, 12.5 µl Sybr Green Master Mix (Stratagene), forward and reverse primer (300 nM each) and MilliQ water up to 25 µl was used. Quantitative PCR was performed in a 72-well Rotor-Gene™ centrifugal real-time thermal cycler (Rotor-Gene 2000 Corbett Research). Following cycling conditions were used: one incubation step of 10 min at 95°C; followed by 45 cycles of 20 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C; an incubation for 1 min at 60°C was followed by a melting curve from 60°C to 99°C in steps of 1°C with 5 sec waiting. At the end of each cycle and during the waiting steps in the melting curve fluorescence intensities were measured. Raw data were analysed using the comparative quantitation of the Rotor-Gene Analysis Software V5.0. PCR values (R) were calculated [1 dividing by the average efficiency (per gene and run) to the power of the take off value] and correlated to the microscopic counting. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The PCR products of both *T. borreli* and *T. carassii* were checked by sequencing.

ELISA for carp IgM against T. borreli

ELISA plates were coated overnight at RT in a humid chamber with 3×10^4 lysed *T. borreli* / well (100 µl per well). Lysate was diluted in a 25 mM (bi)carbonate buffer pH 9.6. Plates were blocked with 200 µl 1% BSA in T-TBS (20 mM TrisHCl, 500 mM NaCl, 0.1 % Tween-20). Sera were pre-diluted 50 times in T-TBS and measured in three dilutions (100, 200 and 400 times diluted) in duplicate. A standard serum with a high antibody titer was included on every plate to normalise the data. Plates were incubated with 100 µl WCI12 (1:250, mouse anti-carp Ig (Koumans-van Diepen *et al.*, 1995; van Diepen *et al.*, 1991)), and subsequently with 100 µl goat anti-mouse-HRP (Dako, 1:2000). Plates were washed three times and incubated for 30 min at RT in the dark with 100 µl 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution (Roche). OD was read at 405 nm with 492 as reference. In between all incubation steps plates were washed twice under running tap water. Incubation steps were for 1 h at 37°C in a humid chamber unless stated otherwise.

Western blotting

Twenty-one µl lysate containing 1×10^6 *T. borreli* or 1.4×10^6 *T. carassii* (heated to 96°C for 10 min with 7 µl loading buffer containing β-mercaptoethanol) was resolved on a 12.5% SDS-PAGE. Proteins were subsequently electro-transferred to a nitro-cellulose membrane (Protran®, Schleicher & Schuell, Bioscience GmbH) and incubated overnight at RT in blocking buffer (3% BSA in TBS, 10mM Tris-HCl, pH 7.5, 150mM NaCl). Immune serum containing (cross-reactive) anti-parasite antibodies was obtained by combining the serum isolated from n=4 fish, 6 weeks post-infection. Membranes were incubated with immune serum (1:50) in TBS-Tween (20mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% (v/v) Tween-20) for 1 h at RT, with WCI-12 (mouse anti-carp Ig) (1:50) in 3% BSA in TBS for 1 h at RT and with goat anti-mouse HRP (1:1000) in 3% BSA in TBS for 1 h at RT. After each incubation step, membranes were washed twice with TBS-Tween/Triton (TBS-Tween, 0.2% (v/v) Triton X-100) and once with TBS, for 10 min at RT. Signal was detected by development with a chemoluminescence kit (Amersham) according to the manufacturer's protocol and visualised by the use of Lumni-fil chemiluminescent Detection Film (Roche). Controls without primary antibody were negative.

Statistics

For parasitaemia levels and antibody concentrations, data were log-transformed and analysed by a repeated measurement model (PROC MIXED, SAS, Version 9.1, SAS Institute) with treatment included as a main effect and the interaction of treatment with week included to test whether the effect of treatment changed with time. For each treatment, the pattern of survival probability over weeks was computed using the Kaplan-Meier method (Kleinbaum, 1996), after which a log-rank test was performed to test for differences between treatments (PROC LIFETEST, SAS, Version 9.1, SAS Institute).

A value of $P \leq 0.05$ was accepted as significant.

Results

Pilot experiment

In a pilot infection experiment, carp were i.p. injected with PBS as a negative control, with a mono-parasitic infection with *T. borreli* or *T. carassii* or with a mixture of both parasites. To confirm that the fish developed mixed infections blood smears were made and stained with parasite-specific antibodies. *T. carassii* was visualised with DAB, giving a brown/red colour (open arrow) and *T. borreli* was visualised with NBT-BCIP giving a blue colour (solid arrow) (Fig. 6.1). All fish receiving an injection with both parasites developed a mixed parasitaemia. Fish injected with PBS did not develop any parasitaemia and all survived.
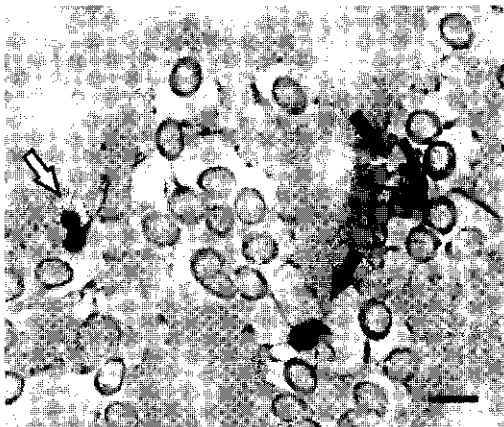
Fig. 6.1 Light microscopic photograph (bar is 10 μm) of *T. borreli* (solid arrows) and *T. carassii* (open arrow) stained with specific antibodies, visualised with NBT-BCIP / blue for *T. borreli* and DAB / brown/red for *T. carassii*.

Parasitaemia in mono-parasitic infections were higher in fish receiving a high infection dose (10,000) than in fish receiving an intermediate infection dose (5,000) (Fig. 6.2A). Peak parasitaemia in *T. borreli*-infected fish (for both 5,000 and 10,000 infection dose) was approximately 100-fold higher than the parasitaemia in *T. carassii*-infected fish. Parasitaemia in fish receiving a mixed infection (ratio 1:1; 5,000 *T. borreli* and 5,000 *T. carassii*) was intermediate to the parasitaemia in fish infected with 5,000 or 10,000 *T. borreli*.

Although parasitaemia was not obviously influenced by the mixed infection, survival (Fig. 6.2B) was increased. *T. carassii* infections were not lethal, while survival of fish receiving a mixed infection rose to 29%, compared to 14% in the mono-parasitic *T. borreli* infection (both doses).

A
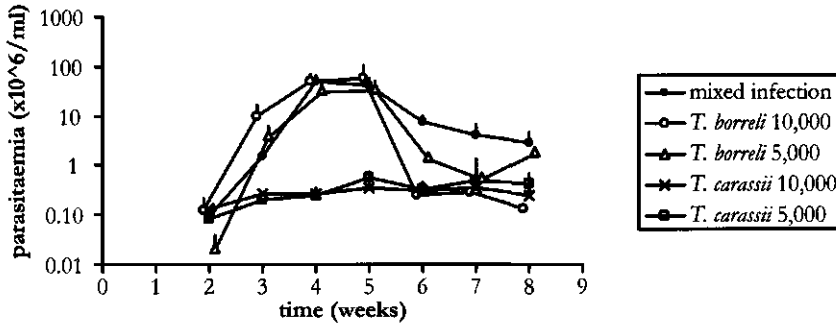
B

Fig. 6.2 Parasitaemia (A), and survival (B) of pilot mixed infection. Fourteen fish received a mixture of 5,000 *T. borreli* and 5,000 *T. carassii* (●) and n=28 fish received a mono-parasitic infection; 7 with 10,000 *T. borreli* (○), 7 with 5,000 *T. borreli* (Δ), 7 with 10,000 *T. carassii* (x), and 7 with 5,000 *T. carassii* (□). Blood samples were taken weekly from week 2 until week 8 and parasitaemia levels were monitored using a Bürker counting chamber. Lethargic fish were removed from the experiment and survival was recorded weekly. For parasitaemia average and SEM of n=7 (mono-parasitic infections), or n=14 (mixed infection) fish are shown.

This indicated a protective effect of co-infection with *T. carassii*. Because of this protective effect, we designed a new infection experiment with a ratio of 100:1 for the two parasite species (10,000 *T. carassii* and 100 *T. borreli*). Control groups were injected with 10,000 *T. carassii* or 100 *T. borreli* or with PBS. To differentiate between *T. borreli*- and *T. carassii*-specific parasitaemia within mixed infected fish we used two different methods.

A

B
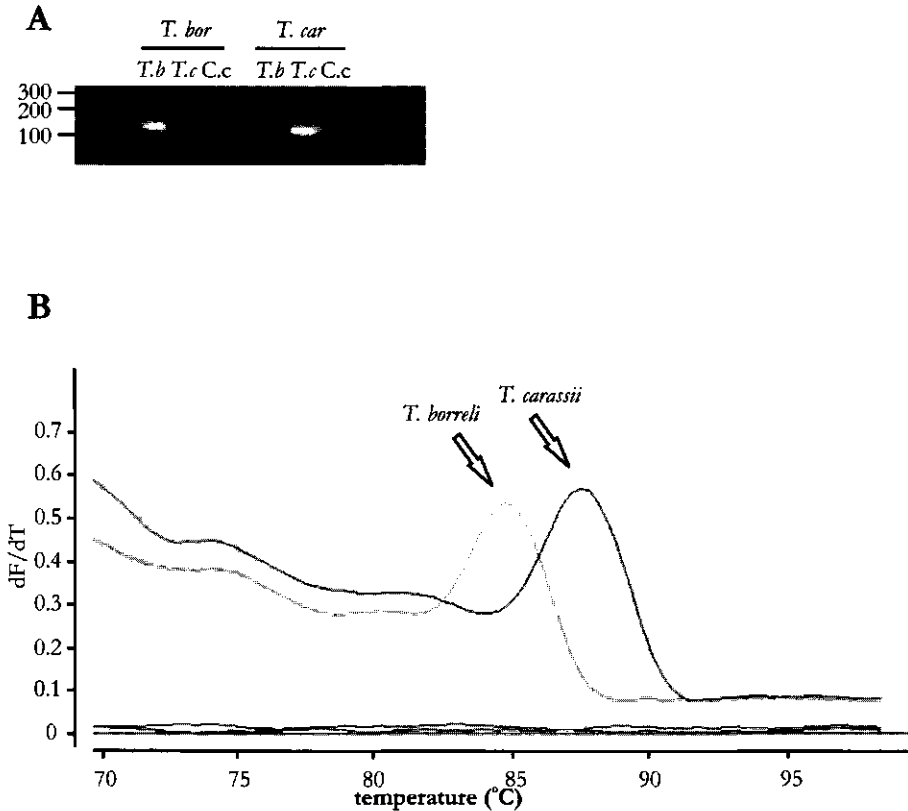
Fig. 6.3 PCR products (A), and melting peaks (B). PCR was performed on *T. borreli* (*T. b*) (lane 2 and 6), *T. carassii* (*T. c*) (lane 3 and 7), and carp (C. c) (lane 4 and 8) cDNA libraries to ensure specificity. Lane 1 and 9: marker, lane 2-4: *T. borreli* HSP70 primers, lane 5: empty, lane 6-8: *T. carassii* HSP70 primers. Real-time quantitative PCR was performed on DNA released by the HotSHOT method for single and mixed infections, melting peaks are shown.

Differential counting based on morphological differences and PCR

To monitor total parasitaemia levels, blood samples were 10 times-diluted with 1mM EDTA RPMI and parasites were counted using a Bürker counting chamber. To determine the *T. borreli*- and *T. carassii*-specific parasitaemia separately in mixed infected fish, two different methods were used. Distinction was not only made on morphology and movement but also on real-time quantitative PCR to determine the amount of parasite-specific DNA present. Real-time quantitative PCR was performed on total DNA released from blood samples by the HotSHOT method. PCR was specific as demonstrated by gel electrophoresis and melting-peak analysis (Figs 6.3). Melting peak analysis was performed for each sample and showed species-specific reactions in each PCR performed. PCR values (R) were plotted against the microscopically-obtained countings, for each week post-infection separately. Presence or absence of parasites could be determined by PCR from week 2 post-infection onwards (data not shown). High correlations between microscopic countings and PCR results could be found starting week 5 post-infection for *T borreli* (df$_{1,8}$ F=83.6 *P*=1.7x10^{-5} R^2=0.91) and *T. carassii* (df$_{1,8}$ F=23 *P*=0.001 R^2=0.74). Although both the microscopic countings and the PCR method yielded comparable results, we decided to continue with the microscopic countings based on morphological differences to exclude false positives by the PCR method that could also amplify DNA from dead parasites.

Parasitaemia and mortality are lower in mixed infected fish

Mixed-infected fish were injected with 100 *T. borreli* and 10,000 *T. carassii* while control fish were injected with either 100 *T. borreli* or 10,000 *T. carassii*, or with PBS. Weekly taken blood samples were analysed for parasitaemia using a Bürker counting chamber. Lethargic fish were removed and recorded as deaths. A clear positive effect was observed in the mixed-infected group for both total parasitaemia and survival.

Total parasitaemia in mixed infected fish (Fig 6.4A) was significantly lower than the parasitaemia in *T. borreli*-infected fish, although the fish with a mixed infection were injected with an additional 10,000 *T. carassii*. Also, a clear although not significant protective effect of the mixed infection was observed on survival which increased from 60% (6/10) in *T. borreli*-infected fish to 86% (19/22) in fish with a mixed infection (Fig. 6.4B). All *T. carassii*-infected fish (10/10) survived the infection.
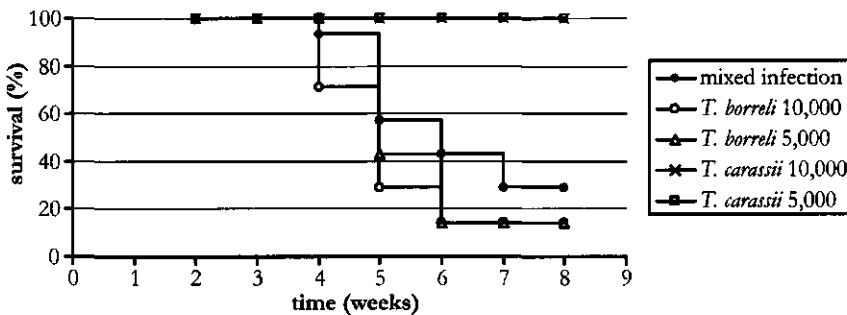
A
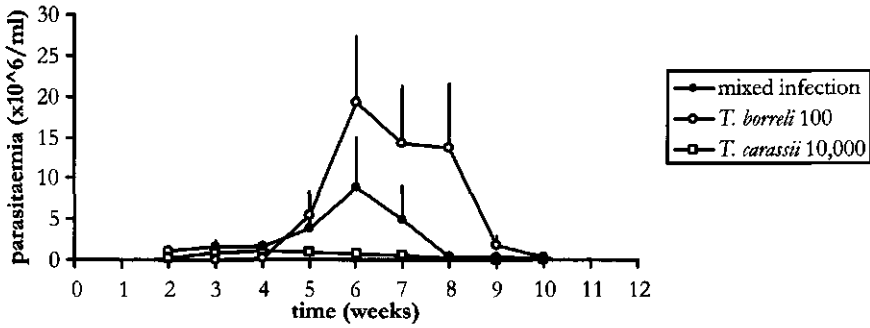
B
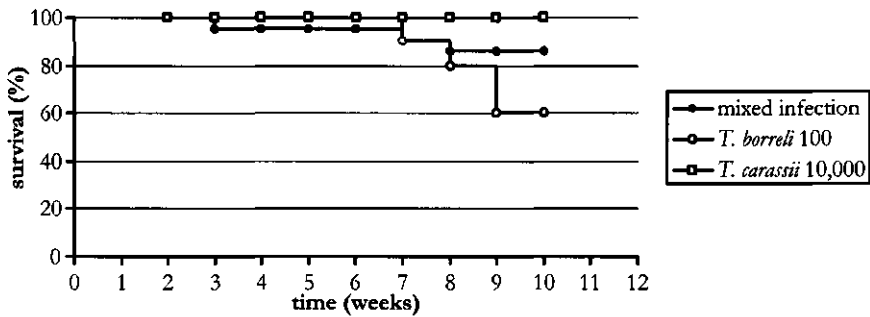
Fig. 6.4 Parasitaemia (A), and survival (B) of mixed infection. Twenty-two fish received a mixture of 100 *T. borreli* and 10,000 *T. carassii* (●) and 20 fish received a mono-parastic infection; n=10 with 100 *T. borreli* (○), and n=10 with 10,000 *T. carassii* (□). Blood samples were taken weekly from week 2 until week 10 and parasitaemia levels were monitored using a Bürker counting chamber. Lethargic fish were removed from the experiment and survival was recorded weekly. For parasitaemia average and SEM of n=10 (mono-parasitic infections), or n=22 (mixed infection) fish are shown.

Parasite-specific parasitaemia in mixed infected fish (Fig. 6.5) was determined using morphological differences between the two species. From week 5 onwards total parasitaemia could be divided into *T. borreli*- and *T. carassii*-specific parasitaemia. The *T. carassii*-specific parasitaemia within the mixed infection (Fig. 6.5A) was equal to the *T. carassii* mono-parasitic parasitaemia (Fig. 6.5C). The *T. borreli*-specific parasitaemia within the mixed infection was significantly lower and decreased faster in the mixed infection (Fig. 6.5B) than in the *T. borreli* mono-parasitic infection (Fig. 6.5D).
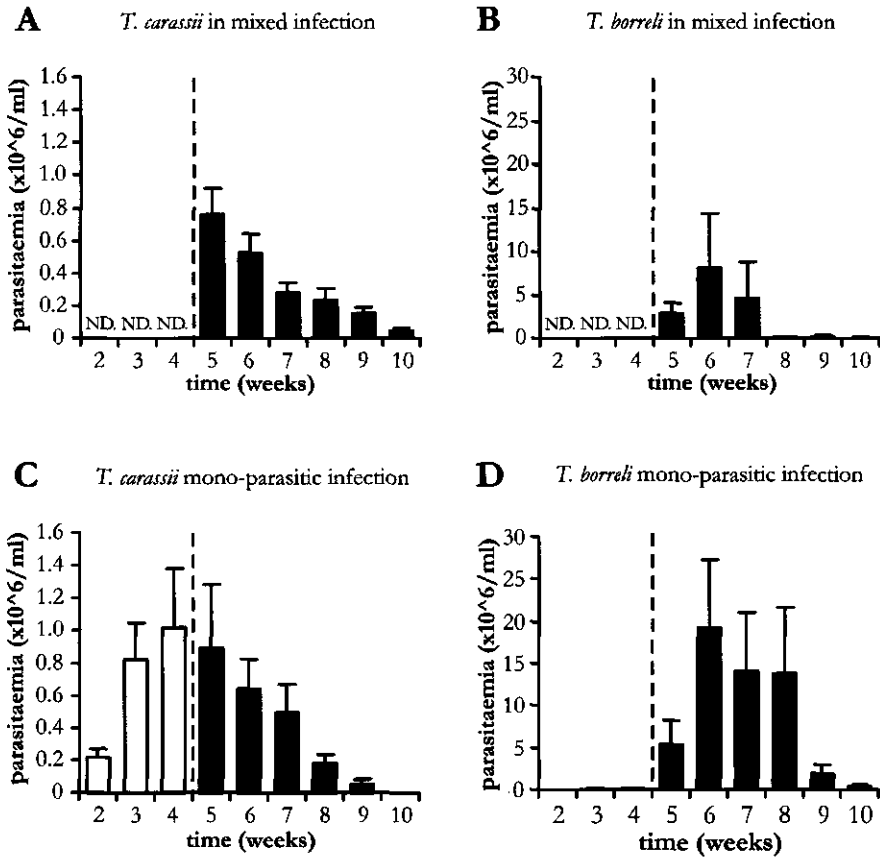
Fig. 6.5 Parasitaemia per parasite species based on morphological differences. *T. carassii* specific parasitaemia in mixed infection (A), and mono-parasitic infection (C). *T. borreli* specific parasitaemia in mixed infection (B), and mono-parasitic infection (D). In the early weeks of infection it was not possible to determine the parasite-species specific parasitaemia (ND.). Average and SEM of n=22 (mixed infection), or n=10 (mono-parasitic infections) fish are shown.

Antibody titers against T. borreli *are higher in mixed infected fish*

We measured the amount of antibodies reactive against *T. borreli* by ELISA. Antibody titers were significantly lower in *T. borreli*-infected fish than in *T. carassii*- and mixed-infected fish. Furthermore, antibody titers in *T. borreli*-infected fish started to increase only at week 9 post-infection (Fig. 6.6A). Surprisingly, in *T. carassii* infected fish, antibody titers (against *T. borreli*) were moderate to high (Fig. 6.6B). In mixed infected fish, *T. borreli*-reactive antibody titers were relatively high and steadily increased from week 5 onwards (Fig. 6.6C). Although individual variation was high, each individual fish in this group was shown to produce antibodies.
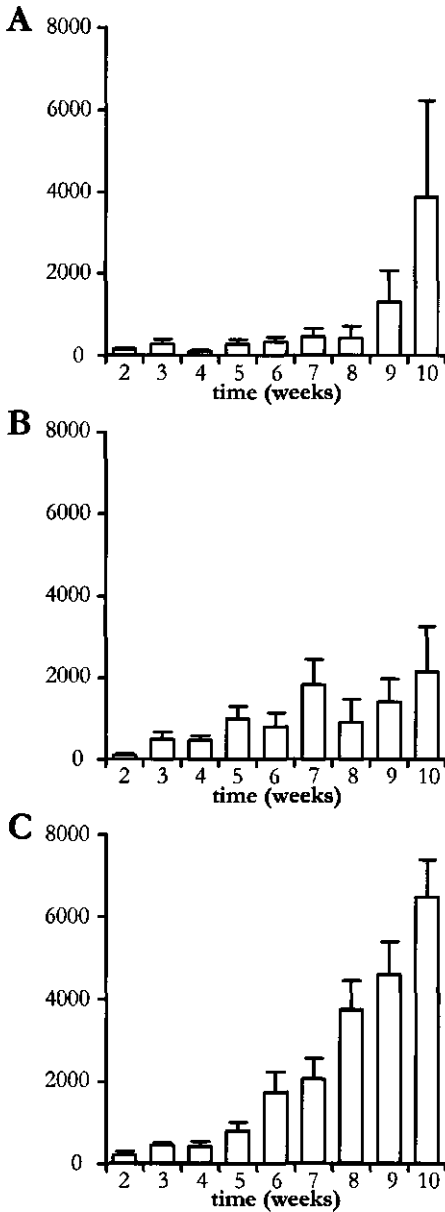
Fig. 6.6 Antibody titers reactive against *T. borreli*. *T. borreli* mono-parasitic infection (A), *T. carassii* mono-parasitic infection (B), and mixed infection (C). Values were normalised against a positive sample which was included on each plate. Average and SEM of n=10 (mono-parasitic infections), of n=20 (mixed infection) fish are shown.

Western blotting confirms cross-reactive antibodies

Western blotting (Fig. 6.7) confirmed cross-reactivity between immune sera obtained from mono-parasitic infections. Immune serum from *T. borreli*-infected fish detected several major bands in *T. borreli* lysate (lane 2) but also in *T. carassii* lysate (lane 4). Likewise, immune serum from *T. carassii*-infected fish recognised several major bands in *T. carassii* lysate (lane 5) but also in *T. borreli* lysate (lane 3).

Both immune sera recognised two protein bands of approximately 80 kDa in the homologous parasite lysates. *T. carassii* immune serum was cross-reactive with the lower of these two bands (lane 3), while *T. borreli* immune serum was cross-reactive with the upper of the two bands (lane 4). Both *T. borreli* and *T. carassii* immune sera recognised two further bands of approximately 33 and 35 kDa in the *T. borreli* lysate.
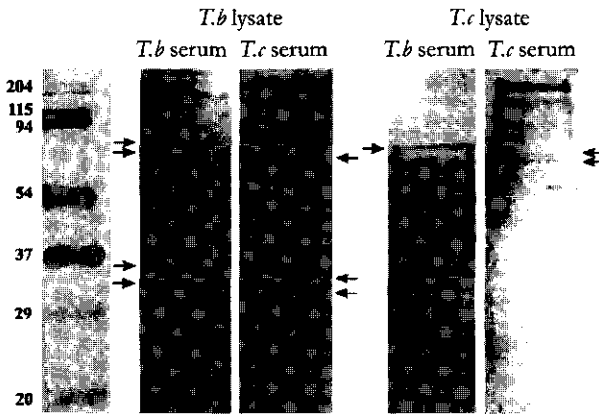
Fig. 6.7 Western blotting of (cross-reactive) antibodies in *T. carassii* and *T. borreli* immune serum. A broad-range marker (lane 1) with sizes indicated on the left in kDa, and 28 µl lysate containing 1×10^6 *T. borreli* (lane 2 and 3) or 1.4×10^6 *T. carassii* (lane 4 and 5) were resolved on a 12.5% SDS-PAGE. Proteins were electro-transferred to a nitro-cellulose membrane and anti-parasite antibodies were detected with immune serum (1:50) from *T. borreli*-infected fish (lane 2 and 4) or *T. carassii*-infected fish (lane 3 and 5). Signal was detected with a chemoluminescence kit and visualised by the use of Lumni-fil chemiluminescent Detection Film. Bands with comparable size, recognised by different immune sera are indicated with arrows.

After T. carassii *infection fish are protected against* T. borreli

To determine if *T. carassii* could protect carp against *T. borreli* over prolonged periods, we re-infected fish that recovered from a *T. carassii* infection with *T. borreli*. Weekly taken blood samples were used to monitor parasitaemia after re-infection. All fish survived the infection. Parasitaemia in re-infected fish was significantly lower than in fish receiving their first infection (Fig. 6.8), and from a number of the re-infected fish (3/14) parasitaemia was undetectable. This indicates a protective effect induced by *T. carassii* infection.
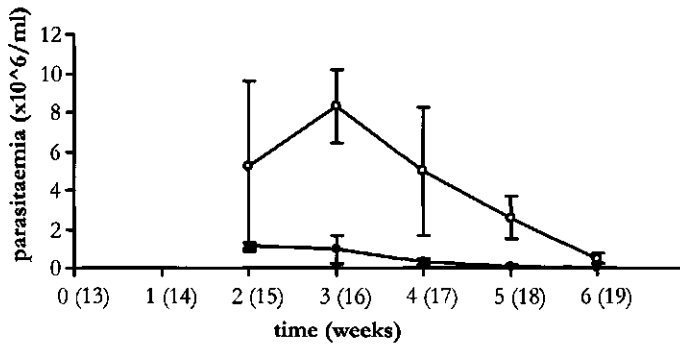
Fig. 6.8 Parasitaemia after re-infection with *T. borreli*. Thirteen weeks after infection with *T. carassii* fish were infected with 5,000 *T. borreli* (●). Controls were naïve fish infected with 5,000 *T. borreli* (○). Blood samples were taken weekly from week 15 until week 19 (week 2 until week 6 after re-infection) and parasitaemia levels were monitored using a Bürker counting chamber. Average and SEM of n=14 (re-infected) and n=3 (control) fish are shown.

Discussion

Our experiments show that co-infection with *T. carassii* can protect carp against *T. borreli* infections. Carp with a mixed *T. borreli* / *T. carassii* infection had significantly lower parasitaemia levels, a tendency for higher survival rates, and significantly earlier and higher antibody production than carp with a mono-parasitic *T. borreli* infection. At least part of the protection seems to be based on the production of cross-reactive antibodies.

Mixed *T. borreli* / *T. carassii* infections are commonly found in nature (Lom and Dyková, 1992) and can be caused by two separate blood meals of infected leeches or by a blood meal of a single leech infected with both parasites. *T. carassii* can develop in *Hemiclepsis marginata* but also in *Piscicola geometra* while *T. borreli* is only found in *P. geometra*. While *T. carassii* trypomastigotes can persist in the leech for several months (Lom, 1979), the longest period *T. borreli* was

observed to persist in the leech was 11 days only (Kruse *et al.*, 1989). *T. carassii* can multiply to several hundreds in *P. geometra* and to tens of thousands in *H. marginata* (Lom, 1979). These findings suggest that the infection dose given by the leech in nature often must be higher for *T. carassii* than for *T. borreli*. This would not only be true for fish infected by two different leeches but also for fish infected by a single leech carrying both parasites. This implies that the ratio of 1:100 for *T. borreli* : *T. carassii* that we used in the mixed infection experiment could be close to the natural situation.

In mono-parasitic *T. carassii* infections parasites were detectable from week 2 onwards while in mono-parasitic *T. borreli* infections hardly any parasites could be detected before week 5 post-infection (Fig. 6.5C and D). In mixed infections, *T. carassii* was detected by PCR in all fish starting week 2 post-infection. In contrast, it took until week 5 post-infection to detect *T. borreli* in all mixed infected fish. Thus, also in the mixed infection, a difference in the development of the two species-specific parasitaemias was seen. The presence of *T. carassii* prior to *T. borreli* suggests that cross-reactive antibodies induced by *T. carassii* could provide (partial) protection against *T. borreli*. Cross-reactive antibodies were shown in a Western blot (Fig. 6.7) with immune serum from both infections and identified antigenic proteins in lysates of *T. borreli* of approximately 33, 35 and 80 kDa which could be of special interest. Cross-reactive antibodies in serum of *T. carassii*-infected fish were also detected by ELISA (Fig. 6.6). Furthermore, in *T. carassii*- and mixed-infected fish, antibodies significantly increased at a much earlier time point (week 5 vs. week 9) than in *T. borreli* mono-parasitic infection. Since parasitaemia was lower in the mixed infection group this suggests a protective role for cross-reactive antibodies against *T. borreli*. Cross-reactive antibodies, recognising different kinetoplastid species, have been shown in humans (Chiller *et al.*, 1990) and might be present in cattle since they can carry mixed kinetoplastid infections (Van den Bossche *et al.*, 2004b; Van den Bossche *et al.*, 2005), but their (protective) role remains to be elucidated.

Other studies on mono-parasitic infections with either *T. borreli* or *T. carassii* previously indicated a role for antibodies in the protection against these parasites. Passive immunisation of susceptible carp with immune serum from *T. borreli*-infected fish was partially protective (Wiegertjes *et al.*, 1995). Passive immunisation with IgM purified from *T. carassii*- infected carp was protective against *T. carassii* infections in carp (Overath *et al.*, 1999). Furthermore, both goldfish and young carp could not be re-infected with *T. borreli* or *T. carassii* at the moment the parasites of the first homologous infection were undetectable

(Lom, 1979), indicating the presence of protective antibodies. Antibodies can act either together with complement to induce direct lysis or as opsonins to facilitate phagocytosis. Both systems have been shown to be active against African trypanosomes (Balber *et al.*, 1979; Dempsey and Mansfield, 1983). For *T. borreli*, *in vitro* killing has been shown with immune serum in the presence of complement (Saeij *et al.*, 2003a; Scharsack *et al.*, 2004). Furthermore, gene expression studies on complement factor 3 showed an increased expression during a *T. borreli* infection (Saeij *et al.*, 2003a). In contrast, although early studies have reported phagocytosis of blood flagellates by fish macrophages, we have no evidence for phagocytosis of *T. borreli* or *T. carassii* by carp macrophages (Saeij *et al.*, 2003b).

In conclusion, we demonstrated that in a mixed kinetoplastid infection, the outcome of infection can be considerably different from the outcome of the respective mono-parasitic infections. We observed a protective effect of co-infection or prior infection with *T. carassii* on resistance to *T. borreli*. Possibly, co-evolution of *T. borreli* and *T. carassii* in the same carp host has been beneficial especially to *T. borreli*, reducing severity and lethality compared with mono-parasitic infection with *T. borreli*.

Acknowledgements

Chapter 7

Identification of *Trypanoplasma borreli*
and *Trypanosoma carassii* antigens:
screening λTriplEx2 expression libraries
with common carp (*Cyprinus carpio* L.)
immune serum

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Trudi Hermsen, Huub F.J. Savelkoul, Geert F. Wiegerjtes
In preparation

Abstract

Trypanoplasma borreli and *Trypanosoma carassii* are kinetoplastid parasites infecting common carp (*Cyprinus carpio* L.). Antibodies have been shown to play a protective role in the immune response of carp against these parasites. Furthermore, antibodies directed against *T. carassii* seem to be cross-reactive and cross-protective against *T. borreli* (Joerink *et al.*, 2006b). In order to identify *T. borreli* and *T. carassii* antigens we constructed and screened a λTriplEx2 expression library of *T. borreli* and a λTriplEx2 expression library of *T. carassii* with homologous immune serum from carp. Serum was collected 6 weeks post infection and pooled from four fish per infection. The screening of the *T. borreli* library revealed ribosomal proteins only. The screening of the *T. carassii* library revealed ubiquitin and activated protein kinase C receptor as antigens next to ribosomal proteins. The possibility that antibodies, directed against ubiquitin, activated protein kinase C receptor, and ribosomal proteins are protective antibodies is discussed.

Introduction

Trypanosoma carassii (syn. *T. danilewskyi* (Lom and Dyková, 1992)) and *Trypanoplasma borreli* are kinetoplastid parasites infecting cyprinid fish, such as carp, goldfish, crucian carp, and tench, as well as some members of non cyprinid families (Overath *et al.*, 1998; Steinhagen *et al.*, 1989). Both parasites are believed to live exclusively extracellularly in the blood and tissue fluids of the host. Blood-sucking leeches (*Piscicola geometra* and *Hemiclepsis marginata*) act as vectors for transmitting *T. carassii*. Within the digestive tract of the leech *T. carassii* transforms into epimastigotes, which multiply and change into metacyclic trypomastigotes. The trypomastigotes are transferred to other fish during a next blood meal. Within the fish host *T. carassii* replicate in the trypomastigote stage (Lom and Dyková, 1992). During the trypomastigote stage *T. carassii* has a carbohydrate-rich coat of glycosyl-phosphatidylinositol (GPI)-anchored mucin-like proteins, which were shown to be antigenic in carp (Lischke *et al.*, 2000). *T. borreli* is only transmitted by *P. geometra*, which functions only as a vector since *T. borreli* does not transform within the leech. Less data is available on the surface coat of *T. borreli*, although, electron microscopy suggests a much more massive surface coat than found for *T. carassii* (Lom and Nohýnková, 1977).

A protective role for antibodies during *T. carassii* and *T. borreli* infections in fish has been suggested before (Lom, 1979; Overath *et al.*, 1999; Scharsack *et al.*, 2004; Wiegertjes *et al.*, 1995). Recently we showed cross-reactivity of anti-*T. carassii* antibodies against *T. borreli* by ELISA and western blotting. Furthermore, carp which survived a *T. carassii* infection were partially protected against a subsequent *T. borreli* infection (Joerink *et al.*, 2006b).

In order to identify the antigenic proteins of *T. carassii* and *T. borreli* we constructed and screened λTriplEx2 expression libraries of *T. carassii* and *T. borreli* with immune serum from carp who survived a homologous infection. The *T. borreli* library contained an excess of ribosomal sequences, consequently all positive phages were identified as ribosomal proteins. Two more antigens were, however, identified from the *T. carassii* library, ubiquitin and activated protein kinase C receptor. The possibility that antibodies directed against these antigens are protective is discussed

Materials and Methods

Animals and parasites

Common carp (*Cyprinus carpio* L.) were reared in the central fish facility 'De Haar-Vissen' at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. R3 x R8 heterozygous carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow, 1995). This carp strain (R3 x R8) is relatively resistant to infections with *T. borreli* (Saeij *et al.*, 2003b) and *T. carassii* (unpublished observations). Carp were between 6 and 8 months old.

Trypanoplasma borreli was cloned and characterised by Steinhagen *et al.* (Steinhagen *et al.*, 1989). *Trypanosoma carassii* was cloned and characterised by Overath *et al.* (Overath *et al.*, 1998). Parasites were maintained by syringe passage through carp. Infections were performed i.p. with intervals of three weeks and infection dose was 10,000 parasites per fish. Parasitaemia was monitored using a Bürker counting chamber. The minimum detection limit by this method was 10^4 parasites/ml.

Parasites were isolated from the blood of three weeks infected carp by centrifugation as described by Steinhagen *et al.* (Steinhagen *et al.*, 2000). *T. borreli* was further purified by column chromatography (Overath *et al.*, 1998) before RNA isolation. *T. carassii* was cultured for four weeks as described by Overath *et al.* (Overath *et al.*, 1998) before RNA isolation.

RNA isolation and cDNA library construction

RNA was isolated from the parasites as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) briefly: parasites were homogenised in lysis buffer (4 M guanidium thiocyanide, 25 mM sodium citrate pH 7.0, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) followed by phenol/chloroform extraction. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry (Genequant, Amersham Pharmacia Biotech AB) and 3 µl was analysed on a 1% agarose gel to check the integrity. RNA was stored at -80 °C until further use. A λTriplEx2 expression library was constructed from 1 µg total RNA using the SMART™ cDNA library construction kit (Clontech) according to the manufacturer's protocol.

Carp anti-serum

Carp were i.p. infected with 10,000 *T. carassii* per fish or with 10,000 *T. borreli* per fish. Generally, carp surviving the infection have produced antibodies (Lischke *et al.*, 2000; Saeij *et al.*, 2003a). Six weeks post-infection four surviving fish, of each parasitic infection, were bled by syringe from the caudal vein. Blood was kept at 7°C for 48 hours, spun (15 min 800 x g), and serum collected. Serum was pooled, for *T. borreli* and *T. carassii* separately, and stored at -20°C in 1 ml aliquots.

Library screening

E. coli strain XL-Blue was grown overnight in 2 ml liquid LB medium containing 10 mM $MgSO_4$ and 0.2% maltose at 37°C while shacking. Bacteria were spun down and resuspended in 10 mM $MgSO_4$ to an OD600 of 0.5. cDNA library was diluted in SM buffer (100 mM NaCl, 8 mM $MgSO_4$.$7H_2O$, 50 mM TrisHCl (pH 7.5) 0.01% gelatin) to 10,000 plaque forming unites (pfu) per 20 µl. Plating the λTriplEx2 cDNA libraries was performed according to the Strategene protocol using the *E. coli* strain XL-Blue and LB media.

When small plaques were visible plates were incubated with nitrocellulose membranes pre-soaked with 0.01 M isopropyl-beta-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Blots were washed (3 times TBS-T, 20mM TrisHCl, 150 mM NaCl, 0.5% Tween-20, pH 7.5), blocked (3% w/v bovine serum albumin (BSA) in TBS), and incubated with three consecutive antibodies (carp anti-*T.carassii*-serum or anti-*T. borreli*-serum (1:50), WCI12 (mouse anti-carp Ig) (Koumans-van Diepen *et al.*, 1995; van Diepen *et al.*, 1991) (1:50) and goat anti-mouse HRP (Dako)(1:1000)). Antibodies were diluted in TBS containing 3% w/v BSA and carp anti-sera were pre-absorbed for 30 min. at RT with XL-Blue lysate (1:50). Blots were washed (3 times TBS-T) in between all incubation steps. Incubation steps were for 1 h at RT. For detection of positive plaques the ECLTM Western Blot Detection Kit (Amersham BioScience) was used according to the manufacturer's protocol.

Positive plaques were cut out of the plates and the phages were eluted in 1 ml SM buffer. The eluted phages were used in a secondary screening with 50 pfu per plate. After secondary screening single plaques were cut out and eluted in 1 ml SM buffer. The inserts from the positive phages were amplified by PCR, using phage specific primers Tr3.5'-ATACGACTCACTATAGGGC GAATTGGCC-3' and Tr5.5'-GTACCCGGGAATTCGGCCATT-3'. PCR reactions were performed in Taq buffer, using 2 U Taq polymerase (Goldstar/ Eurogentec S.A.) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 µM) and

primers (400 nM each) in a total volume of 50 µl. PCR was carried out under the following conditions: one cycle of 2 min at 94°C; followed by 35 cycles of 15 sec at 94°C, 30 sec at 55°C and 2 min at 72°C; and final extension for 7 min at 72°C. PCR products were run on a 1% agarose gel to check for single inserts and extracted out of gel with a gel extraction kit (Qiagen) according to the manufacturer's protocol. The phage specific primer Tr3 was used in a sequence reaction, using the ABI PrismTM Bigdye™ Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 377 automatic sequencer. Searches for similar sequences within the database were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) and Swissprot, EMBL and Genbank databases using SRS 7.1 from the EBI (European Bioinformatics Institute) site (www.ebi.ac.uk). Comparisons between sequences were performed using the CLUSTAL W1.7 software (Thompson *et al.*, 1994) (http://align.genome.jp), with minor optimisations made by hand.

Results

cDNA libraries

For *T. borreli* 2×10^9 parasites were isolated from carp and RNA isolation yielded 302 µg RNA in 400 µl. From this RNA 1.3 µl was used to construct the cDNA library. First strand cDNA was amplified for 18 cycles (Fig. 7.1). The unamplified library had a titer of 1.6×10^6 pfu/ml. The amplified library had a titer of 4.3×10^9 pfu/ml and 96% of the clones contained an insert.

After four weeks of *in vitro* culture 1×10^8 *T. carassii* were obtained and used to isolate RNA, this yielded 22 µg RNA in 60 µl. From this RNA 2.7 µl was used to construct the cDNA library. First strand cDNA was amplified for 24 cycles (Fig. 7.1). The unamplified library had a titer of 5.5×10^6 pfu/ml. The amplified library had a titer of 7.5×10^9 pfu/ml and 92% of the clones contained an insert.
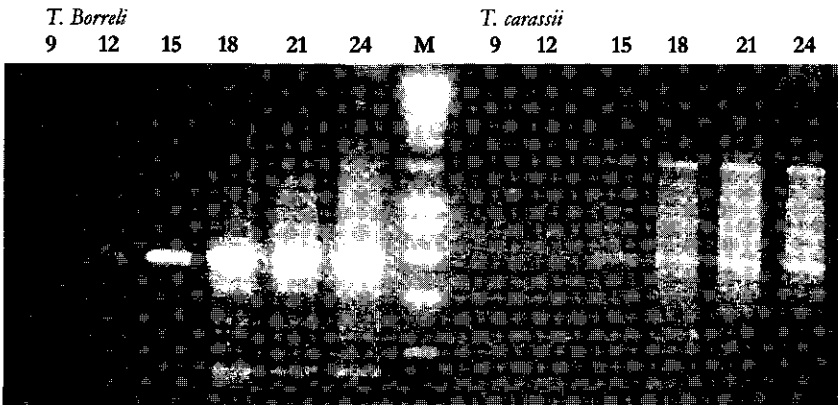
Fig. 7.1 cDNA after different cycles of PCR amplification, and molecular weight lader (M) SmartLader 200-10,000 bp (Eurogentec). RNA was isolated from *T. borreli* and *T. carassii*, first strand cDNA was transcribed while adding extensions. Extensions where used to amplify the cDNA, according to the SMART™ technology. For *T. borreli* 18 cycles and for *T. carassii* 24 cycles of amplification were used to continue with the library.

Screening

Screening of the *T. borreli* library revealed ribosomal proteins only. Therefore we decided to sequence the major band of approximately 600 bp present in the *T. borreli* cDNA. This band was identified as 18S rRNA. Because of this over representation of the ribosomal protein, which was less in the *T. carassii* library (Fig. 7.1) we decided to continue with the *T. carassii* library.

Primary screening of the *T. carassii* library, with in total 6 plates each containing 10,000 plaques, revealed 31 positive plaques. After secondary screening 30 positive plaques were obtained.

From the positive plaques of the secondary screening; 2 were empty, 2 were unknown kinetoplastid sequences containing a part of the mini-exon, 22 (73%) contained ribosomal proteins, 1 encoded for ubiquitin (Fig. 7.2) and 3 for the C-terminal part of the activated protein kinase C receptor (Fig. 7.4).

The translated sequences were aligned with known sequences from the database for ubiquitin (Fig. 7.3) and for the activated protein kinase C receptor (Fig. 7.5).

```
DNA:  CCAAGGCTATTATTGATACAGTTTCTGTACTATATTGCACTAGGCAGAAGC
      P   R   L   L   L   I   Q   F   L   Y   Y   I   A   L   G   R   S

DNA:  ATGCAGATCTTTGTGAAGACGCTCACGGGCAAGACAATTGCCCTCGAGGTC
      M   Q   I   F   V   K   T   L   T   G   K   T   I   A   L   E   V

DNA:  GAGGCCAGTGACACGATCGAGAATGTGAAGGCGAAGATCCAGGACAAGGAG
      E   A   S   D   T   I   E   N   V   K   A   K   I   Q   D   K   E

DNA:  GGCATCCCCCCGGACCAGCAGCGCCTGATTTTCGCCGGCAAGCAACTGGAG
      G   I   P   P   D   Q   Q   R   L   I   F   A   G   K   Q   L   E

DNA:  GAGGGCCGCACGCTGGCGGACTACAACATCCAGAAGGAGTCCACACTGCAC
      E   G   R   T   L   A   D   Y   N   I   Q   K   E   S   T   L   H

                    Ubiquitin  ————————  Ribosomal tail protein (L40)
DNA:  CTTGTGCTTCGCCTGCGCGGTGGCGTGATGGAGCCGACGCTTGAGGCCCTT
      L   V   L   R   L   R   G   G   V   M   E   P   T   L   E   A   L

DNA:  GCGAAGAAGTTCAATTGGGAAAAGAAGGTGTGCCGCCGCTGCTACGCCCGC
      A   K   K   F   N   W   E   K   K   V   C   R   R   C   Y   A   R

DNA:  CTCCCGCCTCGCGCGACGAACTGCCGCAAGAAGGGTTGCGGCCACTGCTCT
      L   P   P   R   A   T   N   C   R   K   K   G   C   G   H   C   S

DNA:  AACCTCCGCATGAAGAAGAAGCTCCGGTAGAGGAATGACGCGCCTGTTGGT
      N   L   R   M   K   K   K   L   R   *   R   N   D   A   P   V   G

DNA:  TTAGTGCAAATGCTGTTTATTTGTCGTGTTTGTTTTTTCTTTGTATGCAAA
      L   V   Q   M   L   F   I   C   R   V   C   F   F   F   V   C   K

DNA:  AAAAAAAAAAAAAAAAAAAAAAAAAAA
      K   K   K   K   K   K   K   K
```

Fig. 7.2 cDNA and deduced protein sequence of positive clone 65, ubiquitin and tail protein. Start (M) and stop (*) codon are depicted in bold, and the mini-exon sequence is highlighted in grey.

The clone containing the ubiquitin sequence was a virtually full length transcript including the tail protein, start and stop codon, 3'UTR up to the poly-AAA tail and the 5'UTR up to 36 out of 39 bases encoding the mini-exon sequence (Fig. 7.2).

```
T.carassii  MQIFVKTLTGKTIALEVEASDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEEGR
T.carDB     MQIFVKTLRGKTIALEVEASDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEEGR
T.brucei    MQIFVKTLTGKTIALEVEASDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEEGR
T.cruzi     MQIFVKTLTGKTIALEVESSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGR
L.major     MQIFVKTLTGKTIALEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEEGR
OnMy        MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGR
HoSa        MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGR
Fugu        MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGR
DaRe        MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGR
            ********  ****.****.********************************.**


T.carassii  TLADYNIQKESTLHLVLRLRGG VMEPTLEALAKKFNWEKKVCRRCYARLPPRA
T.carDB     TLADYNIQKESTLHLVLRLRGG VMEPTLEALAKKFNWEK
T.brucei    TLADYNIQKESTLHLVLRLRGG VMEPTLEALAKKYNWEKKVCRRCYARLEVRA
T.cruzi     TLADYNIQKESTLHLVLRLRGG VMEPTLEALAKKYNWEKKVCRRCYARLEVRA
L.major     TLSDYNIQKESTLHLVLRLRGG VMEPTLVALAKKYNWEKKVCRRCYARLEVRA
OnMy        TLSDYNIQKESTLHLVLRLRGG IIEPSLRQLAQKYNCDKMICRKCYARLHPRA
HoSa        TLSDYNIQKESTLHLVLRLRGG IIEPSLRQLAQKYNCDKMICRKCYARLHPRA
Fugu        TLSDYNIQKESTLHLVLRLRGG LIEPSLRMLAQKYNCDKMICRKCYARLHPRA
DaRe        TLSDYNIQKESTLHLVLRLRGG IIEPSLRQLAQKYNCEKMICRKCYARLHPRA
            **.***************** ::**:*  **:*:*  :*  :**.***** **


T.carassii  TNCRKKGCGHCSNLRMKKKLR
T.carDB
T.brucei    TNCRKKGCGHCSNLRMKKKLR
T.cruzi     SNCRKKACGHCSNLRMKKKLR
L.major     TNCRKKACGHCSNLRMKKKLR
OnMy        VNCRKKKCGHTNNLRPKKKLK
HoSa        VNCRKKKCGHTNNLRPKKKVK
Fugu        VNCRKKKCGHTNNLRPKKKLK
DaRe        VNCRKKKCGHTNNLRPKKKLK
            ***** *** .*** ***::
```

Fig. 7.3 Multiple amino acid sequence alignment of ubiquitin with the ribosomal tail protein. Sequences were aligned by Clustal W1.7 programme. A vertical line is placed between the ubiquitin and the ribosomal tail protein. Asterisks indicate identity, colons and dots indicate decreasing degrees of conservative substitutions, differences are highlighted in grey. T.carDB is the partial ubiquitin sequence (tca-07121.b1) from the web appendix of (Aguero *et al.*, 2002) the other proteins were taken from the Swissprot database with the following accession numbers: *T. brucei* **CAA39864**, *T. cruzi* **CAA30335**, *L. major* **CAJ08391**, rainbow trout OnMy **BAA88568**, human HoSa **AAI01831**, pufferfish Fugu **CAG00768**, zebrafish DaRe **NP_001032190**.

```
DNA:  CTCTCTGCCGGATCTCCCATCAACCAGATTTGCTTCTCCCCGAACCGCTAC
      L   S   A   G   S   P   I   N   Q   I   C   F   S   P   N   R   Y

DNA:  TGGATGTGTGCCGCGACTGAGAAGGCTATTCGCATCTTCGATCTTGAGAGC
      W   M   C   A   A   T   E   K   A   I   R   I   F   D   L   E   S

DNA:  AAGGACGTGATTGTGGAACTTGTTCCGGAGACGCAGCAGAAGGAGAAGAAG
      K   D   V   I   V   E   L   V   P   E   T   Q   Q   K   E   K   K

DNA:  GCCCCGGAGTGCGTCTCGGTGGCATGGTCTGCTGACGGGACCACACTCTAC
      A   P   E   C   V   S   V   A   W   S   A   D   G   T   T   L   Y

DNA:  TCTGGTCACACAGATAACGTCATCCGCGTGTGGAGCGTGTCGGAGAATGCG
      S   G   H   T   D   N   V   I   R   V   W   S   V   S   E   N   A

DNA:  TAATGCTTGGTAGTGACTTTGGCATTACCGTACGGCATTGTTTAGGCTGTT
      *   C   L   V   V   T   L   A   L   P   Y   G   I   V   *   A   V

DNA:  ACGTGCGTTTGCTCTCGGGGCGTGCATAGCGGCAGGCTAACCTGGAGAAAC
      T   C   V   C   S   R   G   V   H   S   G   R   L   T   W   R   N

DNA:  CGCTTGTAATTAATTATTCATTTTTTTGGTTTTGTTTTGAAAAAAAAAAAA
      R   L   *   L   I   I   H   F   F   G   F   V   L   K   K   K   K

DNA:  AAAAAAAAAAA
      K   K   K   K
```

Fig. 7.4 cDNA and deduced protein sequence of positive clones 129-131, C-terminal part of activated protein kinase C receptor. Stop codon (*) is depicted in bold and a PKC phosphorylation site is highlighted grey.

```
T.carassii      ------------------------------------------------------------
T.cruzi         MAVVYEGQLKGHRGWVTALACPQITETYIKAVSTSRDNTLIAWGSNMDRNSEECE
T.congolense    MAVVVEGQLKGHRGWVTSLACPQAAESSTKVVSASRDKTLLSWAANPVRHSSECD
T.vivax         MAVVYEGHSRGHRGWVTSLVCPQCEQTGINVVSASRDKTLISWSDNPNRHAEEND
                **** **: ;*******;*.*** :: :.**:***:**;:*. * *::.* :


T.carassii      ------------------------------------------------------------
T.cruzi         YGFPERRLEGHSAFVSDVALSNNGDFAVSASWDHSLRLWNLQTGVCQHKFLGHTK
T.congolense    YGLPERRLEGHSAFVSDVALSNNGDFAVSSSWDHSLRLWNLQSGQCQHKFLGHTK
T.vivax         YCIPERRLEGHSAFVSDVALSNNGSFAVSASWDRSMRLWNLQNGQCQYKFLGHTR
                * ;***********;********** ****;***:*:****** .* **;******;


T.carassii      ------------------------------------------------------------
T.cruzi         DVLSVTFSPDNRQIVSGGRDNALRVWNVKGECLHTLGRGAHTDWVSCVRFSPSLE
T.congolense    DVLSVAFSPDNRQIVSGGRDNALRVWNVKGECMHTWTRGAHTDWVSCVRFSPSLE
T.vivax         DVLSVAFSPDNRQIVSGSRDRTLRVWNVKGECMHTLN-GAHSDWVSCVRFSPATD
                *****;****;******.**;:*********;** ***;********;; :


T.carassii      ------------------------------------------------------------
T.cruzi         TPLIVSGGWDNLVKVWDIAS--GRLLTDLKGHTNYITSVTVSPDGSLCASSDKDG
T.congolense    APIIVSGGWDNLVKVWDLAT--GRLVTDLKGHTNYVTSVTVSPDGSLCASSDKDG
T.vivax         KPLIVSGGWDNLVKVWDPAGGVSRVVSELKGHTNYVTSVTVSPDGSLCASSDCDG
                *;************** * ,*:::;*******;********************* **


T.carassii      ---------------LSAGSPINQICFSPNRYWMCAATEKAIRIFDLESKDVIVE
T.cruzi         VARLWDLTKGEALSEMAAGAPINQICFSPNRYWMCAATEKGIRIFDLENKDVIVE
T.congolense    VARLWDLTKGESLSEMAAGAPINQICFSPNRYWMCAATEKVIRIFDLESKDVIVE
T.vivax         VARLWDLAKGDSLFEMSAGAPINQICFSPNRYWMCAATDECIRIFDLENKDVIVE
                *******;**;;* *;;**;*************;; ******.******


T.carassii      LVPETQQKEKKAPECVSVAWSADGTTLYSGHTDNVIRVWSVSENA
T.cruzi         LAPEAQQKSKKTPECMSIAWSADGNTLYSGYTDNVIRVWSVSEHA
T.congolense    LAPETQSNCKTLPECVSIAWSADGSTLYSGYTDNVIRVWSVSDRA
T.vivax         LTPEAHGS--KPPQCQSIAWSADGSTLYSGHADNVIRIWTVSLKA
                *.**:: . . *;* *;******.*****;;*****:*;** .*
```

Fig. 7.5 Multiple amino acid sequence alignment of activated protein kinase C receptor. Sequences were aligned by Clustal W1.7 programme. Asterisks indicate identity, colons and dots indicate decreasing degrees of conservative substitutions. Asterisks, colons, and dots are indicated in grey where the *T. carassii* sequence is lacking. Proteins were taken from the Swissprot database with the following accession numbers: *T. cruzi* **XM_812641**, *T. congolense* **AF100286**, *T. vivax* **AF100287**.

Discussion

Screening the *T. borreli* λTriplEx2 expression library with carp immune serum revealed only ribosomal proteins. This could be partly due to the high prevalence of these genes in the library, since the major cDNA band of approximately 600 bp (Fig. 7.1) was identified as 18S rRNA. Screening the *T. carassii* λTriplEx2 expression library with carp immune serum also revealed a high number (73%) of ribosomal products. Additionally, however, we revealed ubiquitin and activated protein kinase C receptor as antigens.

The first antigen we identified was ubiquitin. In a similar experimental setup Kirchhoff *et al.* screened a λgt11 expression library of *T. cruzi* with serum from a mouse chronically infected with *T. cruzi* and identified ubiquitin as an antigen (Kirchhoff *et al.*, 1988). Furthermore, humans are capable of producing antibodies against *T. cruzi* ubiquitin although there are only 3 amino acids different (14, 19 and 57 Fig. 7.2) (Télles *et al.*, 1999). This indicates a good chance that indeed ubiquitin is a *T. carassii* antigen against which carp can produce antibodies.

Ubiquitin genes are organised into either head-to-tail tandem repeats or a single open reading frame conjugated to a C-terminus extension encoding one or two ribosomal proteins, these are termed polyubiquitin gene and fusion ubiquitin genes, respectively (Horrocks and Newbold, 2000). The clone identified here encodes a fusion ubiquitin gene, however, we expect that further screening of the *T. carassii* library would reveal polyubiquitin genes as well.

Ubiquitin is a 8.5 kDa heat-stable protein present in all eukaryotic cells, either free or bound to other proteins. It is used to label misfolded or damaged proteins as a signal for protein degradation, but also short-lived proteins such as transcription factors and cell cycle regulatory proteins are similarly targeted (Hochstrasser, 1995). Furthermore, ubiquitination can be used by trypanosomes as a signal to endocytose cell surface proteins for subsequent proteolysis in the lysosomes (Haglund *et al.*, 2003). Although, for example for *T. brucei* it has been shown that the ectophosphatase contains three possible ubiquitination sites in the extracellular domain of the protein (Bakalara *et al.*, 2000) it is not very likely to find ubiquitin at the outer membrane of the parasite. Therefore it is not very likely that antibodies directed against ubiquitin can be protective, unless they are taken up by the parasite. Indeed uptake of antibodies by *T. brucei* has been shown, however, these antibodies are immediately degraded by the parasite (Russo *et al.*, 1993). Ubiquitin is constitutively expressed and an essential protein for the parasite, if antibodies could get inside without degradation ubiquitin would be a good target for the carp immune system.

The second antigen we identified had 79% identity, on the nucleotide level, with the C-terminal end of *T. cruzi* activated protein kinase C receptor. In humans a strong antibody response is provoked against the *Leishmania* activated protein kinase C receptor (Kamoun-Essghaier *et al.*, 2005). Activated protein kinase C receptor is involved in signalling processes and able to interact simultaneously with several signalling molecules, such as protein kinases and membrane-bound receptors (Nilsson *et al.*, 2004). Next to this it is part of the ribosomes where it can recruit protein kinase C and link signal-transduction pathways directly to the ribosome. Furthermore activated protein kinase C receptor can bind to membrane-bound receptors in order to keep the ribosome at a site where local translation is required (Nilsson *et al.*, 2004). For *T. brucei* the homologue of the activated protein kinase C receptor was upregulated during the transformation from the bloodstream stage to the tsetse fly midgut procyclic form (Matthews and Gull, 1998). And for *T. brucei rhodesiense* it was shown to be upregulated during ConA-induced apoptosis and in terminally differentiated bloodstream forms of the parasite (Welburn and Murphy, 1998). When the activated protein kinase C receptor is also upregulated in apoptotic or dying *T. carassii* it could well be that a reasonable amount of this protein is released after the trypanosome has died and antibodies can be directed against this abundant and foreign molecule. Whether this antibody can be protective remains to be proven, since contradicting results have been observed with vaccination trials in mice. No protection against *Leishmania chagasi* neither in liver nor in spleen was observed (Marques-da-Silva *et al.*, 2005) while protection in the draining lymph nodes against *Leishmania infantum* was observed (Dondji *et al.*, 2005).

The majority, 100% for *T. borreli* and 73% for *T. carassii*, of the positive clones encoded for different ribosomal proteins (5.8S, 18S, 24S or 28S). Ribosomal proteins are highly expressed in the blood stage form of *T. carassii*, Agüero *et al.* sequenced 1921 clones of an expression library and from the 32% that had a match in the database 69% were ribosomal (Aguero *et al.*, 2002). In order to avoid picking up the ribosomal products excretory/secretory products could be analysed for the presence of antigenic proteins as described for *Trypanosoma danilewskyi* (syn. *T. carassii* (Lom and Dyková, 1992)) (Plouffe and Belosevic, 2006). Ribosomal proteins have, however, been identified as antigens, in similar screening procedures as performed here, for *T. cruzi* and *Plasmodium falciparum* (DaRocha *et al.*, 2002; Lobo *et al.*, 1994). It is proposed that immunogenicity of evolutionary highly conserved proteins such as ribosomal proteins might be the result of factors as the abundance and high stability as well as their increased capacity to be phagocytosed and processed by antigen-presenting cells,

furthermore exposure to similar antigens from other micro-organisms might contribute to the strong immune response (Requena *et al.*, 2000). Interestingly antibodies against the ribosomal P protein of *Plasmodium falciparum* have been shown to inhibit the growth of the parasites *in vitro* (Goswami *et al.*, 1997). Whether the antibodies revealed in this screening are also protective antibodies needs to be examined, for example by means of DNA vaccination.

Acknowledgments

Chapter 8

General discussion

Maaike Joerink

To advance our knowledge on the evolution of the immune system and the phenomenon of macrophage polarisation in teleosts, carp macrophages and their mode of activation in response to parasites were investigated. Previously, *Trypanoplasma borreli* and *Trypanosoma carassii* were shown to induce different immune responses and different pathology in the infected carp host. Therefore, we hypothesised that these parasites are able to polarise the macrophages differently, leading to classically activated macrophages and type I immune responses during a *T. borreli* infection. And leading to alternatively activated macrophages and type II immune responses during a *T. carassii* infection.

8.1 Host-parasite model

Trypanoplasma borreli and *Trypanosoma carassii*, the parasites used in this study, are kinetoplastid parasites infecting cyprinid fish such as carp, goldfish, crucian carp, and tench, as well as some members of non-cyprinid families. Both parasites live extracellular in the blood of the fish. For the experiments described in this thesis we used common carp (*Cyprinus carpio* L.) as a natural host. The majority of the experiments were performed with laboratory-bred carp (R3 x R8) relatively resistant to infections with *T. borreli* (Saeij *et al.*, 2003b; Wiegertjes *et al.*, 1995) and *T. carassii* (unpublished observations). Genetic variation between individual fishes generally is high. The fish we have used in our studies (R3 x R8) are a cross between two different carp lines that each has been inbred by brother-sister mating for 9 generations. In theory, each of these carp lines thus has an inbreeding coefficient of 0.9, close to full inbreeding. By cross-breeding two highly inbred carp lines we obtained heterozygous fish with a common genetic background.

In nature, *T. borreli* and *T. carassii* are transmitted by the blood-sucking leeches *Piscicola geometra* (*T. borreli* and *T. carassii*) and *Hemiclepsis marginata* (*T. carassii*). In our experiments, however, we infected carp by intraperitoneal (i.p.) injection. It has been shown that there is no significant difference between intramuscular (i.m.) and i.p. injection with *T. borreli*, with regard to parasitaemia levels and antibody production (Jones *et al.*, 1993). Both i.m. and i.p. infections mimic the natural infection route by the bite of a leech, since the infection dose can be controlled more precisely during i.p. injection we used this route of infection. In general, infections with *T. borreli* are more severe and potentially lethal, while infections with *T. carassii* are less severe and last 2-3 weeks longer (chapter 5). Almost all experiments described in this thesis have been performed with cells isolated from head kidney. Since head kidney is the major hematopoietic organ in teleosts this suggests a rational choice for the establishment of macrophage

cultures (chapter 4). With regard to the *in vivo* experiments, however, where frequently freshly isolated head kidney cells were used, it could be of interest to also consider the use of splenic macrophages. The spleen is a secondary immune organ and, although it is unknown whether antigen presentation takes place in teleost spleen, the spleen increases considerably in size during *T. borreli* and *T. carassii* infections, indicating that the spleen could be involved in the immune response against these parasites.

Previous research showed a difference in the immune response of carp against these two parasites (Saeij *et al.*, 2002b). High levels of nitrite were shown in response to *T. borreli*, while minimal nitrite is produced in response to *T. carassii*. This lead to the hypothesis, that during these immune responses macrophages were differentially activated / polarised (Saeij, 2002a). Macrophage polarisation, with classical and alternative activation representing the two extremes, has been described for mammals, especially in response to parasitic infections. The presence of classical and alternatively activated macrophages coincides with the presence of T helper (T_H)-1 and T_H2 cells, respectively (Fig. 8.1). We were able to show macrophage polarisation both *in vitro* in response to LPS and cAMP and *in vivo* in response to *T. borreli* and *T. carassii* (chapters 3, 4 and 5). The classification of a true classical activation state during these experiments and the sub-type of alternative activation state require further research.

8.2 Innate or classical macrophage activation

Based on the activation by interferon (IFN)-γ a distinction has been made between innate and classical activation of mammalian macrophages. Innate activation can be induced by a microbial trigger alone, while classical activation is induced by the combination of a microbial trigger and IFNγ (Gordon, 2003). During the research described in this thesis it was not possible to make the same distinction for carp macrophages because carp IFNγ was not yet known. At present, however, sequence information is available for pufferfish and rainbow trout IFNγ (Zou *et al.*, 2005; Zou *et al.*, 2004). Very recently, IFNγ has also been identified for carp by our group (E.H. Stolte, personal communication) opening the way for a distinction between innate and classical activation of carp macrophages. This enables us to study whether IFNγ could be involved in the immune response against *T. borreli* and *T. carassii* infections and to determine if the macrophages would be preferentially innate or classically activated. *In vitro* addition of recombinant IFNγ to LPS-stimulated macrophages could possibly show if carp IFNγ would have a synergistic effect on the production of nitrite, comparable to the mammalian situation.

However, since teleosts can be considered evolutionary older vertebrates, it could also be that teleosts do not possess both innate and classical activated macrophages but instead would show a primordial type I activation state, combining the two phenotypes.

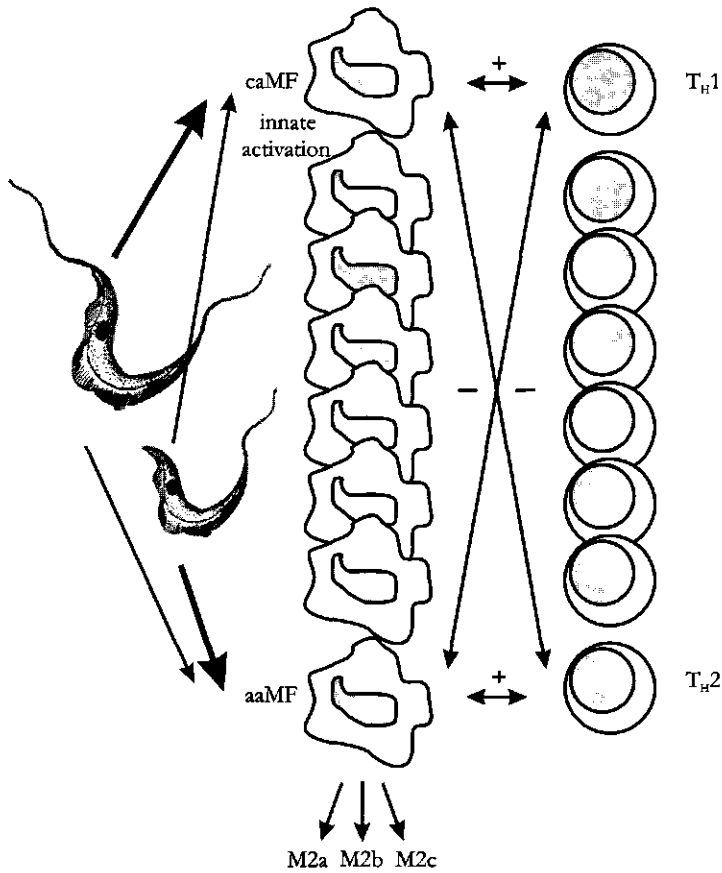Fig. 8.1 Classically activated macrophages (caMF) and alternatively activated macrophages (aaMF) are the extremes of macrophage polarisation. This polarisation is different depending on the parasite. Innate activation is an activation state close to classical activation. Alternative activation can be sub-divided into M2a, b and c. The caMF and aaMF coincide with the polarisation of T helper (T_H)-1 and T_H2 cells.

8.3 Alternative macrophage activation

As suggested by Mantovani *et al.* alternative activation of macrophages can be sub-divided into three different activation states, depending on the stimulating factors or cytokines involved (Mantovani *et al.*, 2004). Interleukin (IL)-4 and IL-13 would then induce M2a or true alternative activation. The combination of immune complexes (IC) and toll-like receptor (TLR) or IL-1R agonist induces M2b or type II activation; while IL-10 induces M2c or a deactivation state. All types of alternatively activated macrophages are involved in T helper (T_H)-2 responses, allergy, immuno-regulation, killing and encapsulation of parasites, matrix deposition and remodelling and promote tumour growth. The M2a type, however, is more specialised in T_H2 responses, type II inflammation, allergy, and killing and encapsulation of parasites. The M2b type is more involved in T_H2 activation and immuno-regulation. The M2c type would be involved especially in immuno-regulation and matrix deposition and tissue remodelling (Mantovani *et al.*, 2004).

From the research presented in this thesis we conclude that alternatively activated macrophages, as characterised by arginase activity, are present in carp. Arginase is mainly upregulated in the M2a and M2c sub-types. However, a sub-division into M2a, b and c sub-types for carp remains to be investigated. IL-4 and IL-13 have not been described for any fish species. So far, synteny searches in pufferfish and zebrafish genome databases did not reveal any candidates either, suggesting that the M2a subtype may not be present in fish. The M2b and M2c subtypes are more likely to be present in fish because their inducers; the combination of immune complexes and TLR or IL-1R agonist, and IL-10, respectively, have been described for several fish species. In addition, the majority of the effector molecules induced in the M2b and M2c macrophages have been described for different teleosts (Fig. 8.2). With regard to M2b sub-type the following effector molecules are known; MHC-II, IL-10, tumour necrosis factor (TNF)-α, and IL-1. With regard to the M2c sub-type; arginase, IL-10, and transforming growth factor (TGF)-β have been described. So far, we have characterised the alternative activation of carp macrophages by the induction of arginase activity.
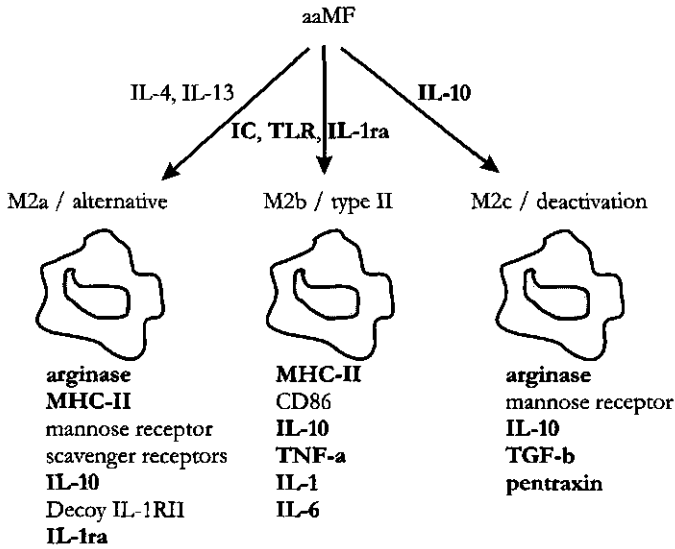
Fig. 8.2 Overview of the sub-types of alternatively activated macrophages, together with their inducers and effector molecules. Molecules known for carp are depicted in bold. (Adjusted from Mantovani *et al.*, 2004.)

A further detailed characterisation to identify sub-types of carp macrophages should be performed using gene expression profiling of cytokines, chemokines and receptors. Beside characterising the known genes one could perform suppression subtractive hybridisation (SSH) to find novel genes upregulated in alternative activated macrophages, such as performed in mice models (Hassanzadeh Ghassabeh *et al.*, 2006). cDNA from differentially stimulated head kidney-derived macrophages or with cDNA obtained from different infections could be starting material for this type of analysis. In order to do so, however, first new stimuli need to be identified. We have used the second messenger, cAMP to induce alternative activation *in vitro* in head kidney-derived macrophages. Although we tested several potential stimulants such as dexamethasone (Morris *et al.*, 1998), phorbol 12-myristate 13-acetate (PMA), concanavalin A, phytohemagglutinin (PHA), and also lysates from *T. borreli* and *T. carassii*, we were not able to significantly induce arginase activity other than by using cAMP.

Cruzipain, a cysteine protease of *Trypanosma cruzi*, has been shown to induce alternatively activated macrophages in mice (Giordanengo *et al.*, 2002). Recently, the cysteine proteases of both *T. borreli* and *T. carassii* have been identified by our group (A. Ruszczyk, unpublished data). This allows for studies on these proteases to determine whether, similar to the protease of *T. cruzi*, these

proteases can induce alternative activation of carp macrophages and to investigate how these proteases are involved in the *T. borreli* and *T. carassii* infections.

Additionally, since infection with *T. carassii* induced only a modest increase of arginase activity and thus alternative activation (chapter 5) another parasitic infection model might be more informative (Wiegertjes *et al.*, 2005). Until present there are no known (molecular) properties of the pathogen that can predict the type of immune response it will induce. Identifying the receptors involved in pathogen recognition could be helpful in clarifying this question.

8.4 Arginase and alternatively activated macrophages in wound healing

Next to a role in parasitic infections arginase activity by alternatively activated macrophages (M2c) has been shown to be involved in the process of wound healing. In a wound healing model for carp, initially set up to characterise gene expression profiles in injured skin, we measured the expression of iNOS and arginase (Fig. 8.3). Expression of arginase 1 was not influenced (data not shown) while expression of arginase 2 (solid bars) was high during the first hours after mechanical skin injury. Expression of iNOS (open bars) increased from 3 hours onwards. We hypothesise that the early increase of arginase 2 could be due to infiltrating macrophages that upon arrival downregulate their arginase expression and increase their iNOS expression. Unfortunately, we were not able to analyse later time points owing to secondary bacterial infections of the wound area influencing gene expression.
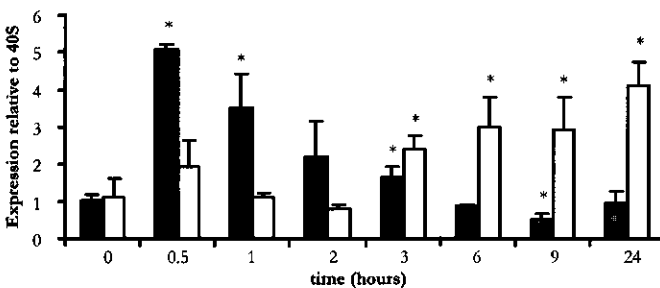
Fig. 8.3 Gene expression of arginase 2 (solid bars) and iNOS (open bars) relative to the expression of 40S, in skin of 'injured' carp. Skin of n=3 carp were used as control (0 h) and skin injury was mechanically induced in n=21 carp. At the indicated time point n=3 carp were sacrificed, skin samples removed, snap frozen in liquid nitrogen and stored at -80 °C. One μg RNA was transcribed into cDNA and five μl of 10 times-diluted cDNA was used for real-time quantitative PCR. Data were analysed using the Pfaffl method and efficiencies were calculated per run per gene (arginase 2; 1.73, iNOS; 1.69 and 40S; 1.77). Average and SD of n=3 carp are shown. * $P \leq 0.05$ Student's *t*-test compared to undamaged controls.
(unpublished data, S.F. Gonzalez and M. Joerink).

In a wound healing model in rats an early (up to three days) increase in iNOS activity was observed, while after the third day arginase activity was increased. Most likely, the iNOS activity induced an early inflammation with a cytotoxic environment, vasodilatation and inhibition of platelet aggregation. Subsequently, arginase activity induced the synthesis of polyamines and proline and thus collagen (Albina *et al.*, 1990; Shearer *et al.*, 1997). Although the skin injury model does not seem to be a suitable model to determine whether arginase expression can play a role during later phases of wound healing in carp, we were able to show an early increase of iNOS activity.

8.5 Macrophage activation during mixed infections

Mixed infections of *T. borreli* and *T. carassii* in carp are commonly found in nature. The majority of laboratory infection experiments, however, are performed with mono-parasitic infections. Since infections with *T. borreli* and *T. carassii* induced different immune responses we proceeded to study the (naturally occurring) mixed-infection. Since the development of the two parasites is different within the leech (Kruse *et al.*, 1989; Lom, 1979) we expect a higher infection dose of *T. carassii* than of *T. borreli* in a natural mixed-infection. Therefore, we simultaneously injected both parasites in the same carp, using different doses (100 times more *T. carassii*) and followed the parasitaemia and antibody production of the fish over time (chapter 6). Interestingly, we observed an interaction between the two parasites whereby mostly the parasitaemia level of *T. borreli* was affected. The decreased *T. borreli*-specific parasitaemia in fish co-infected with *T. carassii* was not based on substrate competition since infections with a higher dose of *T. borreli* resulted in higher parasitaemia. During this mixed infection experiment we took small blood samples weekly, and determined species-specific parasitaemia based on the morphological differences between the two parasites. Since we only had to take small blood samples we could follow the same fish over a 10 week period and obtain data on the overall effect of the mixed infection. We did not study macrophage polarisation in these mixed infections since for this we would have had to sacrifice the fish to measure iNOS and arginase activity or gene expression.

We hypothesise that the observed protective effect of the mixed infection could be caused by the induction of cross-reactive antibodies. Within the mixed infection, but also after the pre-infection with *T. carassii*, antibodies are present at the time when *T. borreli* appears in the blood of the fish. These antibodies, in principle directed against *T. carassii*, can already perform a protective function from the start of the *T. borreli* infection. By Western blotting we

showed the presence of cross-reactive antibodies against *T. borreli* proteins in the serum of fish infected with *T. carassii* (and the reverse as well). Whether the protective effect of co-infection with *T. carassii* is based on specific-antibodies cross-reacting with *T. borreli* antigens, or rather based on a general immuno-stimulatory effect, remains to be investigated. Challenging fish that survived a *T. carassii* infection with an unrelated (bacterial) infection could show if a general immuno-stimulatory effect is involved or not.

8.6 Cross-reactive antibodies as protection

To advance our knowledge about the antibodies most likely involved in protection, we screened expression libraries of both parasites with homologous immune carp serum. The screening of both libraries revealed ribosomal proteins as antigens. Furthermore, the screening of the *T. carassii* expression library revealed activated protein kinase C receptor and ubiquitin as antigens (chapter 7).

We obtained only a partial sequence for the activated protein kinase C receptor making it impossible to estimate the corresponding molecular weight. The homologues proteins from *Leishmania* and *Crithidia fasciculata* are 36 and 35 kDa, respectively. This does correspond in size with one of the protein bands recognised by carp immune serum on Western blot (chapter 6). The *Leishmania* activated protein kinase C receptor has been shown to induce protective immune responses in susceptible mice. This protection is suggested to be partly based on the enhanced binding of the protein to MHC class II molecules, thereby enhancing antigen presentation (Gonzalez-Aseguinolaza *et al.*, 1999). Interestingly, the *Leishmania* activated protein kinase C receptor has been also shown to influence the T_H1/T_H2 balance in mice by inducing a down-regulation of the T_H2 reaction, and increasing host survival (Soldera *et al.*, 1997). It is suggested that this modulatory effect is based on the ability of the activated protein kinase C receptor to bind proteins involved in DNA replication and RNA synthesis, thereby influencing the early immune response (Gonzalez-Aseguinolaza *et al.*, 1999). Since the immune responses of carp against *T. borreli* and *T. carassii* are different, future studies should aim at the determination of putative differences between the activated protein kinase C receptors of these parasites individually.

Another antigen identified during our screening was ubiquitin. In a similar screening of a *T. cruzi* expression library, ubiquitin was identified as major antigen (Kirchhoff *et al.*, 1988). Later, Télles *et al.* described that although *T. cruzi* ubiquitin is only three amino-acids different from the human ubiquitin,

it indeed is immunogenic. Human antibodies most likely recognise amino acids 19 and 57 (see Fig. 8.4), are not auto-reactive and are not cross-reactive with *L. major* (Télles *et al.*, 1999).
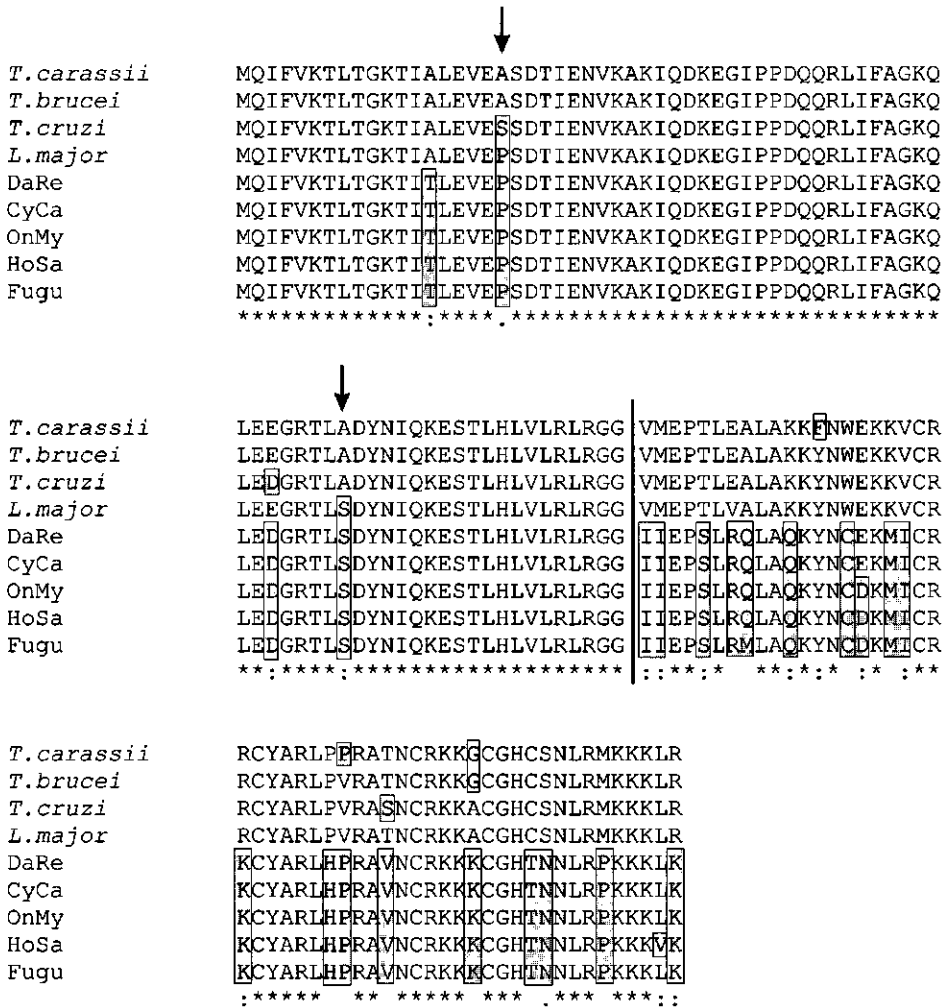
Fig. 8.4 Multiple amino acid sequence alignment of ubiquitin with the L40 ribosomal tail protein. Sequences were aligned by Clustal W1.7 programme. Asterisks indicate identity, colons and dots indicate decreasing degrees of conservative substitutions. Differences are highlighted in grey. Arrows point at amino acid 19 and 57, while a vertical line is placed between the ubiquitin and the L40 ribosomal tail protein. Protein sequences were taken from the Swissprot database with the following accession numbers: *T. brucei* **CAA39864**, *T. cruzi* **CAA30335**, *L. major* **CAJ08391**, zebrafish DaRe **NP_001032190**, rainbow trout OnMy **BAA88568**, human HoSa **AAI01831**, pufferfish Fugu **CAG00768**. CyCa represents the newly sequenced carp ubiquitin sequence.

In order to determine if the carp antibodies against *T. carassii* ubiquitin are likely to recognise the same amino acids we determined the carp ubiquitin coding sequence (Fig. 8.4). *T. carassii* ubiquitin is different from the carp ubiquitin at four amino acids (14, 19, 52, and 57). Amino acid 19 is an alanine in *T. carassii* and a serine in *T. cruzi* while it is a proline in both carp and human. It would be interesting to determine if the carp-anti-*T. carassii* antibodies would be cross reactive with the *T. cruzi* ubiquitin and, vice versa, whether human-anti-*T. cruzi* antibodies would recognise *T. carassii* ubiquitin. Furthermore, it would be of interest to identify the *T. borreli* ubiquitin sequence in order to determine cross-reactivity with carp-anti-*T. carassii* antibodies.

During the screening ribosomal proteins were detected many times. To check if these ribosomal proteins represent real positive proteins also detected by cross-reactive antibodies, phages containing the ribosomal clones (*T. carassii* or *T. borreli*) could be screened with immune and pre-immune sera of both *T. carassii* and *T. borreli* infected fish. Because ribosomal proteins are highly expressed it is likely that carp will indeed produce antibodies against these proteins.

True evidence for the protective nature of the antibodies against the identified antigens could possibly be obtained by means of DNA vaccination.

8.7 In conclusion

Depending on the pathogen entering the body, the macrophages of an infected host will induce different immune responses. After the first polarisation of the macrophages, T cells are highly important in the further development of type I and II responses by the production of different cytokines. Not all cytokines necessary to distinguish type I / T_H1 from type II / T_H2 responses are fully known for carp and T cell markers were lacking at the start of this research. Therefore, we were not able to characterise T cell responses during the infections. With the recently described T cell markers and cytokines, however, it now becomes possible to determine the involvement of T cells. Although we did not consider the T cells, we did measure one of the final outcomes of the immune response by determining the antibody levels, resulting from the activation of B cells.

From the mammalian immune system it is known that depending on the type of the immune response different isotypes of antibodies are produced. During the *T. borreli* and *T. carassii* infections antibodies play a protective role. Not all different isotypes of antibodies have been described for teleosts. IgM is the most prevalent but also IgD and IgT/Z have been identified (Danilova *et al.*, 2005;

Hansen *et al.*, 2005; Hordvik *et al.*, 1999; Savan *et al.*, 2005). Mammalian IgM can activate the classical complement pathway, inducing complement-mediated lysis of the pathogen. Interestingly, *T. borreli* was shown to be killed *in vitro* in the presence of complement (Saeij *et al.*, 2003a; Scharsack *et al.*, 2004) and the gene expression of complement factor 3 was upregulated during a *T. borreli* infection as well (Saeij *et al.*, 2003a). We never observed phagocytosis of the parasites, and the presence of Fc receptors has never been proven for teleosts. Therefore we believe that the complement-mediated lysis induced by parasite specific IgM is the most important and effective mechanism underlying the killing of *T. borreli* and *T. carassii* in carp. This killing is however, only possible when antibodies are produced in time. Since during a *T. carassii* infection macrophages are alternatively activated relatively early in the infection, they will lead to an early type II response. This will also lead to an early antibody production and consequently better protection of the carp. It is intriguing to realise that antibody-antigen complexes are stimulators of alternatively activated macrophages and could lead to a positive feedback. Furthermore, the cross-reactive antibodies produced during a *T. carassii* infection could form antibody-antigen complexes in a secondary *T. borreli* infection. These antibody-antigen complexes could induce aaMF and induce an earlier antibody response, necessary for the complement-mediated lysis of the parasite.

For future studies it would be interesting to see how the antibodies produced during a *T. carassii* infection could be different from antibodies produced during a *T. borreli* infection. Differences could be present at the isotype level or antigen-specificity but also at the level of kinetics, both time and quantity related. This knowledge would shed more light onto the potentially different roles of antibodies during *T. borreli* and *T. carassii* infections, and their relation to macrophage polarisation.

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aaMF,	alternatively activated macrophages
ABTS,	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ANOVA,	analysis of variance
AP,	alkaline phosphatase
BCIP,	5-bromo-4-chloro-3'-indolyphosphate
BLAST,	basic local alignment search tool
BSA,	bovine serum albumin
caMF,	classically activated macrophages
cAMP,	dibutyryl cyclic adenosine mono phosphate
CSP,	carbamoyl phosphate synthase
DAB,	diaminobenzidine tetrahydrochloride
DC's,	dendritic cells
DDC,	duplication-degeneration-complementation
DEPC,	diethyl pyrocarbonat
DHR,	dihydrorhodamine 123
FITC,	fluorescein isothiocyanate
FSC-H,	forward scatter
GABA,	γ-amino butyric acid
GPI,	glycosylphophatidylinositol
HKL,	head kidney leukocytes
HRP,	horseradish peroxidase
HSP,	heat shock protein
IEL,	intraepithelial lymphocytes
IFN,	interferon
IgSF,	immunoglobulin superfamily
IL,	interleukin
i.m.,	intramuscular
iNOS,	inducible nitric oxide synthase
i.p.,	intraperitoneal
IPTG,	isopropyl-beta-D-thiogalactopyranoside
ITAM,	immunoreceptor tyrosine-based activation motif
ITIM,	immunoreceptor tyrosine-based inhibition motif
L-NMMA,	N^{ω}-monomethyl-L-arginine acetate
LPS,	lipopolysaccharide
MTS,	mitochondrial targeting sequence
Mya,	million year ago

NBT,	nitro blue tetrazolium
NCC's,	non-specific cytotoxic cells
NILT,	novel immunoglobulin-like transcript
NK's,	natural killer cells
NO,	nitric oxide
NOHA,	N^ω-hydroxy-L-arginine
OUC,	orntihine-urea cycly
PAMP's,	pathogen-associated molecular patterns
PBL,	peripheral blood leukocytes
PCS,	pooled carp serum
Pfu,	plack forming units
PI,	propidium iodide
PMA,	phorbol 12-myristate 13-acetate
RACE,	rapic amplification of cDNA ends
ROS,	reactive oxygen species
RPE,	R-phycoerythrin
SSC-H,	sideward scatter
SSH,	suppression subtractive hybridisation
TGF,	transforming growth factor
T_H,	T helper cell
TLR,	toll-like receptor
TNF,	tumour necrosis factor
TREM,	triggering receptor expressed on myeloid cells

Summary

Research in the field of comparative immunology, such as investigations on the immune system of carp, provides an excellent opportunity to study the evolution of the immune system. It helps to show what is essential to the complex immune system.

In the studies described in this thesis we used a natural host-parasite model of two parasites (*Trypanoplasma borreli* and *Trypanosoma carassii*) infecting common carp (*Cyprinus carpio* L.), to obtain more knowledge about the phenomenon of macrophage polarisation in 'the evolutionary older' teleosts and the consequences of differential activation for the individual host.

The general aspects of the teleost immune system are very similar to those of the mammalian immune system. Polarisation of macrophages into classically activated macrophages (caMF) or alternatively activated macrophages (aaMF) have been described for mammals, however, not yet for teleosts. These caMF and aaMF are involved in type I and type II immune responses, respectively. Macrophage polarisation has been described during many parasitic infections. Typically, caMF are active early during infection, while aaMF are active late in the infection. *T. borreli* and *T. carassii* are kinetoplastid parasites infecting carp. The immune response of carp against these two parasites is different with regard to the production of nitrite by macrophages. High levels of nitrite are produced during a *T. borreli* infection, while nitrite production is hardly increased during a *T. carassii* infection. We hypothesise that infections with these two parasites in carp provide a good model to study macrophage polarisation in teleosts (chapter 1). Since T helper (T_H)-1 and T_H2 cells and their cytokine profiles are not (fully) characterised for teleosts, immune responses can not easily be assigned type I or type II. However, the polarisation of macrophages into either caMF or aaMF coincides with the type I and type II polarisation, respectively. Therefore, we propose the use of NO production by caMF and arginase activity from aaMF as markers for type I and type II immune responses in teleosts (chapter 2). Since the carp iNOS sequence was known, and an assay to measure nitrite production (Griess reaction) was running these could be readily used as markers to study caMF. To study arginase activity in aaMF we cloned and sequenced the carp arginase genes and optimised an arginase activity assay. Two genes (arginase 1 and 2) are present in carp which are 340 and 347 amino acids respectively, and both are 63% identical to their respective human arginase genes. For arginase 2 three highly homologous genes were identified, each showing only single non-synonymous substitutions. Arginase 1 is mainly expressed in the mid kidney, while arginase 2 is expressed in all immune organs

with the highest expression in liver. Stimulation *in vitro* with the intracellular messenger cAMP, could induce arginase 2 but not arginase 1 upregulation in cultured macrophages. Therefore we proposed that arginase 2 is important in the immune functions of carp, in contrast to arginase 1 being the most important immunological isoform in mammals (chapter 3). To study the caMF and aaMF in more detail we developed and characterised a carp macrophage culture system. Flow cytometric analysis, antibody labelling of cell surface markers, and light microscopy showed the presence of a major population of macrophages with a heterogeneous phenotype after 6 days of culture. These head kidney-derived macrophages can be considered the fish equivalent of mammalian bone marrow-derived macrophages. These macrophages show the ability to phagocytose, produce radicals and are able to polarise into innate/classically activated or alternatively activated macrophages. Gene expression profiling of LPS (innate/classical activated) or cAMP (alternatively activated) stimulated macrophages showed differential gene expression for most of the immune genes presently described for carp. The recently described novel Ig-like transcript 1 (NILT1) and CXCR1 and CXCR2 chemokine receptors were upregulated in cAMP stimulated macrophages (aaMF). Furthermore, NILT1 expression was upregulated in the later phase of a *T. carassii* infection, but not during a *T. borreli* infection. We suggest that NILT1, CXCR1 and CXCR2 could be used as novel markers for aaMF in teleosts (chapter 4). To further investigate the presence of polarised macrophages during parasitic infections in teleosts we infected carp with *T. borreli* and with *T. carassii*. During these infections we followed the NO production and arginase activity in the head kidney leukocytes (HKL) of these carp. Basal nitrite production (read-out for caMF) and arginase activity (read-out for aaMF) were only moderately different between the two infections. Differences were observed, however, after *ex vivo* re-stimulation of HKL. Re-stimulation with LPS and *T. borreli* lysates increased nitrite production by HKL of *T. borreli*-infected carp. Re-stimulation with cAMP increased arginase activity in HKL of *T. carassii*-infected carp. Most likely arginase 1 expression is responsible for this activity, since the expression of arginase 1 is upregulated during the later time points of the *T. carassii* infection. Our data indicate that *T. borreli*-infected carp are more prone to increase nitrite production by caMF, while *T. carassii*-infected carp are more prone to increase arginase activity by aaMF (chapter 5). Continuing on the observation that *T. borreli* and *T. carassii* induce different types of immune responses we co-infected carp with both parasites simultaneously. Total parasitaemia was lower and survival was higher in co-infected carp.

Within the mixed infected fish, species-specific parasitaemia was determined based on morphological differences. *T. carassii*-specific parasitaemia in mixed infections was equal to the parasitaemia in mono-parasitic infection, while *T. borreli*-specific parasitaemia was lower in mixed than in mono-parasitic infections. *T. borreli*-specific antibody levels, determined by ELISA, where higher in mixed and in *T. carassii*-infected carp than in *T. borreli*-infected carp. In mixed infected fish, *T. borreli* was detected from week 5 onwards when antibodies were already present. Furthermore, carp surviving a *T. carassii* infection showed reduced susceptibility to re-infection with *T. borreli*. Our data indicate a protective effect of co-infection with *T. carassii* on the resistance to *T. borreli*, possibly mediated via cross-reactive antibodies (chapter 6). In an attempt to identify the antigenic proteins of *T. borreli* and *T. carassii* we constructed λTriplEx2 expression libraries of both parasites and screened these libraries with homologous carp immune serum. These screenings revealed ribosomal proteins as antigenic proteins for both parasites and ubiquitin and activated protein kinase C receptor as antigenic proteins for *T. carassii*. Whether the antibodies against these proteins are protective and cross-reactive requires further studies (chapter 7).

In conclusion, carp macrophages are able to polarise into classically and alternatively activated macrophages. To some extent this polarisation into caMF and aaMF can be induced by infection with *T. borrreli* and *T. carassii*, respectively. The level to which this polarisation in response to these parasites influences the production of (protective) antibodies remains to be elucidated.

Samenvatting

Vergelijkende immunologie is het onderzoeken van het afweersysteem van andere dieren dan de mens. Het onderzoek aan het afweersysteem van de karper is een vorm van vergelijkende immunologie. Dit type onderzoek wordt verricht om meer te weten te komen over de evolutie van het afweersysteem. Men gaat er vanuit dat moderne beenvissen (waaronder de karper) vrij vroeg in de evolutie zijn ontstaan (ongeveer 450 miljoen jaar geleden). Hierdoor kunnen we met behulp van de kennis over de karper als het ware vrij ver terug in de tijd kijken. Mechanismen die over de tijd bewaard zijn gebleven en nog steeds actief zijn in het menselijke afweersysteem zijn blijkbaar essentieel. Onderdelen die wel bij de mens maar niet bij de karper actief zijn, zijn dan blijkbaar nieuwer en leveren een toegevoegde waarde op voor de mens.

Tijdens het onderzoek, waarvan de resultaten in dit proefschrift staan beschreven, hebben we gebruik gemaakt van een in de natuur voorkomende gastheer-parasiet combinatie als infectie model. De twee parasieten (*Trypanoplasma borreli* en *Trypanosoma carassii*) worden in de natuur door bloedzuigers van vis naar vis overgedragen. Tijdens dit onderzoek hebben we alleen de karper (*Cyprinus carpio* L.) als gastheer gebruikt en hebben we de parasieten met behulp van injecties overgedragen van de ene naar de andere karper. Het uiteindelijke doel van dit onderzoek was om meer inzicht te verkrijgen in de polarisatie toestanden van de macrofagen van de 'evolutionair oude' karpers. Macrofagen zijn een type cel die een belangrijke functie vervullen binnen het afweersysteem, doordat ze (delen van) ziektekiemen (b.v. bacteriën virussen en parasieten) kunnen herkennen en kunnen fagocyteren (opeten). Vervolgens kunnen de macrofagen deze ziektekiem 'verteren' en laten ze onderdelen van de ziektekiem aan andere cellen van het afweersysteem zien, hierna komen ook deze cellen in actie. Afhankelijk van de ziektekiem kan de macrofaag op verschillende manieren reageren, deze verschillende manieren worden ook wel polarisatie toestanden genoemd. Hierbij zijn klassieke en alternatieve activering de twee uiterste vormen van macrofaag polarisatie. Klassieke activering was al aangetoond bij de macrofagen van de karper en andere dieren. Alternatieve activering was echter alleen nog maar bij zoogdieren aangetoond. Tijdens dit onderzoek hebben we bekeken of de alternatieve activering ook bij de karper macrofagen plaats vindt.

In grote lijnen zijn het afweersysteem van de karper (als model voor de moderne beenvissen) en de mens (zoogdieren) gelijk. Of alternatieve activering echter ook bij karper macrofagen plaats kan vinden was niet bekend aan het begin van dit onderzoek. Klassieke activering is over het algemeen betrokken bij de

afweer tegen ziektekiemen die in de cellen van de gastheer zitten en dit type afweer reactie wordt ook wel type I genoemd. Alternatieve activering is juist meestal betrokken bij de afweer tegen de vrij voorkomende ziektekiemen en dit type afweer reactie wordt type II genoemd. Veel van het onderzoek aan de macrofaag polarisatie is gedaan na infectie met parasieten. Het is gebleken dat tijdens veel van de parasitaire infecties, vroeg in de infectie voornamelijk klassiek geactiveerde macrofagen voorkomen terwijl laat in de infectie voornamelijk alternatief geactiveerde macrofagen voorkomen.

T. borreli en *T. carassii* zijn parasieten die in de natuur de karper kunnen infecteren. Uit eerder onderzoek was gebleken dat de afweer reactie van de karper tegen deze twee parasieten verschillend is als het gaat om de productie van radicalen. Radicalen worden vooral geproduceerd door de klassiek geactiveerde macrofagen. Tijdens een infectie met *T. borreli* worden veel en tijdens een infectie met *T. carassii* weinig radicalen geproduceerd. Aangezien de radicalen productie, oftewel de klassieke activering, verschillend is voor de twee infecties was onze hypothese dat ook de alternatieve activering anders is tijdens deze twee infecties. Daarmee zijn deze twee infectie modellen een goede manier om de macrofaag polarisatie van de karper verder te bestuderen (hoofdstuk 1).

De verschillende cel types en signaal moleculen, betrokken bij type I en type II afweer reacties, zijn nog niet goed beschreven voor de karper. Hierdoor kunnen signaal moleculen dan ook niet gebruikt worden om de afweer reactie te benoemen als type I of type II, zoals dit wel bij zoogdieren wordt gebruikt. Aangezien de klassieke activering van de macrofagen overlapt met type I reacties en de alternatieve activering overlapt met type II reacties kunnen de macrofaag polarisatie toestanden worden gebruikt om het onderscheidt tussen type I en type II reacties te maken. Het enzym iNOS (dat het radicaal NO produceert) is voornamelijk actief in klassiek geactiveerde macrofagen terwijl het enzym arginase voornamelijk actief is in alternatief geactiveerde macrofagen. Daarom stellen wij voor om de productie van NO en de activiteit van het enzym arginase als markers te gebruiken voor het aantonen van respectievelijk type I en type II reacties in de karper (hoofdstuk 2).

Hoe een enzym (en alle andere eiwitten) gemaakt moet worden ligt gecodeerd op het genoom (het erfelijke materiaal). Deze code (het gen) was al bekend voor het enzym iNOS en ook een manier (assay) om de productie van NO te meten was al bekend en konden dus gebruikt worden om de mate van klassieke activering te meten. Om op een vergelijkbare manier de activiteit van het enzym arginase te kunnen meten moesten we zowel de genetische

code van dit enzym bepalen als ook een assay voor de activiteit optimaliseren. Net als bij de mens bleek de karper twee arginase genen (codes) te hebben; arginase 1 en arginase 2. Binnen het karper genoom komt het arginase 2 gen echter drie keer voor, waarbij deze drie verschillende genen (arginase 2a, 2b en 2c) slechts op één plaats van elkaar verschillen. Om een enzym te maken moet een gen eerst worden 'overgeschreven' en daarna 'vertaald', hoe vaker een gen wordt overgeschreven en vertaald des te meer enzym is er aanwezig. De hoeveelheid overgeschreven gen is dus een maat voor de hoeveelheid enzym die aanwezig en actief kan zijn in een cel of orgaan. Arginase 1 bleek voornamelijk overgeschreven te worden in de (midden)nier, terwijl arginase 2 (2a, 2b en 2c bij elkaar opgeteld) wordt overgeschreven in alle organen die betrokken zijn bij het afweersysteem, maar met name in de lever. Het stimuleren met (toevoegen van) cAMP (een celeigen boodschapper molecuul) aan gekweekte karper macrofagen verhoogde de overschrijving van het arginase 2, maar niet van het arginase 1 gen. Daarom verwachten wij dat arginase 2 betrokken is bij het afweersysteem van de karper, in tegenstelling tot zoogdieren waarin arginase 1 betrokken is bij het afweersysteem (hoofdstuk 3).

Om de polarisatie toestanden van de karper macrofagen beter te kunnen bestuderen hebben we een kweek systeem ontwikkeld en de cellen tijdens de ontwikkeling gekarakteriseerd. Met behulp van verschillende technieken (flow cytometie, antilichaam kleuring en licht microscopie) konden we aantonen dat de meerderheid van de aanwezige cellen na 6 dagen kweken bestonden uit macrofagen met verschillende vormen. Deze uit de (kop)nier van karpers afkomstige macrofagen kunnen worden vergeleken met de macrofagen uit het beenmerg van zoogdieren. Daarnaast zijn deze macrofagen instaat om: (latex bolletjes) te fagocyteren (op te eten), radicalen te produceren en om te polariseren (hoger productie NO of hogere arginase activiteit). Macrofagen gestimuleerd met LPS (een onderdeel van bacteriën, gebruikt om een klassieke activering te induceren) schrijven andere hoeveelheden over van de genen die een rol spelen binnen het afweersysteem dan de macrofagen die gestimuleerd zijn met cAMP (gebruikt om een alternatieve activering te induceren). Receptoren steken uit een cel en kunnen signaal moleculen herkennen om vervolgens een signaal door te geven naar binnen toe. NILT1, CXCR1 en CXCR2 zijn receptoren die extra werden overgeschreven in cAMP gestimuleerde (alternatief geactiveerde) karper macrofagen. Daarnaast werd NILT1 meer overgeschreven aan het einde van een infectie met *T. carassii* (wanneer er alternatief geactiveerde macrofagen zijn), maar niet tijdens een infectie met *T. borreli* (waarin de macrofagen klassiek geactiveerd zijn). Daarom stellen we voor dat deze receptoren (NILT1, CXCR1

en CXCR2) gebruikt kunnen worden als nieuwe markers voor alternatief geactiveerde macrofagen in de karper (hoofdstuk 4).

Om meer inzicht te verkrijgen in de macrofaag polarisatie tijdens een parasitaire infectie, hebben we karpers geïnfecteerd met *T. borreli* of met *T. carassii*. Gedurende de infectie hebben we de NO (radicaal) productie en de arginase activiteit van de cellen uit de (kop)nier gevolgd. Basale NO productie (maat voor klassieke activering) en arginase activiteit (maat voor alternatieve activering) waren niet erg verschillend tussen de twee infecties. Nadat de cellen buiten het lichaam extra waren gestimuleerd, waren de verschillen groter. Extra stimulatie met LPS of *T. borreli* lysaat (kapot gemaakte parasieten) verhoogde de NO productie van de cellen uit vissen geïnfecteerd met *T. borreli*. Extra stimulatie met cAMP verhoogde de arginase activiteit van de cellen uit vissen geïnfecteerd met *T. carassii*. Waarschijnlijk is arginase 1 verantwoordelijk voor de stijging van de arginase activiteit aangezien arginase 1 meer wordt overgeschreven aan het einde van de *T. carassii* infectie. Onze metingen suggereren dat karpers geïnfecteerd met *T. borreli* geneigd zijn om meer NO te produceren met behulp van klassiek geactiveerde macrofagen, terwijl karpers geïnfecteerd met *T. carassii* geneigd zijn om hun arginase activiteit te verhogen met behulp van alternatief geactiveerde macrofagen (hoofdstuk 5).

Naar aanleiding van de observatie dat *T. borreli* en *T. carassii* verschillende afweerreacties induceren hebben we karpers gelijktijdig geïnfecteerd met beide parasieten. In de dubbel geïnfecteerde karpers waren de aantallen parasieten lager en van deze groep overleefden meer karpers de infectie. Gebaseerd op de uiterlijke verschillen tussen de twee parasieten konden de aantallen van beide parasieten apart worden bepaald in de dubbel geïnfecteerde karpers. *T. carassii* aantallen in de dubbel geïnfecteerde karpers waren gelijk aan de aantallen in de met alleen *T. carassii* geïnfecteerde karpers. Terwijl *T. borreli* aantallen in de dubbel geïnfecteerde karpers lager was dan de aantallen in de met alleen *T. borreli* geïnfecteerde karpers. Dubbel geïnfecteerde karpers en karpers geïnfecteerd met *T. carassii* alleen produceerden meer antistoffen tegen *T. borreli* dan de karpers geïnfecteerd met alleen *T. borreli*. In de dubbel geïnfecteerde karpers werd *T. borreli* gedetecteerd vanaf week 5, op dat moment waren er al antistoffen tegen *T. borreli* aanwezig. Daarnaast waren karpers die eerst een *T. carassii* infectie hadden overleefd minder gevoelig voor een infectie met *T. borreli*. De verlaagde parasiet aantallen, verhoogde overlevingskansen en verhoogde antistof productie in de dubbel geïnfecteerde karpers duiden op een beschermende werking van de infectie met *T. carassii* tegen een infectie met *T. borreli*. Het is goed mogelijk dat deze bescherming berust op de productie

van kruis reagerende antistoffen (hoofdstuk 6).

Om meer over de (kruis reagerende) antistoffen te weten te komen wilden we weten tegen welke eiwitten deze antistoffen gericht zijn (antigene eiwitten). Hiertoe hebben we eerst een expressie bank (λTriplEx2) van beide parasieten gemaakt. In een expressie bank zitten alle eiwitten die worden gebruikt op een zodanige manier dat deze eiwitten door bacteriën kunnen worden geproduceerd. Hierbij produceert iedere bacterie maar één specifiek eiwit. Het serum (bloed zonder cellen) van de karpers die geïnfecteerd zijn geweest met de parasiet bevat de antistoffen waarin we geïnteresseerd zijn. Met behulp van een speciale methode kan men zichtbaar maken tegen welke bacteriën dit serum antistoffen bevat. Vervolgens kan men bepalen welk eiwit door deze bacterie wordt geproduceerd, dit is dan het antigene eiwit. Met behulp van deze methode konden we aantonen dat ribosomale eiwitten antigene eiwitten zijn voor beide parasieten. Daarnaast vonden we nog twee eiwitten, ubiquitine en activated protein kinase C receptor, als antigene eiwitten van *T. carassii*. Of de antistoffen gericht tegen deze antigene eiwitten ook werkelijk bescherming kunnen bieden tegen een infectie en kruis reactief zijn tussen de twee parasieten moet nog verder worden onderzocht (hoofdstuk 7).

In conclusie, karper macrofagen kunnen polariseren tot klassiek geactiveerd en alternatief geactiveerd. Tot op zekere hoogte kunnen deze polarisatie toestanden worden geïnduceerd door de infectie met *T. borrreli* en *T. carassii*. Hierbij induceert *T. borreli* klassiek geactiveerde macrofagen en *T. carassii* alternatief geactiveerde macrofagen. De mate waarin deze polarisatie als reactie of de parasiet invloed heeft op de productie van (beschermende) antistoffen moet nog verder worden onderzocht.

En dan is het zo ver, na vier en een half jaar werk mag 'het boekje' naar de drukker, een vreemd maar toch ook erg prettig idee. En zoals dat voor alle proefschriften geldt, is ook dit proefschrift niet alleen aan de eerste auteur te danken. Daarom ben ik ook blij met dit onderdeel, het dankwoord, waarin ik de gelegenheid heb om al die andere mensen die bijgedragen hebben te bedanken.

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M. Joerink, Groeneveld A., Savelkoul H.F.J., Wiegertjes G.F. Mixed infection with *Trypanoplasma borreli* and *Trypanosoma carassii* induces protection: involvement of cross-reactive antibodies. (Submitted)

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Personalia

Maaike Joerink werd geboren op 4 april 1979 in Heinenoord en verhuisde op haar derde jaar naar de Betuwe. In 1997 behaalde zij haar VWO diploma aan het Hendrik Pierson College te Zetten. In September van datzelfde jaar begon zij aan de studie Moleculaire wetenschappen aan de Wageningen Universiteit. Zij volgde de richting bio-organische chemie met een twee maanden durend afstudeervak bij moleculaire biologie, een zes maanden durend afstudeervak via organische chemie bij Plant Research International, en een zes maanden stage via Moleculaire biologie bij Intervet Norbio in Bergen, Noorwegen. Na het behalen van haar doctoraal diploma eind 2001 begon zij in januari 2002 aan haar promotie-onderzoek bij Celbiologie & Immunologie, Wageningen Universiteit. De resultaten van dit onderzoek, aan het afweersysteem van de karper, zijn in dit proefschrift beschreven. Na haar promotie gaat zij naar het Karolinska Instituut in Zweden om daar als postdoc onderzoek te doen aan de ontwikkeling van allergie.

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Yearly NVVI workshop, one time poster (2003-2005)
Meeting Dutch Parasitologists (2002 and 2005)
Retirement symposium Prof. W van Muiswinkel (2003)
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SCOFDA workshop fish diseases (2002)
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External training period, Edmonton Canada (2002)
Didactic skills training (9.1 ECTS)
Training in presentation skills (2003)
Supervising practicals fish immunology workshop (2003)
Supervising practicals Cell biology I (2004 and 2005)
Supervising five MSc students (2003-2005)
Supervising tutor groups comparative immunology (3003 and 2004)
Managment skills training (9.9 ECTS)
Organising WIAS Science Day (2003 and 3004)
Memberschip of Waps council (2002-2004)