Section I: Keynotes

Macrophage polarization in the immune response to parasites

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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 recognize 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 co-stimulatory activity on professional antigen-presenting cells such as macrophages. The co-stimulation 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 (Th1) and T helper 2 (Th2), typically differ by the cytokine profiles they produce and the functions they perform. For example, these cytokines, produced by the Th cells will influence the activity of the macrophages attracted to the site of infection.

Macrophages have long been recognized 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 recognized that particular cytokines from Th2 cells can induce an 'alternative' activation of macrophages that induces distinct functional activities, integrating these macrophages in the type II response (Mills, 2001; Gordon, 2002; Mantovani *et al*. 2002). It is important to realize that an effective immune response against a particular parasite requires a balanced differentiation between Th1 lymphocytes/ classically activated macrophages (type I response) and Th2 lymphocytes/ alternatively activated macrophages (type II response).

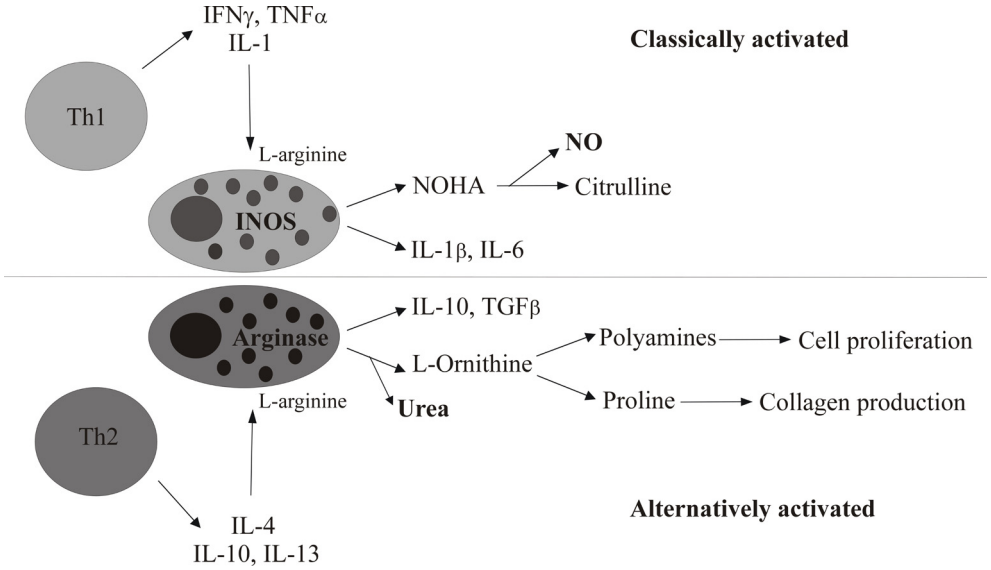

Fig. 1. Macrophages exhibit distinct functional activities in type I and type II immune responses. The classically activated macrophages are associated with the Th1 cells and their cytokines, they have a higher inducible nitric oxide synthese (iNOS) expression and produce more nitric oxide (NO). The alternatively activated macrophages, associated with the Th2 cells and cytokines, upregulate arginase activity important for cell proliferation and wound healing (Adjusted from Sandor *et al*., 2003**).**

Abbreviations: IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; NOHA, N^{ω}-hydroxy-L-arginine; TGF, transforming growth factor.

Macrophage polarization

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 (Vincendeau *et al*., 2003).

What has become clear during the last few years is the central role of the amino acid L-arginine in the polarization 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^{w}-hydroxy-L-arginine (NOHA), which is further oxidized to L-citrulline and NO. NOHA is a competitive inhibitor of arginase and in this way suppresses the alternative activation of macrophages (see Fig. 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 tumor necrosis factor (TNF)á, interleukin (IL)-1â, IL-6 and due to high concentrations of NO. An unregulated alternative activation of macrophages may lead to immuno-suppression induced by anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-â. 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 Th1 and Th2 cells via their secreted cytokine profiles. CaMF develop as part of a type I response with TNFá and interferon (IFN)ã 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. 1). The cytokines produced in the type I or II reaction cross-inhibit each others development (Fiorentino *et al*. 1989; Mills, 2001).

Polarization 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 Th1 and Th2 cells. However, from the Table below it is clear that an assignment 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*. 1995, 1998). Flow cytometric analyses of the *in vitro*-derived carp head kidney cultures showed an enrichment of 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

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

Table 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).

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

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*. 2003). There are two arginase genes and both seem to be activated in (murine) macrophages (Morris *et al*. 1998; Mori *et al*. 2000). Coding sequences for both arginase isoforms have been reported for fish (see Table 1) and we are in the process of identifying the arginase genes in carp. In murine cells, arginase I is located in the cell cytosol (together with ornithine decarboxylase facilitating polyamine synthesis), while arginase II 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 polarization 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.

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 favors 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 polarization 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*. 2002). Carp that are highly susceptible to the parasite, produce immunosuppressive amounts of NO, which is suggestive of an unbalanced immune response. In the late phase, resistant carp produce lytic antibodies (Saeij *et al*. 2003) and overcome the infection. We can now investigate whether carp can and need to switch from a caMF response in the early phase to a aaMF response in the late phase of infection, enabling the production of protective antibodies, similar to what is observed for *T. brucei* infections.

Conclusion

Since evidence for the existence of type I and type II immune responses to fish parasites, based on polarized cytokine profiles secreted by Th1 and Th2 cells is lacking, we propose to use iNOS and arginase activity as markers for polarized immune responses in fish. *In vitro*-derived head kidney myeloid cells from carp readily express iNOS and arginase activity upon specific stimulation and can be of true importance for providing new insights into 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 synthesize 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.

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