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Invited Review

Neospora caninum in non-pregnant and pregnant mouse models: cross-talk between infection and immunity

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ABSTRACT

Neospora caninum is a cyst-forming coccidian which causes abortion in cattle, with a high economic impact globally. Vaccination is considered to be the most cost-effective strategy to control and prevent bovine neosporosis. However, there is no commercial vaccine available to date. To investigate this disease under laboratory conditions, mouse models were developed, and they have been efficiently used as an initial proof-of-concept platform to investigate different immunogenic formulations. We here provide a detailed review on the current knowledge on immunity against neosporosis in non-pregnant as well as pregnant mice, and present a general overview of the most relevant parameters that may be responsible for protective immunity, which in turn could be relevant for vaccine development. Despite the considerable differences in immunity between cattle and mice, it is essential to understand how mice respond immunologically to *Neospora caninum* infection and how this response influences congenital infection and offspring survival. In this context, pregnant mouse models play a key role, and allow correlation of the outcome of congenital neosporosis with specific immune mechanisms which could also be relevant in cattle.

1. Introduction

Neospora caninum is an apicomplexan parasite closely related to *Toxoplasma gondii* that infects canids as definitive hosts and cattle as the intermediate hosts of primary economic concern. Neosporosis is one of the most important infectious causes of abortion in cattle worldwide and based on data from 10 countries, the annual financial losses were calculated to range from US $1.1 million in the New Zealand beef industry to US $546.3 million in the US dairy population (Reichel et al., 2013). The only marketed vaccine, Neoguard™, has been removed due to ambiguous efficacy, and currently there is neither a vaccine nor a drug commercially available for the prevention and treatment of bovine neosporosis. Considering that reproductive losses represent the main economic burden of this disease and that vertical transmission has an important role in maintaining the parasite in cattle herds (Dubey et al., 2017), future vaccines should ideally be able not only to protect against primary infection by oocysts but also be safe during gestation and reduce, if not eliminate, vertical transmission.

To approach vaccine development against *N. caninum* infection rationally, it is important to gain knowledge on the hallmarks of the immunological response against infection, and to define the crucial parameters that determine whether the host can eliminate the invading pathogen or whether the disease is induced. Whilst such investigations should be ideally carried out in cattle as the main host of economic interest, only a few laboratories worldwide have the capacity to do this, and respective studies have been recently reviewed (Horcajo et al., 2016). The majority of investigations on immunity against neosporosis have been carried out in small laboratory animal models such as mice, with extraordinary advantages in terms of the required facilities, costs, time and availability of tools to dissect the immune response. Non-pregnant as well as pregnant mouse models have proven to be useful and versatile for studies on host-parasite interactions, immunity, vaccination trials and novel therapeutic approaches against neosporosis (Hemphill et al., 2016; Dubey et al., 2017). In addition, experimental investigations on neosporosis in mice allow us to draw analogies to similar studies on the closely related *T. gondii,* whose interactions with the murine host are better characterised, and could potentially serve as an inspiration for the design of novel experiments and trouble-shooting to shed more light on how host immunity deals with *N. caninum* infection.

We here provide a review of the last 20 years of research on immunity against experimental neosporosis in mice, by covering the major hallmarks of the immune response in both non-

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pregnant and pregnant mouse models during infection. We also explore the correlation of these immune parameters with protection against infection and disease, and we refer to studies that have aimed to show how the cross-talk between innate and acquired immunity could be exploited for future vaccine development.

2. Immune response to *N. caninum* infection in non-pregnant mice

Experimental *N. caninum* infection has been mostly induced by cell culture-derived tachyzoites, mainly through s.c. or i.p. inoculation (Long et al., 1998; Collantes-Fernandez et al., 2004; Collantes-Fernandez et al., 2006a; Pereira Garcia-Melo et al., 2010) and, in some instances, by intragastric inoculation (Correia et al., 2013; Teixeira et al., 2015). During the acute phase of the experimental infection, the parasites proliferate in several organs of the mouse but the burden diminishes during the second week p.i. and becomes undetectable in most of the organs except the CNS from the third week p.i. onwards, when chronic infection is established (Collantes-Fernandez et al., 2006a). This general picture, which is quite constant despite slight differences depending on the mouse strain (Long et al., 1998; Collantes-Fernandez et al., 2004) and the *N. caninum* isolate (Collantes-Fernandez et al., 2006a; Pereira Garcia-Melo et al., 2010), shows that immunocompetent non-pregnant mice are able to mount an immune response that controls, at least partially, the acute infection. This allows the study of the immune mechanisms leading to protection against a primary infection in non-pregnant mice. Comparison among different mouse strains including genetically modified immunodeficient mice, or the use of mice treated with immunosuppressors or immunomodulators (by cell transfer, depletion experiments, etc.), have been most frequently applied to pinpoint the innate and adaptive immune mechanisms associated with protection in non-pregnant mice. The main findings on innate and adaptive immune responses against primary *N. caninum* infection in non-pregnant mice are summarised in Table 1, which includes the techniques employed in each case.

2.1. Innate immunity against N. caninum primary infection in non-pregnant mice

Immature murine bone-marrow-derived dendritic cells (BMDCs) are readily activated in vitro by exposing them to live or inactivated *N. caninum* tachyzoites or to tachyzoite soluble extract (NSE) suggesting a role of pattern recognition receptors (PRRs) in the initiation of the immune response against *N. caninum*. This results in the up-regulation of CD40, CD80, CD86 co-stimulatory molecules and the major histocompatibility complex (MHC) class II molecules (Mineo et al., 2010; Mansilla et al., 2016), leading to the production of several cytokines including TNFα, IL12p40, IL10 and IL6. The degree of activation depends on the stimulus; thus exposure to live or inactivated tachyzoites leads to much more efficient production of TNFα, IL12p40 and IL10 (Strohbusch et al., 2009; Mineo et al., 2010), whereas incubation with NSE induces more IL10 and IL6 production, but less TNFα (Feng et al., 2010; Mineo et al., 2010; Mansilla et al., 2016).

A thorough investigation of the role(s) of the parasite's pathogen-associated molecular patterns (PAMPs) and respective PRRs is still needed. It has been shown that Toll-like receptor 2 (TLR2) is up-regulated after incubation of immature macrophages with *N. caninum* antigen extract, suggesting the involvement of this receptor in the activation process. Moreover, dendritic cell (DC) activation in *N. caninum*-infected TLR2$^{-/-}$ mice is impaired and the subsequent adaptive immune response loses T helper 1 (Th1) properties compared with wild type (WT) mice, although these changes do not lead to increased mortality (Mineo et al., 2010). In the same study, authors showed that myeloid differentiation primary response gene 88 (MyD88)-deficient mice were much more susceptible to *N. caninum* infection than TLR2$^{-/-}$ mice. The DCs in MyD88$^{-/-}$ mice failed to become activated after *N. caninum* stimulation, and the adaptive immune response was also severely impaired, as shown by a lack of in vitro lymphoproliferation and the loss of the Th1-biased immune response, all leading to mortality of MyD88$^{-/-}$ mice (Mineo et al., 2010). These results demonstrated that the protective Th1 immune response mounted against *N. caninum* relies on MyD88-dependent pathway activation, and the lack of TLR2 in this infection model can be compensated by other mechanisms, probably including other TLRs which also use MyD88-dependent pathways.

Recently, the role of the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) during *N. caninum* infection has been investigated. NOD2 is a PRR expressed in the cytosol of various cells. It recognises PAMPs of intracellular bacteria and protozoa. Engagement of NOD2 induces the transcription of inflammatory cytokines and the activation of the inflammasome, which can initiate cell death in some cases (Kumar et al., 2011). After *N. caninum* infection of C57BL/6 mice, NOD2 is up-regulated in bone-marrow derived macrophages (BMDM), and parasites recruit NOD2 to the vicinity of the parasitophorous vacuole (Davoli-Ferreira et al., 2016). However, NOD2$^{-/-}$ mice infected with *N. caninum* tachyzoites showed increased survival compared with WT mice, even though the parasite burden was higher in most tissues. In addition, these mice exhibited a much lower production of pro-inflammatory cytokines by macrophages (TNFα and IL6), higher expression of anti-inflammatory molecules (IL10, Arginase I), and a down-regulation of the NOD2-associated mitogen-activated protein kinase (MAPK) pathway, which in turn would be responsible for a pro-inflammatory response. This down-regulated inflammatory response to *N. caninum* by NOD2$^{-/-}$ mice appeared to prevent tissue damage and could explain the increased survival rate compared with WT mice in this particular model (Davoli-Ferreira et al., 2016).

In contrast to *T. gondii*, *N. caninum* has been reported to stimulate TLR3, a PRR recognising viral double-stranded RNA (dsRNA). However, the potential relationship between TLR3 stimulation and protection was not investigated (Beiting et al., 2014). By infecting murine macrophages with different TLR deficiencies with *N. caninum* tachyzoites, only TLR3 was found to be essential for induction of a type I IFN response (IFNα and IFNβ). When properly targeted to the endosomes (where TLR3 is naturally located), RNA from *N. caninum*, but not from *T. gondii*, induced a typical anti-viral type I IFN response. Consistent with this observation, Vesicular Stomatitis Virus (VSV) grew normally in *T. gondii*-infected fibroblasts, but viral replication was dramatically impaired in *N. caninum*-infected fibroblasts (Beiting et al., 2014).

C57BL/10ScCr, a mouse strain which lacks TLR4 and a functional IL12 receptor, is highly susceptible to *N. caninum* infection (Botelho et al., 2007; Teixeira et al., 2007). TLR4 is relevant for protection against other protozoan parasites including *T. gondii* (Mun et al., 2005). *Toxoplasma gondii* is also sensed by the intracellular NLRP1 (NOD-, LRR- and pyrin-domain containing 1), and by endosomal TLRs such as TLR7, TLR9 and TLR11 (Yarovinsky, 2014). This last receptor recognises *T. gondii* profilin, an actin-binding protein with particular structural features common to different apicomplexan parasites (Kumpula and Kursula, 2015), and it is a major innate sensor of *T. gondii* in mice (Yarovinsky, 2014). The cloning of *N. caninum* profilin (Jenkins et al., 2010) and experimental immunization with recombinant *N. caninum* profilin has been already reported (Mansilla et al., 2016) but the role of TLR11 recognition in the response to *N. caninum* infection in mice must be still clarified. Is however important to note that TLR11 is functional neither in

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