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Induction of protection in murine experimental models against *Trichinella spiralis*: an up-to-date review

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Abstract

The parasitic nematode *Trichinella spiralis*, an aetiological agent of the disease known as trichinellosis, infects wild and domestic animals through contaminated pig meat, which is the major source for *Trichinella* transmission. Prevention of this disease by interrupting parasite transmission includes vaccine development for livestock; however, major challenges to this strategy are the complexity of the *T. spiralis* life cycle, diversity of stage-specific antigens, immune-evasion strategies and the modulatory effect of host responses. Different approaches have been taken to induce protective immune responses by *T. spiralis* immunogens. These include the use of whole extracts or excretory–secretory antigens, as well as recombinant proteins or synthesized epitopes, using murine experimental models for trichinellosis. Here these schemes are reviewed and discussed, and new proposals envisioned to block the zoonotic transmission of this parasite.

Introduction

Preventive and therapeutic vaccination in definitive or intermediate hosts is an ongoing effort that includes diverse strategies for control of neglected parasitic tropical diseases (NTD) such as malaria, leishmaniasis, Chagas disease, soil-transmitted helminthiasis (STH) and schistosomiasis (Patarroyo *et al.*, 2012; Beaumier *et al.*, 2013). Among these, STHs caused by the hookworms *Ancylostoma duodenale* and *Necator americanus*, *Ascaris lumbricoides* and *Trichuris trichiura* are leading causes of morbidity worldwide (>600 million cases each). Moreover, other helminths, such as *Ascaris suum* or *Trichuris suis*, infect domestic hosts, such as pigs, and are considered a veterinary health problem. Although protozoan infections by kinetoplastids such as *Leishmania* sp. and *Trypanosoma cruzi* have a lower impact (10–12 million cases each) in this NTD list (Beaumier *et al.*, 2013), these are also considered an important health problem. In this article we review the current status of approaches to study protective host responses and immunization strategies aimed at developing vaccines against *Trichinella spiralis* infection, which is the most widespread *Trichinella* species, causing trichinellosis in humans and pigs, which globally exhibits similar burdens to kinetoplastid-caused infections.

Estimating the impact of transmission of pig-to-human trichinellosis

Trichinellosis is transmitted to humans by domestic or wild animals by eating meat contaminated with encysted first-stage larvae. Upon activity of digestive enzymes in the host's stomach, the larvae are released, migrate to the small intestine while they mature and finally harbour in the epithelial cell syncytium where they moult and develop into male/female adults within a period of 30 h. Approximately 5 days after mating, newborn larvae are released continuously from female worms until the worms are expelled from the intestine. Newborn larvae that are released in the gut migrate to striated muscle, where they encapsulate and induce the formation of the nurse cell–larvae complex (NCLC), a process that takes

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around 17 days to be completed. Encysted larvae may survive as long as 25 years in living hosts, in a silent state underlying a tightly controlled inflammatory response around the NCLC (Beiting *et al.*, 2007). Transmission to humans involves the consumption of raw or undercooked meat and its derivatives from domestic (dog, horse, pig) and wild (walruses, wild boars, badgers, bears) animals, with the latter being major reservoirs of this parasite's biomass in contrast to other nematode infections (Pozio, 2013).

The genus *Trichinella* is comprised of nine species and three genotypes divided into two clades that infect more than 100 species of mammals, birds and reptiles. Of these, six species and one genotype have been detected in human infections (Pozio & Zarlenga, 2013). However, the zoonosis, trichinellosis, caused by *T. spiralis* is an eventually fatal infection among a broad range of mammalian hosts, and human outbreaks have been observed in the past two centuries in up to 55 countries (Pozio, 2007; Murrell & Pozio, 2011). In fact, it is estimated that around 11 million people are infected by *Trichinella* sp. worldwide (except Antarctica) (Dupouy-Camet, 2000), and recent surveys suggest an emerging or re-emerging trend for human trichinellosis (Dupouy-Camet, 2000; Pozio, 2007). Of note: pork has the highest consumption rate of any meat by humans (the 2015 forecast is 1 billion pigs) and the demand for its derivatives has increased over recent decades, particularly in fast-growing economies. Half of the pigs are produced under industrial farming conditions, where *Trichinella* testing occurs; however, the other half of pork production occurs in backyard and free-ranging conditions, without specific testing, and these conditions account for the largest portion of *Trichinella* sp. biomass (Pozio, 2014). In this context, the design of control strategies for trichinellosis should include preventing pig-to-human transmission. Therefore the development of therapeutic and prophylactic resources intended for definitive and/or intermediate hosts has been approached using experimental infections in rodent species that are the best-characterized models.

Immunobiology of trichinellosis

The life cycle of *T. spiralis* involves three main stages – infective/muscle larvae, newborn larvae and adults – that display stage-specific antigens with the ability to differentially modulate host responses (Liu *et al.*, 2007; Yépez-Mulia *et al.*, 2009). Also, innate immunity does not usually block the establishment and development of infective larvae to mature adults or the production of the newborn larvae. In this scenario, mucosal and systemic immunity orchestrate protective host responses, and the former has an effect against all three stages while the latter affects the migrating larvae as well as NCLC formation and maintenance. The resistance of the NCLC is partially explained by the production of regulated levels of interleukin (IL)-10 and transforming growth factor (TGF)-β, which influence the surrounding inflammation levels (Beiting *et al.*, 2007). Thus, prophylactic vaccination aimed at inducing mucosal immunity in the small intestine would be more relevant, since the worms live at this site.

Trichinella spiralis adult worm expulsion is regulated mainly by CD4+/T-helper 2 (Th2) cytokines and depends on IL-4/IL-13 production, since their inhibition extends parasite survival (Finkelman *et al.*, 2004). While eosinophilia is a hallmark of parasite infection, both in peripheral blood and tissue, in *T. spiralis*-infected mice (Bruschi *et al.*, 2008), blocking IL-5 does not prevent host protection (Herndon & Kayes, 1992; Dixon *et al.*, 2006). It has been reported that eosinophils from the jejunum may undergo apoptosis once the *T. spiralis* infection is cleared (Bruschi *et al.*, 2008). Interestingly, eosinophils are able to kill larvae *in vitro* and they contribute to resistance to the systemic phase, as the number of larvae in the muscles is almost doubled under eosinophil depletion (Grove *et al.*, 1977; Gurish *et al.*, 2002). More recently, a strikingly distinct role for eosinophils *in vivo* has been reported, as larvae are established in the muscle during eosinophilia, and larval viability is compromised in eosinophil-deficient mice (Fabre *et al.*, 2009). Further work proposed that in chronic murine trichinellosis, parasite growth and survival are supported by eosinophils, which promote accumulation of Th2 (IL-4+) cells and prevent nitric oxide synthase (iNOS) induction in macrophages and neutrophils (Gebreselassie *et al.*, 2012). Eosinophils have been proposed to have an immunoregulatory role in nematode infections as these cells may expand dendritic cells and CD4+ cells to produce IL-10 which, in turn, inhibits iNOS (Huang *et al.*, 2014). Another immune mechanism involves the production of immunoglobulin E (IgE), which promotes parasite expulsion from the small intestine (Gurish *et al.*, 2004). Although treatment of mice with IL-9 enhanced infection-induced jejunal muscle hypercontractility and accelerated worm expulsion, this cytokine does not seem to play a protective role, since its blockage did not impair host protection (Khan *et al.*, 2003).

At the gastrointestinal level, infection by *T. spiralis* promotes a rapid and reversible expansion of the mucosal mast-cell population in the intestinal epithelium. This response is dependent on T cells and it requires the Th2 cytokines IL-4 and IL-13 (Urban *et al.*, 2000; Helmby & Grencis, 2002; Scales *et al.*, 2007). The response is propagated through the IL-4 receptor α (IL-4Rα) by activating STAT6 (signal transducer and activator of transcription 6) (Finkelman *et al.*, 2004; Knight *et al.*, 2008). Consistently, it has been shown that STAT6-deficient mice display impaired IL-4- and IL-13-driven signalling, concomitant with a reduced mast-cell hyperplasia (Urban *et al.*, 2000; Khan *et al.*, 2001a, c). Mast cells in *T. spiralis*-infected mice are negatively regulated by the Th1-type cytokine IL-18, thereby delaying worm expulsion (Helmby & Grencis, 2002). Transcriptomic analyses performed on the jejunal epithelial compartment from *T. spiralis*-infected and -uninfected mice (Urban *et al.*, 2000) revealed that, in mast cells from these animals, the most highly up-regulated transcripts were B-chimases, which include mouse mast-cell protease-1 (Mcpt-1) and -2 (Mcpt-2).

Intestinal infections are usually associated with a high accumulation of fluid in the gut lumen, which is also a hallmark of *T. spiralis* primary and secondary infections. Indeed, increased muscle contractility and fluid accumulation have been shown to promote worm expulsion (Madden *et al.*, 2004). Augmentation of fluid accumulation is due to increased epithelial secretion

(STAT6-dependent prostaglandins and histamines), enteric nerves and increased paracellular epithelial permeability, which may explain the diarrhoea, electrolyte and protein loss that develop during *Trichinella* infections (Field, 2003). In this context, it was suggested initially that mast-cell B-chimases were responsible for the increased paracellular permeability (Scudamore *et al.*, 1995) and further work demonstrated that the deletion of the *mcpt-1* gene was associated with delayed *T. spiralis* worm expulsion (Knight *et al.*, 2000), which supported its role in host protection.

In recent studies macrophages have been reported to play a role in the Th2-type response mediating host defence against *T. spiralis* infection (Dzik *et al.*, 2004). In particular, alternatively activated macrophages (AAMΦ) have been detected using the marker arginase-1. AAMΦ also produce other molecules, including IL-4Rα, chitinase and Fizz family members (ChaFFs), chitinases, FIZZ1/RELMα (found in inflammatory zone/resistin-like molecule alpha) Ym1 (also known as CHI3L3 (chitinase-3-like protein 3) or ECF-L (eosinophil chemotactic factor-L)) and Amcase (acidic mammalian chitinase), among others. Thus it is conceivable that AAMΦ may contribute to control of Th1-driven responses, hence promoting a polarized Th2-type profile (Kreider *et al.*, 2007).

Intestinal infection by *T. spiralis* also induces Th2 immune responses mediated by IL-4 and IL-13 as well as goblet-cell hyperplasia and increased mucus secretion (Moncada *et al.*, 2003; Knight *et al.*, 2004, 2008). Although goblet cells may be abrogated by the Th1-type cytokines and IL-27 (Khan *et al.*, 2001b) during acute intestinal infection, these cells secrete high levels of mucins. Mucins (MUC) are monomers composed of polypeptide backbones glycosylated at serine and threonine residues and linked by disulphide bridges to form a layer of mucus that coats and protects the epithelium (Knight *et al.*, 2008). This layer is largely composed of MUC1, MUC2, MUC3, MUC17, trefoil factor peptides (TFF), intelectin 2 (a galactofuranose-binding lectin, also produced by intestinal Paneth cells), resistin-like molecule beta (RELMβ) and Fc-gamma binding protein (Fcγbp) (Kim & Ho, 2010). In particular, MUC2 plays an important role in host protection, since *Muc2*-deficient mice strains display a significantly delayed worm expulsion when infected by the whipworm *Trichuris muris*, and a good correlation with an increase of MUC2 expression was found in resistant mice (Hasnain *et al.*, 2010). Likewise, an increase of MUC3 and RELMβ was determined by transcriptome analyses of *T. spiralis*-infected mice (Urban *et al.*, 2000). Further proteomic analyses carried out on *T. spiralis*-infected mice showed increased levels of intelectin-2 (Pemberton *et al.*, 2004a, b). In fact RELMβ was identified as one of the most highly up-regulated transcripts during *T. spiralis* infection (Knight *et al.*, 2004) and, based on experimental data obtained in mice infected with *T. muris* and *Strongyloides stercoralis*, it was suggested that RELMβ has potential anti-parasitic activity, since it binds to chemosensory components of nematodes (Artis *et al.*, 2004). Thus, goblet-cell products, such as RELMβ and intelectins, may contribute to worm expulsion by altering mucus composition or by a direct anti-parasitic function (Knight *et al.*, 2008). Consistently, in the lumen-dwelling parasites *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* expulsion by RELMβ is either spontaneous or induced by IL-4 (Herbert *et al.*, 2009). In contrast, RELMβ does not contribute to immunity against the gut-dwelling parasite *T. spiralis*. Although MUC5 is not produced in the intestinal mucosa, it has been reported that its absence caused a significant delay in the expulsion of two gut-dwelling nematodes (*T. spiralis* and *N. brasiliensis*) (Hasnain *et al.*, 2011).

Different intestinal regions may display specific regulatory responses upon *Trichinella* infection. Indeed, recent studies have shown that IL-17 levels and Th17 cells were increased in the jejunum of *T. spiralis*-infected mice 2 weeks post-infection. Further, it has been reported that jejunal muscle contractions elicited by acetylcholine were enhanced by IL-17 in a dose-dependent manner (Fu *et al.*, 2009). Also, jejunal tissue displays a swollen and oedematous appearance during worm expulsion.

A Th2-type response involving IL-4/IL-13 signalling may increase epithelial cell resistance and Na^+-linked glucose absorption, leading to increased levels of luminal fluids (Madden *et al.*, 2004). As mentioned above, during *T. spiralis* infection AAMΦ may contribute to Th2-polarized host response by controlling the Th1 response, while in other intestinal parasites, such as *N. brasiliensis*, AAMΦ may also affect intestinal physiology by promoting smooth muscle contractility (Zhao *et al.*, 2008). Several studies involving mast-cell depletion in mice have shown that mast cells are crucial for worm expulsion and intestinal epithelial cell permeability during *T. spiralis* infection (Ha *et al.*, 1983; Alizadeh & Murrell, 1984; Donaldson *et al.*, 1996; Finkelman *et al.*, 2004). The serine protease mMcpt1 also plays a role, as mMcpt1-deficient mice were unable both to increase their epithelial permeability and to expel worms normally (Knight *et al.*, 2000, 2008). This observation was confirmed in rats (Suzuki *et al.*, 2008). Increased paracellular permeability is likely mediated by mMcpt1 degradation of intercellular junctional proteins, such as occludin located at tight junctions (McDermott *et al.*, 2003).

In summary, the Th2 response is of crucial importance during *T. spiralis* infection, particularly at the intestinal level, since the Th2 cytokines, such as IL-4 and IL-9, promote mastocytosis (Finkelman *et al.*, 2004). In fact IL-9 transgenic mice displayed both increased mast-cell populations and intestinal permeability (Faulkner *et al.*, 1997; Temann *et al.*, 2002, 2007). During the first hours after infection, basophils and mast cells are important suppliers of Th2 cytokines, and their activation may occur by IgE cross-linking. As the immune response progresses, the Th2 cytokines seem to be important in increasing the susceptibility to mast-cell mediators such as mMcpt1 (Strait *et al.*, 2003; Finkelman *et al.*, 2004). Therefore, Th2 cytokines may act on mast cells during *T. spiralis* infection by promoting both mast-cell activation and hyperplasia, as well as by modulating intestinal functions such as mucin secretion and paracellular permeability.

Vaccine development against *T. spiralis* infection

Induction of therapeutic and protective responses in *T. spiralis* infections should activate both innate and acquired immunological mechanisms in order to: (1) block the establishment of infective larvae at the intestinal epithelium; (2) interrupt the development of larvae to

adult worms, hence production of newborn larvae (NBL); (3) prevent the migration of NBL to avoid NCLC formation; and (4) expel adult worms from the intestine

In trichinellosis, host–parasite interplay is complicated by the *T. spiralis* life cycle, which includes a diversity of stage-specific antigens, immune evasion strategies and modulatory effects on host responses. These features make it challenging to achieve effective protective responses. Nevertheless the development of effective mucosal and systemic immune responses is required to control the intestinal phase of the parasite, by promoting the expulsion of adults and preventing NBL release and their migration to the muscle. In this context, during the past two decades most efforts have been focused on immunizing hosts against *T. spiralis* infection using progressively more refined parasite components. These efforts began with complex parasite extracts and more recently have included recombinant proteins or epitope-based immunogens.

Induction of protective immunity by whole extracts and excretory–secretory products

It has been observed consistently that whenever whole-larval extracts are used there is better host protection than when using any isolated parasite antigen, as seen by the reduction in larval or adult worm burden (Behnke *et al.*, 1994; Dea-Ayuela *et al.*, 2000, 2006a, b; Eissa *et al.*, 2003; Deville *et al.*, 2005) (table 1). These responses are within the rage of 50–80% protection in most cases. These studies have included mainly mice, rats and hamsters: from these reports it is known that (a) mouse strains varied in response to *Trichinella* infection (low and high responders) (Robinson *et al.*, 1995); (b) rats are rapid responders, since they exhibit worm expulsion within approximately 2 weeks, although there are also faster (BN) and slower (F344) responder strains (Suzuki *et al.*, 2008); and (c) although *T. spiralis* intestinal infections in pigs and humans are more prolonged than those observed in mice and rats (Murrell, 1985), the most important features of human infections are mirrored in these rodents that are also natural hosts for *T. spiralis*. The mouse strain most widely used has been the BALB/c strain and the outcome of these studies has been quite useful for understanding host immunity against *T. spiralis* infections.

In this context, mucosal immune responses have been analysed in experimental models immunized initially with whole-larval extracts. These studies have shown increased Th1 (interferon-gamma (IFN-γ)) and Th2 (secretory immunoglobulin A (sIgA)) responses from mesenteric lymph nodes as well as an increase in CD4+ lymphocyte numbers in Peyer's patches (Dea-Ayuela *et al.*, 2006b) and intestinal mastocytosis (Behnke *et al.*, 1994). A number of reports have also shown the activation of systemic immune responses, as indicated by elevated levels of total and specific IgG, IgA, IgM, IgG1, IgE (Th1 response) (Behnke *et al.*, 1994; Goyal *et al.*, 1997; McGuire *et al.*, 2002; Abdel-Rahman *et al.*, 2005; Deville *et al.*, 2005; Dea-Ayuela *et al.*, 2006a, b; Dvorožňáková *et al.*, 2011), IgG2a (Th2 response) (McGuire *et al.*, 2002; Dvorožňáková *et al.*, 2011) and even by the increase of IgG3, a subclass isotype considered as a carbohydrate epitope-associated immunoglobulin (Dea-Ayuela *et al.*, 2000). In spite of their relatively high levels of protection, whole-larval extracts may display some toxicity or immunopathogeny derived from an exacerbated Th2 response, an aspect not yet addressed. *Trichinella spiralis* antigens-group 1 from muscle larvae (TSL1 antigens) and excretory–secretory products (mainly composed of the 49, 53, 57 and 65 kDa *T. spiralis* antigens; Bolás-Fernandez & Corral Bezara, 2006) have exhibited good protection against *T. spiralis* (Ortega-Pierres *et al.*, 1989); nevertheless, more refined vaccine formulations, which include different adjuvants, purified antigens, recombinant proteins and synthesized epitopes, have subsequently been evaluated.

Adjuvants and immunization routes used in the induction of protection against T. spiralis

Adjuvants have been used to enhance the humoral and/or cell-mediated immune responses against *T. spiralis* antigens, as well as to protect the antigens from being diluted, degraded and eliminated by the host (Stills, 2005). Thus various adjuvants have been tested in these studies, including the following: IFA (incomplete Freund's adjuvant), CFA (complete Freund's adjuvant), Bacille Calmette–Guerin (BCG), methacrylic acid copolymers, Montanide™ IMS1312 and Montanide™ ISA720 (Ortega-Pierres *et al.*, 1989; Behnke *et al.*, 1994; Goyal *et al.*, 1997; Dea-Ayuela *et al.*, 2000, 2006a, b; McGuire *et al.*, 2002; Eissa *et al.*, 2003; Deville *et al.*, 2005) (table 1). While CFA, a water-in-oil (W/O) emulsion that contains heat-killed and dried mycobacterial cells, has been widely used and is the most effective adjuvant for experimental antibody production, its use has been limited lately due to the side-effects and variety of lesions that it causes. These lesions include granulomas at the injection site, liver, kidney and sub-pleura, as well as necrotizing dermatitis (Billiau & Matthys, 2001; Stills, 2005). Similarly, the use of cholera toxin, which also enhances antigenicity of *Trichinella* sp. extracts, causes gastrointestinal disorders (Williams *et al.*, 1999). Thus, alternative adjuvants have emerged and these have been evaluated in experimental trichinellosis over the past few years. These include other W/O emulsions such as ISA (incomplete Seppic adjuvants), IFA (with the same composition as CFA except that this does not contain mycobacteria) and aqueous formulations with nanoparticles such as Montanide™ IMS1312 (commercialized by Seppic (Paris, France), contains an injectable pharmaceutical mineral oil and a refined emulsifier derived from mannitol and purified oleic acid of vegetable origin), and copolymers of methacrylic acid (based on dimethylaminoethyl methacrylate and neutral methacrylic acid esters). Although the latter are very promising, the efficiency of immunization still depends on the delivery route and the type of antigen used to trigger significant antibody responses.

Indeed, vaccine delivery encompasses: (1) administration of the vaccine formulation to specific sites of the body; and (2) delivery of the antigen to, and activation of, relevant cells of the immune system. The infection model with *Trichinella* initially involves the intestinal mucosal compartment, which targets the induction of local immune responses. Therefore the routes of antigen delivery should involve mucosal tissues or should be deposited directly in accessible mucosal surfaces.

Table 1. Immunization protocols and immunological/parasitological responses using whole extracts and purified antigens of *T. spiralis*.

Antigen	Model/adjuvant/route	% Reduction (ML/worms)	Systemic responses	Mucosal responses	Worm fecundity	Reference
ESPs or 49+55 kDa proteins	Mice/CFA/intraperitoneal	65–70/45–50	ND	ND	Reduced by ~50%	Gamble (1985)
NBL extracts	Pig/CFA/intraperitoneal	78/ND	ND	ND	ND	Marti *et al.* (1987)
ML ESPs	Pig/CFA/ intraperitoneal	40/ND				
TSL-1 antigens (47, 52, 65 and 72 kDa)	Mice/CFA/intraperitoneal	~50/~55	↑ Igs anti-TSL-1	ND	ND	Ortega-Pierres *et al.* (1989)
ML homogenate	Mice/CTB/intranasal	ND/75	↑ IgG1 and IgG2a, ↑ IFN, ↑ IL-5 (spleen)	↑ Intestinal IgA, ↑ IL-5 (MNLC)	ND	McGuire *et al.* (2002)
T. spiralis or *T. britovi* ESPs	Mice/IFA/sbc	ND/~70	↑ Specific IgG1 and total IgG, IgA and IgM	↑ IL-5 (MNLC)	ND	Goyal *et al.* (1997)
Autoclaved larvae	Mice/BCG/intradermic	79/80	ND	ND	RCI reduced	Eissa *et al.* (2003)
ML homogenate	Hamster/CFA/sbc	ND/>80	↑ IgG (serum)	↑ Intestinal mastocytosis	RCI severely suppressed	Behnke *et al.* (1994)
Crude larval extracts	Mice/none/intramuscular	ND/ND	↑ IgG3 (serum)	ND	ND	Dea-Ayuela *et al.* (2000)
Ts/Fg cross- reactive fraction	Rat/CFA/sbc Rabbit/CFA/sbc	ND/74 ND/47.8	↑ IgG (serum)	ND	ND	Abdel-Rahman *et al.* (2005)
ML total antigen	Mice/ISA720, IMS1312/sbc	78–85/ND	↑ IgG1 and IgE (serum)	ND	ND	Deville *et al.* (2005)
L1 larvae	Mice/MAC/oral	53/45	↑ IgG1 (serum)	ND	ND	Dea-Ayuela *et al.* (2006a)
Crude larval extracts	Mice/MAC/oral	ND/29–73	↑ IFN, ↑ IL-4 ↑ CD4+ (spleen) ↑ IgG1 and IgA (serum)	↑ IFN, ↑ IL-4 and ↑ IgA (MLN), ↑ CD4+ (PP)	ND	Dea-Ayuela *et al.* (2006b)
ML	Mice/no adjuvant/oral	ND/ND	↑ Specific IgG1 and IgG2a, ↑ IgM (serum)	ND	ND	Dvorožňáková *et al.* (2010)

BCG, Bacille Calmette–Guerin; CFA, complete Freund's adjuvant; CTB, cholera toxin subunit B; ESPs, excretory–secretory products; Fg, *Fasciola gigantica*; IFA, incomplete Freund's adjuvant; IFN, interferon; IL, interleukin; IMS1312, Montanide IMS1312; ISA720, Montanide ISA720; MAC, methacrylic acid copolymer; ML, muscle larvae; MLN, mesenteric lymph nodes; MNLC, mesenteric lymph node cells; NBL, newborn larvae; ND, not determined; PP, Peyer's patches; RCI, reproductive capacity index (ratio of the total number of larvae recovered from an animal to the number of larvae administered); sbc, subcutaneous; Ts, *Trichinella spiralis*; TSL-1, *T. spiralis* antigens-group 1 from muscle larvae.

An optimal mucosal vaccine strategy can activate local innate immune responses that translate into the generation of neutralizing sIgA Abs, polyfunctional CD8+ cytotoxic T-lymphocytes (CTLs) in effector target sites, and CD4+T-cell effector memory responses, whereas systemic vaccine strategies are much less effective in eliciting the compartmentalized mucosal immunity necessary for these protective responses. In addition, mucosal vaccines can offer needle-free delivery, thereby improving accessibility, safety and cost-effectiveness (for a review, see Belyakov & Ahlers, 2009). Thus, in *Trichinella* vaccination, mucosal immunization through oral or intranasal routes can be more effective, and these routes can induce generalized mucosal immune responses not only at the portals of entry of this parasite but in distant mucosal effector sites as well, although additional constraints on mucosal compartmentalization are evidently dependent upon the adjuvant and the antigen delivery vehicle

In some recent studies the importance of selecting adjuvants to improve vaccine efficacy has been highlighted. Pioneer studies using *Trichinella* excretory–secretory (TES) products administered subcutaneously in high- and low-responder mice did not show a direct influence of adjuvants such as CFA, alum or commercial preparations (ISCOM^{TM} or Titermax^{TM}) on the qualitative profile of antigen-specific antibodies, IL-5 and IFN-γ, although quantitative levels were higher (Robinson *et al.*, 1994). Other studies with Montanide-ISA adjuvants (water-in-oil, W/O; oil-in-water, O/W, and multiphasic) and microbeads demonstrated that the use of adjuvants enhanced immune responses against *T. spiralis* L1 antigens (L1) when administered subcutaneously in mice, since elevated humoral responses were observed with all the adjuvants tested. However, IgG1 and IgG2a levels increased to a greater extent when using microbeads and W/O emulsions containing mineral oil instead of metabolizable oils (Aucouturier *et al.*, 2001). Furthermore, in female OF1 mice immunized with total soluble antigens of *T. spiralis* muscle larvae (ML), two types of Montane adjuvants were tested: ISA70 (W/O emulsion) and IMS1312VG (nanoparticles) along with aluminium hydroxide (alum) and saline as control adjuvant and vehicle, respectively. The three adjuvants contributed to the induction of significantly higher levels of anti-*T. spiralis* IgG1 and IgE antibodies, as compared to saline. In these assays Montane formulations have the highest response. In terms of protection measured by the determination of ML burdens, a more drastic decrease in ML was observed when ISA70 was used, while animals treated with alum and IMS1312VG had a higher number of ML, demonstrating the utility of W/O emulsions in enhancing protection against *Trichinella* (Deville *et al.*, 2005). In a different approach, microparticles of methacrylic acid copolymers (Eudragit) were used as a delivery system for L1 total antigens and *Trichinella* excretory–secretory antigens (TES), in order to avoid tolerance to their oral administration and to target antigens at the mucosal lymphoid organs (Dea-Ayuela *et al.*, 2006a, b). These particles are biocompatible as they exhibit resistance to acidic pH but deliver antigens in the neutral and basic pH range, enabling access of antigen to parenteral/systemic compartments. In these studies female BALB/c and NIH mice were orally vaccinated with larval antigens microencapsulated in Eudragit T100, then mucosal and systemic immune cell populations and cytokines were evaluated. In the vaccinated mice, increased levels of IFN-γ in spleen and mesenteric lymph nodes were observed, while low levels of splenic IL-4, CD4+ and CD19+ cells were detected in these animals, as compared with the non-vaccinated controls. These changes were associated with increased CD4+ cells and decreased CD19+ cells in Peyer's patches (PP). Somatic and TES-specific serum IgG1, IgA and mesenteric lymph node (MLN) IgA were elevated in the immunized mice. Regarding protection, in mice strains both L1- and TES-containing microcapsules provoked a reduction of adult worms at days 3–9 post-infection, although female fecundity was not affected. Nevertheless, protection at the intestine was 45% and 53% at the muscle stage. These observations suggest that the presence of balanced local and systemic Th1/Th2 responses. Thus, formulations with different and complex antigen preparations in mice of a distinct background will produce partial protection.

Recombinant proteins and subunit epitopes used to induce protective responses against T. spiralis

In recent years genetic engineering, including recombinant DNA technology and genome-wide sequencing, has provided useful strategies for the identification and characterization of molecules with potential use in the so-called second- and third-generation vaccines. Some sequences coding for molecules involved in the host–*Trichinella* relationship have been cloned from the incompletely annotated, 64-megabase *T. spiralis* genome containing approximately 15,800 open reading frames (Mitreva *et al.*, 2011). These have been used to design second-generation vaccines, based on either recombinant *T. spiralis* proteins or on peptide epitopes expressed by live and attenuated vectors, such as *Salmonella* sp., which are exposed on the bacterial surface by means of bacterial autotransporters (table 2).

Regarding recombinant proteins or synthesized peptides, while these may be immunogenic, their immunogenicity may be enhanced by using an adjuvant that will either lengthen their half-life or increase innate immune responses. An example is *T. spiralis* paramyosin, which is not only a structural protein but also has a protective role for the worm, preventing its attack by host complement components. For example, C9 has been cloned and used as a vaccine in conjunction with CFA, to immunize BALB/c mice prior to challenge with *T. spiralis* ML. In the immunized animals IgG-specific antibodies with a concomitant Th1/Th2 response associated with a 36% reduction in ML burden were induced (Yang *et al.*, 2008). Due to the CFA toxicity, other adjuvants such as Montanide^{TM} ISA206 and ISA720 were also tested, producing similar levels of reduction in ML burdens (34 and 36%, respectively). Likewise, a Th1/Th2 response was induced using these formulations (Yang *et al.*, 2010a).

In a similar approach, enzymes from *T. spiralis* have been used as prophylactic antigens. Aminopeptidase, a 54.7 kDa cytosolic protein located at the cuticle and in internal organs of ML, NBL, adults and pre-encapsulated larvae, was cloned, purified in a recombinant form from *Escherichia coli* and used to immunize BALB/c mice before challenge with infective larvae. In these assays

Table 2. Second- and third-generation vaccines used in experimental *T. spiralis* infections in mice.

Immunogen	Administration route	Immuno- and parasitological effect	Reference
40-mer of 43 kDa antigen in CFA	Subcutaneous	Faster worm expulsion	Robinson *et al.* (1995)
30-mer of 43 kDa antigen with CT	Intranasal	Th2 response, ↓ parasite burden	McGuire *et al.* (2002)
DNA vaccine of TspE1	Intramuscular	↑ Cellular and humoral response, ↓ worm burden	Wang *et al.* (2006)
Phage-displayed Ts87 peptides	Subcutaneous	↓ Parasite burden	Gu *et al.* (2008)
rTsPmy in ISA106 or ISA720	Subcutaneous	Th2 response, ↓ parasite burden	Yang *et al.* (2010a)
Salmonella-delivered DNA vaccine of Ts87 gene	Oral	Mixed Th1/Th2 response, ↓ ML and worm burdens	Yang *et al.* (2010b)
Salmonella-expressed 30-mer of p43 antigen (ShdA)	Intranasal	↑ IgG1 & IL-5, ↓ worm burden (61%)	Pompa-Mera *et al.* (2011)
DNA vaccine of pVAX1–TsMIF–TsMCD-1	Intramuscular	↑ IFN-γ (serum), ↑ CD4+ and CD8+ cells, ↓ worm burden (23%)	Tang *et al.* (2012)
DNA vaccine of pVAX1–TsMIF–TsMCD-1–mUb	Intramuscular	↑ IFN-γ (serum) & T-cell cytotoxicity ↓ worm burden (39%)	Tang *et al.* (2013)
Salmonella-expressed 30-mer of p43 antigen (MisL)	Intranasal *Salmonella*-30mer +boost with intraperitoneal 30mer	Th2 response, ↑ intestinal mucus, ↓ worm burden (76%)	Castillo-Alvarez *et al.* (2013)
rTs-APase	Subcutaneous	↓ Parasite burden	Zhang *et al.* (2013)
rTs-Adsp in alum	Subcutaneous	Mixed Th1/Th2 response, ↓ ML burden	Feng *et al.* (2013)
rTspSP-1.3	Subcutaneous	↑ IgG and IgE, mixed Th1/Th2 response, ↓ ML burden	Li *et al.* (2013)
Phage-displayed rTsp10	Subcutaneous	Th2 response, ↓ worm and ML burdens	Cui *et al.* (2013)
Salmonella-delivered Ts-cystatin	Oral	Accelerated worm expulsion, ↓ worm fecundity	Liu *et al.* (2014)
Salmonella-delivered Ts87 DNA vaccine+rTs87	Oral DNA vaccine + intramuscular boost with rTs87	Enhanced Th1/Th2 response, ↓ ML burden (46%)	Gu *et al.* (2014)
Salmonella-surface-anchored and secreted 30-mer of p43 antigen (ShdA) fused to $P28_3$	Intranasal	Mixed Th1/Th2 response with Th2 predominance (↑ systemic IgG1, IL-5 and ↑ intestinal IgA), ↓ worm burden (92% secreted, 42% surface-anchored)	Pompa-Mera *et al.* (2014)

APase, aminopeptidase; CFA, complete Freund's adjuvant; CT, cholera toxin; ISA106, Montanide ISA106; ISA720, Montanide ISA720; MCD-1, multi-cystatin domain-1; MIF, migration inhibitory factor; MisL, autotransporter MisL; ML, muscle larvae; mUb, mouse ubiquitin; $P28_3$, three copies of the minimal binding domain of Cd3; Pmy, paramyosin; pVAX1, plasmid VAX1; r, recombinant; ShdA, autotransporter ShdA; Ts, *Trichinella spiralis*; Ts-Adsp and TspSP-1.3, *T. spiralis* serine proteases; Tsp10, *T. spiralis* 31 kDa antigen.

intestinal protection of 38% (reduction of adult worm burden) and systemic protection of 59% (reduction in ML burden) were determined (Zhang *et al.*, 2013). In two independent studies, two serine protease-like proteins from *T. spiralis* were obtained in recombinant form and used to immunize BALB/c mice, which were then challenged with infective larva. One of these, referred to as rTs-Adsp, was given together with alum adjuvant and this scheme induced a mixed Th1 (IFN-γ, IL-2)–Th2 (IL-4, IL-10 and IL-13) response, with the latter predominating. High levels of anti-rTsAdsp IgG and IgE and a reduction of 46% in ML burden (Feng *et al.*, 2013) were detected. The other serine protease, referred to as TspSP-1.3, had a higher expression in ML and adult worms as compared with NBL and, when used to immunize mice, also induced higher IgG and IgE antibody levels and mixed Th1/Th2 responses along with a 39% reduction in ML burden (Li *et al.*, 2013).

In recent studies the potential efficacy of peptide vaccines administered via mucosal (oral or intranasal) routes has been tested. However, in these cases there was a need to use adjuvants, and even one or more boosts, to enhance antigen-specific humoral and cellular responses. An example of these studies is the immunogenic 30-mer peptide (residues 210–239; Gold *et al.*, 1990) from the *T. spiralis* 43 kDa antigen, which is a member of the TSL-1 antigen family that in an earlier study was administered intranasally to NIH mice together with cholera toxin as adjuvant. This immunization protocol induced high titres of serum IgG and intestinal IgA and a Th2 cytokine profile, associated with a modestly accelerated adult expulsion by day 8 post-infection (McGuire *et al.*, 2002). As an alternative strategy, this 30-mer was expressed in the surface of attenuated *Salmonella enterica* serovar Typhimurium SL3261 by cloning into a plasmid containing the ShdA autotransporter, and the transformed bacteria were given intranasally to BALB/c mice prior to challenge with infective larva. Vaccinated mice exhibited a 61% reduction in intestinal worms and a Th2 response involving anti-30mer IgG1 antibodies along with increased IL-5 levels (Pompa-Mera *et al.*, 2011). An improvement of this vaccination strategy included the use of the 30-mer peptide cloned in frame with the MisL autotransporter to express the peptide on the surface of attenuated *Salmonella*. Further, these bacteria were given intranasally to BALB/c mice followed by an intraperitoneal boost with the recombinant 30-mer. Antibodies induced by this immunization were directed towards the ML surface. Vaccinated mice displayed a Th2-type response and, importantly, a greater reduction of intestinal burden of adult worms (76%) along with enhanced mucus production that entrapped adults, thus preventing parasite attachment and promoting their expulsion (Castillo-Alvarez *et al.*, 2013). This strategy showed a better protection induced by immunizing and boosting the animals with the 30-mer peptide prior to infection with *T. spiralis* muscle larvae. Furthermore, recent studies with this 30-mer peptide, now fused to three copies of the molecular adjuvant P28 (minimal binding domain of C3d that, upon interaction with B cells, triggers a Th2 response) expressed in the attenuated strain of *S. enterica*, was either displayed on the bacterial surface or secreted. The results demonstrate that displayed 30-mer induced higher IgG1 titres in BALB/c mice when compared to secreted peptide. In contrast, secreted 30-mer promoted a higher degree of earlier mucosal anti-30mer IgA levels and IL-5 secretion by cells of Peyer's patch. Likewise, greater protection was obtained with the secreted peptide (92% in adult worm reduction) compared to that obtained with the surface-anchored peptide (42%) (Pompa-Mera *et al.*, 2014).

Other peptides, such as the 40-mer comprising residues 40–80 of the 43 kDa glycoprotein, included in the TSL-1 antigens, induced a partial protective effect promoting accelerated worm expulsion in infected mice (Robinson *et al.*, 1995). Also, other *Salmonella* typhimurium strains with modified regulons for sensor kinases (PhoP/PhoQ null mutants), which are markedly attenuated in BALB/c mice, are effective vaccines with *T. spiralis* antigens such as cystatin-like protein (Ts-cystatin). In these studies, mice immunized with Ts-cystatin displayed a mixed Th1/Th2 response without systemic protection; however, adult expulsion was accelerated by 10% and worm fecundity was dramatically decreased (91%), suggesting a role for Ts-cystatin in *Trichinella* resistance to rapid expulsion by the infected host (Liu *et al.*, 2014).

Another strategy to express recombinant proteins and epitopes is the construction and screening of phage-display peptide libraries to find immunogenic peptides with antibodies. The monoclonal antibody 5A3, which recognizes the Ts87 protein, an excreted-secreted product from *T. spiralis* worms, was used to select four phage clones that, in turn, were used to subcutaneously immunize BALB/c mice (Gu *et al.* 2008). Of these, two phage clones induced specific anti-Ts87 antibodies and when these clones were co-administered, a 28.7% worm burden reduction was obtained. This approach was also used to identify T7 phage clones expressing recombinant Tsp10, a polypeptide localized at the stichosome of infective larvae with the ability to bind to mouse epithelial cells. Mice immunized with phage T7-Tsp10 displayed noticeably high reductions of adult worms (62%) and ML (78%) burdens and a predominant Th2 response after challenge with ML (Cui *et al.*, 2013). These data strongly support this approach as promising to achieve effective protection against *T. spiralis* infection using single peptides.

DNA vaccines against T. spiralis

The third-generation vaccines are made of plasmids containing sequences coding for foreign proteins, which upon injection and access to host cells are translated, processed and expressed on their surface, promoting antibody responses, cytotoxic T-lymphocyte activation and Th1-type cytokines. DNA vaccines are usually well tolerated and thereby safe for use in the induction of protection in experimental trichinellosis (table 2). In a murine model using BALB/c mice, a DNA vaccine (plasmid PcDNA3-TspE1) designed with a sequence coding for a 31 kDa-sized *T. spiralis* antigen (sharing 98% identity to the 45–50 kDa antigen from infective larvae) was injected intramuscularly. This preparation induced a cellular response associated with 37% reduction of adult worms at the intestine (Wang *et al.*, 2006). Another DNA vaccine was engineered for delivery by an attenuated *Salmonella* typhimurium strain SL7207 that

was transformed with an expression plasmid (pVAX1) encoding the aforementioned Ts87 protein. After oral administration of this vaccine, the BALB/c mice expressed the Ts87 protein at mesenteric lymph nodes and showed an intestinal IgA and a balanced systemic Th1 (IFN-γ)/Th2 (IL-5, IL-6 and IL-10) response. Challenge with muscle larva resulted in a 29% reduction in adult worm burden and a 34% reduction in ML burden (Yang *et al.*, 2010b).

In addition, a heterologous vaccination regimen using Ts87 DNA vaccine to prime, combined with rTS87 protein boost, along with homologous DNA–DNA or protein–protein prime/boost protocols, was evaluated in BALB/c mice. Interestingly, the DNA prime/protein boost protocol induced an enhanced Th1/Th2 mixed response that surpassed that obtained with homologous prime/boost protocols. ML burdens were diminished by the heterologous regimen by 46%, while for homologous (DNA and protein) protocols it was 36% and 24%, respectively (Gu *et al.*, 2014). This study showed the usefulness of DNA vaccines combined to recombinant proteins to potentiate both immunogenicity and protection towards a given antigen. In another approach, the pVAX1 plasmid vector was used to express both the macrophage migration inhibitory factor (MIF) (TsMIF) and a multicystatin-like domain protein (MCD-1) of *T. spiralis* (TsMCD-1), which is a pro-inflammatory factor from intracellular stages of adult worms. These recombinant plasmids were given to BALB/c mice and it was observed that the co-expression of TsMIF and TsMCD-1 produced more serum IFN-γ and enhanced CD4+ and CD8+T-cell levels compared to the response observed for either DNA vaccine on its own. The immunization with pVAX1–TsMIF–TsMCD-1 reduced worm burdens by 23% (Tang *et al.*, 2012). This latter plasmid, now containing a third component (mouse ubiquitin, mUb) in order to promote antigen ubiquitination and presentation by major histocompatibility complex I (MHCI) to induce stronger Th1-type responses, was tested again as a DNA vaccine in BALB/c mice. Animals vaccinated with pVAX1–TsMIF–TsMCD-1–mUb certainly increased IFN-γ levels and T-cell cytotoxicity and, moreover, showed a greater degree of reduction in worm burden (39%) than that in the absence of mUb (Tang *et al.*, 2013).

Based on the data reported on vaccination against *T. spiralis,* it is still difficult to conclude that DNA vaccines could have advantages over recombinant proteins/epitope-based peptides, or vice-versa. This comparison is difficult because different groups have employed different antigens, routes of infection and parameters for assessment of vaccine effectiveness. Thus a certain level of standardization would represent a great improvement to compare the antigens used.

Perspectives on Trichinella *vaccination*

Up to now, the efficacy of *Trichinella* proteins used for vaccination has been optimally adapted to rodent species, therefore these require verification in pigs and in the human setting, so that parasite effects can be reproduced by these or other *Trichinella* molecules containing the minimal effective parasite-encoded motifs required to interact with key host molecules. Furthermore, in *T. spiralis*-derived therapeutic agents, the molecular motifs that are responsible for protection from immunopathology should be identified, and the host pathways that are targeted likewise defined.

Most importantly, one has to keep in mind that in order to eliminate parasites from the gut, the immune response induced by vaccines must disable, degrade and dislodge the parasites (Maizels *et al.*, 2012). Disabling parasites can be achieved by neutralizing antibodies, and by components such as resistin-like molecule-β. Cumulative damage to the parasite can be achieved by granulocyte attack promoted by antibodies or complement/serum components, as well as mast-cell or eosinophil responses. Also macrophages and neutrophils may release nitric oxide. Further dislodging of parasites can be achieved by making the intestinal tract unbearable. In this process, alarmins IL-25 and IL-13 play an important role by promoting changes in intestinal structure and function. IL-13 also induces a switch from the predominant MUC2 to MUC5ac, which is required for normal expulsion of intestinal parasites (Hasnain *et al.*, 2011). Interestingly, the participation of the innate immune response through epithelial cells, by expressing alarmins such as IL-25 and IL-33 (Scalfone *et al.*, 2013) when the parasite has caused tissue damage, has been demonstrated to be crucial for triggering a Th2 response, as alarm-driven innate lymphocytes are then recruited and release IL-4 and IL-13, thereby promoting an early Th2-skewed response.

Interestingly, most of these mechanisms are activated upon *Trichinella* infection. As mentioned above, *Trichinella* induces a polarized Th2-type response, which is protective and responsible for parasite expulsion (Allen & Maizels, 2011). Although CD4+ Th2 cells are key players in this type of immune response, other non-T cells also contribute to enhance Th2-type immunity (Allen & Maizels, 2011). Thus, activation of a Th2-type immunity by defined parasite antigens should be a priority. In this context, other parasite antigens, different from the ones already used, may be tested. Thus, antigens such as cystatins, serpins and glycans, which could influence antigen processing, presentation and subsequent T-cell polarization, could be tested. These antigens have been shown, in many parasites, to trigger an innate and adaptive immunity response in a tuned orchestration followed by an induction of B- and T-regulatory cells to ameliorate the damage to the host and to the parasite. Further specific adult parasite antigens may also be tested to induce this type of response.

Whenever recombinant proteins are used, special attention should be paid to avoid incorrectly folded proteins and/or lack of critical post-translational modifications, particularly glycans (e.g. tyvelose), that are attached to several of the native parasite antigens and that have been shown to be immunogenic. Therefore, improvements in the protection induced by recombinant proteins could be achieved using methods for single-point attachment of polysaccharides and oligosaccharides to protein carriers. Indeed, contemporary approaches involve synthetic oligosaccharides with linker or tether chemistry designed for compatibility with synthetic strategies. Improvements for vaccination against *Trichinella* could also include genes encoding molecules with adjuvant- or immunomodulating activity, and

multi-epitope formulations. Most importantly, one has to keep in mind that a good vaccine must stimulate protective immune responses that will play a role in limiting parasite proliferation in hosts infected by *Trichinella.*

Conclusions

Approaches to vaccination against *T. spiralis* primarily using murine experimental models have used a wide range of strategies that include whole extracts and excretory–secretory products, recombinant proteins, epitopes–peptides and DNA vaccines, along with various adjuvants and different routes of antigen administration.

In general, the levels of protection observed in most experimental trials may not be sufficient to provide full disease control. Thus critical analysis is required to assess whether partially effective vaccines against *T. spiralis* may be useful for the protection of both individuals and whole populations. Their usefulness in the agricultural sector should be defined to minimize the economic impact of the disease.

To eliminate parasites while minimizing unwanted collateral effects during *Trichinella* infection results in redundancy and parallelism between innate and adaptive effector responses, and the prominence of regulatory populations and mechanisms to focus and restrain host immunity. Clearly, more basic research on the molecular nature and function of selected *Trichinella* antigens is needed. Specifically, there is a need to evaluate whether potential vaccine candidates have protection-inducing qualities that outperform the immune response of natural, primary infection. These approaches will provide insights for the proposal of new strategies for the control of infections caused by *Trichinella*.

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Conflict of interest

None.

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