Review

The immunoregulatory effects of co-infection with *Fasciola hepatica*: from bovine tuberculosis to Johne's disease

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Abstract

Fasciola hepatica is a parasite prevalent in much of the world, that causes the economically-important disease of fasciolosis in livestock. The threat this disease poses extends beyond its direct effects, due to the parasite's immunomodulatory effects. Research at this laboratory is focussing on whether this immunoregulation can, in animals infected with liver fluke, exert a bystander effect on concurrent infections in the host. It has already been established that *F. hepatica* infection reduces cell mediated immune responses to *Mycobacterium bovis* in cattle, and that the interaction between the two pathogens can be detected on an epidemiological scale. This review explores the immunological consequences of co-infection between *F. hepatica* and other bacterial infections. Arguments are presented suggesting that immunity of cattle to *Mycobacterium avium* subsp *paratuberculosis*, is also likely to be affected.

Keywords: Bovine tuberculosis; Johne's disease; Fasciolosis; Immunomodulation; Co-infection.

Introduction

Most helminthic parasites are detrimental to the health of their hosts, to some extent. The pathophysiology of helminth infections usually involves host tissue damage resulting from the worms' migratory and feeding behaviour, diversion of the hosts' resources to their own growth and reproduction. If and how well these effects are tolerated depends largely on the biology of the parasite, its predilection site in the host and the fitness of the host. In addition, by polarising the host's immune response to a T helper 2 (Th2)-/ Treg response, parasitic worms can downregulate protective T helper 1 (Th1)-type responses against concurrent infections, thereby increasing the host's susceptibility to many bacterial, viral and protozoal pathogens (Salgame et al., 2013). This Th2 shift can also potentially interfere with diagnostic tests which rely on an effective Th1 response, resulting in false negative results (Flynn et al., 2007b, 2009; Claridge et al., 2012).

By the same token, immune polarisation by parasitic worms can be of benefit in those conditions where the host immune and inflammatory response is, in itself, a significant contributor to disease, a discovery that gave rise to concepts such as the 'hygiene hypothesis' and 'helminth therapy', a treatment approach for inflammatory (mostly autoimmune) diseases which involves the deliberate infection with intestinal worms (Maizels et al., 2014).

The "bystander immunregulatory effect" of helminth infections on concurrent bacterial or viral infections in the same host has been a subject of significant interest. The outcome in such co-infections is complex, depending on such factors as timing of

each infectio, and species involved. By far, the most complex scenario is where a microbial pathogen uses elements of the host's immune response to gain access and disseminate throughout the organism, as is the case with intracellular infections such as those caused by mycobacteria, where macrophages are infected. Whether in these cases helminth-induced immune modulation promotes or inhibits onset of disease, has been the subject of debate. One such parasite- microbe co-infection to gain significant interest, is the co-occurrence of the liver fluke, *Fasciola hepatica* (*F. hepatica*) and bovine tuberculosis (BTB) caused by *Mycobacterium bovis* in cattle (Flynn et al., 2009; Claridge et al., 2012; Garza-Cuartero et al., 2016). In this case, cell-mediated immune responses against *M. bovis* are downregulated in animals also infected with liver fluke. Yet, contrary to what one may expect, reduced mycobacterial burdens were also shown in these animals (Laura Garza-Cuartero et al., 2016). In this review we explore whether a similar interaction is likely to occur between *F. hepatica* and a close relation of *M. bovis*, *Mycobacterium avium* subsp *paratuberculosis* (MAP), the causative agent of Johne's disease in cattle.

Bovine tuberculosis and Johne's disease

BTB and Johne´s disease are both caused by bacteria of the genus *Mycobacteria*. Granulomatous lesions are characteristics of both diseases, although the pathology and resulting clinical signs differ considerably. This section deals with similarities and differences between the two infections..

Pathogens and disease

Mycobacteria are aerobic, acid-fast microorganisms characterised by a thick, lipid-rich cell wall containing mycolic acids, which confers durability in the

environment and resistance to many disinfectants and antibiotics (Rogall et al., 1990; Whittington et al., 2012). While interspecies similarities within the *Mycobacterium* genus are relatively high, there is a clear separation between two clusters: slowly and rapidly growing groups. Both *M. bovis* and MAP are classified within the 'slow growers', a group that, unlike the 'rapid growers', appears to have a monophyletic origin (Rogall et al., 1990; Devulder et al., 2005; Mignard and Flandrois, 2008; Tortoli, 2012).

The final target of these two pathogens is fundamentally different. While BTB affects primarily lungs and tracheobronchial and mediastinal lymph nodes, MAP has predilection for the ileum, ileocecal valve, jejunum and associated lymph nodes (Palmer et al., 2002; González et al., 2005). The main route of infection for each disease is, therefore, related to the target organs; aerosol exposure for BTB, and oral ingestion of contaminated faeces, milk or colostrum for Johne's disease, although *in utero* infection is also possible (Pollock et al., 1996; Cassidy et al., 1999; Stabel et al., 2009). BTB most commonly becomes established in cattle aged between 1 and 2 years (Brooks-Pollock et al., 2013), while animals under 6 months of age are most susceptible to infection with MAP. This raised susceptibility to MAP in neonates has been attributed to various factors including increased intestinal permeability, a weakness of the mucosal barrier or differential distribution and/or predominance of distinct T cell subpopulations in younger animals (Chiodini et al., 1984; Sweeney et al., 1992; Rideout et al., 2003; Windsor and Whittington, 2010). Both diseases are considered to have a chronic course.. However, while patent BTB usually takes a couple of weeks or months to develop, lesions due to Johne's disease typically do not appear for 2 to 5 years after infection (Tiwari et al., 2006).

Despite these striking differences between BTB and Johne´s disease, there are also some important similarities with regard to the pathological changes and immune response developed by the hosts. During BTB, a failure to fully control the pathogen leads to formation of granulomas, mainly in lungs and associated lymph nodes, although intestines, liver, spleen, pleura, and peritoneum may also be affected (Cosma et al., 2003; Rohde et al., 2007; Harris et al., 2009; OIE, 2009). Granulomas are compact, organized aggregates of macrophages that undergo specialised transformation into epithelioid or multinucleated giant cells (Cosma et al., 2003; Ramakrishnan, 2012). Although their function is still debated, these aggregates are thought to localize and contain the pathogen, as well as concentrate the immune response to a limited area thus diminishing tissue damage (Ramakrishnan, 2012; Cambier et al., 2014). Johne´s disease also induces the formation of granulomatous lesions, although in this case they are located in the intestinal *lamina propria* and associated lymphoid tissues (González et al., 2005). Histologically, Johne's disease is characterised by granulomatous lesions which, in contrast to BTB lesions, do not caseate and lack a fibrous capsule (Whittington et al., 2012). Furthermore, MAP granulomas from animals showing clinical signs tend to be more diffuse, although focalised granulomas may also be observed (Fernández et al., 2016; González et al., 2005). Macrophages focal lesions in MAP-infected animals have been related to either initial phases or latent stages of the disease (Fernández et al., 2016; Palmer et al., 2007). In human tuberculosis, individuals capable of mounting an effective response may experience a decrease in granuloma cellularity, leading to a latent infection (Guirado and Schlesinger, 2013). Some authors have suggested that a latent stage is also possible in bovine tuberculosis (Cassidy, 2006; L. Garza-Cuartero et al., 2016; Pollock and Neill, 2002). These infections can reactivate

as a result of immunosuppression, resulting in more severe lesions and development of clinical signs (Flynn and Chan, 2001).

The development of granulomatous lesions in the distinct organs leads to associated clinical signs. During BTB, these signs vary depending on the severity and distribution of lesions: cough and dyspnoea may be manifested when lungs are severely affected, and other signs like diarrhoea or constipation may indicate digestive tract involvement (Cassidy, 2006; OIE, 2009). However, in other cases cattle may not show clinical signs in spite of severe lung pathology visible at post-mortem examination (Menin et al., 2013). In Johne's disease, the inflammatory process leads to a thickened and corrugated intestinal mucosa producing diarrhoea and the characteristic malnutrition syndrome associated (Harris, 2001).

Invasion and immune response

BTB and MAP are both obligate intracellular pathogens, which infect, reside and replicate inside the host's macrophages (Rue-Albrecht et al., 2014). At a cellular level, MAP crosses the intestinal barrier mainly via the M-cells (microfold epithelioid cells), although enterocytes have also been proven to translocate the bacteria (Bermudez et al., 2010). In order to attach to the ileal epithelium, MAP has fibronectin attachment protein (FPA) in its cell wall, which may be activated during the journey through the digestive system (Bannantine and Bermudez, 2013), and contributes to the uptake by M cells (Arsenault et al., 2014; Secott et al., 2004). These are specialized epithelial cells of the mucosa-associated lymphoid tissue, which transport extraneous material from the intestinal lumen across the epithelial barrier to sentinel cells, mainly macrophages and dendritic cells (DC) situated in the Peyer's patches (Momotani et al.,

1988; Tessema et al., 2001). In BTB, inhaled bacteria are phagocytosed by alveolar macrophages in the lungs (Cosma et al., 2003). The invasion of macrophages by both pathogens involves cell surface receptor molecules including complement receptors (CR1, CR3, CR4), mannose receptors (MR), scavenger receptors, or Toll-like receptors (TLRs) (Arsenault et al., 2014; Guirado et al., 2013; Woo and Czuprynski, 2008)

Macrophages can be activates through various pathways: innate, classical, or alternative. The first time a pathogen encounters the host' immune system, macrophages are activated through the innate pathway. The resulting cells promote phagocytosis, endocytosis, and antigen presentation, and produce pro-inflammatory molecules including IFN-α/β, triggering a typical Th1 immune response (Gordon, 2003). Th1 lymphocytes in turn release inflammatory cytokines, principally IFN-γ which classically activate macrophages to the M1 phenotype (Guirado et al., 2013). M1 macrophages are characterised by increased major histocompatibility complex (MHC) class II-mediated antigen presentation capability, and enhanced microbicidal activity mainly through the generation of nitric oxide (NO), which is crucial to develop a protective response (Gordon, 2003; Guirado et al., 2013; Italiani and Boraschi, 2014). However, virulent *M. bovis* strains have been shown to modulate the host response by switching macrophage phenotypes from M1 to M2 (alternatively activated macrophages), or a mixed environment (Andrade et al., 2012). Alternative activation is mediated by IL-4 and IL-13, acting through a common receptor chain (IL-4Rα). These cells have a reduced cellular responsiveness to IFN-γ, use preferentially arginase-1 instead of inducible nitric oxide (iNOS) for metabolism of nitrogen, secrete anti-inflammatory molecules and are more related to tissue repair and humoral immunity (Guirado et al., 2013). Recruitment of blood mononuclear cells and proliferation of

208 tissue-resident macrophages are strategies used to fight the loss of resident cells during
209 the initial phase of an inflammatory reaction (Italiani and Boraschi, 2014).
210
211 Both MAP and *M. bovis* induce an early Th1 response in the host, which later
212 in the disease shifts to a Th2 biased or mixed (Th1/Th2) response (Rhodes et al., 2000b;
213 Stabel, J.R., 2000; Coussens et al., 2004; Welsh et al., 2005). In a comparative study,
214 monocyte derived macrophages (MDM) were infected with either *M. bovis* or MAP,
215 and pan-genomic gene expression was generated using the Affymetrix® GeneChip®
216 Bovine Genome microarray platform (Rue-Albrecht et al., 2014). The timing of highest
217 number of differentially expressed genes (as compared to non-infected control MDM)
218 varied between the two pathogens. The highest number of differentially expressed
219 genes for *M. bovis* was reached at 24 h post-infection (hpi), while MAP reached it at 2
220 hpi (MacHugh et al., 2012; Magee et al., 2012). Proinflammatory genes like IL-1A, IL-
221 1B, TNF, NFKB1, and NFKB2 were up-regulated at one or more time points in both
222 infected MDM. However, *M. bovis* was a much greater inducer of proinflammatory
223 genes. Th2 cells, in contrast, secrete IL-4, IL-5, IL-10 and IL-13, which induce M2
224 cells and activate B cells to produce immunoglobulins (Mills et al., 2000). While in
225 cattle Johne's disease appears to follow this Th1/Th2 switching pattern, another profile
226 is possible in sheep, where both IFN-γ and antibody production increase at the same
227 time (Begg et al., 2011; De Silva et al., 2011; Roussey et al., 2014). It is worth
228 mentioning that antibody production has also been detetced in early infection in cattle
229 (Stabel et al., 2011; Waters et al., 2003, 1999). However, in these studies both memory
230 T and B cell proliferation and production of non-protective IgG1 antibody increased
231 gradually throughout the subclinical stage of disease. Increased antibody levels are
232 probably related to disease progression rather than switch to clinical disease given that

233 animals with clinical Johne's disease show T and B cell unresponsiveness (Roussey et
234 al., 2014; Waters et al., 1999). The role of antibody in BTB and Johne's disease is still
235 unclear (Jacobs et al., 2016; Schiller et al., 2010). In fact, during BTB, increased
236 antimycobacterial IgG1 antibody levels during late disease are generally associated
237 with a lack of cell-mediated immunity, an exacerbation of clinical signs and an increase
238 in bacterial shedding in nasal mucus (Welsh et al., 2005).
239
240 *Mechanisms of immune subversion by mycobacteria*
241 Mycobacteria have the capacity to subvert the killing activity of macrophage by
242 inhibiting phagosome maturation using a number of well-described mechanisms
243 (Brumell and Scidmore, 2007). One of these involves Rab7, which is one of a group of
244 small GTPase proteins that have been implicated in the regulation and maturation of
245 endosomal vesicles. This protein is involved in the recruitment and fusion of lysosomes
246 in the late phagosome compartment (Vanlandingham and Ceresa, 2009). Via et al.
247 (1997) who compared mouse macrophages infected with *M.bovis* BCG (an avirulent
248 strain of *M. bovis* commonly used for human vaccination) with macrophages infected
249 with latex beads, reported that phagosomes containing mycobacteria lacked Rab7.
250 Other studies reported that Rab7 may be recruited during mycobacterial infection, but
251 not activated due to a deactivating factor expressed by the pathogen (Sun et al., 2007).
252 As a result, the protein is unable to recruit RILP, its effector protein. A more recent
253 study using a human monocytic cell line (THP-1) found that both MAP and *M. bovis*
254 interfered with the recruitment of RILP (Keown et al., 2012). In this case, expression
255 of RILP relative to expression of Rab7 was significantly reduced at 48h post-infection
256 with live bacteria compared to phagosomes containing heat-killed bacteria. Regulation
257 of apoptosis is another mechanism employed by mycobacteria to evade the host

response. Virulent strains may initially inhibit apoptosis of infected cells thereby reducing bacterial destruction and antigen presentation, and facilitating initial replication. Later on in the infection, the pathogen induces necrotic cell death, which enables to exit the cell and disseminate to neighbouring cells (Kabara and Coussens, 2012; Lee et al., 2009; Tessema et al., 2001; Whittington et al., 2012).

Mycobacterial survival following phagocytosis may also be enhanced through secretion of IL-10. IL-10 is an immunoregulatory cytokine produced by monocytes, macrophages, Th1, Th2 and T regulatory (Treg) cells. Its chief function is to reduce inflammation and tissue damage by neutralizing the effects of IFN-γ (Sabat et al., 2010). O'Leary et al. (2011) showed that *M. tuberculosis*-infected human macrophages were able to overcome the inhibition of phagosome maturation provoked by the pathogen when IL-10 was blocked, while the addition of IL-10 facilitated mycobacterial survival and growth (O'Leary et al., 2011). Likewise, bovine macrophages produce relatively large amounts of IL-10 when they are incubated with MAP, compared to unstimulated macrophages (Weiss et al., 2005; 2006). It is possible that IL-10 leads to a partial inhibition of acidification of the endocytic compartments as reported in MAP-infected macrophages, *M. tuberculosis*, and *M. bovis* BCG (Kuehnel et al., 2001; O'Leary et al., 2011; Rhodes et al., 2000a; Russell, 2001; Stober et al., 2001; Weiss et al., 2005).

Co-infection of Mycobacterium spp and helminth parasites

As discussed above, protective immune mechanisms against *M. bovis* and *M. tuberculosis* are mainly characterized by a Th1-type immune response, involving antimicrobial activity of M1 macrophages (Cooper and Khader, 2012) while

dysregulation of this response is associated with chronic disease, or reactivation of latent infections (Rue-Albrecht et al., 2014; Schreiber et al., 2009). On the other hand, helminths induce a strong Th2 response, by activation of innate and adaptive mechanisms that contribute to parasite resistance and host tolerance, including attraction of eosinophils that secrete Th2 inducing molecules, and production of IgE. It is now well established that one of the factors that can cause a dysregulation of the response to maycobacterial infection is co-infection with helminth parasites (Salgame et al., 2013).

Specifically, Potian and colleagues (2011) reported a transient increase of *M. tuberculosis* burden in mice co-infected with *Nippostrongylus brasiliensis*, a gastrointestinal helminth and *M. tuberculosis*. These animals also had more IL-4 producing cells in lungs and lymph nodes, and higher levels of alternatively-activated macrophages in the lungs (Potian et al., 2011). Moreover, a sustained Th2 milieu created by re-infection of co-infected mice led to exacerbation of *M. tuberculosis* infection manifested as an increase in the bacterial burden and the size of granulomas in the lungs. *N. brasiliensis*, briefly migrates through the lungs up to 50 hrs before being coughed up and re-swallowed. The authors suggested that the short sojourn of the parasite in the lungs and the tissue lesions caused by its migration induced a Th2 response in the tissues and their associated lymph nodes, which affected the course of the bacterial infection. Elias et al. (2005) showed that *Schistosoma mansoni*, a parasitic trematode, which also has a migration period through the lungs (Wilson et al., 1986), lead to higher *M. bovis* BCG bacterial loads in concurrently infected mice, and impaired proliferative and IFN-γ responses from splenocytes to specific purified protein derivative (PPD) (Elias et al., 2005). More recently co-infection with *S. mansoni* was

shown to impair Th1 responses to *Mycobacterium tuberculosis* resulting an accumulation of M2 macrophages in the lungs, formation of type 2 granulomas, which are formed by arginase-1–expressing macrophages (M2), and exacerbated fibrosis (Monin et al., 2015). The same results were observed when *M. tuberculosis*- infected mice were immunized with *S. mansoni* Egg Antigen (SEA). These authors also reported a correlation between the levels of arginase-1 activity in the serum from humans with active tuberculosis and the lung inflammatory damage as assessed by chest radiographs (Monin et al., 2015). *S. mansoni* effects were also studied by Sacco et al. (2002), showing that liver lesions from mice co-infected with *S. mansoni* and *M. avium* had larger numbers of eosinophils, and serum levels of IgG2 were significantly lower, indicating downregulation of Th1 responses (Sacco et al., 2002). In addition, PPD-specific IFN-γ was completely abrogated in isolated spleen cells. Studies in humans revealed similar findings with respect to immune responses to parasitic infections. For instance, infection with the nematode *Necator americanus* leads to a strong systemic and mucosal Th2 and regulatory response (Gaze et al., 2012). PBMC and intestinal mucosal biopsies from volunteers infected with L3 larvae were stimulated with *N. americanus* Excretory/Secretory proteins (NaES), showing increased production of IL-4, IL-5, IL-10 and IL-13 both at the secreted protein level and RNA transcripts, as compared to uninfected controls (Gaze et al., 2012).

These effects exerted by parasites are likely to have epidemiological consequences. Indeed, several studies have revealed strong association between helminth infections and tuberculosis in humans (Elias et al., 2006), farm animals and wildlife (Claridge et al., 2012; Ezenwa et al., 2010; Jolles et al., 2008).

The specific case of *F.hepatica*

Fasciolosis in livestock results in significant economic losses worldwide (Bloemhoff et al., 2015). The life cycle of the parasite involves intermediate mud snails hosts (Taylor et al., 2016). Animal ingest encysted cercariae form pasture, and juvenile liver flukes excyst in the duodenum, migrating afterwards through the gut wall and peritoneal cavity towards the liver. Once in the liver, they make their way to the bile ducts, where they mature and establish as adult parasites producing massive numbers of eggs,that are shed to the environment. Miracidia hatch from the eggs and penetrate the snail, closing the life cycle of the parasite (Skuce and Zadoks, 2013; Taylor et al., 2016). Liver damage can be variable, from haemorrhage in sheep due to migration through the liver parenchyma of the juveniles, to calcification of the bile ducts and enlargement of the gallbladder in cattle (Taylor et al., 2016). Acute liver disease and mortality are far more common in sheep than cattle. All in all, the infection leads to losses in milk production, reduced weight gain, lower fat content and condemnation of a large proportion of cattle livers at slaughter (Skuce and Zadoks, 2013). Like other helminth parasites, *F. hepatica* induces a Th2/Treg response in the host, with the potential to exert a bystander effect on concurrent infections.

Fasciola hepatica immunomodulatory molecules

There are a variety of multiple immunomodulatory mechanisms employed by the parasite, most of which are not fully understood. Little is known about the local effect of the migrating liver fluke cercariae on the intestinal mucosa. There is evidence of a Th2 response in the *lamina propria*, with raised levels of eosinophils in cattle (McCole et al., 1998), and IgG, IgE and mast cells in rats (Pfister and Meierhofer, 1986; Van Milligen et al., 1999). Nevertheless, we do know that the newly excysted juvenile

(NEJ) excretory/secretory (ES) products contain a cocktail of molecules for tissue degradation, feeding and immunomodulation including cathepsin L3 and L4 proteases, cathepsin B endopeptidases, and the antioxidant molecule peroxiredoxin (Robinson et al., 2008, 2009; Molina-Hernández et al., 2015). Both cathepsins have been found to have a role in digesting host collagen, thus facilitating parasite migration (McGonigle et al., 2008; Molina-Hernández et al., 2015). However, peroxiredoxin drives Th2 responses in the host involving, amongst other things, alternative activation of macrophages (Donnelly et al., 2008, 2005).

Adult flukes producem in addition, a range of immunomodulatory chemicals, which are released as part of the excretory/secretory (ES) fraction.. For instance, cathepsin L proteases were shown to supress *B. pertussis*- specific IFN-γ *in vivo* (O'Neill et al., 2001). In addition, Glutathione- S-transferase (GST), inhibits the proliferation of rat spleen cells in response to Con A stimulation in vitro. *F. hepatica* tegumental Ags (glycocalyx proteins) have been shown to significantly suppress serum levels of IFN-γ and IL-12p70 in a murine model of septic shock, and to impair bone-marrow dendritic cells (DC) function by inhibiting their phagocytic capacity and ability to prime T cells (Cervi et al., 1999; Hamilton et al., 2009). Furthermore, a recent study on *F. hepatica* fatty acid binding protein (FABP) showed that injection of Fh12 (native form of FABP´s) before administration of LPS reduced serum levels of IFN-γ, TNF-α, GM-CSF, IL-12p70, IL-3, and IL-15 (Martin et al., 2015). *In vitro* treatment of macrophages with Fh12 before exposure to LPS suppressed the expression of IL-12, TNF-a, IL-6, and IL-1b cytokines, inducible iNOS2 and their phagocytic ability (Martin et al., 2015). Altogether, these molecules switch the host immune response towards Th2, and have been shown to exert an effect in concurrent bacterial diseases.

F. hepatica effect on concurrent bacterial infection

For instance, cattle co-infected with *F. hepatica* and *Salmonella* died following infection with smaller doses of bacteria, excreted larger numbers of bacteria for a longer period of time, and showed a greater extent of tissue infection than animals that were not infected with liver fluke (Aitken et al., 1978b). The authors suggested that the effects of the helminth infection on intravenously induced salmonellosis was associated with liver damage, because only the chronic phase of *F. hepatica* infection enhanced the clinical signs of salmonellosis (Aitken et al., 1978a). On the other hand, when *S. Dublin* was given orally, susceptibility to salmonellosis was not increased (Hall et al., 1981). During an epidemiological study on dairy farms from the Netherlands, a logistic regression model was developed to assess the relationship between *S. Dublin* and various risk factors. Liver fluke infection was found to be highly associated with *S. Dublin* in the model (Vaessen et al., 1998). In a study on a co-infection with another pathogen, *Bordetella pertussis*, a delayed clearance of the bacteria due to suppression of protective Th1 responses was observed in *F. hepatica* co-infected mice (Brady et al., 1999; O'Neill et al., 2000). The immune polarisation was shown to be dependent on IL-4 production, as *F. hepatica* infection did not suppress IFN-γ or elevate IL-4 production by *B. pertussis*- specific T cells in IL-4 knock out (K.O.) mice. However, higher parasitic doses completely suppressed Th1 cytokines in IL-4 K.O. mice, thus the involvement of immunomodulatory molecules secreted by the parasite was thought to be responsible for this outcome. In later studies, both the Excretory/Secretory (ES) products from the parasite and purified cathepsin L proteinases (FheCL), which are the major components in the ES products, were proven to supress the *B. pertussis* specific

IFN-γ production by a mechanism mediated, at least in part, by IL-4 (O'Neill et al., 2001).

Co-infection of F.hepatica and BTB

During co-infection with *F. hepatica* and BTB, it has been shown that macrophage phenotype differs from that seen in animals infected with *M. bovis* only (Flynn et al., 2007a, 2007b; Laura Garza-Cuartero et al., 2016). Macrophages in co-infected animals are more commonly activated through the alternative pathway (Flynn et al., 2007a, 2007b; Laura Garza-Cuartero et al., 2016). This shift in macrophage phenotype leads to a reduction of microbicidal properties of these cells as phagosome maturation is blocked, facilitating mycobacterial survival and growth (O'Leary et al., 2011). It would be reasonable to expect that these changes result in an increase in the bacterial burden and/or lesion distribution in co-infected animals. Paradoxically, the opposite appears to be the case as co-infected cattle had fewer BTB lesions at slaughter and fewer culture-positive tissue samples than animals that were infected with *M. bovis* alone (Flynn et al., 2009). A more recent study found that the bacterial distribution in the organism was similar in both the *M. bovis* only and the co-infected groups; most bacteria were isolated from bronchial, mediastinal and cervical lymph nodes in both groups, and no quantitative or qualitative differences were observed with respect to tuberculous lesions. In addition, a reduction in the number of culture-positive tissues and total bacterial burden was recorded in the co-infected group (Garza-Cuartero et al., 2016). Collectively, these results indicate the complexity inherent in the effect of helminth immunomodulation on mycobacterial infections, depending on whether or not systemic or local effects predominate.

Failure to detect BTB

The standard method for the detection of BTB is the Single Comparative Intradermal Tuberculin (SCITT) Test, which is based on a delayed hypersensitivity reaction. The SCITT test involves measuring the skin thickness 72 hours after injecting bovine tuberculin intradermally. The other most commonly used test for international trade is the IFN-γ assay, an *in vitro* test which is based on the release of IFN-γ from sensitised lymphocytes during a 16 to 24 hour incubation period of whole blood with a PPD-tuberculin antigen (OIE, 2009). Because both tests detect Th1 cell-mediated immune responses against *M. bovis* antigens, their sensitivity may be affected by immune modulatory effects induced by *F. hepatica*, compromising their efficacy in co-infected animals (Ameni and Medhin, 2000; Flynn et al., 2007b; Claridge et al., 2012). We have previously observed that animals experimentally exposed to both *F. hepatica* and *M. bovis* BCG (an avirulent strain of *M. bovis* commonly used for human vaccination), had lower SCITT and IFN-γ test scores than those that were immunised with BCG only (Flynn et al., 2007b). Similar results were obtained during an experimental co-infection conducted with virulent *M. bovis* bacteria (Claridge et al., 2012).

Although the UK has had an eradication programme for BTB since the 1950s, the incidence of bovine tuberculosis has actually increased in recent years (de la Rua-Domenech et al., 2006; Gibbens, 2011). Claridge et al. (2012), who observed a negative spatial association between exposure to *F. hepatica* and diagnosis of BTB, suggested that one of the reasons for the recent set-back in the UK eradication programme might be continued trade with BTB-infected animals that show false negative results to both the SCITT and IFN-γ tests due to *F. hepatica* suppression of the Th1 immune responses

in co-infected animals (Claridge et al., 2012). In Ireland, a compulsory eradication programme has been in place since 1962 (Good, 2006). Even though considerable progress was made during the early years, the standardized annual herd prevalence in 2010 was still as high as 7.4 %, far from the EU's statutory target of 0.1% for a country to be designated with the official status of being free of BTB, although there has been a slow but steady decrease in prevalence since this study was published (Abernethy et al., 2013; Food and Veterinary Office, 2015). Whether there is a negative spatial correlation in Ireland between *F. hepatica* prevalence and detection of BTB as described for the UK is yet to be determined. Clearly, this potential interaction must be considered on a background of other factors such as transmission of *M. bovis* infection from wildlife to cattle, such as the badger in Ireland and the UK (Gormley and Corner, 2013).

Potential effects of *F. hepatica* infection on Johne's disease

As described above, infection with MAP typically occurs soon after birth. On the other hand, exposure to *F. hepatica* tends to peak every year in late summer and early autumn in animals grazed on pasture. Consequently, cattle on MAP-positive farms are likely to already carry the bacteria in their gut mucosa before they are infected with *F. hepatica*.

Local effect

Ruminants become infected with liver fluke by ingesting mectacercariae encysted on pasture. Once in the small intestine, NEJ migrate through the gut wall, in order to cross the peritoneum and penetrate the liver capsule. As mentioned above, this migration is facilitated by a cocktail of ES chemicals produced by the NEJ (Robinson

et al., 2008, 2009; Molina-Hernández et al., 2015). A Th2 response becomes evident in the *lamina propria*, with raised levels of eosinophils in cattle (McCole et al., 1998). We hypothesize that this local Th2 shift stimulated by *F. hepatica* NEJ is likely to affect the progression of Johne's disease by reducing the microbicidal activity of macrophages further and may result in enhanced bacterial replication in local granulomas. In animals that progress to clinical disease, diffuse lesions are more common than localised ones (González et al., 2005). In addition, macrophages in diffuse lesions show a M2 phenotype, with high expression of CD163, IL-10, and TGF-β, and low iNOS and TNF-α (Fernández et al., 2016; Hostetter et al., 2005). An accelerated formation of diffuse lesions due to alternative activation of macrophages induced by the migrating parasite is likely to occur. This hypothesis has previously been proposed by Rafi et al. (2012) and Potian et al. (2011) for human tuberculosis, claiming that parasitic larval migration through the lung is the primary inducer of a skewed local immune response that enhances the activation of Th2 cells (Potian et al., 2011; Rafi et al., 2012)

Systemic effect

The systemic Th2 milieu stimulated by hepatic stages of *F. hepatica* is likely to suppress the MAP-specific Th1 response. Monocyte contribution to resident macrophages is highly tissue-dependent. In fact, the lamina propria is considered to be one of the tissues with a greater contribution of monocyte-derived macrophages, as compared to the epidermis for example, with a higher proportion of tissue-resident macrophages (Italiani and Boraschi, 2014). As shown by Flynn et al. (2007), animals infected with *F.hepatica*, and animals co-infected with *F.hepatica* and *M. bovis* BCG show greater levels of arginase production in blood monocyte-derived macrophages

without further stimulation, than those only infected with BCG (Flynn et al., 2007b). We propose that recruited monocytes to the site of infection during co-infection of *F. hepatica* and MAP may be less responsive to mycobacterial antigens, probably resulting in increased bacterial burdens and formation of diffuse lesions in the intestinal *lamina propria* and associated lymph nodes, that are associated to clinical disease.

Conclusions

Several studies indicate that regulation of the cell-mediated immune response by *F. hepatica* infection can affect susceptibility and development of concurrent bacterial diseases. Surprisingly, in BTB this is not associated with an increase in bacterial burdens. In spite of the similarities between *M. bovis* and MAP we predict that *F. hepatica* may affect Johne's disease differently because, in this case, a short sojourn of the parasite through the intestine is involved. Thus, early in the infection, the effect of *F. hepatica* is likely to be restricted to the gut and mediated by immunomodulatory molecules excreted by NEJ as they migrate through the intestinal wall. This local effect may lead to a loss of host control on bacterial numbers. A systemic Th2 polarisation would be expected to further increase bacterial burdens in the intestine, producing greater inflammatory infiltrate, thickening of the mucosa and contributing to the formation of diffuse lesions with its characteristic M2 cells. Therefore, we hypothesise that co-infection with *F. hepatica*, may result in accelerated development of Johne's disease, and appearance of associated clinical signs. Experimental co-infections will be required to confirm this hypothesis.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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