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Review Article

The host immune response to gastrointestinal nematode infection in sheep

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SUMMARY

Gastrointestinal nematode infection represents a major threat to the health, welfare and productivity of sheep populations worldwide. Infected lambs have a reduced ability to absorb nutrients from the gastrointestinal tract, resulting in morbidity and occasional mortality. The current chemo-dominant approach to nematode control is considered unsustainable due to the increasing incidence of anthelmintic resistance. In addition there is growing consumer demand for food products from animals not subjected chemical treatment. Future mechanisms of nematode control must rely on alternative, sustainable strategies such as vaccination or selective breeding of resistant animals. Such strategies take advantage of the host's natural immune response to nematodes. The ability to resist gastrointestinal nematode infection is considered to be dependent on the development of a protective acquired immune response; although the precise immune mechanisms involved in initiating this process remain to be fully elucidated. In this paper current knowledge on the innate and acquired host immune response to gastrointestinal nematode infection in sheep and the development of resistance is reviewed.

Keywords: gastrointestinal nematode, sheep, innate immunity, protective antibodies

INTRODUCTION

Gastrointestinal nematode (GIN) parasitism is a major constraint affecting sheep production systems. Naïve lambs are exposed to infection when grazing contaminated pasture. Consequently, infections are generally comprised of a mix of species, which infect both the abomasum and intestine. The species of infective larvae on pasture is dependent on a number of factors including temperature and moisture and therefore often displays a seasonal distribution (1). As GIN are highly aggregated within the host population, susceptible individuals can harbour thousands of worms, which in turn leads to increased pasture contamination. Current sheep production systems are highly dependent on the availability of efficacious anthelmintic products and are threatened by the increasing incidence of anthelmintic resistance. Resistance to all anthelmintic classes has now been reported, with the exception of derquantel, which first came to market in 2010 (2-5). The looming spectre of widespread anthelmintic resistance has led to renewed interest in alternative nematode control strategies such as vaccination, breeding for resistance and immunomodulatory anthelmintics. Many of these strategies exploit the natural host immune response to GIN. The major host defence mechanism against GIN is considered to be acquired immunity (6), which develops over time in response to challenge and is dependent on the age of the animal, nutritional status and genotype (7-9). A current challenge for sheep producers is to allow stock sufficient exposure to GIN in order to develop immunity without impairing production.

MANIFESTATIONS OF IMMUNITY

The development of immunity to GIN is complex and highly variable. The rate of development of immunity depends on the breed of sheep, the nematode species to which they are exposed and the intensity of infection. While lambs rapidly develop the ability to control GIN such as *Nematodirus battus* (10), resistance to other species, such as *Teladorsagia*

circumcincta, is much slower to develop (9). Immune competence can be observed through prevention of establishment of most incoming infective larvae, suppressed GIN growth (and therefore fecundity), the expulsion of adult worms, or a mixture of the above (6, 11, 12). Lambs start to demonstrate immune competence from 2 to 3 months of age (13), with regular exposure to larval challenge allowing the immune response to develop until a significant protective immune capability is developed by 10 to 12 months of age (1, 11). Adult sheep tend to remain relatively resistant to infection, harbouring only a few adult worms, although regular exposure to some level of infection is required to retain immunity (14). An alternative view is that immunity develops in two stages; suppression of worm growth precedes suppression of worm establishment and survival (15). Immunity to intestinal worms also develops more rapidly than immunity to abomasal worms (16).

Nutritional stress, ill-health and pregnancy can all influence an individual's immune status. It has been observed that the nutritional status of the host during GIN infection is important, with the provision of additional protein to growing sheep during infection resulting in enhanced immunity to GIN (17, 18). A relaxation in host immunity to GIN is observed in ewes during the periparturient period, from approximately 2 weeks before lambing to approximately 6 weeks post lambing, although this timing is very variable. It is largely due to nutritional stress in the ewe and can be prevented by supplementary feeding (19). The increase in faecal egg count (FEC) is known as the periparturient rise (20), and is a major contributor to pasture larval contamination encountered by lambs (21).

THE INNATE IMMUNE RESPONSE

The immune system of vertebrates is composed of two arms, the innate (non-specific) immune response and the adaptive (specific) response, the various cellular and biochemical

components of which work together to protect vertebrates from a range of threats. The first line of defence against GIN is the innate immune system, which plays a role in sensing GIN, then initiating and driving the acquired immune response. Of particular relevance are innate physical barriers to the establishment and survival of GIN, and subsequently the process by which the host recognises the presence of GIN and activates an immune response.

Physical barriers to the establishment and survival of GIN

The inner surface of the gastrointestinal tract is covered with a layer of mucus, primarily produced by epithelial goblet cells (22). This is the front line of the innate defence against ingested food and pathogens in the gastrointestinal tract. The primary component of mucus is mucin, however it also contains an array of bioactive molecules such as defensins and trefoil factors (23). Many of these bioactive molecules have been shown to be anti-microbial, or to stimulate inflammation (24). Both increased mucus production and the presence of inhibitory substances in the mucus have consistently been observed during the development of immunity to GIN (25-27).

Enteric smooth muscle contractility has been shown to play an important role in mediating nematode resistance in mice, with changes in intestinal motility reported to be responsible for parasite expulsion (28). However, its role in GIN expulsion in sheep is less clear. An up-regulation of genes related to the structure and function of the enteric smooth muscle was observed in lambs selected for resistance to GIN when compared to their susceptible counterparts (29). Additionally, the concentration of bradykinin, a physiologically active peptide which can promote vasodilation and smooth muscle contraction was negatively correlated with the number of adult *T. circumcincta* worms in immune sheep (30). Contrary to this, however, it has been reported that susceptible Suffolk lambs showed greater duodenal contractile force compared to resistant lambs in response to *T. circumcincta* infection (31).

Pattern recognition receptors (PRRs)

Among the earliest systems for the detection of pathogens are the germline-encoded pattern recognition receptors (PRRs) such as C-type lectin receptors (CLRs) and toll-like receptors (TLRs). CLRs and TLRs are expressed by many cell types, including the cells of mucosal surfaces and tissue immune cells such macrophages and dendritic cells, the major antigen presenting cells (APCs) (32, 33). PRR proteins identify both pathogen-associated molecular patterns (PAMPs; pathogen molecular structures not found in the host), and damage associated molecular patterns (DAMPs; molecules released from damaged or stressed cells). Both PAMPs and DAMPs can result in the initiation and perpetuation of the inflammatory response. As well as being the first line of defence, PRRs play an important role in the induction of cytokines and other signals responsible for the activation and manipulation of the adaptive immune system (34).

While viral, bacterial and fungal ligands which act as potent PAMPs and are recognised by mammalian PRRs are well described, less is known about the role of PRRs in the response to nematode infection. TLR genes (*TLR2*, *TLR4* and *TLR9*) have been found to be more abundantly expressed in the gut mucosa of genetically resistant sheep following GIN challenge (35). CLRs are also candidates for innate recognition of surface carbohydrate present on nematodes The mannose receptor (a CLR) has been shown to bind to excretory/secretory proteins of the mouse nematode *Trichuris muris*, but was not essential for protective immunity (36).

Tissue phagocytic cells such as dendritic cells and macrophages play a critical role in innate immunity, but also help initiate acquired immunity through their ability to sample antigens, migrate to secondary lymphoid tissue and activate antigen-specific T cells within this tissue. M1 (classically activated) macrophages are activated through TLRs and interferon-gamma (IFN-γ), whereas M2 (alternatively activated) macrophages are stimulated by the interleukins (IL) IL-4 or IL- 13. These states are not static however, with ovine M1 and M2

patterns capable of reverting from one to the other according to cytokine availability (37). M2 macrophages have three main functions during helminth infection: regulation of the immune response, healing of damaged tissue, and resistance to parasite invasion (38). During a Th2-type response to nematode infection, M2 macrophages express chitinase and FIZZ family member proteins (ChaFFs), suggesting an effector or wound-repair role for the molecules at the site of nematode infection (39). Chitinases degrade chitin, a molecule present in the exoskeletal elements of some animals, including helminth larvae (40). A joint role for macrophages and neutrophils in preventing establishment of *H. contortus* larvae has also been suggested (41). Macrophage-like cells were also occasionally observed associated with completely destroyed *H. contortus* larvae from sensitized sheep (42).

Cytotoxic and proinflammatory cells

At the site of infection in the gastrointestinal tract mast cells are recruited by the release of chemokines and other inflammatory mediators by innate immune cells. Although best known for their role in the allergic response, increased numbers of tissue mast cells have also been observed during helminth infection. Mast cells are inflammatory cells that can both respond directly to pathogens and send signals to other tissues to modulate both the innate and adaptive immune responses (43). Two subsets of mast cells have been described based on their location: connective tissue mast cells (CTMCs) and mucosal mast cells (MMCs) (44). Mast cells appear uniformly scattered in tissue and activation of mast cells occurs primarily through antigen induced stimulation of the high-affinity immunoglobulin E (IgE) receptor (FcεRIs) expressed at the mast-cell surface (45). Mast cells can also be activated by directly interacting with PAMPs through PRRs (43). Mast cells store a number of inflammatory mediators (including histamine, leukotrienes and proteases) that are released upon degranulation into the surrounding tissues (46, 47). The effects of these chemical mediators are characteristic of type 1 hypersensitivity, and include smooth muscle contraction, increased vascular permeability and local blood flow, and enhanced mucus secretion. In response to

GIN infection, mast cells also produce Th2 cytokines such as IL-13, IL-4 and IL-5 in addition to chemotactic factors which contribute to the recruitment of multiple inflammatory cells including eosinophils, natural killer (NK) cells, and neutrophils (43). In sheep, nematode-induced activation of mast cells has been associated with the acquired immune response (48, 49). An important mechanism controlling the number of adult *T. circumcincta* in previously sensitised animals appears to be IgE-dependent mast cell degranulation (12), with sheep mast cell proteinase systemically released during nematode infections (50).

In addition to an increase in the numbers of mast cells, an increase in eosinophils is also characteristic of infection with nematode parasites. Eosinophils develop in the bone marrow from haematopoietic stem cells (51) in response to the Th2 cytokines IL-3, IL-5, and GM-CSF (52). Following infection, eosinophils proliferate in the blood in a process known as eosinophilia. Mature eosinophils are activated and migrate to the site of infection in response to various chemoattractants, such as IL-5 and members of the eotaxin family of chemokines CCL11, CCL24 and CCL26 (53). In tissue, eosinophils can show directional migration toward a parasite target (54). Following activation, the effector functions of eosinophils include immune regulation, resistance to parasitic invasion through degranulation and the release of eosinophil secondary granule proteins (EPGPs) and healing damaged tissue. The effector functions result in the damage and killing of larval stages of many helminth parasites (42, 55, 56).

Eosinophils have been shown to play a significant role in the development of resistance to multiple species of GIN in sheep (42, 57-59). A reduction in peripheral blood eosinophilia has been observed during primary infection with *T. circumcincta*, which was hypothesised to be a result of recruitment of cells into the intestinal epithelium (60). However, the relationship between peripheral blood eosinophilia and tissue eosinophilia is reasonably

weak, with only a proportion of circulating eosinophils moving into the abomasal mucosa in response to GIN infection (58). Increases in tissue eosinophils have been observed during *Haemonchus contortus* infection of both naïve (61) and previously sensitised (42, 62) sheep, resistant Romney selection line animals with a naturally acquired mixed infection (63) and Suffolk and Texel lambs infected with *T. circumcincta* (64).

THE ADAPTIVE IMMUNE RESPONSE

On encountering a foreign antigen, antigen presenting cells (APCs) such as activated dendritic cells and macrophages migrate to the regional lymph nodes via the afferent lymphatic system where they display the antigens to their cognate T cell receptor via MHC class I or II carrier molecules. The activation of the naïve T cell by APCs initiates the adaptive immune response and results in the release of cytokines, leading to both T cell differentiation and the proliferation of further T cells.

Antigen processing and presentation

Thymus-derived T cells play a central role in the cell-mediated immune response. T cells are differentiated from other lymphocytes by the presence of a T cell receptor (TCR) on the cell surface. There are several types of T cell, including cytotoxic, helper and regulatory T cells. Cytotoxic T cells (Tc) kill cells that are infected with viruses or other intracellular pathogens or damaged cells. They are also known as CD8 T cells as they express the CD8 glycoprotein at their surface. T helper cells (Th) express the surface protein CD4, and provide essential additional signals to activate maturation of B cells, Tc cells, and macrophages. Th cells can be further classified as Th1, Th2 or Th17 cells depending on the cytokines they produce. CD8 and CD4 T cells bind MHC class I and MHC class II molecules respectively. Regulatory T cells (Treg) suppress the activity of other lymphocytes, and are critical for the maintenance of immunological tolerance.

213 *The T cell response*

214 The Th1 response has been traditionally associated with the immune response to intracellular
215 bacteria, protozoa and viruses. The Th1 cascade is triggered by the production of IL-12 by
216 dendritic cells, macrophages and B cells (65), which stimulates the production of the pro-
217 inflammatory cytokine IFN-γ by natural killer (NK) cells (66). IFN-γ is important for
218 differentiation of naive $CD4^+$ T cells into IFN-γ-producing Th1 cells (67). The T-box
219 transcription factor T-bet plays a critical role in this process, accounting for Th1 cell
220 development and the Th1 cell-specific IFN-γ production (68, 69). Both IL-12 and IFN-γ also
221 inhibit the production of the Th2 cytokine IL-4 in mice infected with intestinal nematodes
222 (70). The effector molecules of the Th1 response are specialised to stimulate proliferation of
223 $CD8^+$ Tc cells and activate macrophages and increased expression of these effectors has been
224 associated with GIN susceptibility in sheep in a number of studies (71-73).

225 An antibody-stimulating protective Th2-type response is commonly elicited by
226 helminth parasites. Common features include expression of Th2-type cytokines (IL-4, IL-5
227 and IL-13), infiltration of eosinophils, basophils and mast cells (all of which can produce
228 several types of Th2-type cytokines), and IgE production (74). The presence of IL-4 early in
229 *Trichuris muris* infection has been shown to be critical for the activation of the protective Th2
230 response in mice (75). IL-4, through activation of STAT6, up-regulates GATA3 expression,
231 inducing differentiation of naïve Th cells to Th2 cells while suppressing differentiation into
232 Th1 cells (76). Upon activation, Th2 cells produce additional IL-4 in a positive feedback loop,
233 along with other Th2 cytokines including IL-5, IL-9, IL-13 and IL-25. IL-4 induces class
234 switching in activated B cells, leading to production of IgE (77). The antibody IgE primes the
235 IgE-mediated type 1 hypersensitivity response by binding to Fc (FcεRI and II) receptors on
236 the surface of mast cells and basophils (78). When helminth antigen binds to cell bound IgE it
237 leads to mast cell degranulation, and the release of soluble mediators (74). The sensitivity of
238 target cells to mast cell and basophil-derived mediators is increased by IL-4 and IL-1 3

signalling. In mice it has been shown that together the two cytokines promote increased contractility of smooth muscle cells (79), increased permeability of epithelial cells (80), and elevated goblet-cell hyperplasia during nematode infection (81). The presence of IL-4 in extravascular tissue induces alternative activation of resident tissue macrophages, which function in wound healing and tissue repair. IL-5, aside from triggering eosinophilia, enhances secretion of IgA by B cells (82). The Th2 cytokine IL-1 3 induces epithelial cell repair and mucus production, and together with IL-9 recruits and activates mucosal mast cells. In sheep, the timely induction of a Th2 response to GIN infection, characterised by mast cell hyperplasia, eosinophilia, recruitment of IgA/IgE producing cells and the expression of Th2 cytokines, is considered to promote the development of resistance (83, 84).

The roles of the more recently discovered Th1 7 and Treg cells in the ovine response to GIN remains to be elucidated. Th17 cells promote inflammation through the recruitment of neutrophils and macrophages to the site of infection. Early in infection IL-6, produced by dendritic cells, acts with TGF-β (also required for the differentiation of regulatory T cells) to produce the Th17 response. This results in the production of IL-17 family members and IL-21, a subset of cytokines particularly important in clearing pathogens during host defence responses and in inducing tissue inflammation in autoimmune disease (85). Later, dendritic cells along with other antigen-presenting cells produce cytokines to promote either Th1 or Th2 development, and suppress Th17 development. Increased expression of Th17-associated genes has been associated with both susceptibility (86) and resistance (87) to GIN in sheep depending on the experimental model. Treg cells are a subpopulation of T cells that are involved in the maintenance of immunological self-tolerance and homeostasis through immune suppression (88). Expression of the forkhead transcription factor FOXP3 is critical for the development and function of Treg cells (89). Treg ($CD4^+CD25^+Foxp3^+$) cells are an important "self-check" in the immune system, and have been shown to be activated and expanded during helminth infection in mice (90-92). A faster switch from a Th1 to a

Th2/Treg response was found in resistant Suffolk lambs compared to susceptible lambs (93).

The human T cell response may be more functionally diverse than previously thought. Pathogen stimulation of naïve T cells may give rise to multiple T cell subtypes, suggesting that Th cell polarisation could be the results of preferential expansion of particular clones rather than preferential priming (94). The implication of this for sheep Th cell polarisation remains to be determined.

Antibody response

The principal function of B cells is to make antibodies (immunoglobulins) against antigens. The binding of an antigen to a naïve B cell, coupled with the accessory signals from Th cells, stimulates the lymphocyte to proliferate and differentiate into plasma cells, which secrete large amounts of antibodies. A number of antibodies isotypes have been shown to be correlated with GIN resistance in sheep, including IgA, IgG1 and IgE. IgA, which has both circulating and secretory isoforms, is the isotype most closely associated with intestinal mucosal immune responses. Increased levels of IgA have been positively associated with resistance to *T. circumcincta*, regulating both worm length and fecundity (95-98). This resistance is regulated through suppressed parasite growth, development and fecundity, and is mediated by IgA activity against 4th-stage larvae. In Scottish Blackface lambs the presence of arrested L4 larvae has been shown to be positively associated with both worm burden and the size of the local IgA immune response (12). Elevated levels of both IgA and IgG1 were observed in *T. colubriformis*-challenged sheep (99).

Increased levels of IgG1 and IgE have also been negatively correlated with FEC in Romney selection line sheep in New Zealand (100-102), although IgE was positively correlated with breech soiling (102). IgE mediates mast cell, eosinophil and basophil

degranulation in response to GIN and elevation of total and/or parasite-specific IgE serum antibodies have been reported during infection with *H. contortus* (103), *T. colubriformis* (104) and *T. circumcincta* (105, 106). In addition, an association between a polymorphism at the 5' end of the sheep IgE gene and resistance to *T. colubriformis* has been reported, although attempts to confirm this finding in other flocks failed (107).

A significant number of activated antigen-specific B cells and T cells persist after an antigen has been eliminated, and these are known as memory cells. These cells form the basis of immunological memory and can be reactivated much more quickly than naïve lymphocytes, and usually provide lasting protective immunity.

DEVELOPMENT OF RESISTANCE TO GIN IN SHEEP

Studies comparing naïve and previously infected animals have shown that development of immunity to GIN is associated with a predominantly Th2 response, characterised by an increase in Th2 cytokines, recruitment of eosinophils, mast cells and globule leucocytes, and increased production of parasite-specific IgA, IgG1 and IgE (108-110). However, there is conflicting evidence on whether a Th2 response can be used to select resistant or susceptible animals. While an increase in inflammatory cells and parasite-specific IgA were generally inversely associated with *H. contortus* worm burden and FEC in three breeds of sheep, mean values were not found to differ between the resistant (Santa Ines) and susceptible (Suffolk and Ile de France) breeds (111). This is in contrast to a study comparing genetically resistant sheep with random-bred Merino lambs, which found resistant lambs had increased *IL-5* expression, increased IgG1 and IgE antibody production, and higher densities of mucosal mast cells and eosinophils in response to *H. contortus* infection (71). During repeated experimental infections with *T. colubriformis*, genetically resistant sheep were also able to respond earlier than susceptible animals with nematode-specific IgA and IgG2 (112).

Resistant Barbados Black Belly lambs have also been shown to develop a more rapid Th2-type response than the susceptible INRA 401 lambs after a primary infection with *H. contortus* (113). A differential interplay between Th1/Th2 and Treg genes has also been proposed to modulate the immune response to GIN rather than a straightforward Th1 or Th2 pathway (93) and failure to observe consistent gene expression profiles between resistant and susceptible animals could be due to variation in response time between studies. Additionally, multiple studies have suggested that the mechanisms of resistance may vary between animals with different genetic backgrounds, and may be parasite-specific (111, 114).

CONCLUSION

The host-parasite interaction is a complex relationship which determines the outcome of infection. Sheep GIN display a variety of surface and secretory/excretory antigens which can be stage-specific. Such molecules trigger the host's immune response generally resulting in the development of a protective immune response, although the level of immunity is dependent on age, nutritional status and genotype. Increased mucus and bioactive molecule production, activation of mast cells, eosinophilia, polarisation of the immune response to a Th2 response and the production of anti-nematode antibodies are all associated with the development of immunity. A protective immune response can be considered an expression of resistance and a detailed understanding of the genes and biological mechanisms involved in protective immunity will aid the development of non-chemical effective and sustainable nematode control methods. Understanding the genetic and molecular basis of disease resistance also has many advantages and applications such as the development of novel genetic markers for inclusion in genetic improvement programmes.

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