Characterizing physiological and genetic differences in the early immune response to *Haemonchus contortus* in resistant and susceptible sheep

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Abstract

This dissertation compares immune responses of resistant and susceptible sheep to infection with *Haemonchus contortus* during the peri-parturient period and larval stage of infection. Identification of immunological events resulting in parasite resistance in St. Croix hair sheep may provide better targets for differential gene expression analysis and eventual discovery of selectable markers for parasite resistance. Antibody levels of hair ewes and composite Dorset x Finnsheep-Rambouillet wool ewes were measured during breeding and again after parturition. Results demonstrated that hair ewes had higher levels immunoglobulin-A after infection and maintained a higher level of circulating antigen-specific antibody when compared to wool ewes. To characterize immune responses to the larval stage of infection, hair and wool lambs were sacrificed at 0, 3, 5, and 7 d after infection with *H. contortus*. Neutrophil migration to abomasal mucosa and lymph node development were higher in hair sheep than in wool sheep. Gene expression analysis indicated no difference in the abomasal lymph node as both breeds expressed a general T-helper cell type 2 (T_H2) response. However, profound differences in T_H2 responses were observed in the abomasal mucosa, where hair sheep expressed more IL-4, -13 and -33 than wool sheep. These data thus document the presence of immunological differences between the breeds. Immune responses to larval parasite infection in wool sheep are generally suppressed and may increase the magnitude and duration of infection whereas immune responses to larval infection in hair sheep was more robust and more strongly polarized towards a T_H2 response.

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Introduction

Resistance to anthelmintics has caused a shift in the methods of small ruminant production worldwide. Instead of relying on drugs to treat gastrointestinal nematode (GIN) parasites, producers have had to re-evaluate potential solutions to this parasite problem. Treatment strategies have been employed that improve the efficacy of some anthelmintics and prolong the building of resistance within the worm population. Modified grazing plans have been implemented to reduce exposure of sheep to harmful parasites (Whittier et al., 2003). In some cases, selective breeding has shown promise in combating GIN parasitism (Vanimisetti et al., 2004a). However, the best tool seems to be the integrated use of all the practices listed above to effectively manage parasitism in sheep.

The identification of genetic markers that are indicative of parasite resistance could enhance the effectiveness of selection to enhance parasite resistance. To date, however, no specific functional markers have been consistently and empirically associated with parasite resistance in sheep. Quantitative trait loci (QTL) data document the involvement of immune function genes and microarray analysis indicated differential expression of these genes in parasite-resistant sheep (MacKinnon et al., 2009).

Differential expression of immune genes in parasite-resistant sheep leads to a reduced level of infection or expulsion of the parasite. The nature of the immune response to GIN does not appear to differ between resistant and susceptible sheep, but differences in levels of production of various immune effectors in response to the parasite have been reported. However, previous studies have focused largely on the immune responses to the adult parasite

infection as opposed to the larval parasite. This difference is important, as the immune response to the larval infection promotes the differentiation of immunological phenotypes towards one which aids in the removal of the infection (Gause et al., 2003).

This review will not focus on the immunological response to parasites in murine models, as this model is not an absolute reflection of the immune response to *H. contortus* in sheep. However, immunological pathways discovered using mice will be discussed. Studies from murine models of infection have demonstrated that innate immune mechanisms play a larger role than previously thought (Anthony et al., 2007). Innate immunity is responsible for recognition of foreign invaders and for directing the development of the appropriate immune phenotype (Kreider et al., 2007). The infiltration of cells such as neutrophils and macrophages during the pre-patent period has a clear impact on the intensity and duration of GIN infection (Anthony et al., 2006). Recent findings in sheep have also alluded to the importance of certain aspects of the innate immune response in differentiating resistant and susceptible sheep (de Veer et al., 2007; Ingham et al., 2008).

The immune response to the larval parasite and its role in the development of a more rapid and robust immune response to the patent infection is an important component in parasite resistance. This study focuses on the immune response of St. Croix sheep (resistant) compared to crossbred wool sheep (susceptible) to the GIN *Haemonchus contortus*, during the first 7 days of infection. In addition, the expression levels of candidate genes involved in T-helper cell type 2 (T_H2) inflammation including, cytokines, chemokines and transcription factors were measured in the abomasal mucosa and lymph nodes.

The goal of this dissertation was to characterize the early cellular response to *H. contortus* and specifically to determine whether resistant sheep develop a T_H2 response more quickly and with higher expression levels of soluble mediators that aid in the recruitment of innate cells to the site of infection and polarize immune phenotypes in the local lymph nodes.

Chapter 1 **: Literature Review**

Gastrointestinal nematode parasitism in sheep

Anthelmintic resistance

Gastro-intestinal nematode parasitism has long been considered to be a manageable problem for sheep producers. Parasitism has been managed with chemo-therapeutics thus producers have not needed other control strategies. However, dependence on anthelmintics has lead to the rapid and pervasive development of anthelmintic-resistant parasites. Anthelmintic resistance has now reached worldwide status occurring in the US (Miller and Barras, 1994), the UK (Bishop and Stear, 2001), Europe (Schallig, 2000), Africa (Matika et al., 2003) and Australia (Overend et al., 1994). There are only three anthelmintic drug classes available to treat GINs, the imidazothiazoles, benzimidazoles and macrolides (Bowman, 2003). Resistance has developed in parasite populations to each of these three drug classes (Howell et al., 2008). Although research and dosage trials are currently being conducted on a new class of dewormer (Kaminsky et al., 2009), it is difficult to say when that particular anthelmintic will be available in the US. In order to maintain drug efficacy and reduce the impact of parasitism on production, producers have implemented management strategies that incorporate selective deworming, pasture management, and selection of resistant individuals (Eady et al., 2003).

Trichostrongylidae parasites

Sheep are affected by a multitude of parasites ranging from external lice, ticks and mange to internal nematodes and intracellular parasites. The greatest concern to most sheep producers

are the GIN in the family Trichostrongylidae. These include but are not limited to *Haemonchus contortus, Trichostrongylus columbriformis* and *Teladorsagia circumcinta.* These three species cause the vast majority of parasite problems in the US and abroad. Formerly referred to as the HOT complex, these parasites are responsible for weight loss, anemia, reduced performance, and, if left untreated, death of the host. These genera not only affect sheep but also infect cattle, deer, goats and other grazing species (Bowman, 2003).

Haemonchus contortus and *T. circumcinta* are parasites that affect the abomasum or "true stomach" of the ruminant host. Although both reside at the same location, they have quite different effects on the host. *Teladorsagia*, formerly known as *Ostertagia*, causes a disruption in the gastric pits of the abomasum, reducing the animals' ability to convert pepsin to pepsinogen, and thereby raising the pH in the abomasum and decreasing the hosts' ability to properly digest feedstuffs(Bowman, 2003). The resulting symptoms of *Teladorsagia* infection include weight loss, diarrhea, and anemia due to malnutrition. This parasite has its highest economic impact on growing lambs. The efficient utilization of feed and subsequent time on feed, prior to slaughter, can affect the profitability of lamb production. Moreover *Teladorsagia*, in the adult ewe, can prevent maintenance of proper body condition causing difficulty in breeding, metabolic problems around parturition and a general lack the ability to efficiently utilize forage resources.

Haemonchus contortus is one of the most problematic worms worldwide from both pathologic and economic standpoints (Miller et al., 1998). This worm feeds on blood and its pathologic effects are more direct than those of other species. A single adult female is capable of removing 0.05 ml of blood and producing approximately 10,000 eggs each day (Bowman, 2003). Host exsanguination causes an anemic and hypoproteinemic condition. The animal can quickly lose body weight and, if left untreated, die from infection (Miller et al., 1998). This effect of

exsanguination is amplified in the youngest and oldest individuals, as they are less able to mount an effective immune response to the parasite. The economic losses resulting from this parasite are two-fold involving the loss of productive ewes (equity) and the loss of lambs or lambs with reduced growth rate, yielding more days on feed and loss of potential receipts.

The effects of *T. colubriformis* are commonly seen in countries where sheep production is the dominant livestock enterprise. Countries such as New Zealand, Australia and the UK struggle with this parasite. However, this parasite is not limited to areas of high sheep production and, is also found in other locations worldwide. This species, unlike those of the previous genera, resides in the small intestine and disrupts the ability of the small intestine to absorb nutrients. The signs associated with this parasite include rapid weight loss, inability to efficiently gain weight, diarrhea and death. Most research on parasite resistance, from the southern hemisphere, has focused largely on *Trichostrongylus* due to its effect on lamb survival and growth (Kemper et al., 2009). Time on feed is one of the largest costs of livestock production, and the resulting cost to get an animal to its logical slaughter endpoint is of great concern to producers. Managing this parasite is critical for efficient production of lamb.

Life cycle of *H. contortus*

The life cycle of *H. contortus* is shared with the two previously discussed species and infection with *H. contortus* has three distinguishable stages: pre-patent period, patent period and hypobiosis (Bowman, 2003). Eggs shed by the host onto pasture via fecal matter will hatch and go through two larval molts, arriving at larval stage 3 (Balic et al., 2006) within 7 days. The L_3 larvae are the infective stage and are consumed by the grazing host. Larvae have specific environmental requirements critical to survival. A warm and humid environment with

temperatures not exceeding 50°C (Zajac, 2006) will aid larval survival. L_3 larvae are sensitive to desiccation and, in order to ensure survival during varying relative humidity, are protected by a sheath that aids in maintaining osmotic pressure. The pre-patent period occurs within the animal, lasts from 2 to 3 wk and is the period when larvae are developing prior to becoming adults. Once consumed, L_3 larvae move with the digesta through the first three compartments of the ruminant stomach, reaching their final tissue destination in the abomasum and beginning to feed on blood within 7 d or less (Colditz and Le Jambre, 2008). The L_3 larvae molt to the L_4 stage in the abomasum, and shortly after become adults capable of reproduction. The appearance of the adult worms marks the end of the pre-patent period and the start of the patent period. The patent period is denoted as when adults begin to reproduce (Whittier et al., 2003).

During the patent period, effects of parasitism are visible in the host. In the case of *H. contortus* infection, signs include anemia, weight loss, hypoproteinemia, and death (Zajac, 2006). The adult parasites feed on blood by using a lancet, found at the end of the mouth. This lancet is used to pierce the abomasal mucosa and cause bleeding from the host. All of the blood lost from the host is not consumed by the parasite; residual blood mixes with the contents of the abomasum, resulting in a bloody stool. During this period, male and female *H. contortus* mate. The female is capable of producing up to 10,000 eggs per day (Bowman, 2003). Eggs from the adult worms must pass though the digestive tract, develop on pastures and be ingested by another or the same host animal, before they can become pathogenic.

Haemonchus, Trichostrongylus and *Teladorsagia* have developed an incredible adaptive mechanism, referred to as hypobiosis, to survive during harsh environmental conditions (Bowman, 2003). Hypobiosis is a developmental stage when the parasite becomes dormant, does not cause disease, and is not metabolically active. At the L_4 stage, larvae may be cued to

initiate hypobiosis and remain in the abomasum. The larvae will reside at this location until environmental conditions are appropriate for emergence and reproduction. The cues that initiate and terminate hypobiosis are largely unknown, due to the inability to simulate hypobiosis in vitro. Relaxation of immunity in late gestation or suppression of immunity by reproductive endocrine activity are theorized to play a role in emergence from hypobiosis (Courtney et al., 1985). Hypobiosis is a driving element in the condition known as spring rise. Sheep reproduction is seasonal, ewes typically breed in fall and lamb in spring. During winter months, most L_3 larvae on pasture will die and adult worms inside of the host will senesce, leaving the only surviving parasites as hypobiotic L_4 larvae. Typically peri-parturient ewes will begin to produce eggs in feces, even if they were not shedding prior to this time. About 30 d after introduction to pasture, the ewes, and more importantly lambs will begin to have high fecal egg output as a result of hypobiotic larvae emerging from the ewes and acting as a source of infection for the lambs (Sargison et al., 2007). Hypobiotic larvae may be treated using macrolide anthelmintics, but this is the only class anthelmintics that is effective against this type of larvae (Bowman, 2003). The emergence of anthelmintic resistance in worm populations reduces the level of control of hypobiotic larvae that was once available.

Knowledge of the lifecycle, environmental conditions, and species of parasite provides producers with a number of tools to manage parasite infection in their lambs and ewes. In efforts to aid the efficacy of these control strategies, many producers in the southeastern US have begun using breeds of sheep that have an innate ability to resist GIN parasitism (Miller et al., 1998).

Genetics of Parasite Resistance

Breeding for parasite resistance depends on the identification of highly resistant individuals. A convenient measurement of parasite resistance to *H. contortus*, and other strongylid parasites, is the fecal egg output. To quantify fecal egg output, a McMaster's fecal egg count (FEC) is performed that measures the number of eggs per gram of feces (Whitlock, 1948). Fecal egg output is an indirect measurement of actual infection, but indicates relative levels of infection (Gray, 1997). A measurement that is more specific to *H. contortus* is the hematocrit or packed cell volume (PCV) which provides a measurement of anemia. Selection criteria to improve parasite resistance have commonly been based on one or both of these two measures.

Breeding for parasite resistance

Selection for parasite resistance has been conducted for many years in Australia, through selection for low FEC in response to both natural and artificial infections (Kemper et al., 2009). Selection based on FEC has improved parasite resistance, but within-breed improvement requires many generations. If improvement in parasite resistance can be made by selection for low FEC, then parasite resistance is a genetically mediated trait. Selection has also been attempted based on PCV, but has been less effective than selection based on FEC. Genetic selection strategies discussed in this section will only be based on selection for low FEC in lambs.

Prediction of the potential response to selection requires knowledge of genetic parameters such as heritabilities and genetic correlations. Heritability is defined as the proportion of phenotypic variation within a population that is attributable to genetic variation among individuals (Falconer and Mackay, 1996). The square of the genetic correlation is the proportion

of variance that two traits share due to common genetic factors. Research has focused on FEC and its relationship to growth to determine whether selection for parasite resistance will have a positive or negative an impact on growth rate.

Heritability of FEC, or parasite resistance, has been calculated for different breeds of sheep. In Polish long-wool sheep, the heritability of parasite resistance varied between 0.20 and 0.33 depending on the time of year (Bouix et al., 1998) with a favorable negative genetic correlation (-0.61) between FEC and average daily gain. Selection for growth in an infected environment would be expected to indirectly also improve parasite resistance of lambs (Bouix et al., 1998). In France, researchers evaluated differences in parasite resistance of Rhön and Merinoland sheep (Gauly et al., 2002). Rhön sheep had a higher heritability (0.35) of FEC than Merinoland (0.17) (Gauly and Erhardt, 2002). These studies and others note that animals that have lower FEC early in the grazing season also have lower FECs later in the grazing season (Bouix et al., 1998; Gauly and Erhardt, 2002; Gauly et al., 2002).

In the UK, Scottish Blackface sheep were used to estimate heritability of parasite resistance. Heritability estimates varied with the age of the lambs, slowly increasing from an average of 0.01 at 1 month to 0.22 at six months (Bishop et al., 1996). Heritability determined from mean FEC from month 3 to month 6 was 0.33, indicating a moderate level of heritability. The genetic correlation between FEC and live weight in lambs older than 3 months of age was close to –1.0 (Bishop et al., 1996), which is consistent with results in the Polish longwool sheep.

In Australia, the estimated heritability of FEC in Merino sheep divergently selected for parasite resistance was 0.29 across sexes, years and infection levels (Woolaston and Piper, 1996). Heritability of FEC on Merino sheep selected for parasite resistance since 1988 was 0.24

and the genetic correlation between FEC and growth was virtually null (Kemper et al., 2009). These data indicate that selection for parasite resistance should not impact expression of other economically important traits.

In the US, heritability estimates for FEC are very consistent with those from other countries. In a group of crossbred sheep not selected for parasite resistance, heritability for FEC was 0.31 at the peak of infection (Vanimisetti et al., 2004a). Genetic correlation data from this study indicated that lambs with high genetic merit for body weight had a higher level of parasite resistance.

Heritability estimates for FEC in lambs ranging from 0.20 to 0.35 can be considered moderate. Meaningful genetic improvement will therefore require multiple generations of selection for low FEC. However, there are sources of concentrated genetics for parasite resistance, located in breeds that have developed in equatorial climates. The incorporation of those breeds into a program selecting for parasite resistance would have promise. For example, the heritability of FEC in Suffolk x Gulf Coast Native crossbred lambs was 0.22, but heritability in their purebred Suffolk ancestors was only 0.12 (Miller et al., 2006).

Breeds with natural resistance to *H. contortus*

Breeds of sheep originating from humid, tropical climates are hypothesized to have evolved parasite resistance in order to survive under constant exposure to *H. contortus*. In the US, the Florida Native has demonstrated higher weight gains and lower establishment of adult parasites when compared to Rambouillet sheep (Bradley et al., 1973). The Gulf Coast Native, another breed indigenous to the US, also had lower FEC and higher PCV when naturally infected with *H. contortus* compared to Suffolk sheep(Miller et al., 1998). Many studies have been

conducted using these breeds; however breeds originating from the Caribbean also provide a useful model for studying natural parasite resistance.

The Barbados Blackbelly (Yazwinski et al., 1979), St. Croix (Courtney et al., 1985) and Katahdin (Burke and Miller, 2002) breeds have been noted for their resistance to GIN. When compared to the Dorset (Yazwinski et al., 1979) or Rambouillet-Dorset crossbred (Zajac et al., 1990), Barbados Blackbelly sheep had consistently lower FEC and higher PCV. St. Croix sheep have shown similar results to those reported for Barbados Blackbelly and therefore these Caribbean hair-type sheep are superior in parasite resistance to domesticated sheep originating from Europe or the UK (Vanimisetti et al., 2004b; Zajac et al., 1990). Concerns over use of Caribbean hair sheep in cross-breeding programs arises, however, due to their small mature size, slow growth rate and lower carcass weights (Wildeus, 1997).

In the late 1950s, a commercial sheep breeder in Northern Maine created a breed using Caribbean hair sheep and British wooled sheep (Parker et al., 1991). After years of breeding for a similar phenotype, the resulting breed was named Katahdin. The intent behind the creation of the Katahdin breed was to create a meat animal that did not require shearing, and the Katahdin has recently gained favor in the Southeastern US as a low-input maternal ewe breed (Burke and Miller, 2002). The mature size of the Katahdin ewe is significantly larger than that of Caribbean breeds, and Katahdin lambs typically have better carcass merit (Vanimisetti et al., 2004b; Wildeus, 1997). In practical lamb production scenarios, Katahdin ewes would be mated to terminal sires to produce offspring that can both survive on infected pastures and maintain carcass merit. The Katahdin breed is a very functional ewe breed as they have complementarity with black-faced terminal sires and are nearly as parasite resistant as Caribbean breeds (Burke and Miller, 2002; Vanimisetti et al., 2004b).

Breeding for parasite resistance can bring about changes within a flock in a relative short period of time, especially if breeds like the Katahdin or St. Croix are introduced into the flock. However, crossbreeding is not applicable in purebred flocks, and purebred producers must rely on the identification of parasite-resistant individuals within their breed. Identification of parasite resistant sheep is a difficult task that requires multiple FEC to reliably confirm reduced infection levels through seasons of high and low parasite infectivity. Over the past decade, there has been a trend in livestock selection to focus on the identification of genetic markers and implementation of genetic marker assisted selection (GMAS). This principle of GMAS relies on identification of variable alleles in regions of DNA that are specifically associated with the desired phenotype. The use of GMAS to identify parasite-resistant individuals would be very useful and may eliminate the need for labor-intensive FEC determinations.

Genetic markers for parasite resistance

Resistance to parasites is a highly variable quantitative trait which researchers lack the ability to divide into discrete genetic units and therefore has been described statistically via heritability (Beh and Maddox, 1996). The additive effect of many genes is responsible for the phenotype of quantitative traits (Falconer and Mackay, 1996), but the question is; what genes control parasite resistance? The resulting parasite resistant phenotype could be due to small effects of many genes or major genes whose effects may be obfuscated by additive genetic variation and environmental effects (Beh and Maddox, 1996).

QTLs of parasite resistance

Fecal egg counts and hematological measurements have proven to be fairly accurate at identifying parasite-resistant individuals and there is clearly variation among breeds for parasite

resistance. The first attempts at genetic marker identification used quantitative trait loci (QTL) analysis to locate markers for parasite resistance (Charon, 2004). Most of such studies have yielded similar results suggesting that QTL for resistance to strongyle-type parasites are located on chromosome-3 near the interferon-gamma (IFNγ) gene (Beh et al., 2002; Coltman et al., 2001; Davies et al., 2006; Gasbarre et al., 2001), and on chromosome-20 near the major histocompatibility complex (MHC) (Charon, 2004; Davies et al., 2006; Paterson, 1998; Schwaiger et al., 1995). The cumulative effect of these QTLs has 98% reduction in FEC among resistant genotypes in some studies (Schwaiger et al., 1995) , but has had no significant effect on FEC reduction in others (Marshall et al., 2009). These QTL studies have consistently indicated that the regions of the genome associated with parasite resistance are typically also involved in immune responses.

Microarray technology used to discover parasite resistant genes

New technology has allowed researchers to evaluate what genes are differentially expressed during infection. These microarray assays have typically been conducted on bovine cDNA microchips as no ovine cDNA microchips were available. The first published report using this technology stated that over 100 genes were differentially expressed in susceptible and resistant sheep. Two pathways that were in particular represented by differentially expressed genes were involved in development of the acquired immune response and related to the structure of intestinal smooth muscle (Diez-Tascon et al., 2005). Many up-regulated genes were associated with MHC class II components, which are a critical component in antigen presentation. Initial QTL and microarray analyses are indicating that immunity-related genes are involved in parasite resistance.

A later microarray study attempted to reduce variation in gene up- or down-regulation by evaluating naïve sheep that were also genetically resistant to parasites. In this particular scenario, it was expected that different genes were expected to be up-regulated since these individuals had not been exposed to parasites and had not developed immune memory response (Keane et al., 2006). This study was the first to use an ovine cDNA microarray. The authors indicate that intestines of resistant sheep do not appear to become stressed due to parasitic infection, whereas the intestine of susceptible sheep expressed significantly more stress genes (Keane et al., 2006). The gene ontology (GO) terms associated with resistance were cellular processes, whereas the GO term associated with susceptibility was response to stress. However both of these studies are limited because responses were not assessed until 120 d (Diez-Tascon et al., 2005) and 84 d after initial infection (Keane et al., 2006).

Two reports compared infection at 3 versus 27 d (MacKinnon et al., 2009) or from 1to 45d after infection (Rowe et al., 2009). MacKinnon et al. (2009) reported increased expression of genes related to neutrophil cell markers, migration of immune cells, blood flow to site of infection and expression of IL-4Rα and IL-12Rβ1 at 3 d after infection (MacKinnon et al., 2009). By 27 d, the profile of expressed genes in the resistant sheep had shifted from inflammatory or innate genes to genes responsible for protective responses including increased expression of IL-4 and decreased expression of IFNγR. There was also an increase in the expression of smooth muscle genes in the resistant sheep suggesting that those individuals may have been attempting to physically expel the parasite. Rowe and colleagues reported that intelectin 2 was up-regulated over all time points, and has a variety of functions ranging from effects on mucus viscosity (Arranz-Plaza et al., 2002) to a protective anti-bacterial role (Tsugi et al., 2001) and phagocyte activation (Abe et al., 1999). Other differentially expressed genes shifted in expression from

being associated with innate immune responses at 3 to 10 d after infection to a later expression profile consistent with the protective effects of an acquired immune response. Immunoglobulin genes dominated the expression profile on 22 and 45 d after infection supporting development of an acquired response (Rowe et al., 2009). Although these two reports used sheep that have natural parasite resistance (MacKinnon et al., 2009) and sheep that are susceptible to parasites (Rowe et al., 2009), both authors reported consistent results in that the substantial numbers of genes related to the immune response were activated during both the pre-patent and patent periods of parasite infection.

Both QTL and microarray studies provide clear and consistent evidence about the type of genes that are involved in parasite resistance. However, evidence for a small number of major genes associated with these responses has not been reported. Thus a better understanding of genetic differences among various sheep types will benefit from enhanced understanding of differences in immune function in order to assist in identification of logical pathways and potential candidate genes.

Immunology of Parasite Resistance

Characterizing the immune response to adult parasites

The immune response to nematode parasitism in the sheep is best characterized in two stages: the immune response to infective larvae and the immune response to adult parasites. A tremendous amount of literature exists on immune responses to infection with *H. contortus* (Adams, 1982; Fox, 1997; Gamble and Zajac, 1992; Mansfield and Gamble, 1995; Meeusen et al., 2005; Miller and Horohov, 2006; Pernthaner et al., 2006; Pfeffer et al., 1996; Rowe et al.,

2009; Schallig, 2000). In all cases, the immune response to the adult parasite is best described as a T-helper cell type 2 (T_H2) response (Lacroux et al., 2006; Shakya et al., 2009) with high levels of immunoglobulins-A(IgA), E (IgE) and G (IgG) (Pernthaner et al., 2006) and of mastocytosis and eosinophilia at the site of infection (Balic et al., 2006; Henderson and Stear, 2006; Mansfield and Gamble, 1995). Moreover there is clear evidence that both naturally resistant breeds and sheep selected for parasite resistance do not have an innately different type of immune response when compared to susceptible breeds (Bowdridge et al., 2008). The difference between resistant and non-resistant sheep is in the magnitude of their immune responses and in the time required for them to mount an effective immune response (Bowdridge et al., 2008). Sheep that were immune to *H. contortus* infection but subsequently were prevented from developing an acquired immune response were still able to suppress larval establishment (Adams, 1982). In that study, the production of effector cells associated with the acquired immune response were blocked, thereby, demonstrated the importance of innate immunity in parasite expulsion. The generally more robust immune response to *H. contortus* in resistant breeds is thought to limit larval establishment, as previously infected sheep typically have fewer established larvae in the abomasum (Kemp et al., 2009). Other studies report a correlation between host lymphocyte counts and parasite fecundity, further supporting the role of T_H2 immune responses (Rowe et al., 2008). Magnitude and timing of immune response seem to be the only immunological differences seen between the resistant and susceptible phenotypes during the patent infection. To detect genetic mechanisms underlying parasite resistance, studies should perhaps shift emphasis to analysis of the immune response to the larval parasite.

The immune response to larval parasites

Specific antigens are present in L_3 (Raleigh and Meeusen, 1996) and L_4 (Balic et al., 2003) larvae that are not present in adults, suggesting that each stage may be considered as a distinct antigenic organism by the host's immune system (Meeusen et al., 2005). To accurately characterize the pre-patent immune response, the next three sections will review cellular responses, the role of cytokines and antibody production. In most of these experiments, sheep were previously exposed to the parasite in question and observed immune responses cannot be considered those of a naïve host.

Cellular immune response during larval infection

After infection with *H. contortus* larvae migrate to the abomasum. By day 4 after infection the L_4 larvae will be feeding on blood (Colditz and Le Jambre, 2008) and should elicit a local immune response. This response will occur at the site of infection and in local lymph nodes, where T-cells and B-cells become antigen specific and B-cells are cloned forming antibody-producing plasma cells (Elgert, 1996). Studies of the primary immune response to *H. contortus* indicate that the percentage of $CD4^+$ T-cells in the abomasal lymph node (ALN) increased by 3 d after infection and lymph node weights increased two-fold by 5 d after infection (Balic et al., 2000). Weights of ALN likewise were higher during the patent infection. The $CD4^+$T-cell populations in the ALN co-expressed MHC class II but not CD25 which indicates that this T-cell population is becoming antigen-specific and is not of the T-regulatory (Treg) phenotype (Balic et al., 2000). In abomasal tissue, increased numbers of eosinophils, $CD4^+$ and $\gamma\delta^+$ T-cells were found as early 5 d after infection, which is consistent with other findings (Lacroux et al., 2006; Pfeffer et al., 1996). The $\gamma\delta^+$ T-cells can account for up to 60% of the lymphocyte population in ruminants, especially in calves and lambs (Tizard, 2004). These cells

are thought to bind a variety of antigen and may play a role in acquired immunity. They are located beneath and between enterocytes and thus are referred to as intraepithelial lymphocytes (IEL). They are thought to play a key role in defense of the gastrointestinal tract (Tizard, 2004).

The role of eosinophils in the immune response to parasites has been questioned, especially in immunized sheep where eosinophils appear to play a role in larval killing but to have no effect on the expulsion of adult parasites (Meeusen and Balic, 2000). To study effects of eosinophils on larval killing, researchers used previously immunized lambs, introduced 10^7 *H. contortus* larvae directly into the abomasum, and sacrificed the lambs at 24 and 48h after infection (Balic et al., 2006). Results suggest that eosinophils are recruited when larvae reach their tissue niche, but tissue eosinophils were not consistently in contact with the larvae. This indicates that the presence of tissue eosinophils alone is not sufficient to cause eosinophil-mediated attack on invading larvae (Balic et al., 2006). Numbers of eosinophils in the abomasum during the early immune response are typically low, but increase dramatically during the adult parasite infection (Pernthaner et al., 2005a; Shakya et al., 2009). Data are consistent in that eosinophils must be present in order for larval killing to occur, but may require activation from other sources to become fully functional (Balic et al., 2006).

Two other effector cell types involved in the cellular immune response include mast cells and globule leukocytes (Meeusen et al., 2005). Gloubule leukocytes (GL) are degranulated mast cells and tend to be located in the upper two-thirds of the abomasal mucosa after infection with *H. contortus* (Balic et al., 2006). The GL do not make up a large component of cellular infiltrate into abomasal mucosa. However differences have been found in the presence on these cell types between resistant and susceptible sheep during the adult parasite infection (Shakya et al., 2009). During the larval stages, research indicates that there is typically no significant increase of this

cell population (Balic et al., 2006; Kemp et al., 2009; Lacroux et al., 2006) but GL are known to mediate rapid rejection of larvae (Balic et al., 2006). The degranulation of mast cells may affect the cytokine milieu in the abomasal mucosa. GL counts are positively correlated with IL-4 mRNA in the abomasum, and IL-4 can activate eosinophils *in vitro* (Lacroux et al., 2006). The infiltration of mast cells into the abomasal mucosa far exceeds that of eosinophils and GL during early *H. contortus* infection (Kemp et al., 2009; Lacroux et al., 2006). Mast cells secrete the cytokines IL-4, -5, -6, and-13,which promote a T_H2 response or TNFα promoting a proinflammatory response (Tizard, 2004). The increased counts of mast cells in the abomasum of immunized sheep may indicate the role that mast cells play in orchestrating the protective immune response by producing cytokine and chemokines that recruit other cells to the site of infection and direct the acquired immune response.

Cytokine production during larval infection

Cytokines are critical mediators of any immunological response and add directionality to stimulate $CD4^+T_H$ cells to develop into different lineages (Anthony et al., 2007). Of particular importance are the cytokines that are involved in the development of an appropriate immune response to parasites (Meeusen et al., 2005). The immune response to the adult parasite is referred to as T_H2 response, with a specific expression pattern of cytokines both at the site of infection and in the ALN (Lacroux et al., 2006). The naïve T_H cell is directed towards a lineage that is dependent on both transcription factors and master cytokines (Figure 1.1) (Anthony et al., 2007). Cytokines necessary for development of a T_H2 lineage are IL-4,-25, and -2 and thymic stromal lymphopoeitin (TSLP). Cytokines secreted from T_H2 cells include but are not limited to IL-4, IL-5, IL-10 and IL-13.

In sheep previously infected with *H. contortus*, expression of IL-4 and IL-5 increases in abomasal lymph nodes after secondary infection (Balic et al., 2006; Craig et al., 2007; Kemp et al., 2009; Pernthaner et al., 2006; Pernthaner et al., 2005a). Expression of IL-13 was also increased after secondary exposure in both the ALN and abomasal mucosa (Craig et al., 2007; Lacroux et al., 2006; Pernthaner et al., 2006). These observations indicate that polarization of the immune response in immunized sheep occurs early in the infection cycle. Interestingly, expression of IFNγ was not down-regulated and no difference was found between resistant and susceptible or immunized sheep compared with naïve sheep (Craig et al., 2007; Pernthaner et al., 2005b). Moreover TNFα expression was upregulated in resistant sheep, which is consistent with the influx of more inflammatory cells in the abomasal mucosa of infected sheep, which may lead to the strength of T_H2 polarization (Pernthaner et al., 2005b). To date no study has clearly identified that a single cytokine genes that are differentially expressed between resistant and susceptible types early in infection, and therefore have a potential role as a selectable marker. The importance of studying cytokine profiles in the ALN and abomasal mucosa is to clarify the specific immune responses that develop in early infection between resistant and susceptible lines.

Antibody production during larval infection

Production of specific antibodies to larval infection requires that the animal has been previously exposed to the parasite and is capable of recognizing larval antigen (Balic et al., 2006). From 0 to 10 d after infection, production of IgA, IgG and IgE in immunized or resistant animals is quite low (Lacroux et al., 2006; Pernthaner et al., 2006). Although antigen specific Ig levels were measured, no clearly significant differences were found between secondarily challenged and primarily infected animals until 15 d after infection (Lacroux et al., 2006). An additional study reported that increases in antibody production occurred at an average of around

28 d after infection (Pernthaner et al., 2006). These data indicate that production of antibody to larval-specific antigen is very low during the pre-patent period, and should play a greater role during the patent infection.

Summary

Based on QTL and microarray data, several immune-response genes clearly differ in expression between parasite-resistant and parasite-susceptible sheep. However, the timing of expression of these genes also needs to be considered. As discussed above, qualitative differences have not been reported between the immune responses of susceptible and resistant breeds of sheep. However, resistant breeds have the capacity to prevent larval establishment during early infection, documenting a genetically mediated quantitative effect on immune responses in these animals (Figure 1.2).

In order to identify specific genes that merit further study, the precise mechanisms of immunity to nematode parasites must be understood. Knowledge of these immune mechanisms will prevent fishing for relevant genes and allow the narrow targeting of pathways towards immunity. Additionally, relevant findings from mouse models can be helpful as a guide to explore the migration of different cells, expression of cytokines and other soluble mediators, and their role in parasite resistance in the sheep.

The ultimate goal of this research is to aid sheep producers in the battle with parasites. The problem of GIN parasitism in the southeastern US has become so pervasive that sheep producers have become accustomed to experiencing death losses due to parasites. Although many producers are implementing rotational grazing strategies, smart drenching and use of

parasite-resistance sheep breeds, one more tool that producers need is a method to empirically identify parasite-resistant individuals in any breed.

The data presented in this dissertation thus expands knowledge of the specific physiological and immune responses that occur early in *H. contortus* infection in sheep, and may contribute to the future of genetic selection for parasite resistance.

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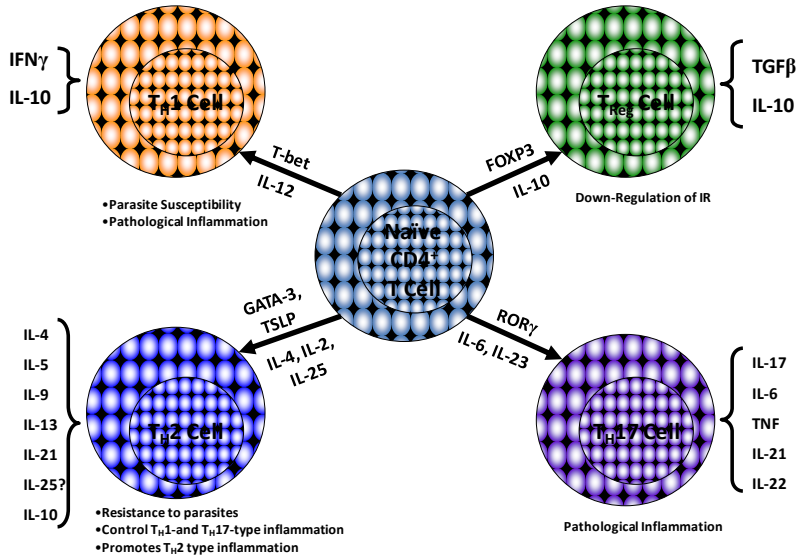

Figure 1.1: New model of CD4$^+$ T-cell differentiation in helminth infections.

Adapted from Anthony et al. (2007)

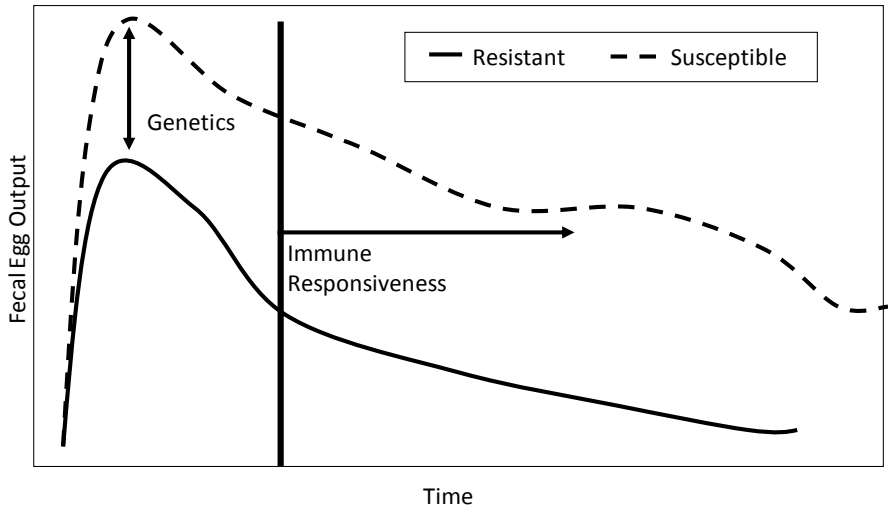

Figure 1.2: Theoretical relationship of genetics and immunity in parasite resistance.

Chapter 2 **:Humoral immune response to nematode parasite infection is accelerated and longer-lived in hair-type sheep.**

Abstract

Immunoglobulin-A (IgA) levels in wool and hair-type sheep infected with *Haemonchus contortus* were monitored for 40 d following the end of a 45-d autumn breeding season and subsequently for 6 wk after parturition. Ten, first-parity ewes of each type were infected with 12,000 *H. contortus* L_3 larvae at 5 days following the end of breeding (day 0). IgA levels were assessed at d 0, 3, 5, 7, 14, 21, 28, 35 d p.i., and weeks 0 3 and 6 after parturition. Antigen-specific IgA assays used adult *H. contortus* crude worm antigen (CWA) to measure antigen-specific IgA (CWA-IgA). Serum IgA in hair ewes was higher than in wool ewes and peaked twice; once at d 5 (13.2 mg ml^{-1}), and again at d 28 (17.3 mg ml^{-1}). The two breeds differed in average peri-parturient fecal egg count (FEC) ($P < 0.05$). Wool ewes had a FEC of 2,588 eggs g^{-1} at 3 weeks before parturition and maintained a high FEC until 1 week prior to parturition. Higher total IgA (0.903 mg ml^{-1}) and lower CWA-IgA in serum of wool ewes at parturition is presumably due to recent infection. Hair sheep had much lower FEC prior to parturition and maintained antibodies for *H. contortus* with 4-fold higher serum CWA-IgA at parturition ($P < 0.05$) but no higher CWA-IgA in colostrum. These results suggest that hair-type sheep reduce infection quicker, generate greater antibody responses more rapidly, and maintain circulating antigen-specific antibodies in the absence of a significant infection.

Keywords: peri-parturient, colostrum, IgA, *H. contortus*

Introduction

Hair-type sheep are well known for their resistance to gastro-intestinal nematode parasites, however the biological mechanisms of resistance have yet to be fully characterized. St. Croix sheep, originating in the Caribbean, have developed a natural resistance to parasites that is primarily mediated by their increased humoral immune response to helminth parasites (Gamble and Zajac, 1992). Parasite-resistant sheep have a distinct advantage in development of an acquired immune response as evidenced by an increase in circulating and local antibodies specific for both parasite adult and larval antigen (Pfeffer et al., 2005).

The parasite *Haemonchus contortus* has an adaptive mechanism whereby it enters a hypobiotic state inside the host when environmental conditions are not favorable for reproduction (Kahn et al., 2003). Hypobiosis may subsequently lead to a condition that occurs around lambing called peri-parturient rise. Peri-parturient rise (PPR) results from stimulation of hypobiotic L_4 larvae to develop into adult worms which undergo reproduction inside the ewe thereby increasing the fecal egg count without requiring ingestion of infective L_3 larvae from the environment (Courtney et al., 1984). When ewes and lambs are sent to graze, ewes will then immediately shed parasite eggs on the pasture allowing lambs to consume L_3 larvae and show signs of parasitism prior to weaning. Typically occurring in the spring, this condition has been given the moniker of "spring rise". Levamisole and ivermectin are anthelmintics that are effective against hypobiotic larvae, and if given approximately 2 weeks prior to lambing, may reduce the impact of PPR (Whittier et al., 2003). Abrogation of PPR can also be accomplished through protein supplementation (Kahn et al., 2003). If ewes experience a significant parasite challenge prior to parturition, they may also be able to tolerate an infection (Courtney et al.,

1983) and develop enough antigen-specific antibody to provide a measure of protection to the neonate.

Antibody transfer from the mother to the fetus generally does not occur in livestock (Butler, 1999), but the amount of immunoglobulins (Ig) G and A in colostrum is substantial. In the neonatal lamb, pinocytotic absorption of antibodies provides passive immunity but declines rapidly, as does antibody concentration in milk (Butler, 1999). The antibodies IgG and IgA remain in milk of sheep for some time and may provide protection to the newborn (Pfeffer et al., 2005). IgA is typically found in surface secretions and in high levels in saliva, intestinal fluid, milk, colostrum and urine (Tizard, 2004). The major role of IgA is immune exclusion, as it is secreted in the mucus of the small intestine and protects the lumen from invasion of viruses, bacteria or parasites (Tizard, 2004). Increased production of IgA is a hallmark of parasite resistance (Amarante et al., 2005; Martinez-Valladares et al., 2005; Pfeffer et al., 2005), especially during secondary infection.

This experiment compared immune responses of hair and wool type ewe lambs to challenge with *H. contortus* following first breeding at approximately 7 months of age. After the initial challenge, ewe lambs were maintained in drylot and further characterized for fecal egg counts and immunoglobulin levels around the time of parturition.

Materials and Methods

Sheep and experimental design

Ten St. Croix (hair sheep) ewe lambs and 10 ewe lambs of a composite line of wool sheep developed at Virginia Tech were used for this study. Ewes of the composite line were made up of ½ Dorset, ¼ Finnsheep and ¼ Rambouillet breeding and, due to their composition were considered to be susceptible to the parasite. Ewes were born in January 2006 and exposed to rams for 45d beginning at approximately 7 months of age in August and early September 2006. Ewes were housed indoors from the end of breeding in September 2006 through lambing and early lactation. At 50 d following ram removal, trans-abdominal ultrasound was used to determine pregnancy and only pregnant ewes were selected for study. Pregnant ewes were fed a 16% CP corn-soybean ration, grass hay and provided water *ad lib*.

The ewe lambs were infected with 12,000 *H. contortus* L_3 larvae 5 d after the end of breeding to measure total IgA antibody production in response to secondary exposure of *H. contortus*. This initial infection was allowed to persist until the peri-parturient period. No additional infection was allowed, as the animals were maintained in drylot from initial infection until after (Figure 2.1).

Parasitological Techniques:

Adult *H. contortus* were collected from sheep maintained on pastures known to be infected with *H. contortus*. Worms were pulverized in an ice-cold glass tissue homogenizer to release developing eggs. Homogenate was mixed with egg-free feces to obtain a mono-specific

larval culture. The Baermann technique (Zajac and Conboy, 2006) was used to collect *H. contortus* L_3 larvae. These larvae were used to infect two worm-free donor wethers. At least 21 days after infection, feces were collected from donor lambs and cultured at 30^{o}C for 7 to 8 days. Larvae were collected as described above, stored at 4^{o}C in deionized water, and used within one month to orally infect experimental animals. Fecal egg counts were determined by the modified McMaster's technique (Whitlock, 1948). Each egg observed represented 50 eggs per gram of feces. Fecal egg counts were conducted on d 0, 14, 21, 28 and 35 after infection. Beginning at the end of December 2006 and ending in early March 2007, FEC measurements of peri-parturient ewes were taken every 5 to 7 days.

Blood collection, antigen preparation and extraction of milk immunoglobulins:

Blood was collected, via jugular venipuncture, into a 10 ml vacutainer tube (BD) eight times after infection, at parturition and at exactly 3 and 6 weeks after parturition for serum antibody analysis. Blood samples were allowed to clot and then centrifuged at 2,000 x *g* for 20minutes at 4^{o}C. Serum was then aliquoted into 1.5ml centrifuge tubes. Additional blood samples were collected into 4 ml EDTA-treated vacutainer tubes (BD) at the same time as above to measure packed cell volume (PCV). Crude worm antigen (CWA) was made by removing adult *H. contortus* worms from a previously infected donor and freezing at -20^{o}C. Worms were washed twice in PBS (pH 7.4), diluted to a final volume of 7 ml in PBS (pH7.4) and homogenized in an ice-cold glass Dounce homogenizer. Homogenate was centrifuged at 15,000 x *g* at 4^{o}C for one hour, supernatant was removed, protein content was measured using the BCA assay (Pierce, Rockford, IL) and samples were stored at -80^{o}C. Serum was diluted 1:1000 for Total IgA and CWA-IgA ELISA analysis.

Milk samples (10 ml from each half of the udder) were collected at parturition and at exactly 3 and 6 weeks after parturition. Milk samples of each ewe were pooled and diluted 1:1 in PBS (pH 7.4) and stored at -20^{o}C for later analysis. To effectively analyze milk IgA without inappropriate binding of fat or large proteins, diluted milk was precipitated in 36% ammonium sulfate and centrifuged at 3,000 x *g* for 20min at 4^{o}C; the whey portion of sample was then removed and buffer was exchanged using a 3,000 MW membrane dialysis cassette (Pierce, Rockford, IL) in 1L of PBS (pH 7.4) at 4^{o}C overnight.

Total and antigen specific IgA ELISA:

To determine concentrations of capture and detection antibodies, checkerboard titrations were performed using serum of an infected ewe. Capture antibodies, anti-sheep IgG_1 (Sigma-Aldrich, St. Louis, MO) and polyclonal anti-sheep IgA (Bethyl, Montgomery, TX), diluted to 1:100 in carbonate-bicarbonate buffer (pH 9.6) were placed in each well of a 96-well plate (Nunc, Rochester, NY) and incubated overnight at 4^{o}C. The plate was then washed 3 times in PBS with 0.05% Tween-20 and blotted dry. Plates were blocked with 200µl of PBS with 0.1%BSA and allowed to incubate for 1 hour at room temperature (RT). After another wash step, diluted serum samples (1:1000) and milk samples (1:20) in PBS with 1%BSA were added in duplicate and allowed to incubate for 2 hrs at RT. Polyclonal anti-sheep IgA conjugated with HRP (Bethyl Laboratories Inc., Montgomery, TX.) was diluted 1:1500 and 100 µl were added to each well on the plate. After incubation at RT for 1hour in the dark and a final wash step, 100 µl of TMB substrate (Pierce, Rockford, IL) was added to each well to produce a colorimetric response that was allowed to develop for 20 minutes. Plates were read at a wavelength of 450 nm, on a Biotek EL-311 spectrophotometer. Total IgA values were compared to a sheep IgA

standard curve (Accurate Chemical Co., Westbury, NY). Data from antigen-specific assays are reported in absorbance values.

Statistical analysis

Fecal egg counts and immunoglobulin data were not normally distributed and were transformed as either $\log_{10}$(FEC+25) (LFEC) or $\log_{10}$(ABS+1). Data from the experiment were then analyzed using the general lineal model of SAS (SAS Institute, Cary, NC). Fixed effects in the model included breed, time and two-way interactions. Significant differences in LS means analysis were compared using t-tests with critical probability values calculated using the Bonferroni adjustment for comparisons among more than two means. Means of transformed FEC and absorbance values were then back-transformed for reporting.

Results

Parasitological measurements

Fecal egg counts increased in both groups and no difference existed between the breed types in FEC during the first 35 d after infection (Figure 2.2a). During the peri-parturient period however, the average FEC of wool ewes across all time points was 565 eggs g^{-1} and significantly higher than that of hair ewes at 148 eggs g^{-1} ($P < 0.05$) (Figure 2.2b). At this time no type x time interaction was observed, but at 3 weeks prior to parturition, FEC of hair sheep was quite low with a mean of 318 eggs g^{-1} and this low-level was maintained for the remainder of the trial (Figure 2.2b). Wool sheep had higher FEC than hair sheep at 3 weeks before parturition with a mean of 2,587 eggs g^{-1} and were not able to reduce their infection until one week prior to parturition (Figure 2.2b).

During initial infection, PCV did not differ between the breed types but did differ between day 0, 21 and day 35 ($P < 0.05$) (Figure 2.2c), reflecting observed increases of FEC. There was no significant effect of time or type x time interaction during the peri-parturient period. However, a significant effect of type was observed as hair ewes had a higher PCV (31.6%) than wool ewes (28.5%), when averaged across all time points ($P < 0.05$) (Figure 2.2d). These data are consistent with higher FEC observed in wool ewes during the peri-parturient period.

Serum total (T-IgA) and crude worm antigen-specific IgA (CWA-IgA)

Only T-IgA was measured following initial infection, and results suggested a two-stage antibody response (Figure 2.3). T-IgA increased significantly in hair sheep from 5.99 mg ml^{-1} at d 0 to 13.18 mg ml^{-1} at d 5 ($P < 0.01$) but returned to baseline level by d 21. The second period of increased IgA production occurred at d 28 as T-IgA again increased to 17.30 mg ml^{-1}. Across all time points T-IgA was higher in hair sheep ($P < 0.05$) indicating a greater humoral immune response in this breed.

At parturition, wool sheep had significantly higher T-IgA in serum, and maintained higher values until 3 weeks after parturition ($P < 0.01$) likely due to the presence of adult worms in the gut at 2 and 3 week prior to parturition (Figure 2.4a). In contrast, hair sheep had low serum levels of T-IgA, presumably as a result of reduced level of infection. The higher IgA level of wool ewes observed in serum did not reflect T-IgA level in colostrum and milk, as there were no differences between breeds (Figure 2.4b).

Higher levels of serum CWA-IgA at all time points in hair ewes ($P < 0.05$) (Figure 2.5a) indicates maintenance of a potent memory response. Wool sheep had significantly less serum CWA-IgA, which may be due in part to the sequestering of CWA-IgA to the abomasum. Production of CWA-IgA in the colostrum and milk did not different between the groups (Figure 2.5b). However, the presence of CWA-IgA in milk suggests that both breeds have some potential to confer protection to the neonate.

Discussion

There were no differences between breeds in FEC up to 35 d following experimental infection, which is uncommon as hair sheep typically have lower FEC following an infection of *H. contortus* (Notter et al., 2003). The effect of nutritional supplementation on reducing parasitism are well-known (Eady et al., 2003), in particular effects of protein supplementation on FEC reduction (Fox, 1997; Kahn et al., 2003). Ewes in this trial were bred to lamb at one year of age and thus were maintained on a concentrate and high quality forage diet. The absence of differences in early gestational FEC and PCV thus may be accounted for by the diet these ewes were fed.

Differences in FEC between breed types were not observed until the peri-parturient period and no FEC measurements were obtained during the time in between trials. Therefore, the possibility of larvae becoming hypobiotic and re-emerging 30 d later, resulting in higher FEC of wool ewes cannot be disproven by these data. Hypobiosis could have been precluded in parasite-resistant hair ewes (Courtney et al., 1984) and may have accounted for the low FEC observed during the peri-parturient period. Therefore, had data been collected during the time in between trials it may have added useful information.

The rapid decline of FEC in wool ewes from 3 to 1 week prior to parturition could be due to senesce of the adult worm. It is not unreasonable to expect an adult *H. contortus* worm to survive in the host for 12 to 16 weeks (Courtney et al., 1984; Behnke, 1987) but without re-infection egg shedding will halt as adult parasites die. Based on this information, timing of parasite senescence would occur between 1 and 2 weeks prior to parturition, in accordance with wool ewe FEC data. The reduced FEC observed in the hair sheep prior to parturition is more likely due to prior immune-mediated parasite killing.

The humoral immune response to secondary infection typically occurs faster, at a higher magnitude, withdraws quickly and retains memory for the pathogen (Tizard, 2004). When infected with identical doses of *H. contortus,* during a secondary challenge, wool ewes failed to generate the same concentration of T-IgA as hair ewes after infection. Low IgA concentrations in wool ewes may play a role in their inability to reduce infection. Antibodies in the abomasal mucus may immobilize infective larvae (Harrison et al., 2003) or aid in adult killing via antigen-dependant cell-mediated cytotoxicity (Kooyman et al., 1997). Therefore, reduced antibody production may be partially responsible for the length of infection observed in wool ewes. Parasite-resistant hair sheep seemed to mount a two-stage antibody response that coincides with different stages of the parasite. Moreover, higher IgA production in hair ewes was consistent with reports of other parasite resistant breeds, that had increased antibody production upon secondary exposure to GINs (Gill et al., 1993; Schallig et al., 1994).

At parturition and at week 3 of lactation, wool ewes had more T-IgA in their serum than hair ewes. An increase in serum concentration of IgA should be reflective of concentrations of IgA in colostrum and milk (Jertborn et al., 1986). However, no differences existed in concentrations of IgA in the milk or colostrum of hair and wool ewes. Prior to parturition, wool

ewes had higher FEC than hair ewes and thus the higher concentration of T-IgA in the serum at parturition may be more reflective of infection status than an ability to mobilize IgA to milk. The reduced level of T-IgA observed in hair sheep after parturition is consistent with a normal IgA serum concentration (Tizard, 2004), supporting the conclusion that hair sheep had a low peri-parturient FEC and thus did not require elevated antibody production. Yet with lower T-IgA serum concentrations, hair ewes matched the T-IgA production in milk, and therefore circulating antibody concentration may not be reflective of antibody concentration in milk.

Parasite-specific antibody levels of hair sheep were significantly higher than those of wool sheep at parturition and through 6 weeks after parturition. Higher CWA-IgA levels in hair sheep are presumably due to immunological memory because, unlike wool ewes, hair ewes did not show evidence of substantial infection shortly before parturition. Lower serum CWA-IgA levels of wool ewes may be accounted for by their infection level prior to parturition. Interestingly, their FEC was reduced dramatically from 2 to 1 week prior to parturition. However, this FEC reduction was thought to be most likely due to parasite senescence rather than to immunologically mediated inhibition. Colostrum and milk levels of CWA-IgA did not differ between breeds, suggesting that serum antibody levels do not predict colostrum and milk antibody levels.

Immunizing ewes prior to lambing can have protective effect on the neonate via passive transfer of antibodies through colostrum (Butler, 1999). Although there is a typical lactation-associated relaxation of immunity, mice infected with *Nemadospiroides dubius* and ewes infected with *H. contortus* remained able to passively transfer immunity to neonates (Shubber et al., 1981). Maternal transfer of IgA to the neonate has been observed in Romney ewes infected with *T. colubriformis* but no difference in IgA level existed between colostrum and milk 30 d

after parturition (Pfeffer et al., 2005). Alternatively, IgE appears to have a greater effect on passage of immunity to the neonatal lamb with significant correlations between colostrum and plasma concentration in the ewe and plasma concentration in the lamb. These data indicate that ovine colostrum can contain high levels of IgE (Pfeffer et al., 2005). The possibility of other isotypes such as IgE and IgG1 should be considered if evaluating antibody differences between parasite-resistant and susceptible sheep. The lack of availability of commercially produced anti-sheep IgE has limited the scope of parasite resistance research based on humoral immune responses. Evaluation of total and specific antibody levels in the lamb should have been evaluated to examine passage of those specific antibodies to the neonate.

This study documents differences in production IgA in parasite-resistant hair and parasite-susceptible wool sheep infected with *H. contortus*. Upon secondary infection, hair ewes have a greater and more rapid immune response. These parasite-resistant ewes were able to expediently reduce infection levels yet maintain a significantly higher circulating level of antigen-specific IgA well after infection. Higher levels of circulating antibodies were not reflective of similar antibody levels in colostrum or milk yet the absence of difference in antigen-specific and total IgA in the colostrum and milk of both types suggests at least an equal ability to provide potential protection to the neonate.

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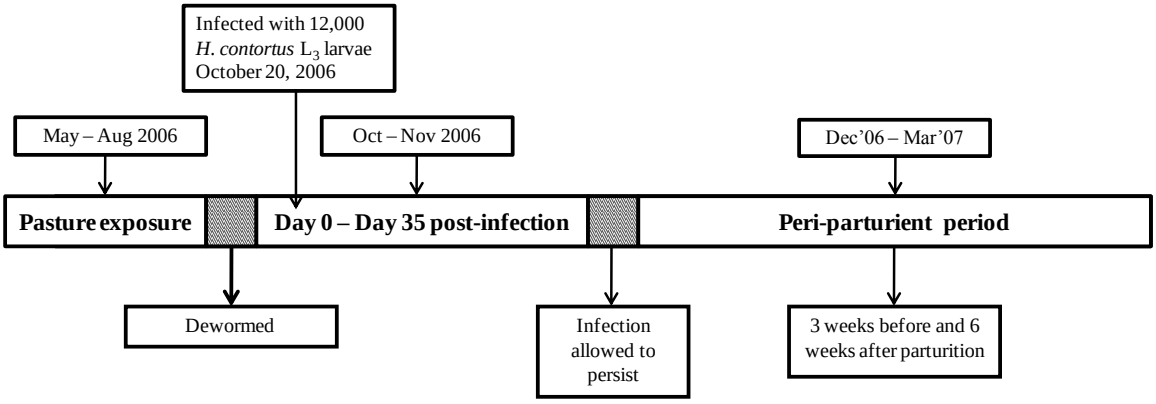

Figure 2.1: Schematic timeline of experimental events

The first trial was conducted from day 0 to day 35 after infection with *H. contortus*. The second trial was initiated at least 3 weeks prior to parturition, which began on January 17, 2006. Milk samples were collected at 3 and 6 weeks following parturition.

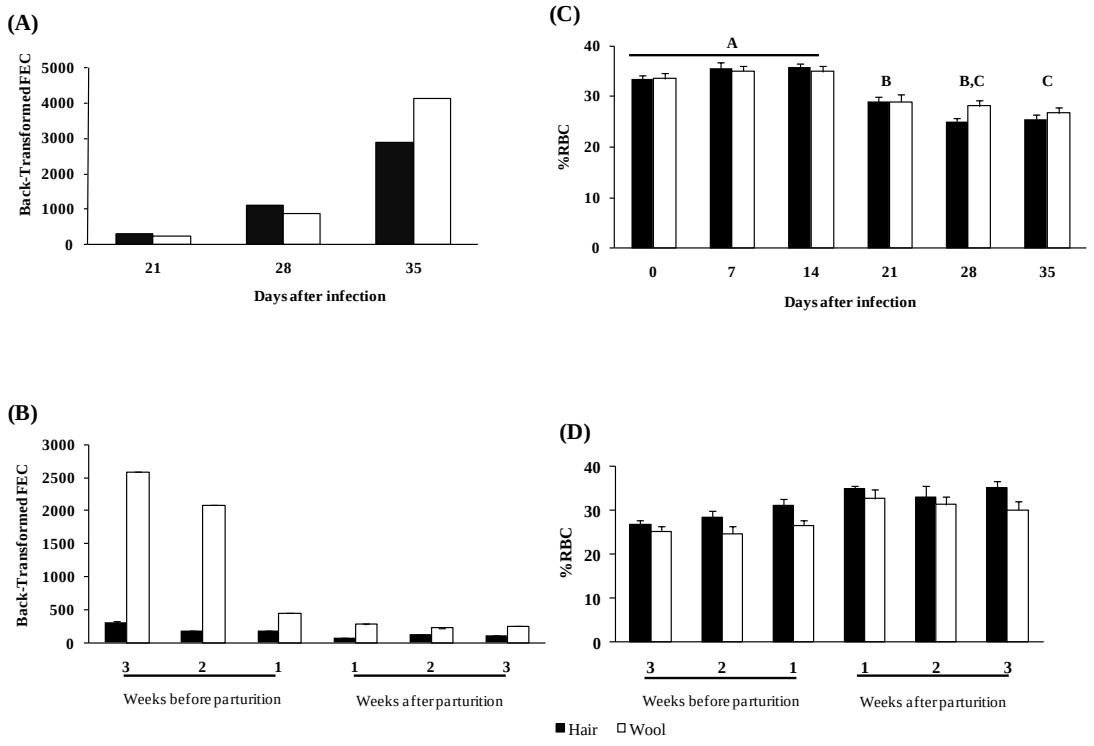

Figure 2.2: Parasitological measures of ewes immediately following infection and during the peri-parturient period

Data from hair ewes are in solid bars and wool ewe data in open bars. Fecal egg counts of hair and wool ewes immediately following infection (A) were not significantly different. During the peri-parturient period (B), FEC averaged over all time points was significantly different between types ($P < 0.05$) but no type x time interaction was observed. Packed cell volume measured as the percent of red blood cells (%RBC) did not differ immediately following infection (C) between the types, but a significant day effect was observed (means with different letters differ significantly at $P < 0.05$. No type x time interaction existed during the peri-parturient period (D), however, when averaged across all time points PCV was significantly higher in hair sheep ($P < 0.05$)

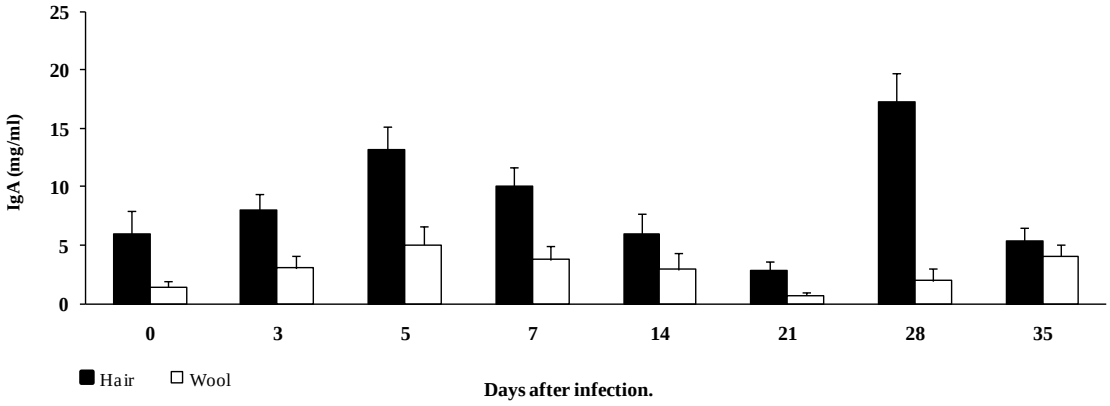

Figure 2.3: Total serum IgA levels following secondary infection with 12,000 *H. contortus* L_3 larvae on Day 0

T-IgA levels differed significantly between the breeds on all days except d 14 and 35.

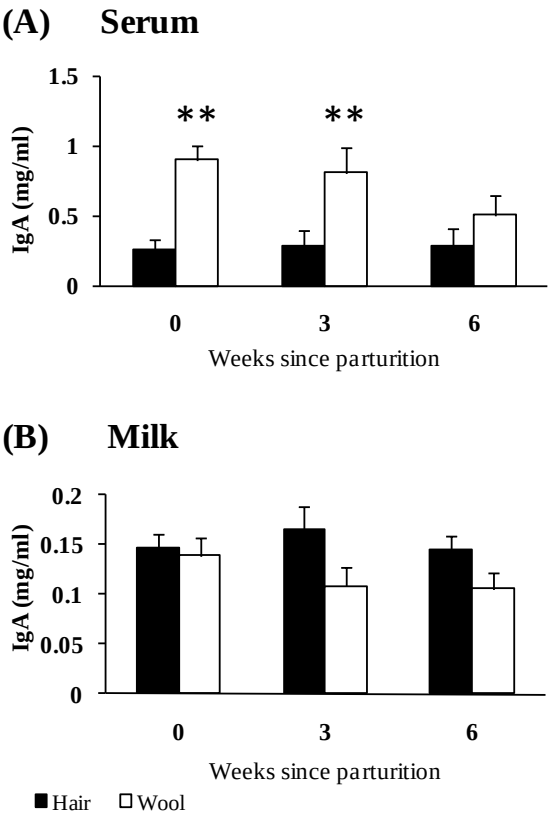

Figure 2.4: Serum and milk total IgA in the weeks following parturition

Concentrations of serum T-IgA (A) in wool sheep were significantly different from those of hair sheep at 0 and 3 but not 6 weeks after parturition. No significant differences were found for milk T-IgA (B). ** $P < 0.01$

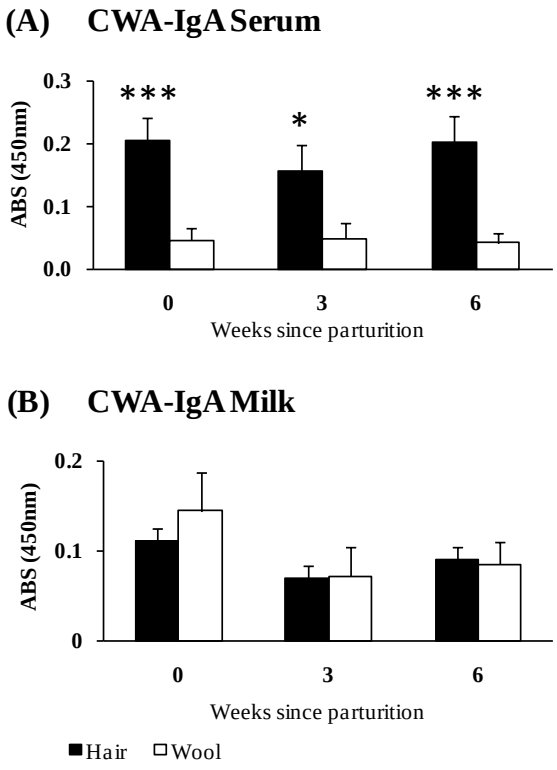

Figure 2.5: Absorbance values of antigen-specific IgA in serum and milk

Crude worm antigen (CWA) IgA absorbance values for serum differed significantly between hair and wool sheep (A) No differences between hair and wool sheep were detected for milk CWA-IgA (B). * $P < 0.05$; *** $P < 0.001$

Chapter 3 **: The early physiological and cellular immune responses to *Haemonchus contortus* in parasite-resistant and susceptible breeds of sheep.**

Abstract

Peripheral and local cellular immune responses were evaluated in 24 St. Croix hair lambs and 24 Dorset x (Finn -Rambouillet) wool lambs at 0, 3, 5 and 7 d after infection with 10,000 L_3 *Haemonchus contortus* larvae. Blood samples, taken immediately before harvest, revealed no differences in circulating effector cell populations, yet there were significant differences in levels of circulating neutrophils. Hair lambs had a higher average circulating neutrophil concentration (3,018 cells/μl) than wool lambs across all time points (1818 cells/μl; $P < 0.05$). Breeds did not differ in eosinophil or globule leukocyte (GL) counts in abomasal tissue, yet infiltration of these cell populations increased with time. GL counts peaked at day 3 after infection whereas eosinophils continued to increase to day 7 after infection. Abomasal neutrophil counts were higher in hair lambs (831cells/mm^2), than wool lambs (561 cells/mm^2; $P < 0.0001$) when averaged across all time points. Total abomasal lymph node (ALN) weight increased exponentially from 2.60 g at day 0 to 6.57 g at day 7 in hair lambs, whereas ALN weight only increased from 1.88 to 2.67 g over this period in wool lambs ($P = 0.0003$). This result suggests a greater expansion of lymphocytes in the ALN promoting early development of antigen-specific acquired immune responses in hair lambs. Greater infiltration of immune cells to the abomasal mucosa at an earlier stage of infection may limit establishment of adult parasites and thereby shorten the duration and/or severity of infection.

Keywords: Parasite resistance, cellular immune response, neutrophils

Introduction

The problem of GIN parasitism in sheep production is well-known and has been intensely studied during adult worm infection. Regulating the impact of parasitism on production may be achieved by selection for parasite resistance or the incorporation of breeds that already possess substantial levels of parasite resistance (Vanimisetti et al., 2004). Genetic linkage studies indicate that regions of the genome associated with parasite resistance are those involved in the immune response (Coltman et al., 2001). Characterization of immune responses to GIN parasitism may therefore lead to identification of candidate genetic markers that could be useful to identify parasite resistant individuals (Charon, 2004).

The acquired immune response to GIN parasitism in sheep has been well-defined as a classic T_H2 response with high levels of IgG, IgA and IgE, mastocytosis and eosinophilia at the site of infection (Lacroux et al., 2006). Unlike mouse models where susceptible mice have an inappropriate T_H1 response to GIN parasites (Else et al., 1993), susceptible sheep are able to mount an appropriate type of immune response (Shakya et al., 2009). In sheep, susceptibility appears to result from a reduced level (Bowdridge et al., 2008) and delayed timing of immune responses (Rowe et al., 2009) which facilitates establishment of the adult parasite. The difference between parasite resistance and susceptibility therefore lies in the magnitude of immune response to GIN during secondary infection.

Innate immune responses to larval GIN infection involve initiating factors that direct the adaptive immune response and are less clearly understood (McNeilly et al., 2009). The migration of eosinophils, mast cells, and other granulocytes to the site of infection during the secondary immune response aids in polarization of T-cells towards the T_H2 response in helminth

infections (Perrigoue et al., 2008). During a primary infection with *H. contortus*, numbers of eosinophils, $CD4^+$T-cells and B-cells in the abomasal mucosa increased and local lymph node size increased within 5 d after infection (Balic et al., 2000). During secondary challenge, eosinophils, globule leukocytes and mast cells migrate into the abomasal mucosa as early as 3 d after infection (Lacroux et al., 2006).

The aims of this experiment were to determine if there are differences in the cellular infiltrate in the abomasal mucosa between resistant and susceptible sheep and if there are associated increases in abomasal lymph node development within the first 7 d after infection with *H. contortus*.

Materials and Methods

Sheep and experimental design

Twenty-four St. Croix hair sheep and 24 composite wool lambs born in January and February 2007 at the Virginia Tech Sheep Center were used for this experiment. The wool composite lambs were composed of 50% Dorset, 25% Finnsheep and 25% Rambouillet breeding and were considered parasite susceptible. After weaning, lambs grazed naturally infected pastures for 30 d, and to further ensure that all lambs had been previously exposed to *H. contortus*, each additionally received a weekly dose of 2,000 *H. contortus* L_3 larvae for 4 wk before the experiment began. Trickle infection resulted in increased FEC and decreased PCV by 21 d after infection (Figure 3.1). Lambs were then treated with levamisole (8mg/kg) and maintained in drylot. Fecal egg counts were monitored for 3 consecutive weeks before starting the experiment to ensure that fecal egg counts were less than 50 eggs/g.

Lambs were randomly divided into blocks of 4 lambs of each breed and assigned to treatments based on sex within blocks (Table 3.1). Blocking was necessary because all lambs could not be euthanized on a single day. Lambs within each block were either not infected (Day 0) or received 10,000 *H. contortus* L_3 larvae on day on day 0 and were then sacrificed 3, 5, or 7 d after infection (Table 3.1). Lambs were housed on elevated floors from 1 wk before experimental infection until sacrifice, and fed a 16% CP corn-soybean meal ration, grass hay and water *ad libitum* throughout the experiment. Lambs were humanely euthanized using captive bolt-gun stunning followed by exsanguination. All samples were collected at the Virginia Tech Meats Laboratory. Methods of infection, sample collection and euthanasia used in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Parasitological methods

Infective stage *H. contortus* were kindly donated by Dr. James Miller of Louisiana State University and were used to infect two worm-free donor wethers. At least 21 d after infection, feces were collected from donor lambs and cultured at 30^{o}C for 7 to 8 d. Larvae were collected using the Baermann technique (Zajac and Conboy, 2006), stored at 4^{o}C in deionized water, and used within 1 month to orally infect experimental animals. To monitor success of trickle infection fecal egg counts were performed 14, 21 and 28 d after first infection (Figure 3.2), using the modified McMaster's test (Whitlock, 1948).

Hematology

Prior to sacrifice, blood samples were taken via jugular venipuncture and collected in either EDTA-treated 10ml vacutainer (BD) tubes for packed cell volume and white blood cell

differential counts or 10ml vacutainer (BD) tubes with no additive for serum analysis. White blood cell differential counts were performed in the clinical pathology lab of the Virginia Tech Veterinary Medical Teaching Hospital. Whole, anti-coagulated blood was analyzed using a Cell-Dyn 3700 (Abbott) cell counter and results were visually verified by Wright-Giemsa staining. Cell counts are reported in cells/µl of whole blood (Table 3.2).

Abomasal and lymph node tissue histology

All palpable lymph nodes were extracted from the lesser curvature of the abomasum and superficial fat was removed. Lymph nodes were counted and weighed and the largest lymph node was cut longitudinally with a 4mm slice placed in 10% buffered formalin. The abomasum was cut along the greater curvature and contents were removed and washed gently in PBS (pH7.4). A section of the fundic region of the abomasum was removed, including a fold and all associated connective tissue, and placed in 10% buffered formalin. Small, longitudinal slices of both the lymph node and abosmasal mucosa were embedded in paraffin, cut and stained with hematoxylin and eosin (H&E) by the clinical pathology lab of the Virginia Tech Veterinary Medical Teaching Hospital. Eosinophils and neutrophils in the lower two-thirds and globule leukocytes in the upper one-third of the abomasal mucosa epithelium were visualized at 400X. Forty non-sequential views of each sample were counted using a grid reticule, and data are reported as the average cells/mm^2.

Statistical analysis

Data from the experiment were analyzed as a randomized complete block design using the general linear models procedure of SAS (SAS Institute Inc. Cary, NC). Fixed effects of the

statistical model included block, breed, day and all two- and three-way interactions. Due to occasional missing samples, an analysis for an unbalanced design was used. Data from the tissue cell counts were not normally distributed and were transformed by taking the natural logarithm of the cell count plus one before statistical analysis. Means were compared using t-tests with critical probability values calculated using the Bonferroni adjustment for comparisons among more than two means.

Results

Peripheral white blood cell counts

Differential counts of circulating white blood cell revealed a significant breed difference only in the concentration of circulating neutrophils (Table 3.2). Averaged across all time points in circulating blood hair sheep had higher neutrophil concentrations (3,018 cells/µl) compared to wool sheep (1818 cells/µl) ($P < 0.05$).

Abomasal mucosa cell counts

Comparisons of H&E stained abomasal tissue sections at days 0 and 7 revealed greater cellular infiltration of immune cells into both the submucosa and mucosal epithelium of hair sheep by day 7 (Figure 3.3). Extensive presence of immune cells in both the submucosa and mucosal epithelium was greater in hair sheep than in wool sheep. (Figure 3.3b,c). Globule leukocyte counts increased by 3 d after infection in both types ($P < 0.05$) and remained elevated through 7 d (Figure 3.4a) but did not differ between hair and wool sheep. Eosinophil counts

increased exponentially and significantly to day 7($P < 0.001$) but no breed effect or day x breed interaction was observed (Figure 3.4b).

Upon closer examination of the cellular infiltrate into the abomasal mucosa, neutrophils were found to comprise a large proportion of the cellular infiltrate (Figure 3.4c). A significant day x breed interaction ($P = 0.02$) was observed with significant breed differences at both 3 ($P < 0.001$) and 7 d ($P < 0.05$) after infection.

Lymph node development

All palpable abomasal lymph nodes were counted and weighed for each individual lamb. No difference was found in the LN count between hair and wool sheep, but LN weight increased exponentially in hair sheep through 7 d after infection (Figure 3.5). A modest increase was observed for wool sheep but LN weights of infected wool lambs did not differ significantly from those at day 0. A significant day x breed interaction ($P < 0.02$) was detected between days 0 and 7 after infection, hair sheep LN weight increased by 3.97 g whereas wool sheep LN weight only increased by 0.39 g, a ten-fold difference in magnitude of response.

Lymph node H&E sections revealed massive increase of B-cell follicle size in LN of hair sheep but not wool sheep. Antigen presentation to T-cell occurs in the paracortex of the LN and examination of this region revealed cells that phenotypically resembled neutrophils and were in close association with lymphocytes (Figure 3.6). This condition was found to occur only in the LN of hair sheep at days 5 and 7 after infection.

Discussion

No differences in numbers of infiltrating eosinophils or GL in the abomasal mucosa were observed between resistant and susceptible sheep during the early cellular immune response to a secondary challenge with *H. contortus*. The patterns of infiltration observed in this experiment were consistent with other reports of eosinophil and GL migration into the abomasal mucosa during secondary challenge with this parasite (Balic et al., 2000; Lacroux et al., 2006), and therefore were not associated with differences between resistant and susceptible breeds. These findings differ from those involving cellular immune responses to the adult parasite, where eosinophils and GLs are present in higher numbers in resistant sheep (Pfeffer et al., 1996; Shakya et al., 2009). GL appeared to infiltrate into the abomasal mucosa by 3 d after infection, indicating that GL are responding to migration of larvae to its tissue niche and the initiation of blood feeding (Colditz and Le Jambre, 2008). Tissue damage caused by feeding may stimulate eosinophil migration to the abomasal mucosa (Balic et al., 2006). Nonetheless, the infiltration of eosinophils and GL seems to be associated with a normal secondary cellular immune response to GIN parasitism and therefore are not indicative of a difference between resistant and susceptible breeds.

The main physiological difference between resistant and susceptible sheep during the larval stage of secondary infection with *H. contortus* seems to be LN development. The very substantial increase of LN weight in hair sheep indicates the development of an acquired immune response by the clonal expansion of antigen specific B-cells into plasma cells capable of producing antibodies specific to *H. contortus*. The failure of LN development in infected wool sheep compared with uninfected animals (Balic et al., 2000) is perhaps is due to lack of early

antigen recognition. Although at a lower magnitude than hair sheep, there was a marked increase in immune cell numbers at the site of infection in wool sheep. This indicates the initiation of an appropriate immune response however there appears to be a failure of communication to the local lymph nodes preventing the timely initiation of the acquired immune response

Helminth parasites are well-known for their ability to suppress and regulate immune responses in their hosts (Maizels and Yazdanbakhsh, 2003), and down-regulation of host immunity protects the parasite from elimination. While greater susceptibility to immunosuppression by the parasite may be a factor in parasite susceptibility in wool sheep, failure to develop a rapid immune response in susceptible sheep may also be due to a lack of pattern recognition by innate cells. Recognition of pathogen-associated molecular patterns (PAMP) by epithelial cells, granulocytes, dendritic cells and macrophages affect the expression of cytokines, chemokines and other soluble mediators necessary for the development of a T_H2 response (Perrigoue et al., 2008). A lack of PAMP recognition by toll-like receptors in susceptible sheep during larval infection would be consistent with their delayed development of a protective immune response to the adult parasite. Recent data suggest differential expression of toll-like receptors in resistant and susceptible sheep during the first 10 d after infection with *H. contortus* (Ingham et al., 2008)

The increased presence of neutrophils at the site of infection and in the local lymph nodes was neither expected nor characteristic of classic cellular immune response to adult GIN infection. There are no data that suggest a role for neutrophils in larval killing of a helminth of this size, yet there are reports of neutrophil-mediated killing of *Trichinella* larvae in humans (Bass and Szejda, 1979) and in strongyloides infection in mice (Galioto et al., 2006).

Interestingly, during strongyloides infection, neutrophils and eosinophils are needed for the protective innate response but only neutrophils are necessary for the protective adaptive immune response. Neutrophils thus perhaps have the ability to enhance protective immunity in the resistant sheep.

Neutrophil infiltration is not commonly reported in GIN infection in sheep, but is reported frequently in the secondary immune response to *Heligmosomoides polygyrus* infection in mice (Anthony et al., 2007). A notable influx of these cells to the small intestine occurs in mice, and neutrophils are a large component of the granuloma that form around parasitic larvae in the intestinal mucosa (Patel et al., 2009), providing innate protection and aiding in the recruitment of other cells to the site of infection. The phenotype of activated neutrophils in this tissue niche has not been characterized as classically or alternatively activated. Differentiation of these two phenotypes has recently been discovered in mice infected with a bacterial pathogen (Tsuda et al., 2004). It would be interesting to know the phenotype of the neutrophils migrating to the abomasal mucosa during *H. contortus* infection.

The role of neutrophils in the abomasal lymph nodes of resistant sheep and their potential localization to a region of the LN associated with antigen presentation deserves further study. A hallmark feature of antigen-presenting cells (APC) is the expression of MHC class II. The inducibility of MHC class II expression in activated neutrophils has been known for some time (Gosselin et al., 1993) and requires both the presence of antigen and a specific cytokine environment. The role of neutrophils in response to different pathogens is currently under review and some of the latest data suggest that neutrophils can both present antigen to T-cells (Culshaw et al., 2008) and transport antigen from the site of infection to the local lymph nodes (Terasawa et al., 2008).

Data from this experiment indicate that immune cells found to differ in concentration in abomasal mucosa between resistant and susceptible types during adult worm infection do not differ during the larval stage of infection, suggesting that other cell types may have a greater effect on promoting immune responses during this period. The difference in local LN development may be due to unimpaired antigen recognition in hair sheep allowing them to develop a rapid acquired immune response. Interestingly, neutrophils may potentially be playing a critical role in the difference between hair and wool sheep, both at the site of infection and in the local LN. Further analysis and empirical confirmation of the presence and concentrations of neutrophils in local lymph node may provide more information on their role in parasite resistance.

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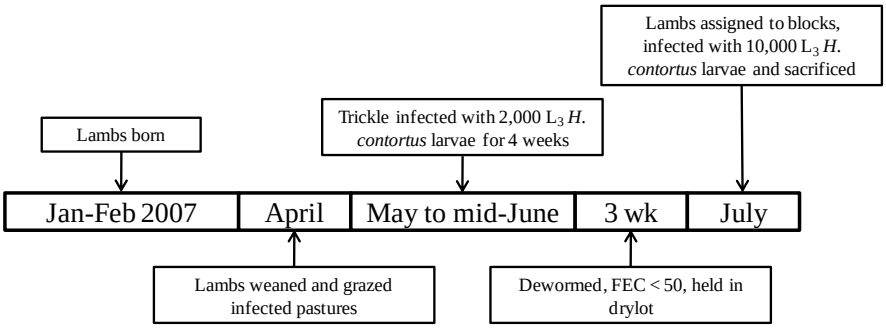

Figure 3.1: Timeline of experimental events

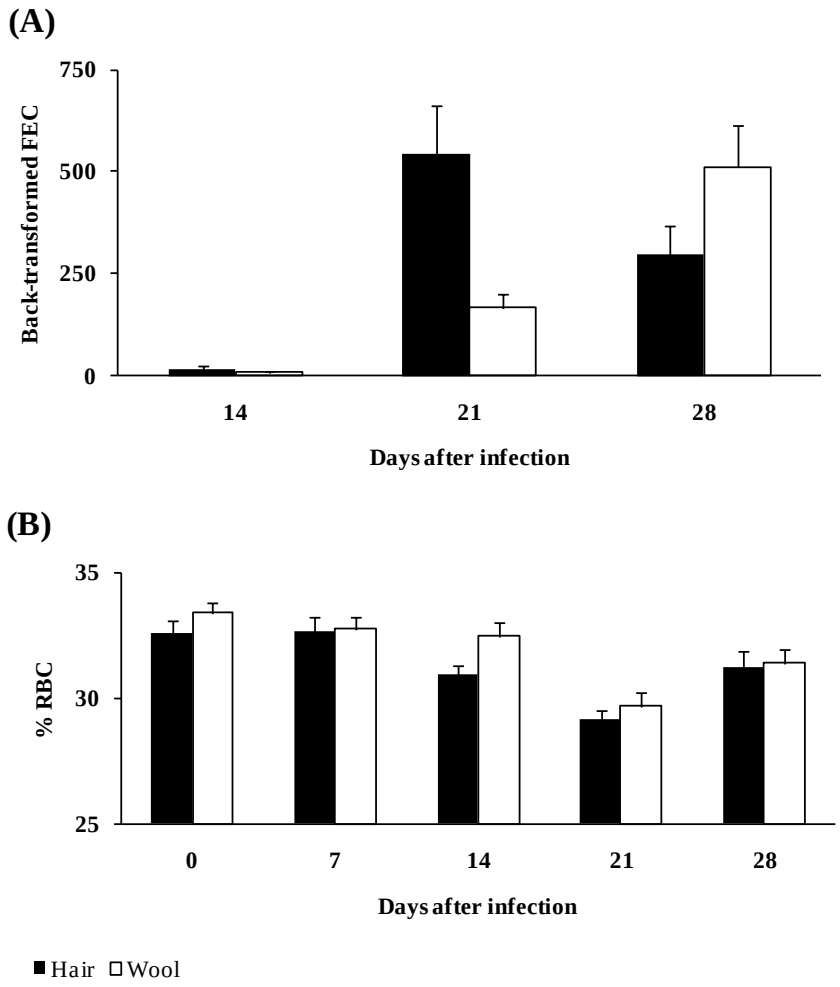

Figure 3.2: Parasitological data during trickle infection

Measurements of parasitism from trickle infection conducted prior to experimental infection. Fecal egg counts (A) exhibited highly significant day x breed interaction ($P < 0.05$). Packed cell volumes (B), expressed as percentage of red blood cells (RBC), did not differ significantly between types but a significant day effect was observed. Lambs of both types had lower PCV at day 21 after infection ($P < 0.05$).

	Type		Days infected prior to sacrifice*				Sacrifice
Block	**Hair (n)**	**Wool (n)**	**Uninfected**	**Day 3**	**Day 5**	**Day 7**	**Date**
A	8	8	2	2	2	2	July 19
B	8	8	2	2	2	2	July 25
C	8	8	2	2	2	2	July 31
	Sex Distribution (♂:♀)		5:1	3:3	3:3	3:3	

* Number of lambs used for each type

Table 3.1: Experimental design, block composition and animal sacrifice dates

Experiment was designed to evaluate immunological responses within the first 7 days following a secondary challenge with *H. contortus*. A total of 24 hair and 24 wool lambs were divided into blocks and assigned to times of sacrifice within blocks as described above.

	Hair Sheep				Wool Sheep			
	Days after infection				Days after infection			
	0	**3**	**5**	**7**	**0**	**3**	**5**	**7**
WBC	8998.3	9458.00	10316.7	10258.3	7530.0	8050.0	7873.3	6500.0
s.e.	(±740.5)	(±1144.1)	(±1636.4)	(±2016.7)	(±1141.97)	(±716.9)	(±1019.6)	(±400.7)
Neutrophils	3654.8	3196.2	3602.5	2486.2	1896.2	2176.8	2640.8	1381.0
s.e.	(±815.4)	(±273.6)	(±591.1)	(±309.5)	(±380.8)	(±343.3)	(±868.4)	(±229.9)
Lymphocytes	5002.0	5963.2	6213.8	7452.7	5211.3	5528.8	4890.3	4832.9
s.e.	(±360.7)	(±1145.9)	(±1183.3)	(±1988.7)	(±859.3)	(±548.0)	(±530.6)	(±320.7)
Monocytes	244.67	257.00	345.33	176.17	295.0	261.00	135.5	228.9
s.e.	(±23.50)	(±62.69)	(±59.93)	(±35.45)	(±81.47)	(±80.24)	(±36.8)	(±21.9)
Eosinophils	13.33	41.6	75.83	101.50	70.67	73.17	150.2	37.0
s.e.	(±13.33)	(±25.80)	(±40.12)	(±33.52)	(±14.80)	(±28.90)	(±92.42)	(±19.06)
Basophils	85.00	0	79.50	41.67	57.00	10.33	26.83	20.42
s.e.	(±31.89)		(±28.73)	(±29.94)	(±34.94)	(±10.33)	(±17.50)	(±13.24)

Table 3.2: Differential white blood cell (WBC) counts of peripheral blood samples

Circulating counts of neutrophils differed between breeds and, when averaged across all time points, were higher in hair sheep ($P < 0.05$). No differences were found in the other cell types either between breeds or among days ($P > 0.05$). Data in table are reported as cells/µl of whole blood. *s.e.* = the standard error of mean

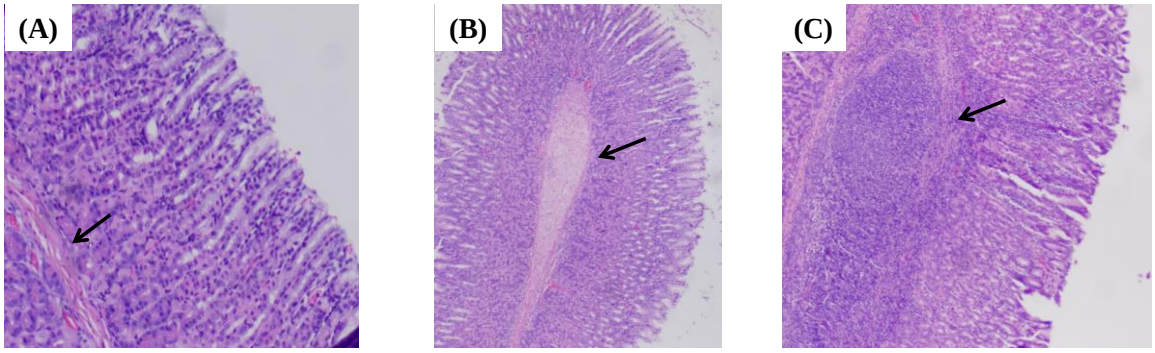

Figure 3.3: H&E stained cross-sections of the abomasal fundic region.

Uninfected sheep (A) at 10X magnification. By 7 d after infection few immune cells are migrating into the submucosa (B) in wool sheep. Hair sheep at 7 d after infection (C) had higher influx of cells into the submucosa and into the mucosal epithelium. Arrows indicate location of muscularis mucosae. Magnification in both panels (B) and (C) was 4X.

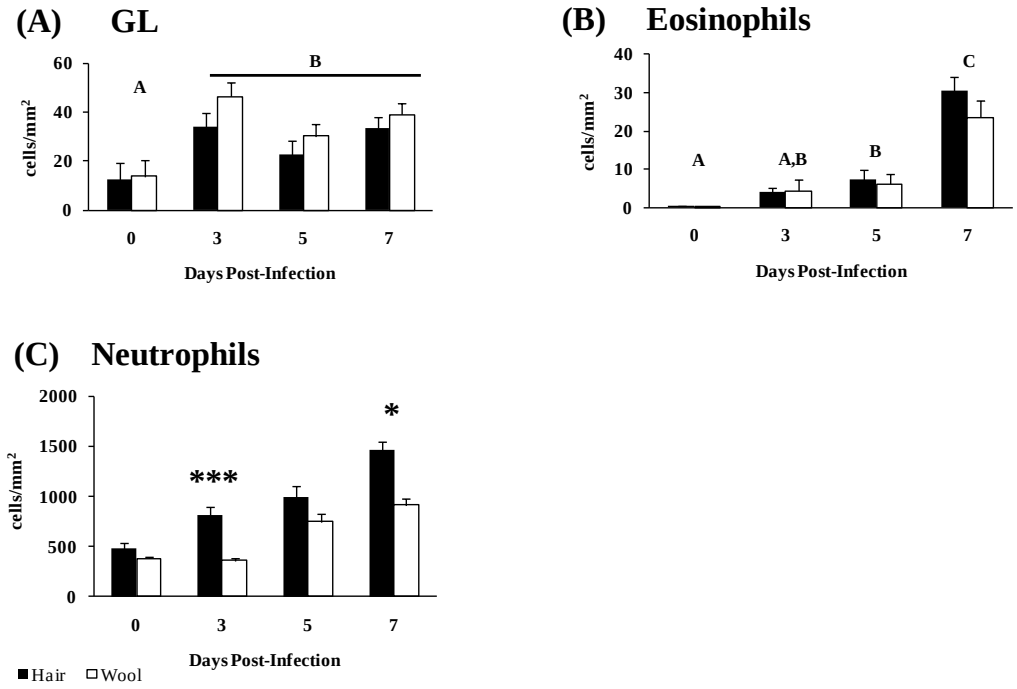

Figure 3.4: Composition of cellular infiltrate into abomasal mucosa

No significant effect of breed was observed for either globule leukocytes (GL) or eosinophils. A significant effect of day was found for GL (A) which increased by day 3 for eosinophils, which increased exponentially through day 7 (B). There was a highly significant day x breed interaction for neutrophil counts (C) in abomasal mucosa, hair sheep differed significantly from wool sheep at days 3 and 7. Means with different letters indicate a significant effect of day ($P < 0.05$). Asterisks indicate a significant effect of breed ($*P < 0.05$; $***P < 0.001$).

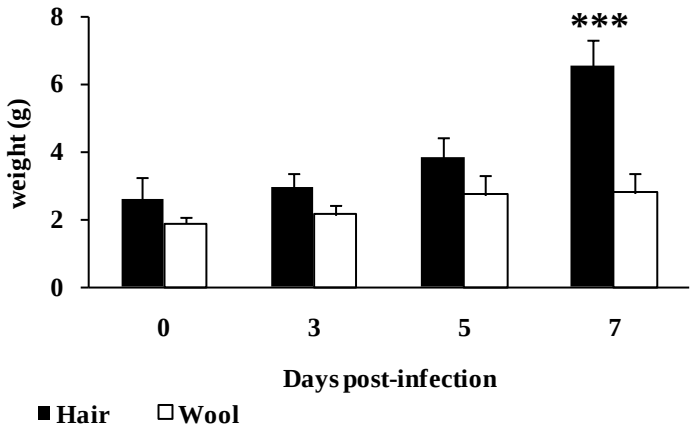

Figure 3.5: Abomasal lymph node (ALN) weight

All palpable lymph nodes were removed from the lesser curvature of the abomasum and weighed. A significant day x breed interaction was observed. LN weight of hair lambs increases significantly through day 7 compared wheras LN of wool lambs had no significant increase in weight following infection. Asterisks indicate breed type differences (***$P < 0.001$).

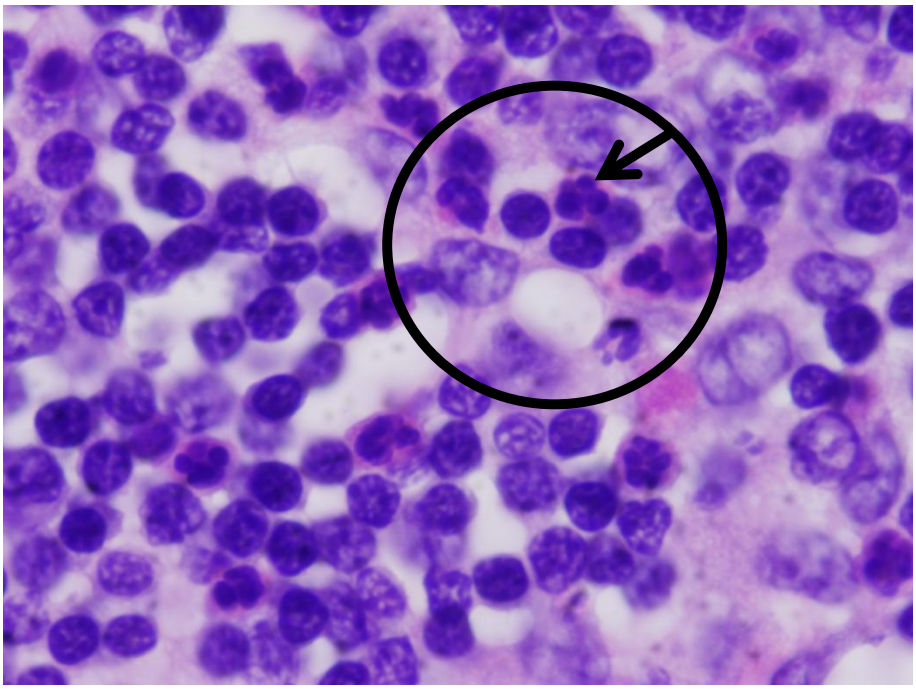

Figure 3.6: H&E stain of abomasal lymph node (400X)

Image above is from the paracortex of a hair lamb at day 7 after infection. Neutrophil appearing cells were seen in the ALN paracortex of all hair lambs at both d 5 and 7 but were never observed in wool lambs. Neutrophils are the multi-lobed cells with pink-staining cytoplasm. Black arrow points to neutrophil in close association with a lymphocyte.

Chapter 4 **: Differential gene expression and antibody responses between parasite-resistant and susceptible sheep during larval *Haemonchus contortus* infection.**

Abstract

Immunological events that accompany larval infection with *H. contortus* are anticipated to be key to understanding genetic differences of parasite resistance in sheep. Twenty-four parasite-resistant St. Croix hair lambs and 24 susceptible Dorset x Finnsheep-Rambouillet crossbred wool lambs were used in this study. Lambs were sacrificed at day 0 (uninfected lambs) or 3, 5, or 7 d after infection with 10,000 L_3 *H. contortus* larvae. No differences between breeds were observed for antibody levels in serum or abomasal mucus. The general pattern of gene expression in the abomasal lymph node (LN) was consistent with a typical T_H2 response for both breeds. In abomasal mucosa, the gene expression pattern indicated a potent localized T_H2 response in hair sheep, with elevated expression of IL-13 and IL-33 at 3 d after infection but suppression by day 5. IL-4 expression increased 40-fold from d 0 to d 7 ($P < 0.001$) in hair sheep and was higher than that observed in wool sheep at d 7 ($P < 0.05$). No changes in expression of IL-4, -13 or -33 in abomasal mucosa were observed in infected wool sheep. These data provide evidence of an earlier and more robust T_H2 response in the abomasal mucosa of hair sheep during the larval stage of gastrointestinal nematode infection. These up-regulated genes may be the cause of the generally greater immune response found in parasite-resistant sheep.

Keywords: *H. contortus*, cytokines, parasite resistance, gene expression

Introduction

Genetic differences between parasite resistant and susceptible sheep are proposed to be associated with genes related to immune function, and this hypothesis is supported by studies of quantitative trait loci associated with parasite resistance (Charon, 2004). Differences between breed types in immune responses to the adult parasite have been reported. Resistant sheep generally exhibit a more robust T_H2 response (Lacroux et al., 2006) and clear infection more rapidly than susceptible sheep (Shakya et al., 2009). Differences in the basic nature of immune responses in resistant and susceptible sheep have not, however, been observed during the adult parasite stage of infection. Analysis of immune function during the larval stage of infection may thus aid in identifying the underlying genetic differences between parasite-resistant and parasite-susceptible sheep.

A classic indicator of immune response to GIN parasitism has been elevated immunoglobulin concentrations in serum and at the site of infection. The antibody response to adult GIN parasite infection is higher both at secondary exposure (Balic et al., 2003) and in parasite-resistant sheep (Gill et al., 1993). Few studies have evaluated antibody production in the first 10 d after infection, but results to date suggest that serum IgE increases by 10 d after infection in resistant sheep (Shakya et al., 2009) and that IgA and IgG_1 in lymph plasma do not significantly differ between the primary and secondary parasite challenge (Pernthaner et al., 2006).

The expression of cytokines and other soluble-mediators can modulate many immune responses and can also initiate numerous cellular inflammatory responses (Budhia et al., 2006). The immune response to adult *H. contortus* is a T_H2 response with elevated levels of IL-4 and reduced levels of IFNγ. Typically IL-4, -5 and -13 are expressed at higher levels in abomasal mucosa during early secondary challenge (Pernthaner et al., 2006; Craig et al., 2007), and resistant sheep generally have greater IL-5, and-13 and TNFα in afferent intestinal lymph cells by 5 d after infection (Pernthaner et al., 2005). Suppression of T_H2 cytokine gene expression in the local LN during the first 5 d of GIN infection has been shown during the primary infection but not during secondary infection (Lacroux et al., 2006; Craig et al., 2007). Therefore, cytokine gene expression appears to be either up- or down-regulated between 3 and 7 d after infection, depending on the level of parasite resistance or on whether or not the animal is experiencing a secondary challenge. Many studies have documented that this is a period of noteable in T_H2 polarization and up-regulation of T_H2 cytokines (Pernthaner et al., 2005; Lacroux et al., 2006; Craig et al., 2007) up-regulation of T_H2 cytokines.

The objectives of this study were to measure early antibody production, determine rate of development of T_H2 response in the local lymph node, and evaluate cytokine and chemokine gene expression profiles in abomasal mucosa through 7 d after infection with *H. contortus* in parasite-resistant and susceptible sheep.

Materials and Methods

Animals, sample collection and RNA extraction

Details of the experimental procedures and parasite culture are outlined in chapter 3. Briefly, 24 hair and 24 wool lambs were randomly divided into 3 blocks and, within the blocks, lambs were sacrificed either 0, 3, 5, or 7 days after infection. For 1 wk before final infection, lambs were housed on elevated floors to prevent parasite exposure and then either left uninfected (day 0 group) or infected with 10,000 L_3 *H. contortus* larvae. Prior to sacrifice, blood samples were taken via jugular venipuncture and collected in 10ml vacutainer (BD, Franklin Lakes, NJ) tubes with no additive for serum analysis. Blood was centrifuged at 2,000 x *g* at 4^{o}C for 20 minutes. Serum was decanted and stored at -20^{o}C until analysis. Methods of infection, sample collection and euthanasia used in this study were approved by the Virginia Tech Institutional Animal Care and Use Committee.

All palpable lymph nodes were extracted from the lining of the lesser curvature of the abomasum. The largest lymph node was cut longitudinally and a 4mm slice was weighed and placed in 10 times volume-to-weight of RNA-later (Ambion, Austin, TX). The abomasum was then cut along the greater curvature and contents were removed and washed gently in PBS (pH 7.4). Mucus from one half of the abomasum was gently scraped with a glass slide, placed in a microcentrifuge tube, weighed, and mixed with an equal volume of PBS (pH 7.4) with 1% sodium azide (w/v). The mucus samples were then centrifuged at 12,000 x *g* for 30 minutes at room temperature. Supernatant was collected and stored at -20^{o}C until analysis. Abomasal

mucosa was collected by scraping a glass slide across the folds of the remaining one half of the abomasum and collecting only yellowish-white tissue without penetrating the muscularis mucosae. Mucosa samples were placed in a 5ml cryotube (Corning, Lowell, MA), weighed, mixed with 10 times volume-to-weight of RNA-later (Ambion, Austin, TX) and stored at -20^{o}C.

Total RNA was extracted from abomasal mucosa and lymph node tissue samples stored in RNA-later. A 20 to 30 mg sample of tissue was blotted dry and RNA extraction was conducted following the RNeasy Miniprep Kit (Qiagen, Valencia, CA) manafacturer's protocol. Total RNA was quantified using the Nano-Drop (Thermo Scientific, Waltham, MA) spectrophometry system and an absorbance ratio (A260:280) of greater than 1.8 was observed in all total RNA samples. This ratio is used to validate purity and to confirm that RNA samples were not contaminated with protein.

Immunoglobulin assays

To determine concentration of capture and detection antibodies, checkerboard titrations were performed using serum of an infected ewe. Capture antibodies, anti-sheep IgG_1 (Sigma-Aldrich, St. Louis, MO) and polyclonal anti-sheep IgA (Bethyl, Montgomery, TX), diluted to 1:100 in carbonate-bicarbonate buffer (pH 9.6) were placed in each well of a 96-well plate (Nunc, Rochester, NY) and incubated overnight at 4^{o}C. The plate was washed 3 times in PBS with 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO) and blotted dry. Plates were blocked using PBS with 0.1%BSA (Sigma-Aldrich, St. Louis, MO) and allowed to incubate for 1 hour at room temperature (RT). After another wash step, serum samples were diluted in PBS with

1%BSA at 1:400 or 1:1000 for serum IgG and IgA, respectively, or at1:20 for mucus IgA analysis. Diluted mucus and serum samples were added in duplicate to the plate, incubated for 2 hrs at RT and then washed. Detection antibodies (HRP conjugated), monoclonal IgG_1 diluted to 1:3,200 (Sigma-Aldrich, St. Louis, MO) and polyclonal anti-sheep IgA (Bethyl, Montogomery, TX) diluted 1:1,500, were added to the plate and incubated at RT for 1 hr in dark. After a final wash step, TMB substrate (Pierce, Rockford, IL) was used to produce a colorimetric response with the reaction allowed to develop for 20 min. Plates were read at 450 nm on a Biotek EL-311 spectrophotometer. For total IgG_1 and IgA, values were compared to a standard sheep IgG_1 (Sigma-Aldrich, St. Louis, MO) and IgA (Accurate Chemical Co., Westbury, NY).

Gene expression analysis

To synthesize first strand cDNA, 2 µg of total RNA was reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. cDNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) and then diluted to 10 ng/µl in RNase-free DEPC water and stored at -20^{o}C. Primer pair sequences used in this study (Table 4.1) were replicated from those of other experiments (Ingham et al., 2008) or designed based on ovine or bovine mRNA sequences. Primer sequences were designed using Primer Express software (Applied Biosystems, Foster City, CA), to span an intron-exon junctions to limit DNA amplification. Primer concentration was optimized by titrating primer pair combinations from a concentration of 200 nM to 900 nM, to determine the most appropriate primer concentration. In this experiment, forward and reverse primer concentration of 500 nM performed well, for all genes and tissues.

Real-time PCR was conducted in a total volume of 20 µl, using 2 µl of sample cDNA (10 ng/µl), 2 µl (5 µM) of each forward and reverse primer, 10 µl of Fast-SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and 4 µl of RNase-free DEPC water. Samples were loaded in duplicate into a 0.1 ml Fast 96-well optical plate (Applied Biosystems, Foster City, CA) and the real-time PCR reaction was carried out in an ABI 7500 Fast sequence detection system (Applied Biosystems, Foster City, CA). The reaction was carried out under the following conditions: 95^{o}C for 20 sec, 40 cycles of 95^{o}C for 3 sec and 60^{o}C for 30 sec, and 60^{o}C for 20 sec. A melt curve was run for each gene to ensure amplification of a single product. A non-template control, with RNase-free water, was used in place of cDNA template for each gene.

Statistical analysis

Either ΔC_T values or log transformed ELISA values were analyzed as a randomized complete block design using the general linear models procedure of SAS (SAS Institute Inc. Cary, NC) including effects of breed, day, block and all two- and three-way interactions. Due to occasional missing samples, an analysis for an unbalanced design was used. Means were compared using t-tests with critical probability values calculated using Tukey's adjustment for comparisons among more than two means. Values were considered significant when $P < 0.05$. Expression of selected genes was compared to expression of GAPDH, which was used as a constitutively expressed or house-keeping (HK) gene, in both tissues. The mean ΔC_T for each individual sheep was compared to the mean ΔC_T of their respective breed at day 0, resulting in $\Delta\Delta C_T$ values for each sheep at each day after infection. Direct interpretation of ΔC_T values is difficult so fold change ($2^{-\Delta\Delta C_T}$) was calculated for each infected lamb and averaged by day for each breed. All tests of significance were based on the analysis of ΔC_T values, but observed

standard errors of fold change means were also calculated for each day and breed type and used to provide a graphical indication of variation among means.

Results

Antibody production

Total IgG_1 levels in serum and IgA levels in both serum and mucus did not differ between resistant and susceptible sheep (Figure 4.1a). Breeds appear to differ slightly in serum IgG_1 and IgA at 3 d after infection, but these differences were not statistically significant.

Gene expression in abomasal lymph nodes

Gene expression analysis in the abomasal lymph nodes indicated no difference between the breeds in expression of cytokines or transcription factors; however, there was a significant effect of day for many genes (Figure 4.2). Development of a T_H2 response requires expression of transcription factor GATA-3 within T-cells in the presence of IL-4. IL-4 expression increased by d 5 ($P < 0.05$) in both breeds and was maintained to d 7, indicating initiation of T_H2 polarization (Figure 4.2a). Suppression of the key T_H1 cytokine IFNγ by day 7 ($P < 0.05$) and lack of differential IL-12p40 expression (Figure 4.2 b,d) contribute to T_H2 polarization and indicate that the T_H1 response is being down-regulated in both breeds. Gene expression of transcription factors also supports T_H2 polarization. There was a significant reduction of T-bet from d5 to d7 ($P < 0.05$), indicating T_H1 suppression by day 7, but no significant increase of GATA-3 was observed (Figure 4.2e,f).

Gene expression in the abomasal mucosa

Differential gene expression between the breeds in abomasal mucosa was observed for the T_H2 cytokines IL-4, IL-13 and IL-33 (Figure 4.3a,b,c). A highly significant day by breed interaction ($P < 0.01$) was observed for expression of IL-4. By 7 d after infection, IL-4 gene expression increased nearly 40-fold compared to d 0 (Figure 4.3a), and was significantly higher at day 7 after infection when compared to wool sheep ($P < 0.05$). The variation associated with IL-4 gene expression in hair sheep at day 7 was associated with one individual with extremely high levels of IL-4 expression. After removal of this outlier, the mean increase of IL-4 expression at day 7 in hair sheep was reduced to 16.65 fold and was no longer significantly different from that of wool sheep. Greater T_H2 polarization in hair sheep was also indicated by the early response of both IL-13 and -33 by d 3 after infection (Figure 4.3b,c). Up-regulation of IL-4 may have been enhanced by early expression of IL-13 and IL-33. Levels of IL-13 and -33 at d 3 did not differ from those at day 0, but both decreased significantly from d 3 to d 5 ($P < 0.001$). Expression of both these genes was likewise higher at d 3 in hair sheep compared to wool sheep ($P < 0.001$). In fact, wool sheep demonstrated no changes at all in IL-4, -13 or -33 over this period. Expression of T_H2 cytokine genes in the wool sheep was thus maintained at a level not different from that at d 0.

Expression of T_H1 cytokines and soluble mediators of inflammation were measured but revealed no differences between breeds or among days (Figure 4.4). Expression of IFNγ and IL-12p40 did not change from the expression levels observed at d 0 in both breeds (Figure 4.4a,c). The massive influx of immune cells to the abomasal mucosa during the early infection suggested gene expression analysis of inflammatory mediators. However, the chemokine IL-8 and pro-

inflammatory cytokine TNFα were not differentially expressed between breeds or among days (Figure 4.4b,d) and therefore may not be involved in cellular recruitment to the abomasal mucosa in response to this pathogen. The early immune response to GIN parasitism seems to suppress expression of classic T_H1 and pro-inflammatory cytokines allowing the development of an appropriate protective immune response.

Discussion

Some studies indicate increases in antibody production as the parasite develops to the adult stage and higher levels of circulating IgG_1 and IgA in sheep that are resistant or during an animal's secondary challenge (Pernthaner et al., 2006). IgA levels in 7-month old ewe lambs (Chapter 2) indicated that hair lambs had greater serum IgA than wool lambs, and this was anticipated to occur in this experiment. However, the trickle infection administered before the main parasite challenge may have primed the immune response of wool lambs, masking differences that might otherwise have been observed. No differences were observed in either IgA or IgG_1 concentrations between the breeds and thus may have been affected by the short time interval between trickle and experimental infection. Nonetheless, production of these antibodies during early infection may not be required for development of an appropriate T_H2 protective immune response.

Differences in cytokine profiles and between the abomasal mucosa and LN were not expected. Hair sheep developed a more rapid and stronger T_H2 response at the site of infection but no difference was found in T_H2 cytokine expression in the LN. This result suggests that the immune response is focused in the abomasal mucosa during the early infection. A similar situation was reported in IL-4 knockout mice infected with *Nippostrongylus brasiliensis,* in

which immune polarization towards a T_H2 response was maintained in the LN when compared to wild-type mice (Liu et al., 2007). In the LN, a step-wise increase in expression of IL-4, IL-13 and IFNγ occurred though day 5 but by 7 d only IL-4 expression remained elevated, indicating a consistent T_H2 response in local lymph nodes of both breeds.

The increased expression of IL-13 in the abomasal mucosa of hair sheep at day 3 may be partially responsible for the influx of immune cells to the site of infection. Although less important than IL-4 in T- and B-cell responses, IL-13 has been implicated in smooth muscle function, and has been associated with expulsion of GIN (Finkleman et al., 1999; Anthony et al., 2007). Hair sheep also had greater expression of IL-33 at day 3. IL-33 is a member of the family of IL-1 cytokines and has been reported to induce IgE-independent production of IL-13 by mouse mast cells (Ho et al., 2007). IL-33 can also operate in combination with thymic stromal lymphopoietin (TSLP) to maximize T_H2-associated cytokines (Allakhverdi et al., 2007). In helminth infections, IL-33 functions as a chemoattractant for T_H2 cells enhancing IL-4, -5 and -13 production and causing changes in mucosal tissue that are considered to be IL-13-dependant (Schmitz et al., 2005). In *Trichuris muris* infections in mice, IL-33 expression in cecal mucosa increased significantly by 3 d after infection (Humphreys et al., 2008) but declined dramatically after that time. Data from this experiment displayed a similar trend and are consistent with the postulate that IL-33 expression by innate immune cells may potentiate establishment of a T_H2 immune environment in the abomasal mucosa.

Thymic stromal lymphopoietin (TSLP) also plays an important role in GIN parasitism. In the mouse, TSLP is produced by epithelial cells and interacts with dendritic cells (DC) to regulate DC homing properties, surface molecule expression, and production of pro-inflammatory cytokines (Artis and Grencis, 2008). Due to the role of TSLP in influencing local

immune responses, TSLP gene expression was assessed in the abomasal mucosa, but measureable levels were not detected. It was concluded that PCR conditions may not have been appropriate or there was a lack of TSLP expression in both breeds. In recent studies, TSLP was reported to be differentially expressed throughout the GI tract and not typically expressed at high levels in the stomach and small intestine of mice (Taylor et al., 2009). A second study recently suggested that TSLP may be a redundant cytokine during helminth infection whose only role is to suppress IL-12 production and thereby promote T_H2 responses (Massacand et al., 2009).

The most striking result of this experiment is the profound increase in IL-4 gene expression in the abomasal mucosa of infected hair sheep. Cells that produce IL-4 include eosinophils, mast cells (Artis and Grencis, 2008), alternatively activated macrophages (Anthony et al., 2006), T-cells (Miller and Horohov, 2006) and neutrophils (Nathan, 2006). The cell-surface marker granulocyte differentiation antigen 1 (Gr1) is expressed on both neutrophils and macrophages (Culshaw et al., 2008). Recent data suggest that $Gr1^+$IL-4-producing innate immune cells are involved in the development of a protective T_H2 response (McKee et al., 2008) and thus the type of cell producing IL-4 at the site of infection remains unclear.

Up-regulation of IL-4, -13 and -33 in the abomasal mucosa of hair sheep during the early stage of GIN infection provides more information about the development of immunological resistance in these animals. This early immune response may be responsible for expelling parasites and/or affecting the fecundity of the adult parasite, resulting in lower fecal egg counts and shortened duration of the patent infection. The failure of wool sheep to express either a T_H1 or T_H2 cytokine environment in the gut mucosa may indicate that the immune response in these animals was suppressed, thereby increasing the duration of infection.

Data from this experiment demonstrate significant up-regulation of T_H2 cytokine expression in the abomasal mucosa of hair sheep, but no such changes were observed in wool sheep. Up-regulation of IL-4 in hair sheep may have been augumented by the early expression of IL-13 and IL-33, as these two cytokine genes were suppressed in wool sheep. To further characterize immune responses to the larval parasite, analysis of additional cytokines may be warranted. Expression of IL-6, -10, -17 and TGFβ as well as alternatively activated macrophage products such as arginase-1 and FIZZ may be involved in the polarization immune responses of parasite-resistant sheep and require further investigation.

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Gene	GenBank Accession	Forward Sequence*	Reverse Sequence*	Amplicon T_m (°C)
GAPDH†	DQ152956	CCTGGAGAAACCTGCCAAGT	GCCAAATTCATTGTCGTACCA	81.56
GATA-3†	DQ152958	TCACCACCCCTCCAGTATGG	ATGATACTGCTCCTGCGAAAATG	82.61
IL-4†	AF172168	GAGCTGCCTGTAGCAGACG	TCGTCTTGGCTTCATTCACA	80.57
IL-8‡	X78306	AGAAGTCCTCTGGGACAGCA	CACACCTCTTTTCCGTTGGT	79.61
IL-12p40†	DQ152965	ATTGAGGTCGTGATGGAAGC	TTGTTCTTTCCCTGGACCTG	80.02
IL-13‡	DQ679798	ACACCTGGTGGAGGAGACAC	TGGCTGTCAGACAAGAGTGG	81.59
IL-33†	BC123562	CCCAAGTTGAGAAAATCCCA	GGTTTGTTCTGGCAACTGGT	81.53
IFNγ†	DQ152961	TAAGGGTGGGCCTCTTTTCT	CATCCACCGGAATTTGAATC	76.22
T-BET†	DQ152994	CCCACCATGTCCTACTACCG	GGACACACGCCTCCTCTTAG	85.76
TNFα†	DQ153000	CAGGGCTCCAGAAGTTGCT	GGGCTACCGGCTTGTTATTT	82.85

* Sequences are listed in 5'- 3' direction

† Published primer sequences (Ingham et al., 2008)

‡ Primer sequences were obtained using Primer Express Software (Applied Biosystems) based on ovine mRNA sequence for IL-8 and bovine mRNA sequence for IL-33

Table 4.1: Real-time PCR primer sequences and amplicon melting points

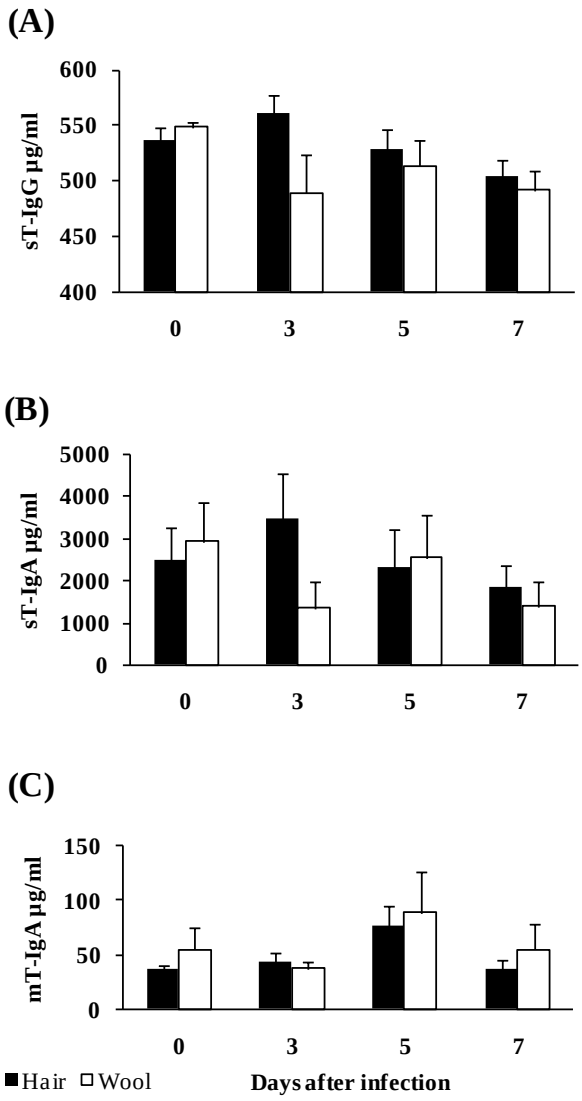

Figure 4.1: Levels of serum and mucus antibodies during the first 7d following infection

Total serum IgG_1 (A), serum IgA (B) and abomasal mucus IgA (C) were determined using ELISA No significant differences were observed between breed types or among days.

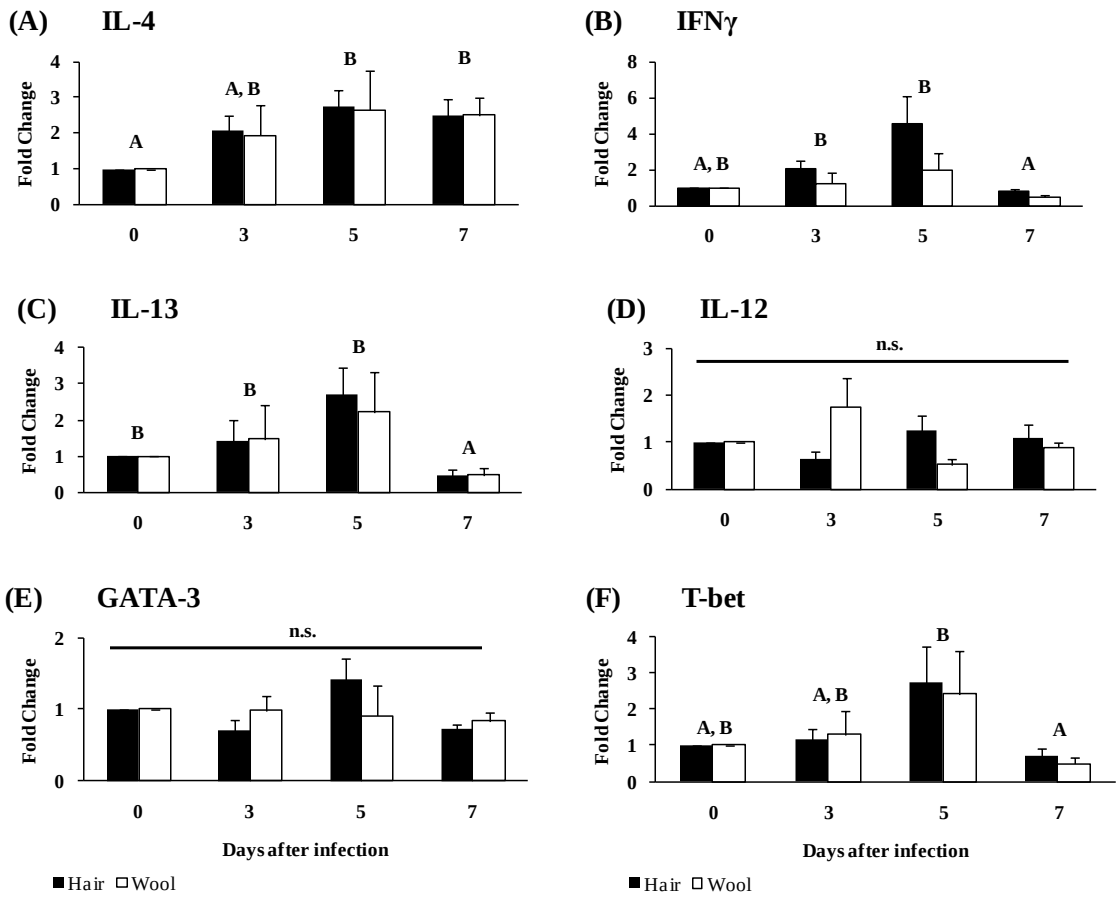

Figure 4.2: Differential gene expression in abomasal lymph node

Day means with different letters are significantly different ($P < 0.05$). No significant breed differences or breed x day interactions were observed. Error bars represent standard errors of means and were derived directly from individual fold-changes for each lamb relative to the mean of that breed at day 0. .

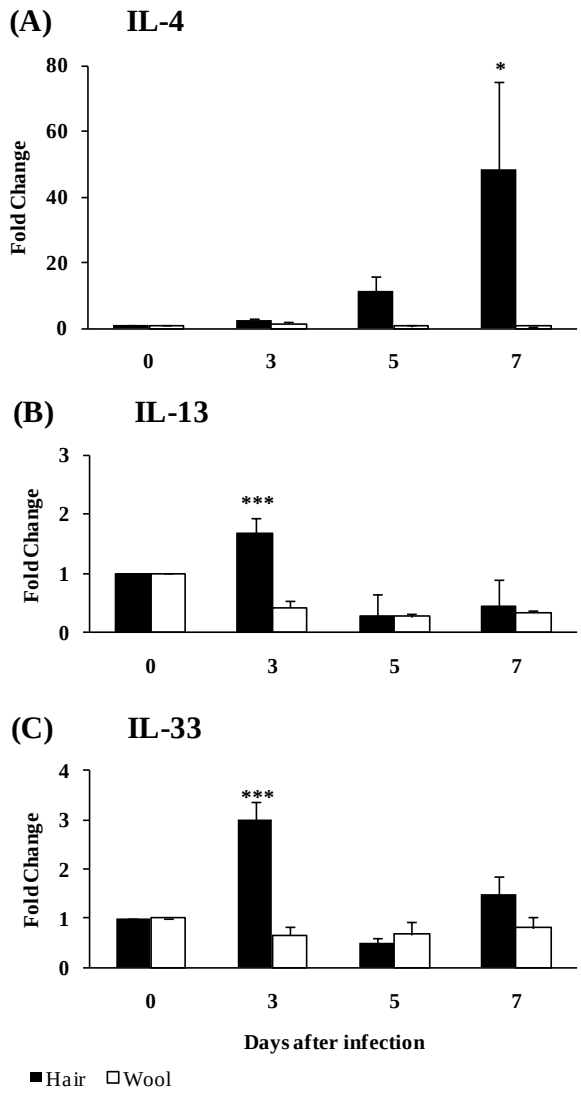

Figure 4.3: Differential T_H2 cytokine gene expression in the abomasal mucosa

Significant day x breed interaction was observed for all cytokines. Breeds differed among days at $P < 0.05$ (*) or $P < 0.001$ (***). Error bars represent standard errors of means and were derived directly from individual fold changes for each lamb relative to the mean of that breed at day 0

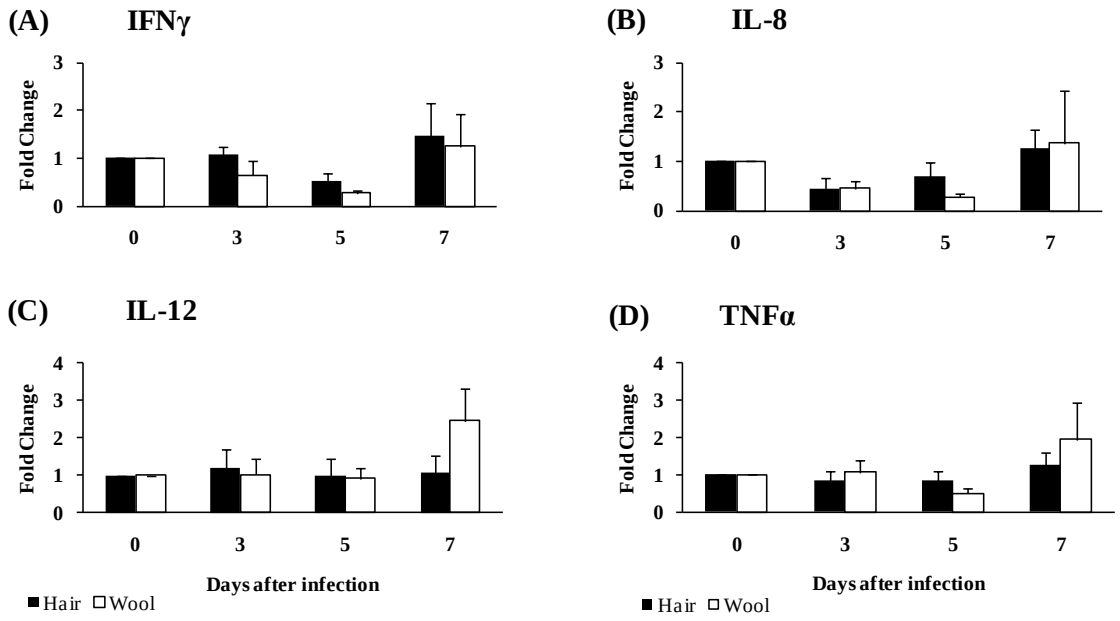

Figure 4.4: T_H1 and pro-inflammatory cytokine gene expression in abomasal mucosa

No significant differences were observed between breeds or among days. Error bars represent standard errors of means and were derived directly from individual fold changes for each lamb relative to the mean of that breed at day 0.

Chapter 5 **: Summary and identification of future areas of research**

Parasite-resistant Caribbean hair sheep develop a greater acquired immune response upon secondary infection. The magnitude of these responses during adult parasite infection was hypothesized to be a function of immune responses during larval infection. Characteristics of immunity to the larval parasite have not been clearly defined between parasite-resistant and susceptible sheep. Within the first 7 d after infection with *Haemonchus contortus*, hair sheep have greater cellular infiltration to the abomasal mucosa, abomasal lymph node development, and expression of cytokines IL-4, -13 and -33 in the abomasal mucosa. The combination of these events results in development of a highly polarized T_H2 response in the abomasum, yielding an inhospitable environment for development of *H. contortus*. Failure to observe differences in antibody levels within the first 10 d following infection does not preclude their involvement during immune response to the adult parasite. However, immune responses to the larval parasite appear to primarily involve cellular rather than humoral immunity.

The greater T_H2 response observed in hair sheep warrants a detailed analysis of contributing immunological events occurring in the abomasal mucosa. Specific identification of cellular infiltrate, via immuno-histochemistry, would better characterize the cellular influx into the abomasal mucosa. Antibodies to cell surface markers that specifically identify neutrophils, macrophages, eosinophils, and T and B-cells would aid in the localization of these cells in the abomasal mucosa and sub-mucosa. Measurement of additional cytokine production would potentially better characterize T_H cell polarization. Characterization of expression levels for

cytokines such as CCL5, IL-6, IL-10, IL-17 and TGFβ would provide more information about the development of the T_H2 response. Analysis of adhesion molecules belonging to the integrin and selectin families could verify the migration of immune cells to the site of infection. A specific lineage of macrophages has been identified which is exclusively involved in immune responses to helminth parasites. These cells produce specific products and therefore gene expression analysis of arginase-1 and FIZZ may illuminate the role of these cells in helminth infection of sheep.

Failure of general immune responses of wool sheep within the first 7 d after *H. contortus* infection cannot be ignored and may serve as a therapeutic target. Consistently reduced immune responses over a variety of immunologic measurements indicate that wool sheep either experience immune suppression due to antigen-masking by the parasite larvae or lack ability to recognize parasite antigen. This may be a scenario where immunomodulation may aid host ability to mount an effective immune response. Administration of an immunomodulator prior to parasite exposure may improve immune responses. Successful application of immunomodulation would allow continuation of forage resource utilization by commercial breeds of sheep without the incorporation of parasite-resistant sheep.

Characterization of immune response to larval parasite infection has not yielded the identification of a selectable marker or a single key candidate gene for parasite resistance in sheep. Genetic analysis of toll-like receptor expression has been implicated in differences between parasite-resistant and susceptible sheep in the southern hemisphere but analysis has yet

to be conducted on St. Croix hair sheep. Analysis of diversity within the MHCII complex may reveal genetic differences between types and also warrants further study.

The impetus of this dissertation was to assist sheep producers in the southeastern US, with the management of GIN parasitism. Results of this project are not directly applicable to the producer, but the parasite-resistance of hair sheep has been further confirmed and maintains their potential in cross-breeding programs to reduce the effects of parasitism. This particular result does not aid the purebred producer as they remain in wait for a selectable marker. However, improved characterization of immune responses to *H. contortus* may assist in detection of genes that are differentially expressed and that warrant further detection and evaluation of polymorphisms associated with those genes that may serve as selectable markers. The future of selection of parasite-resistant sheep will therefore benefit from a more complete knowledge of immune responses to GIN infection.