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Exploring the transcriptomic changes underlying recombinant vaccine efficacy against *Teladorsagia circumcincta* in 3-month-old lambs

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ABSTRACT

Teladorsagia circumcincta is an abomasal parasitic nematode that can cause serious issues in small ruminant production, which are aggravated by drug resistance. Vaccines have been suggested as a feasible, long-lasting alternative for control since adaptation to the host's immune mechanisms by helminths develops at a much slower pace than anthelmintic resistance. Recently, a *T. circumcincta* recombinant subunit vaccine yielded over a 60% reduction in egg excretion and worm burden and induced strong humoral and cellular anti-helminth responses in vaccinated 3-month-old Canaria Hair Breed (CHB) lambs, but Canaria Sheep (CS) of a similar age were not protected by the vaccine. Here, we compared the transcriptomic profiles in the abomasal lymph nodes of such 3-month-old CHB and CS vaccinates 40 days after infection with *T. circumcincta* to understand differences in responsiveness at the molecular level. In the CS, differentially expressed genes (DEG) identified were related to general immunity processes such as antigen presentation or antimicrobial proteins and down-regulation of inflammation and immune response through regulatory T cell-associated genes. However, upregulated genes in CHB vaccinates were associated with type-2 oriented immune responses, i.e., immunoglobulin production, activation of eosinophils, as well as tissue structure and wound repair-related genes and protein metabolism pathways such as DNA and RNA processing. These results highlight potentially more optimal timing and orientation of immune responses in CHB sheep compared to CS associated with vaccine-induced protection. The data obtained in this study thus deepens our understanding of variations in responsiveness to vaccination in young lamb and provides insights for vaccine refinement strategies.

1. Introduction

Climate change-driven expansion of diseases such as gastrointestinal nematode (GIN) infections threatens the profitability of ruminant livestock farming (Mavrot et al., 2015), contributing to food insecurity in an increasing human population scenario (Fitzpatrick, 2013). The pathogenic effects of GIN and their ability to induce economic loss is particularly concerning in lamb production (Nieuwhof and Bishop, 2005). At the same time, we have historically relied on anthelmintics as the main control strategy, but their efficacy has been steadily decreasing due to an increase in drug resistance in nematode populations across the globe (Gilleard et al., 2021).

Vaccines are considered a sustainable alternative because of their long-lasting protective effect in the host without residues in the final product. However, GIN vaccine development has been slow since the 1970s, yielding one commercially available vaccine based on native *Haemonchus contortus* antigens: Barbervax® (Nisbet et al., 2016b). The main constraints to vaccine development have been attributed to: 1) difficulty in expressing complex nematode antigens as recombinant proteins, limiting product standardization and safe, large-scale production; and 2) variability in host protective immune responses, which may be influenced by physiological status, nutrition, genetic variation, sex, and age (Nisbet et al., 2016b).

Young lambs have a limited ability to mount full anti-nematode

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responses during their first grazing season when their immune system is still maturing (Greer and Hamie, 2016; Stear et al., 2000). Aligned with this, is the observation that 3–6-month-old vaccinated lambs of certain breeds develop incomplete immune responses that may not control worm infection (Kooyman et al., 2000; Nisbet et al., 2013), which can lead to clinical symptoms and subsequent economic loss. A recent study demonstrated that a recombinant subunit *T. circumcincta* vaccine induced a protective immune response to helminth infection in 3-month-old resistant Canaria Hair Breed (CHB) lambs, while stabilizing lamb growth during infection (Pérez-Hernández et al., 2022). In contrast, age-matched susceptible Canaria Sheep (CS) counterparts were not protected by vaccination. Pinpointing the mechanisms behind protective responses to vaccination in young sheep is thus critical to improve vaccination strategies in young sheep of different breeds. In this sense, transcriptomic analysis has been successfully used to explore and compare changes in gene expression during GIN infection between different sheep lineages or breeds with marked phenotypic differences in their susceptibility to GIN (Aboshady et al., 2020; McRae et al., 2016). In this study, we explored the differences in the transcriptomic signatures of CHB and CS vaccinates after infection with *T. circumcincta* by comparing their gene expression profiles in draining abomasal lymph node tissue samples.

2. Materials and methods

2.1. Experimental design

The vaccination and challenge protocol followed in this study has previously been published in detail (Pérez-Hernández et al., 2022). Briefly, 3-month-old CHB (N = 24) and CS (N = 24) were purchased, dewormed on arrival, and kept in conditions that prevented strongyle infection. The study was designed as a 2 (breed) x 2 (treatment) factorial arrangement, assigning animals randomly to vaccinated (CHB-VAC, CS-VAC) or control (CHB-Control, CS-Control) groups (N = 12). During the study, two animals in group CHB-VAC were excluded from the experiment because of reasons not related to the procedure. For this study, only the data obtained from CHB (N = 10) and CS (N = 12) vaccinated (VAC) groups were used.

The prototype recombinant vaccine, described previously by (Nisbet et al., 2013), consisted of a cocktail of 8 recombinant proteins. The protein mixture (400 μg) was administered with Quil A and PBS on days 0, 21 and 42 of the experiment. Simultaneously, control groups received PBS/Quil A injections. From day 42 until day 68, sheep were infected orally with *T. circumcincta* 2000 L3 three times a week (Nisbet et al., 2013). On days 82–85, animals were euthanised to collect abomasal lymph node samples, which were stored in E.Z.N.A. RNA Lock Reagent (Omega Bio-tek) at − 80 °C prior to RNA extraction.

2.2. RNA extraction and sequencing

The extraction protocol has been described in Pérez-Hernández et al., 2022. Briefly, total RNA was extracted from lymph node samples using RNeasy mini-isolation kits (Qiagen Ltd, UK) following the manufacturers' protocol. RNA quantity and integrity were assessed prior to sequencing on an Illumina HiSeq 4000 at The Centre for Genome Research (CGR) at the University of Liverpool, UK, generating 2 × 150 bp strand-specific, paired-end reads.

2.3. RNA-seq quality control and alignment

R version 3.4.4 was used for the RNAseq data quality control (QC) based on samples obtained from CHB and CS vaccinates with the array QualityMetrics package. Sequences were processed, then aligned to the *Ovis aries* genome assembly Oar_v3.1 (GCA_000298735.1) using the STAR aligner and the numbers of mapped read pairs counted based on the *Ovis aries* genome annotation (Ensembl release 91), also within STAR (Dobin et al., 2013). Following data QC, a subset of 18 RNAseq samples (CHB: N = 9; CS: N = 9) was considered for further analyses.

2.4. Statistical analysis

For the RNAseq analysis, count data for the samples were normalized using TMM (Trimmed Mean of M-value) and transformed with VOOM (Law et al., 2014) to log2-counts per million with associated precision weights. A comparison between CHB vaccinates versus CS vaccinates in abomasal lymph node tissue was performed using linear modelling. Subsequently, empirical Bayesian analysis was applied including adjustment for multiple testing, which controls for false discovery rate (FDR) based on an FDR p-value cut-off of < 0.01 and a 1.2-fold change in gene expression. The null hypothesis was that there was no difference between the groups being compared. The Bioconductor package, Limma (Ritchie et al., 2015), was used to identify differentially expressed genes and gene lists were then annotated based on the *Ovis aries* genome, with further manual curation of genes to the gene symbol level.

Gene network analysis annotations of the differentially expressed genes (DEG) identified in the CHB versus CS vaccinates in abomasal lymph node tissue were manually obtained using different tools. DAVID v6.8 ("DAVID: Database for Annotation, Visualization and Integrated Discovery," 2021) was used to annotate some gene symbols of *Ovis aries* species. The remaining gene symbols were studied through Ensembl Oar v3.1 (Ensemble, 2022), Uniprot ("UniProt: Universal Protein Knowledgebase," 2021) and BLAST ("BLAST Tool: Basic Local Alignment," 2021), whilst biological process, cellular component and molecular function were investigated using GeneCards webpage ("GeneCards: The Human Gene Database," 2021).

2.5. Pathway analysis

To explore potential functions of the DEG in the vaccinated CHB and the CS breeds, a cluster annotation analysis was performed using the CTD bioinformatics tool ("CTD: The Comparative Toxicogenomics Database," 2022), classifying these genes into pathways using KEGG ("KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway Database," 2022) and the REACT ("Reactome Pathway Database," 2022) directories.

3. Results

3.1. Parasitology and immunology

The results of the effect of the vaccine on parasitological and immunological parameters have been described in detail previously (Pérez-Hernández et al., 2022). In brief, the *T. circumcincta* recombinant sub-unit vaccine elicited protection in CHB lambs but not in CS lambs. Vaccinated CHB had significant lower egg excretion in faeces than vaccinated CS (CHB: 711 ± 254 EPG; CS: 4278 ± 1037 EPG) ($p < 0.05$) and lower abomasal worm counts (CHB: 1147 ± 374; CS: 4613 ± 652) ($p < 0.05$) than vaccinated CS. Worms were also significantly shorter (CHB: 7.75 ± 0.1 mm; CS: 7.95 ± 0.07 mm) ($p < 0.01$) and had fewer eggs in utero (CHB: 11 ± 0.48 eggs in utero; CS: 15 ± 0.6 eggs in utero) ($p < 0.01$) in the CHB vaccinates. The expression of antigen-specific IgA and IgG2, combined with the presence of $CD4^+$, $CD8^+$, $CD45RA^+$ cells and globule leukocytes in the abomasal wall, were associated with this protective effect of the vaccine in the CHB lambs. On the other hand, in CS lambs, antigen-specific IgG1 levels and $CD45RA^+$ cells, globule leukocytes, mast cells were negatively associated with parasitological parameters (Pérez-Hernández et al., 2022).

3.2. Transcriptomic analysis

A total of 239 significantly DEG were identified between vaccinated CHB and CS lamb lymph node tissue after establishing an FDR-adjusted

p-value threshold of < 0.01 and a FC cut-off of > 1.2. When comparing breeds, 91 DEG were upregulated in the vaccinated CHB lambs compared to CS vaccinates (Additional file 1) and 148 DEG were upregulated in the vaccinated CS lambs in comparison with CHB vaccinates (Additional file 2).

In the CHB group a considerable number of upregulated genes were immune-related. For instance, several genes identified are known to be involved in the regulation of the immune system, such as antigen presentation through Class I Major Histocompatibility Complex (MHCI) molecules (RNF14, HECW2, ASB2, BTNL2) and activation of type I interferon responses (TMEM173). Similarly, other upregulated genes had a role in direct killing of infectious and parasitic agents (for example, SC5, LYZ, HMGN2, SCG2 genes). Genes regulating immunity (HGSNAT) were also upregulated in the CHB lambs, with one set of genes involved in up-regulation of inflammatory processes (GBP1, GBP2, MTHFD2, TCAF2, SLCO3A1, HS1BP3, MGB1, IL-36β), whereas others had anti-inflammatory functions (BTNL2, OSGIN1, RGS22, NLRP12, RPL13A). Genes encoding lymphocyte (LY6L) antigens, T cell receptor (TCR) (TCRB- T cell beta chain, TCRG- T cell Gamma chain) and immunoglobulins (JCHAIN, FCGR2A- Fc Gamma Receptor IIa) were highly expressed in this breed. A further set of identified DEG was related to effector cells (MBP2, GNLY), or had tissue structure and wound repair-related functions, such as keratinocyte differentiation (ETV4), protection of the epithelial barrier (SERPINB12), angiogenesis (ECM1) and fibrinolysis (PLAU).

In the CS breed, a considerable proportion of the identified upregulated DEG were involved in general immune processes such as antigen presentation (RNF144B, ABCC4, HLA-B- Class I MHC antigen B alpha chain, SLA-DQA- Class II MHC antigen DQ alpha 1 chain) and damage to infectious and parasitic agents (CST9L, DUOXA2, PRSS2, DEFB104A). Also identified as DEG, were up-regulated genes participating in inflammation and immune regulation and signaling (GPNMB, PTEN), with some genes involved in pro-inflammatory (FANCC, RHEBL1, PELI3, GBP1, MSMP, USP29, MAPK10, GBP4) and anti-inflammatory processes (LIPH, CSMD1, RNF144B, RASAL3, PARG, ALOX15B, LCTL, MYADML, CST9L). Also, some genes encoding αβ (TCRA, TRAV24, TCRB), γδ (TCRD, WC1, CD163L1) and T reg (ART1) population markers were upregulated. Also, NK cell (B3GAT1) markers, B-cells (TLE3, DLK1) and immunoglobulin components (IGKV2D-26) were found to be highly expressed in this group. A further subset of genes involved in maintaining tissue architecture were also upregulated in the CS lambs (MYADML & AFDN).

In the cluster annotation analysis, a total of 35 and 21 pathways were obtained from the DEG in CHB and CS lambs, respectively. In CHB lambs, the clusters with the largest numbers of genes were associated with the immune system, neutrophil degranulation, the innate immune system, metabolism of proteins and metabolism (Table 1). Similarly, in the CS lambs, a high proportion of DEG were clustered in the immune system, the innate immune system, metabolism and signal transduction (Table 2).

4. Discussion

Developing stable, long-lasting protection against *T. circumcincta* for lambs to reduce the economic impact of this disease and ensure animal welfare has been a long-term driver for vaccine development against this parasitic nematode. However, attempts to protect young lambs by vaccination against challenge have yielded inconsistent results due to difficulties in inducing appropriate protective, immune responses after vaccination in various breeds. The recombinant prototype vaccine against *T. circumcincta* has been found to induce significant protection against challenge in periparturient Texel crossbred ewes (Nisbet et al., 2016a) and in lambs (Nisbet et al., 2013), albeit with considerable age-dependent variability in vaccine efficacy (Liu et al., 2022). An exception to this was the favourable outcome obtained previously in 3-month-old CHB lambs, a GIN-resistant breed (González et al., 2008),

Table 1
Enriched pathways identified from differentially expressed genes (DEG) in CHB lambs in comparison with CS lambs, both vaccinated with a recombinant subunit vaccine against *Teladorsagia circumcincta* and subsequently challenged with the parasite (N = 9).

Pathway	Pathway ID	Annotated Genes Quantity	Annotated Genes
Immune System	REACT:R-HSA-168256	17	ASB2, BTNL2, DNAJC3, FCGR2A, GBP1, GBP2, GNLY, HECW2, HGSNAT, IL36B, LYZ, PLAU, PRG3, RNF14, SERPINB12, STING1, TKFC
Neutrophil degranulation	REACT:R-HSA-6798695	8	DNAJC3, FCGR2A, HGSNAT, LYZ, PLAU, PRG3, SERPINB12, STING1
Viral mRNA Translation	REACT:R-HSA-192823	4	DNAJC3, RPL13A, RPL7, RPS26
Influenza Viral RNA Transcription and Replication	REACT:R-HSA-168273	4	DNAJC3, RPL13A, RPL7, RPS26
Influenza Life Cycle	REACT:R-HSA-168255	4	DNAJC3, RPL13A, RPL7, RPS26
Innate Immune System	REACT:R-HSA-168249	10	DNAJC3, FCGR2A, GNLY, HGSNAT, LYZ, PLAU, PRG3, SERPINB12, STING1, TKFC
Influenza Infection	REACT:R-HSA-168254	4	DNAJC3, RPL13A, RPL7, RPS26
NOD-like receptor signaling pathway	KEGG: hsa04621	4	GBP1, GBP2, NLRP12, STING1
Ribosome, eukaryotes	KEGG: hsa_M00177	3	RPL13A, RPL7, RPS26
Peptide chain elongation	REACT:R-HSA-156902	3	RPL13A, RPL7, RPS26
Selenocysteine synthesis	REACT:R-HSA-2408557	3	RPL13A, RPL7, RPS26
Eukaryotic Translation Termination	REACT:R-HSA-72764	3	RPL13A, RPL7, RPS26
Eukaryotic Translation Elongation	REACT:R-HSA-156842	3	RPL13A, RPL7, RPS26
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	REACT:R-HSA-975956	3	RPL13A, RPL7, RPS26
Formation of a pool of free 40 S subunits	REACT:R-HSA-72689	3	RPL13A, RPL7, RPS26
Metabolism of proteins	REACT:R-HSA-392499	10	ABCA3, CNIH2, DNAJC3, GNG8, LYZ, RPL13A, RPL7, RPS26, UGGT2, WIPI1
SRP-dependent cotranslational protein targeting to membrane	REACT:R-HSA-1799339	3	RPL13A, RPL7, RPS26
GTP hydrolysis and joining of the 60 S ribosomal subunit	REACT:R-HSA-72706	3	RPL13A, RPL7, RPS26
L13a-mediated translational silencing of Ceruloplasmin expression	REACT:R-HSA-156827	3	RPL13A, RPL7, RPS26
Nonsense-Mediated Decay (NMD)	REACT:R-HSA-927802	3	RPL13A, RPL7, RPS26
Nonsense Mediated Decay (NMD) enhanced by the Exon	REACT:R-HSA-975957	3	RPL13A, RPL7, RPS26

(*continued on next page*)

Table 1 (*continued*)

Pathway	Pathway ID	Annotated Genes Quantity	Annotated Genes
Junction Complex (EJC)			
Selenoamino acid metabolism	REACT:R-HSA-2408522	3	RPL13A, RPL7, RPS26
Cap-dependent Translation Initiation	REACT:R-HSA-72737	3	RPL13A, RPL7, RPS26
Eukaryotic Translation Initiation	REACT:R-HSA-72613	3	RPL13A, RPL7, RPS26
Ribosome	KEGG: hsa03010	3	RPL13A, RPL7, RPS26
Hemostasis	REACT:R-HSA-109582	5	AK3, ECM1, GNG8, JCHAIN, PLAU
Infectious disease	REACT:R-HSA-5663205	4	DNAJC3, RPL13A, RPL7, RPS26
IRE1alpha activates chaperones	REACT:R-HSA-381070	2	DNAJC3, WIPI1
Major pathway of rRNA processing in the nucleolus and cytosol	REACT:R-HSA-6791226	3	RPL13A, RPL7, RPS26
Metabolism	REACT:R-HSA-1430728	10	BDH1, CYP2J2, DPYS, GNG8, GNPDA1, MTHFD2, RPL13A, RPL7, RPS26, TKFC
RIG-I-like receptor signaling pathway	KEGG: hsa04622	2	STING1, TKFC
rRNA processing	REACT:R-HSA-72312	3	RPL13A, RPL7, RPS26
rRNA processing in the nucleus and cytosol	REACT:R-HSA-8868773	3	RPL13A, RPL7, RPS26
Translation	REACT:R-HSA-72766	3	RPL13A, RPL7, RPS26
XBP1(S) activates chaperone genes	REACT:R-HSA-381038	2	DNAJC3, WIPI1

where immunization impacted egg excretion, abomasal worm establishment and yielded shorter and less prolific worms (Pérez-Hernández et al., 2022). Vaccination did not lead to reductions in these parasitological measurements in 3-month-old CS lambs, a more GIN-susceptible breed. After analyzing the immune responses at the infection site, these differences seem to be due to a more rapid immune response in vaccinated CHB group, in which $CD4^+$ and $CD8^+$ T lymphocytes are likely to play a role in coordinating a protective response, along with globular leukocytes, B lymphocytes, and *T. circumcincta* specific IgA and IgG2 (Pérez-Hernández et al., 2022). However, there is still a lack of knowledge about the dynamics of the immune and non-immune related pathways that could predict a protective response in young individuals. In this study, we sought to obtain more knowledge of response variability after vaccination and challenge with *T. circumcincta* in young lambs by exploring breed differences in the transcriptome profile in the abomasal lymph node in coordinating host response at the site of infection.

The transcriptomic analysis showed substantial numbers of DEG between breeds (239), possibly reflecting the considerable differences in parasite viability following vaccination. The number of genes that were more highly expressed between breeds was higher in the CS lambs (148) than in the CHB lambs (91). In both breeds, a major proportion of DEG were involved in the immune system process, which may be a consequence of the responses induced by the vaccine, subsequent exposure to the parasite and the intrinsic nature of the sampled tissue.

Some genes involved in general immunity were identified as DEG in both breeds, such as those involved in antigen presentation through MHC molecules. Additionally, in the CS breed, some genes were implicated in dendritic cell migration (ABCC4) (van de Ven et al., 2009) and

Table 2
Enriched pathways identified from differentially expressed genes (DEG) in CS lambs in comparison with CS lambs, both vaccinated with a recombinant subunit vaccine against Teladorsagia circumcincta and subsequently challenged with the parasite (N = 9).

Pathway	Pathway ID	Annotated Genes Quantity	Annotated Genes
Alpha-defensins	REACT:R-HSA-1462054	2	ART1, PRSS2
Immune System	REACT:R-HSA-168256	15	ANAPC10, ART1, DERA, DNM1, GBP1, GBP4, HLA-B, KLHL41, MAPK10, PELI3, PRKCG, PRSS2, PTEN, RASAL3, RNF144B
Thyroid hormone synthesis	KEGG: hsa04918	3	DUOXA2, PRKCG, TPO
Autoimmune thyroid disease	KEGG: hsa05320	2	HLA-B, TPO
Autophagy - animal	KEGG: hsa04140	3	DEPTOR, MAPK10, PTEN
cAMP signaling pathway	KEGG: hsa04024	4	ABCC4, AFDN, CHRM2, MAPK10
Cholinergic synapse	KEGG: hsa04725	3	CHRM2, KCNQ3, PRKCG
Defensins	REACT:R-HSA-1461973	2	ART1, PRSS2
Endocrine and other factor-regulated calcium reabsorption	KEGG: hsa04961	2	DNM1, PRKCG
Focal adhesion	KEGG: hsa04510	4	MAPK10, PRKCG, PTEN, TNC
Hepatitis B	KEGG: hsa05161	3	MAPK10, PRKCG, PTEN
Innate Immune System	REACT:R-HSA-168249	10	ART1, DERA, DNM1, HLA-B, MAPK10, PELI3, PRKCG, PRSS2, PTEN, RASAL3
Interferon gamma signaling	REACT:R-HSA-877300	3	GBP1, GBP4, HLA-B
Metabolism	REACT:R-HSA-1430728	13	ADHFE1, AHR, ALOX15B, B3GAT1, CYP2F1, DERA, HSD17B13, LIPH, NAXD, PTEN, RPL22L1, TPO, UCP1
mTOR signaling pathway	KEGG: hsa04150	3	DEPTOR, PRKCG, PTEN
Ras signaling pathway	KEGG: hsa04014	4	AFDN, MAPK10, PRKCG, RASAL3
Signal Transduction	REACT:R-HSA-162582	15	ARHGAP44, CHRM2, CRH, DLK1, DNM1, ESRP2, GPNMB, GUCY2F, HECW1, NDE1, PLPPR3, PRKCG, PTEN, RASAL3, TLE3
Sphingolipid signaling pathway	KEGG: hsa04071	3	MAPK10, PRKCG, PTEN
Toll Like Receptor 4 (TLR4) Cascade	REACT:R-HSA-166016	3	DNM1, MAPK10, PELI3
Toll-Like Receptors Cascades	REACT:R-HSA-168898	3	DNM1, MAPK10, PELI3
Xenobiotics	REACT:R-HSA-211981	2	AHR, CYP2F1

Toll-Like receptor activation (PELI3, USP29). Toll-like receptors are an innate pathogen recognition system with an important role in the induction of cytokines, and other trigger signals, for the adaptive immune response (McRae et al., 2015). Similarly, dendritic cells are able to sample foreign antigens, migrate to local lymph nodes and present antigens to naïve T cells through an MHC molecule, initiating the adaptive immune response (Abbas et al., 1999). These results could represent the effect of pathogen infection on activation of immune mechanisms.

Similarly, some genes encoding proteins with antibiotic, antifungal and, in some cases, antiprotozoal activity in mucus had a high level of expression in both breeds after vaccination. Some examples in the CHB lambs include secretogranin II (SCG2) (Shooshtarizadeh et al., 2010), antibacterial peptide SMAP-29 (SC5) (Giacometti et al., 2003; Skerlavaj et al., 1999) and lysozyme (LYZ), all of which are characterized as defensins in the mucosal barrier. In CS vaccinated lambs, DEG identified include cystatin 9 (CST9L), which has demonstrated antimicrobial activity (Eaves-Pyles et al., 2013), and DUOXA 2, which is reportedly expressed in respiratory tract epithelium and in gastrointestinal mucosa, where it plays a role in antimicrobial defense by participating in generation of H_2O_2 (Carré et al., 2015). There is growing interest in the potential role of these proteins in parasitic nematode infections due to their ability to mediate host-parasite interactions. For example, local expression of galectins and intelectins is characteristic of GIN infections and is known to influence nematode survival, probably by modifying mucus properties or by influencing mast cell degranulation (Artis, 2006; Donskow-Łysoniewska et al., 2022, 2021). Future studies should clarify the source of antimicrobial proteins' transcripts in the regional lymph node and how the expression of some of these molecules is related to their activity at the mucosal barrier.

A high number of upregulated DEG with immunomodulatory properties were identified in both breeds. In the CHB lambs, some of these genes encode proteins that activate cell signaling, chemotaxis and cell proliferation (MGB1, MTHFD2, TCAF2) (Acharya et al., 2021; Sugiura et al., 2022) or control differentiation and proliferation through cell death (OSGIN1), whereas other molecules suppress immune responses (NLRP12) and T cell activity (BTNL2) (Abeler-Dörner et al., 2012). Interestingly, in vaccinated CS lambs, regulatory genes were numerous. Some of these are known to increase regulatory T cells such as ART1 (Cortés-Garcia et al., 2016) and LIPH, which reduces $CD8^+$ populations (Zhuang et al., 2022). Others, such as GPNMB, reduce T cell functionality (Saade et al., 2021), and B3GAT1, which is a marker of NK and T cells with reduced functionality and inability to proliferate (Kared et al., 2016). Several of the identified DEG in the vaccinated CS lambs (PARG, ALOX15B, MYADML, CST9L) are known to participate in anti-inflammatory responses (Aranda et al., 2013; Eaves-Pyles et al., 2013; Snodgrass and Brüne, 2019; Wang et al., 2019). Other DEG, such as CSMD1, may inhibit complement (Blom, 2017), and RASAL3, which negatively regulates neutrophils and NK cells (Saito et al., 2021).

Several genes (GBP1, GBP2, GPB4, FANCC) that participate in interferon-γ (INF-γ) activation were significantly differentially expressed in both breeds. IFN-γ is a molecule traditionally associated with responses against intracellular pathogens (Type 1 responses) and not with protection against GINs (Type 2 responses) (Gill et al., 2000; Spellberg and Edwards, 2001). However, there is increasing evidence that GIN-infected animals display a concomitant activation of type 1 and 2 pathways (Aboshady et al., 2020; Liu et al., 2022). This could be due to the ability of some animals to balance immune responses as observed in mixed infections with intracellular and extracellular pathogens in wild ruminants (Corripio-Miyar et al., 2021). Furthermore, experimental assays have highlighted the importance of an early and wide activation of Type 1 and Type 2 responses in parasitic nematode rejection in animals rendered immune by exposure, but also by vaccination (Aboshady et al., 2022; Liu et al., 2022).

In the CHB lambs, several identified DEG were T lymphocyte associated. Some encoded parts of T-cell receptor (TCR) structure (β and γ chains), whilst others were T cell- activating proteins such as TCAF2 and MTHFD2. TCAF2 is a surface Ca^{+2} channel in T cells, recently described as an important regulator of TCR activation and differentiation to effector cells (Acharya et al., 2021), while MTHFD2 is an enzyme belonging to the *de novo* purine synthesis pathway that participates in signaling in activated T cells to promote proliferation and inflammatory cytokine production (Sugiura et al., 2022). In addition, interleukin-36β is involved in promoting dendritic cell maturation and inducing type 1 responses (Dong et al., 2022), but also induces type 2 responses and downregulates T cell (Treg) responses in mice and humans with inflammatory bowel disease (Zhu et al., 2022). In the CS lambs, putative immune mechanisms involved in responses to vaccination and challenge involving T cells were more limited. In fact, sequences encoding TCR α (TCRA and TRAV24), β (TCRB) and δ chains (TCRD), as well as WC1 antigen, a γδ T cell subpopulation marker, which is a cell associated with innate responses, were identified as DEG in this breed. Moreover, DLK1, a gene instrumental for B cell development (Raghunandan et al., 2008), and MSMP, a peripheral blood monocyte- and lymphocyte-attracting chemokine (Pei et al., 2014) were more highly expressed in this breed.

These results highlight an important component of T cell presence, activation, differentiation, and cytokine production, which has long been recognized as a main control mechanism against parasitic nematode establishment, fecundity and egg output through B-cell activation and IgA and IgE production that stimulate eosinophil and mast cell degranulation (Gill et al., 1993; McRae et al., 2015). The predominant role of T cells, described here, is in keeping with the previous histological analysis, especially in CHB lambs, where $CD4^+$ T cells were involved in stimulating a strong adaptive type 2 response (Pérez-Hernández et al., 2022). Regarding the marked differences between breeds in T cell involvement, it is possible that the balance of genes stimulating T-cell proliferation and activation may be different between breeds at this age, resulting in differences in vaccine efficacy. This interpretation is based on the results obtained in 6-month-old CS lambs, where vaccinates had higher IgA and IgG2 levels and a higher CD4/CD8 ratio than control CS lambs (Quil A-only recipients) (Machín et al., 2021). Also, subsequent transcriptomic analysis demonstrated TCR alpha variable (TRAV41) and delta locus (TRD) were more highly expressed in CS vaccinates than in controls (Machín, 2021), suggesting the limited participation of T cells at 3 months may be age-related.

Certainly, the effects of breed differences after vaccination were most evident in effector responses against the parasite. In CHB vaccinates, increased expression of MBP2 compared to CS, associated with activation of eosinophil granules, was identified. This protein has an important role in synthesis of histamine, which is present in mast cells granules. Eosinophils, mast cells and globule leukocytes are cells associated with worm growth and fecundity impairment and burden regulation in *T. circumcincta* (Stear et al., 1995) and *H. contortus* (Nisbet et al., 2016b) infection. Here, DEG were observed that are linked with IgG receptor expression (FCGR2A) and a J-chain protein (JCHAIN), key in the formation of IgA dimers and IgM pentamers. As alluded to above, IgA has a main role in protection against *T. circumcincta* (Stear et al., 2004), especially against L4, which is the parasitic stage against which most of the proteins in this vaccine cocktail were developed (Ellis et al., 2014). Interestingly, in the more susceptible breed, only one gene (IGKV2D-26), which encodes a region of light immunoglobulin chains and participates in antigen recognition, was upregulated.

Epithelial integrity appears to be important in the GIN-resistant breed (CHB), represented by the increased expression of SERPINB12, which is a epithelial protein that has the ability to protect cells from the damaging action of proteases and maintaining barrier function (Niehaus et al., 2015). Moreover, production of extracellular matrix proteins, angiogenesis (ECM1) and keratinocyte migration (ETV4), typically occur during classical stages of wound repair and regeneration (Gurtner et al., 2008). Previous studies have suggested heightened cell proliferation and tissue repair contributing to host resistance to GIN infection in this breed (Guo et al., 2016). However, how the expression of these intracellular proteins in the draining lymph node affects the mucosal

barrier repair is unclear.

Pathway analysis demonstrated enriched immune responses, innate immunity and cell signaling pathways were upregulated in both breeds. In CS lambs, innate immunity, defensins and TLR routes were particularly numerous. In the CHB lambs, enriched routes included haemostasis and protein metabolism processes such as ribosome metabolism, RNA transcription and DNA replication.

The study of abomasal lymph nodes transcripts here suggests that young vaccinated CHB lambs are able to coordinate T cells more efficiently than age-matched vaccinated CS lambs, triggering a wider range of humoral and cellular responses, based on participation of immunoglobulins and effector cell populations as identified in the DEG analysis. Interestingly, CHB lambs responded with enhanced innate responses to intracellular agents, which may be a suitable formula for balancing the protective response in the context of an increased type 2 response and may be important in maintaining balance with gut microbiota. In CS sheep, vaccination generated differences in expression of innate protective responses and was predominantly oriented towards intracellular pathogens such as viruses or bacteria, which is presumably not an adequate response to protect against GIN such as *T. circumcincta*. The upregulation of immunoregulatory cells (Treg) and components in this breed is noteworthy. Perhaps including adjuvants that control this type of immunoregulation could be useful to reverse a lack of response in young lambs of more nematode-susceptible breeds.

Other studies on resistant breeds have highlighted that timing of the anti-parasitic response could be key in resistance. Since this study evaluated transcriptome dynamics after 40 days of infection, this could explain why both breeds showed an array of immune mechanisms, but the protective effect on parasitological parameters was only evident in CHB lambs. It is possible that susceptible CS lambs have a higher stimulation threshold, taking them longer to develop a protective response. Another aspect to consider is that this study was performed on lymph node tissue and may not accurately represent all effector mechanisms participating at the site of infection in the abomasal wall. Future studies should focus on following development of immune responses using sequential biopsies, as has been done previously in Texel-cross lambs (Liu et al., 2022), and investigating the effect of the immunogens in pathway activation in each breed. This could elucidate differences between responder and non-responder individuals and provide new approaches to improve protection elicited by the vaccine in young lambs.

Ethics approval and consent to participate

The experiment was designed and performed in accordance with the Spanish Legislation (RD 53/2013) and was subsequently approved by the Animal Welfare Ethics Committee of the Universidad de Las Palmas de Gran Canaria (OEBA_ULPGC_003_2014) and ratified by the local competent authority.

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CRediT authorship contribution statement

Tara P é rez-Hernández: Formal analysis, Investigation, Writing – original draft, Visualization. **Julia N. Hernández:** Investigation, Writing – original draft, Supervision, Project administration. **Cynthia Machín:** Investigation. **Tom N. McNeilly:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Alasdair J. Nisbet:** Conceptualization, Methodology, Writing – review & editing, Project administration. **Jacqueline B. Matthews:** Conceptualization, Methodology, Funding acquisition, Project administration. **Stewart T.G. Burgess:** Conceptualization, Methodology, Writing – original draft, Project administration. **Jorge F. González:** Conceptualization, Methodology, Writing – original draft, Supervision, Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jacqueline B. Matthews reports financial support was provided by European Commission. Tara Perez-Hernandez reports financial support was provided by Cabildo de Gran Canaria. Cynthia Machin reports financial support was provided by Agencia Canaria de Investigación, Innovación y Sociedad de la Información and European Social Fund. Julia N. Hernandez reports financial support was provided by Agencia Canaria de Investigación, Innovación y Sociedad de la Información. Tom N. McNeilly, Alasdair J. Nisbet, Stewart T. G. Burgess reports financial support was provided by Scottish Government Rural & Environment Science & Analytical Services Strategic Research Programme 2022–2027.

Availability of data and materials

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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Consent for publication

All authors have read and agreed to the published version of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109960.

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