Tesis doctoral

Immune response of Canaria Hair Breed and Canaria Sheep vaccinated against *Teladorsagia circumcincta*

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Doctorado en Sanidad Animal y Seguridad Alimentaria

Arucas, Diciembre 2021

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Respuesta inmune de las razas ovinas Canaria de Pelo y Canaria vacunadas frente a *Teladorsagia circumcincta*

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D ANTONIO JESÚS FERNÁNDEZ RODRÍGUEZ COORDINADOR DEL PROGRAMA DE DOCTORADO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA (SASA) DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha / / tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "**Immune response of Canaria Hair Breed (CHB) and Canaria Sheep (CS) vaccinated against *Teladorsagia circumcincta***" presentada por la doctoranda **Dª Cynthia Machín Pérez** y dirigida por los **Doctores Jorge Fco. González Pérez** y **Julia N. Hernández Vega**.

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 04/03/2019) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a de de 2021.

ÍNDICE

1. INTRODUCCIÓN

Teladorsagia circumcincta es uno de los nematodos gastrointestinales más importantes en los pequeños rumiantes debido a las pérdidas productivas que genera. El desarrollo de resistencias a los antihelmínticos y la mayor demanda de productos de origen animal debido al incremento poblacional hace que sea necesario desarrollar y/o implantar métodos alternativos/complementarios al tradicional uso de fármacos para su control.

Una opción respetuosa con el medioambiente que puede suponer una gran ventaja en la lucha frente a NGI, al favorecer la respuesta inmunitaria por parte del hospedador, es la vacunación. Desafortunadamente hay muy pocas vacunas comercializadas frente a ellos debido, en mayor medida, a la dificultad de encontrar inmunógenos efectivos y de producir la versión recombinante de estos antígenos, lo que ayudaría a mejorar su producción y comercialización. No obstante, en los últimos años, se ha estudiado un prototipo vacunal de 8 proteínas recombinantes frente a *T. circumcincta* (**Nisbet *et al.,* 2013**) que ha obtenido resultados prometedores, confiriendo protección en corderos, aunque observándose cierta variabilidad individual en respuesta a la vacunación. Esto comprometería su comercialización, por lo que nuevos estudios que aporten información sobre cómo mejorar dicho prototipo serían necesarios.

Otro método sostenible que se practica es la selección de animales genéticamente resistentes, utilizando como sementales aquellos animales más resistentes para favorecer la transmisión de ese carácter a su descendencia, aprovechando la heredabilidad de ésta. Con ello se consigue reducir paulatinamente la susceptibilidad en el rebaño y disminuir las cargas ambientales.

Varias razas ovinas locales están descritas como resistentes a algunos nematodos gastrointestinales debido a su adaptación al medio. El desarrollo de trabajos encaminados a estudiar su respuesta inmune puede ayudar en la búsqueda de nuevas dianas farmacológicas. En este sentido, en las Islas Canarias (España) contamos con dos razas locales (Canaria de Pelo -CAP- y Canaria -CAN-) que han presentado diferencias en susceptibilidad frente a NGI, considerándose la raza CAP resistente a *T. circumcincta* en infecciones naturales mixtas (**Hernández, 2015**) y a *H. contortus* en infecciones experimentales (**González *et al.,* 2008**).

Por todo ello, la finalidad de esta tesis ha sido el utilizar corderos de 6 meses de edad de estas dos razas locales (CAP y CAN) como modelo biotecnológico en el que observar el efecto del prototipo vacunal recombinante de *T. circumcincta* desarrollado por **Nisbet *et al.,* 2013**, estudiando la respuesta inmune y genómica generada en los animales vacunados y controles de ambas razas.

2. OBJETIVOS

1. Comparar la eficacia del prototipo vacunal recombinante frente a *Teladorsagia circumcincta* en corderos de las razas locales Canaria y Canaria de Pelo de las Islas Canarias (España), que han sido sometidos a inoculaciones seriadas de 2000 L3 de este parásito.

Objetivos específicos

Investigar el efecto de la vacunación comparando el grupo vacunado y control de cada raza, mediante la valoración de:

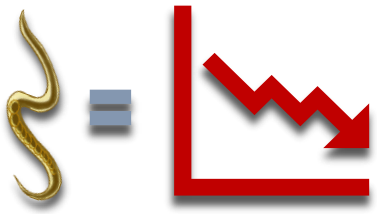
- Los datos parasitológicos: número de huevos por gramo de heces (HPG), carga parasitaria, longitud y recuento de huevos intrauterinos de los vermes.
- La respuesta inmune generada por los corderos mediante el estudio de:
  - Inmunoglobulinas específicas frente al parásito (IgA, IgG, IgG_1 y/o, IgG_2) en suero.
  - El recuento de poblaciones celulares en mucosa abomasal (eosinófilos, leucocitos globulares, mastocitos y linfocitos $CD4^+$, $CD8^+$, $\gamma\delta^+$, $\gamma\delta$-$WC1^+$, $CD45RA^+$, MHC-II^+ y Galectina-14^+).
  - Determinación de citoquinas específicas (IFN-γ, IL-4 e IL-17A) frente al parásito y fenotipaje de células producidas por los linfonodos del abomaso.
- La expresión génica cuando se observen diferencias en las variables parasitológicas entre grupo vacunado y control dentro de raza; comparando la expresión génica obtenida de muestras de tejido de abomaso recogidas en el sacrificio.

2. Comparar la resistencia a la infección por *Teladorsagia circumcincta* de corderos de las razas Canaria y Canaria de Pelo, contrastando los datos parasitológicos de los grupos no vacunados de ambas razas.

3. REVISIÓN BIBLIOGRÁFICA

3.1. ¿POR QUÉ LOS NEMATODOS GASTROINTESTINALES SON UN PROBLEMA MUNDIAL?

Figura 3.1 Pérdidas productivas causadas por los NGI



Los nematodos gastrointestinales (NGI) generan graves pérdidas económicas (**Figura 3.1**) en el sector ganadero a nivel mundial al afectar a los rumiantes en pastoreo, principalmente de forma subclínica y crónica (**Charlier *et al.*, 2018; Skuce *et al.*, 2013**). En ovino, a modo de ejemplo, se estiman pérdidas productivas de hasta un 15% para el mantenimiento de la inmunidad frente a estos parásitos (**Charlier *et al.*, 2018; Greer, 2008**). Para mitigar estas pérdidas, en la Unión Europea se generan costes de hasta 400 millones de euros anuales en tratamientos (**Matthews *et al.*, 2016**). Además, se espera que el impacto de las enfermedades por NGI sea aún mayor, vinculado a nuevos escenarios, que se interconectan entre sí, como el cambio climático, la creciente resistencia a antihelmínticos y la mayor demanda de productos de origen animal debido al crecimiento poblacional.

Figura 3.2 Efecto del cambio climático en la distribución de los NGI

En este sentido, el cambio climático parece afectar de forma directa a los parásitos y, por ende, a los parasitismos. Un ejemplo de ello es el aumento de temperatura que ha experimentado el planeta a lo largo de los años, que ha propiciado una redistribución de los NGI (**Figura 3.2**), extendiéndose cada vez más hacia los polos, desarrollándose especies tropicales en climas templados y prolongando su periodo de transmisión significativamente a lo largo de las estaciones (**Fox *et al.*, 2012; McMahon *et al.*, 2012**). Esto, que hasta hace unos años se consideraba una amenaza, ya es visible en diversos estudios de NGI, por ejemplo, en el Reino Unido, (**van Dijk *et al.*, 2010; McMahon *et al.*, 2012; Sargison *et al.*, 2007**) donde se puede apreciar el aumento de la prevalencia de *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp. y *Nematodirus battus*, coincidiendo con aumentos significativos de la temperatura media en los últimos años.

El calentamiento global también podría generar cambios en el patrón de crecimiento de la vegetación, influyendo en el estado físico por la falta de disponibilidad de nutrientes, pudiendo generar alteraciones en el sistema inmune, y en el manejo de los hospedadores debido a cambios en la temporalidad del pastoreo y a la densidad de animales de acuerdo con

la vegetación disponible. Todo ello supondrá afrontar mayores riesgos en el futuro de la producción ganadera y podría contribuir a que los efectos de los NGI lleguen a ser incontrolables si se siguen utilizando las estrategias actuales de control (**van Dijk *et al.*, 2010**).

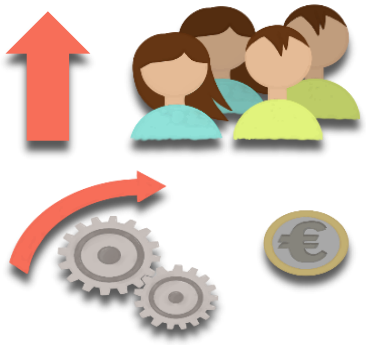
Figura 3.3 Resistencia de los NGI a antihelmínticos

El uso indebido de los antihelmínticos, que se han aplicado como tratamiento profiláctico a lo largo del tiempo, empeora aún más la situación. Hoy en día es muy común encontrar NGI multirresistentes a diferentes moléculas (**Figura 3.3**) presentes en el mercado en casi todo el mundo (**Bordes *et al.*, 2020; Dey *et al.*, 2020; Herrera-Manzanilla *et al.*, 2017; Martínez-Valladares 2006; McIntyre *et al.*, 2018**) y debido a que la industria farmacéutica no es capaz de generar nuevas moléculas para el mercado con la suficiente celeridad, no es un modelo de control sostenible (**McRae *et al.*, 2015; Nari *et al.*, 2003; Vercruysse *et al.*, 2018**). Además, una vez más, el cambio climático podría estar contribuyendo en la propagación de esta resistencia, al favorecer la supervivencia y el desarrollo larvario (**Morgan y van Dijk 2012; Rose *et al.*, 2014, 2016**).

Figura 3.4 Productos de origen animal

Toda esta problemática, a su vez, se ve agravada por la mayor demanda de productos de origen animal (**Figura 3.4**) que existe en la actualidad, debida al incremento de la población mundial (**Figura 3.5**) y al crecimiento económico de países en vías de desarrollo, lo que supone otro reto para la ganadería (**FAO, 2007; Steinfeld *et al.*, 2006; Thornton, 2010**). Se estima que los países en vías de desarrollo triplicaron su producción cárnica total entre 1980 y 2002, coincidiendo con el aumento de demanda que se produjo en ellos, además de la que ya existía en los países desarrollados (**Thornton, 2010**) y, es previsible que esta demanda y la de otros productos de origen animal sea mayor en futuros años (**Banco Mundial, 2009**).

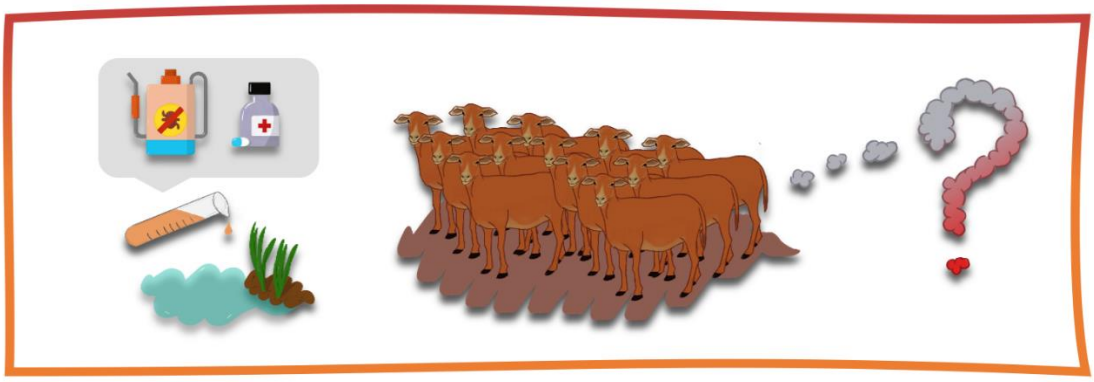
Figura 3.5 Aumento poblacional y desarrollo económico



Debido a los problemas descritos anteriormente, se puede entender la imperiosa necesidad de incrementar los rendimientos productivos, que parece que lleva ineludiblemente

a aumentar el número de animales para conseguir esa mayor producción (**Figura 3.6**). Sin embargo, esto se traduciría en un mayor deterioro del ecosistema. Así, por ejemplo, se estima que, actualmente, el 18% de los gases invernadero de origen antropogénico pertenecen al sector ganadero (**Smith *et al.*, 2008; Steinfeld *et al.*, 2006**) y se ha observado que los parásitos son capaces de incrementar estas emisiones, por ejemplo, en torno a un 33% en corderos (**Fox *et al.*, 2018**). Por otra parte, este sector ganadero ocupa en nuestros días el 30% de la superficie terrestre no cubierta de hielo (**Thornton, 2010**). Por tanto, un aumento indiscriminado en los censos de animales de producción repercutiría en el medioambiente, agravando la contaminación y afectando seriamente la disponibilidad de recursos naturales.

Figura 3.6 Soluciones no sostenibles para afrontar las pérdidas por NGI como: el uso indiscriminado de fármacos, el incremento del censo de animales y el uso de pesticidas para garantizar la alimentación de estos



Estos motivos acentúan el interés en buscar alternativas al manejo clásico del ganado. Actualmente, para poder conseguir una producción eficiente y respetuosa con el medioambiente, se está estudiando la implementación de métodos de control sostenibles (**Figura 3.7**) y se apuesta de forma decidida por la detección temprana de enfermedades y de patógenos (**Thornton, 2010**). Algunos de estos métodos serán analizados a lo largo de la revisión bibliográfica, en especial, el uso de animales genéticamente resistentes y la inmunización frente a NGI. Es fundamental que apliquemos programas de control basados en métodos alternativos y complementarios a los fármacos frente a estos parásitos, para poder afrontar los cambios que se puedan producir en el planeta en el futuro (**van Dijk *et al.*, 2010**).

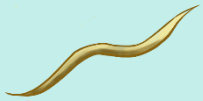
Figura 3.7 Métodos sostenibles que se pueden adoptar para afrontar las pérdidas causadas por NGI como: el diagnóstico preventivo, la selección genética y el uso de vacunas, de forma que no sea necesario aumentar el censo de animales

Figura 3.8 Resumen gráfico de problemas generados por nematodos gastrointestinales, situaciones agravantes y posibles soluciones.

3.2. *TELADORSAGIA CIRCUMCINCTA* Y SU IMPACTO EN OVINOS

Teladorsagia circumcincta es uno de los nematodos gastrointestinales que más pérdidas económicas genera en ganado ovino y caprino (**O'Connor, Walkden-Brown y Kahn, 2006; Stear *et al.,* 2019**). Esta especie pertenece a la superfamilia Trichostrongylidea, orden Strongylida (**Bowman, 2011**). Los estadios que desarrolla durante su ciclo presentan características morfológicas que permiten diferenciarlos de otros nematodos (**Tabla 3.1**).

Tabla 3.1 Características morfológicas de los principales estadios de *Teladorsagia circumcincta*

<table>
<tr><th>Huevo</th><th>L3</th><th colspan="2">Adulto</th></tr>
<tr><td>  </td><td>  </td><td colspan="2">  </td></tr>
<tr><td rowspan="2">· Medidas: 92-110 x 42-50 µm
· Cáscara transparente, lisa y elipsoidal
· Se depositan en fase de mórula</td><td rowspan="2">· Longitud: 795-865 µm
· Longitud vaina: 30-44 µm</td><td colspan="2">· Color parduzco
· Cavidad bucal corta y amplia</td></tr>
<tr><td>Hembra</td><td>Macho</td></tr>
<tr><td></td><td></td><td>· 10-12 mm
· Vulva a mitad del cuerpo
· Solapa vulvar</td><td>· 7-9 mm
· Bolsa copuladora con lóbulos laterales predominantes
· Espículas cortas y gruesas (280-330 µm)
· Gubernáculo 80-90 µm</td></tr>
</table>

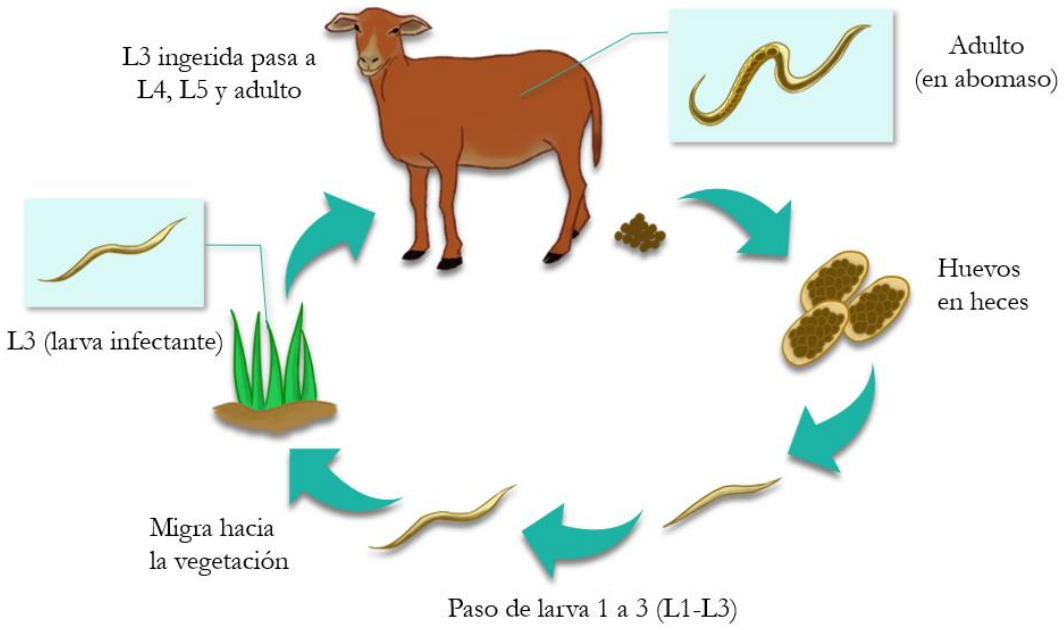
Fuentes: (**Borchert, 1975; Meana y Rojo-Vázquez, 1999; Bowman, 2011; van Wyk y Mayhew, 2013**).

3.2.1 Ciclo biológico

Su ciclo de vida (**Figura 3.9**) es directo y comienza con la diseminación de huevos en fase de mórula (sin embrionar) en las heces del hospedador. De esta mórula eclosiona el primer estadio larvario (L1) que, más tarde, mudará al segundo (L2) (**Bowman, 2011**). Estos dos estadios de vida libre se alimentan de microorganismos fecales. Sin embargo, cuando se produce la segunda muda de L2 hacia la larva de tercer estadio o infectante (L3), la larva mantiene la cutícula del anterior estadio, lo que le impedirá seguir alimentándose del medio, pero a su vez, le servirá como vaina protectora (**Bowman, 2011; O'Connor *et al.,* 2006**). El

proceso del paso de huevo a estadio L3 se produce en 5-14 días si las condiciones climáticas son adecuadas (**Martínez-Valladares, 2006**). Una vez desarrollada la larva infectante, cuando hay suficiente humedad migra hacia la vegetación para favorecer su ingestión por parte del hospedador (**Urquhart *et al.,* 1996**). No obstante, se ha observado que el estadio L3 puede llegar a sobrevivir largos periodos de tiempo en las heces si se mantiene en condiciones adecuadas de luz, temperatura y humedad. Posiblemente, eso se debe a su vaina, que le aporta mayor resistencia a la desecación y al frío (**van Dijk *et al.,* 2010; O'Connor *et al.,* 2006**). A continuación, ya dentro del animal, la larva libera su vaina cuando llega al rumen y se aloja en las glándulas abomasales de la región antropilórica para mudar al estadio L4 (**Meana y Rojo-Vázquez, 1999**). A los 6-10 días post-infección saldrá hacia el lumen y madurará al siguiente estadio (L5 o pre-adulto) (**Martínez-Valladares, 2006**). Por último, alcanzará la fase adulta y se aparearán para dar lugar a los huevos (100-200 diarios), que serán eliminados posteriormente en las heces del hospedador, cerrando de este modo el ciclo (**Meana y Rojo-Vázquez 1999**). Si éste no se detiene, el periodo de prepatencia es de unos 17-21 días (**Bowman, 2011; Meana y, Rojo-Vázquez, 1999**).

Figura 3.9 Representación del ciclo biológico de *Teladorsagia circumcincta*



El desarrollo del ciclo, y por tanto su prevalencia, va a depender de diferentes factores ambientales que condicionan la supervivencia y desarrollo larvario de los estadios de vida libre (**Bowman, 2011; Morgan y van Dijk, 2012**) ; del número de hospedadores infectados con parásitos adultos; de la resistencia que presenten estos al parásito; y del fenómeno

denominado hipobiosis, que consiste en la detención temporal del desarrollo del estadio L4 hacia el L5 hasta que se produzca un estímulo concreto para su reactivación (**Bowman, 2011; Urquhart *et al.,* 1996**). Se cree que esto último puede suceder como mecanismo de resistencia del hospedador o como resultado de la autorregulación generada por el propio parásito, bien porque las condiciones climáticas no son favorables en ese momento o porque la densidad de población del parásito es muy alta para que pueda ser tolerado por el animal (**Martínez-Valladares, 2006; Soulsby, 1987**).

3.2.2 Epidemiología

Tanto la fase de desarrollo embrionario en el huevo, como la larvaria de *T. circumcincta*, sobreviven a temperaturas más bajas que las otras especies de nematodos del abomaso de ovinos como *Haemonchus contortus* (**Waller *et al.,* 2004**). Los huevos sin embrionar poseen una mayor viabilidad entre 0 y 10ºC, pudiendo eclosionar una vez embrionados, a temperaturas menores a 5ºC (**O'Connor *et al.,* 2006**). El estadio pre-infectivo es algo más susceptible al frío, aunque cuando madura a larva infectante adquiere una mayor resistencia (**O'Connor *et al.,* 2006**). Por otro lado, en general no toleran la desecación, aunque varía su susceptibilidad a lo largo de los estadios (**O'Connor *et al.,* 2006**). Por esta razón se distribuyen por regiones húmedas y templadas como, por ejemplo, el norte de Europa, Asia, Norteamérica, Escandinavia y Nueva Zelanda - por encima de los 40º de latitud -, y zonas con clima Mediterráneo de todo el mundo - entre 30 y 40º latitud -. Aunque también se encuentran en regiones más cálidas como el sudeste de Australia (**O'Connor *et al.,* 2006**) o, por ejemplo, en Gran Canaria, donde se ha observado en diferentes regiones de la isla independientemente de los microclimas de la isla (**Hernández, 2015; Molina *et al.,* 1997**). Además, como consecuencia del calentamiento global, este parásito ha experimentado un aumento de su prevalencia en algunas zonas templadas, y ha extendido su ciclo hasta los meses de otoño (**van Dijk *et al.,* 2008, 2010; McMahon *et al.,* 2012**). Por otro lado, se ha observado que el calentamiento global puede a su vez disminuir la población de larvas infectantes que se encuentran latentes durante el invierno. Esto se debe a que, al aumentar la temperatura en esta estación, producen una aceleración del metabolismo de las larvas que, al contar con la vaina que les impide alimentarse del medio, agotan las reservas que poseen y mueren al no tener hospedadores susceptibles a los que infectar (**van Dijk *et al.,* 2010; O'Connor *et al.,* 2006**).

Debido al fenómeno de la hipobiosis, la enfermedad que produce *T. circumcincta* se puede presentar en dos épocas del año en las regiones templadas. Normalmente en esta zona los

partos se producen en primavera, dando lugar a hembras más susceptibles a la infección y jóvenes que entran en contacto por primera vez con el parásito durante el pastoreo, lo cual ayuda a la diseminación de estos (**Bowman, 2011; Urquhart *et al.,* 1996**). Por lo que, desde finales de primavera hasta verano (Abril-Junio) se contaminarán los pastos con L3, infectando a los animales especialmente entre Julio y Octubre, manifestando entonces los síntomas de la enfermedad. Sin embargo, en ocasiones también se pueden manifestar durante el periodo comprendido entre finales de invierno y principios de primavera (**Urquhart *et al.,* 1996**). Esto se atribuye a que se establecen las condiciones climáticas óptimas para que las larvas vuelvan a activar su metabolismo, tanto en el medio ambiente (L3), como las que se encuentran en hipobiosis (L4) dentro del hospedador - en especial en hembras en periodo de periparto, debido a la supresión temporal de la inmunidad - (**Bowman, 2011; van Dijk *et al.,* 2010; Sargison *et al.,* 2007**). Por otro lado, en las regiones más cálidas como las subtropicales, los síntomas de la enfermedad se producen principalmente a finales de invierno, debido a la concentración de lluvias en esta estación (**O'Connor *et al.,* 2006; Urquhart *et al.,* 1996**).

3.2.3 **Patogenia**

Durante la parasitación, las larvas penetran y crecen dentro de las glándulas del abomaso produciendo su dilatación, lo que conlleva a que las células parietales dañadas se sustituyan por células indiferenciadas (**Martínez-Valladares, 2006; Meana y Rojo-Vázquez, 1999**). A diferencia de las células parietales, las indiferenciadas no producen ácido clorhídrico, por lo que se va a ver disminuida su secreción, dando lugar a un aumento del pH en el abomaso, que va a tender a la neutralidad. Esta falta de ácido clorhídrico conlleva a un incremento de la población bacteriana, produciendo diarreas, al aumento de la producción de gastrina (favoreciendo el peristaltismo) y, además, a que se produzca una alteración en la digestión de proteínas (afecta al proceso de degradación del pepsinógeno en pepsina) (**Bowman, 2011; Meana y Rojo-Vázquez, 1999; Soulsby, 1987; Urquhart *et al.,* 1996**).

3.2.4 **Clínica**

Generalmente, la enfermedad cursa de forma subclínica y por ello, supone un problema importante en la producción ganadera, al reducir la producción láctea, calidad de la lana y la ganancia diaria de peso debido a la limitación de proteína disponible (**Meana y Rojo-Vázquez, 1999**). Cuando se producen signos clínicos, es más frecuente que en animales adultos se dé la forma crónica, manifestándose como emaciación debido a la pérdida de apetito, mientras que en jóvenes se produce la forma aguda, consistiendo principalmente en

diarrea y consiguiente deshidratación (**Martínez-Valladares, 2006; Meana y Rojo-Vázquez, 1999**) .

3.3. ¿CÓMO DISMINUIR LAS PÉRDIDAS PRODUCIDAS POR NGI?

Con el fin de reducir el impacto económico que han ejercido los NGI, la búsqueda de métodos de control se ha centrado en interrumpir el ciclo de vida del parásito. De esta manera, siempre se ha tratado de que cumplan alguno de los siguientes objetivos (**Torres-Acosta y Hoste, 2008; Kearney *et al.,* 2016**):

- Eliminar los nematodos en el hospedador

- Disminuir el contacto entre el hospedador y las larvas infectantes de la vegetación.

- Aumentar la resistencia/resiliencia del hospedador.

Hasta ahora, el control más efectivo se realizaba a través de fármacos, pero desde que emergieron parásitos resistentes a ellos y comenzó la preocupación por el efecto de los químicos en nuestra alimentación y el medioambiente, se ha incrementado la búsqueda de nuevos métodos de control (**Matthews *et al.,* 2016**). A continuación, se describirán tanto los métodos químicos, más convencionales, como otros métodos complementarios que se postulan como tendencias a adoptar en el futuro.

3.3.1 Control químico

Los antihelmínticos que podemos encontrar en el mercado se agrupan principalmente en 3 familias. La familia de los benzimidazoles (ej. albendazol), la de los imidazotiazoles (ej. levamisol) y las tetrahidropirimidinas (ej. pirantel), y la familia de las lactonas macrocíclicas (ivermectina y moxidectina, entre otros) (**Charlier *et al.,* 2018; Coles *et al.,* 2006**). Aunque hay dos grupos de fármacos más reciente, uno es el de los derivados de amino-acetronitrilo (Amino-acetronitriles derivatives -AAD-), que se usa en algunos países y en el que se encuentran el monepantel, y el otro grupo son los espiroindoles como el derquantel, comercializado en combinación con abamectina (**Charlier *et al.,* 2018**).

Figura 3.10 Antihelmínticos

El tratamiento del ganado con antihelmínticos resultaba ser la solución más eficaz frente a nematodos, dependiendo de ellos desde la década de 1960 (**Charlier *et al.,* 2018**). Este método era tan conveniente que, en respuesta al gran incremento de la demanda de

productos de origen animal, se abusó de su empleo profiláctico con el fin de aumentar la producción (**Vercruysse *et al.,* 2018**). Ese uso inadecuado y prolongado en el tiempo, eventualmente, dio lugar al desarrollo de resistencia por parte de los parásitos (**Vercruysse *et al.,* 2018**), incluso, frente a una molécula tan reciente como el monepantel (**Bartley *et al.,* 2019; van den Brom *et al.,* 2015**), lo que indica que son capaces de adaptarse con rapidez (**Kearney *et al.,* 2016**) y, actualmente, supone el principal obstáculo en el control de nematodos (**Charlier *et al.,* 2018; Kaplan y Vidyashankar, 2012**). Además, el tratamiento químico acarrea otra serie de consecuencias, que se traducen en el depósito de residuos en la canal del ganado y su posible efecto en los consumidores y el medioambiente (**Torres-Acosta y Hoste, 2008**).

A pesar de ello, los avances tecnológicos en biología molecular podrían contribuir al descubrimiento de nuevos fármacos orientados a proteínas específicas. Esto permitiría conocer cuáles son las mejores combinaciones de moléculas a realizar para ampliar el espectro de actuación del fármaco y, a la vez, ralentizar el desarrollo de resistencia a estos (**Vercruysse *et al.,* 2018**). De hecho, actualmente se continúa el desarrollo de nuevos fármacos y se cree que en el futuro continuaremos dependiendo de ellos, si bien desde una perspectiva más sostenible. Por ejemplo, llevando a cabo tratamientos selectivos, estableciendo previamente planes de manejo, combinándolos con otros métodos y, probablemente, restringiendo el uso de ellos (**Charlier *et al.,* 2018; Vercruysse *et al.,* 2018**).

Tratamiento Selectivo Dirigido y Tratamiento Dirigido

El tratamiento dirigido (TT – *Targeted Treatment*) reduce el desarrollo de resistencia de los nematodos a los antihelmínticos al disminuir la frecuencia de tratamientos y la contaminación de pastos con larvas resistentes, tratando a todo el ganado en un momento determinado atendiendo a la parasitación media que presentan como grupo (**Charlier *et al.,* 2018; Kenyon *et al.,* 2009**). Por ejemplo, es conveniente realizarlo durante el periodo de periparto, cuando existe un mayor riesgo de contagio (**Charlier *et al.,* 2014**). No obstante, para aplicar correctamente este método de control hay que conocer bien la epidemiología de la zona y se recomienda, entre otros métodos de diagnóstico, el recuento de huevos en heces combinado con el estudio del rendimiento productivo del ganado (**Charlier *et al.,* 2014**).

Este método hay que diferenciarlo del tratamiento selectivo dirigido (TST – *Targeted Selective Treatment*), que consiste en el tratamiento individual manteniendo una parte de la población de nematodos en "refugio", sin que entren en contacto con antiparasitarios, con el objetivo de que no desarrollen resistencia y continúen aportando genes de susceptibilidad

a la población total (**Charlier *et al.,* 2018; Jackson *et al.,* 2009; Kenyon y Jackson, 2012; van Wyk *et al.,* 2006**). Debido a que la mayoría de los parásitos de una población se concentran en un bajo porcentaje de animales del rebaño (**Torres-Acosta y Hoste, 2008; van Wyk *et al.,* 2006**), el tratamiento se aplicará sólo a esos animales, considerados los más susceptibles (presentan síntomas/poseen una mayor carga parasitaria), y en el momento adecuado (conociendo la epidemiología) (**Kenyon *et al.,* 2009**). De esta manera, es posible reducir los síntomas que presenten, evitar altas concentraciones de nematodos en heces, ahorrar en el coste de fármacos, disminuir la presión de selección de resistencia de los nematodos (**Charlier *et al.,* 2018; Torres-Acosta y Hoste, 2008**) y, por ende, también las pérdidas productivas. Sin embargo, para saber a qué animales tratar, se debe realizar un diagnóstico previo en el rebaño, mediante marcadores parasitológicos, fisiopatológicos o de producción (**Tabla 3.2**). Los métodos de diagnóstico representados en la tabla son los más usuales, pero se continúa en la búsqueda y aplicación de nuevas tecnologías que puedan mejorar el rigor de los existentes (**Kenyon *et al.,* 2009**). Aunque muchos autores consideran que en el futuro se dependerá más del TST, insisten en una mayor investigación de ellos y de nuevos sistemas de diagnóstico, para mejorar su aceptación por parte de los ganaderos (**Charlier *et al.,* 2014**).

Tabla 3.2 Marcadores comúnmente propuestos para la selección de animales en el tratamiento selectivo

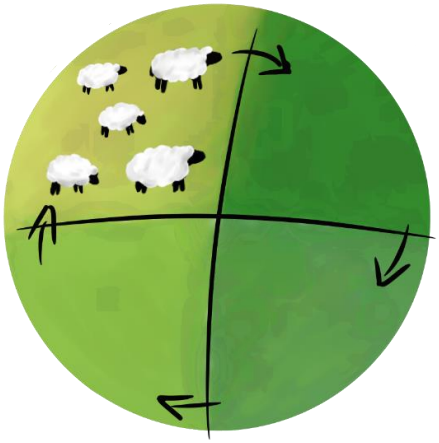
MARCADOR	DESCRIPCIÓN	VENTAJAS	INCONVENIENTES
FAMACHA©	Valoración del grado de anemia según el color de la conjuntiva del ojo (1-5). En general, se aplica tratamiento cuando el valor se encuentra en 3-5.	Técnica efectiva, simple y de bajo coste.	Sólo aplicable a parásitos hematófagos. Otras enfermedades pueden causar pérdida de sangre.
Condición corporal	Estimación visual y por palpación del estado corporal basado en una escala del 1 (muy delgado) al 5 (muy grueso). Algunos autores sugieren tratar cuando llegan al valor 2.	Técnica rápida. Técnica prometedora en ovejas.	Poco sensible. Menos preciso que la ganancia de peso. Otras enfermedades pueden causar pérdida de peso.
Ganancia de peso	Consiste en tratar al grupo de animales con menor tasa de crecimiento del rebaño.	No penaliza el rendimiento de estos, en comparación con lo que ocurriría si no se trataran. Si el proceso de pesaje se automatiza, se ahorra tiempo y trabajo.	Para parásitos no hematófagos. Otros factores pueden causar pérdida de peso. Hay que invertir tiempo en la técnica o dinero en sistemas de pesaje.
Producción láctea	Selección de las hembras con mayor tasa de producción láctea y de las que se encuentran en su primera lactación para el tratamiento, al ser las más susceptibles.	Consigue disminuir la frecuencia de tratamiento antihelmíntico sin afectar negativamente a la producción. Fácil medición.	Marcador poco preciso en vacas.
Recuento de huevos en heces	Administración del tratamiento cuando se estima una cantidad de huevos en heces elevada en la granja.	Resultados positivos en diversos estudios.	Baja correlación con el rendimiento productivo del animal y el grado de infección. Alto coste y laborioso. No hay umbral definido para el tratamiento.

Fuentes: Hoste, Le Frileux y Pommaret, 2002; van Wyk *et al.,* 2006; Jackson *et al.,* 2009; Kenyon *et al.,* 2009; Kenyon y Jackson, 2012; Charlier *et al.,* 2018.

3.3.2 **Manejo de pastos**

Hay diferentes métodos que se centran en el manejo del pasto para impedir o reducir el contacto del ganado con las larvas infectantes presentes en la vegetación (**Bowman, 2011; Hoste y Torres-Acosta, 2011**) valiéndose del hecho de que los estadios de vida libre se encuentran en el medio durante un periodo limitado de tiempo (**Torres-Acosta y Hoste, 2008**).

Figura 3.11 Rotación de pastos



El sistema de rotación de pastos (**Figura 3.11**) se encuentra entre ellos. Consiste en dividir el área de pasto en secciones, de manera que el ganado irá rotando entre ellas en cortos periodos de tiempo, para evitar que entren en contacto con las larvas L3 que puedan surgir de huevos depositados en heces. Para ello, se respetará el tiempo necesario hasta asegurar que la sección esté libre de larvas infectantes y reintroducir al rebaño en ella (**Bowman, 2011**). Los inconvenientes de este método es que, además de ser costoso (**Bowman, 2011**), resulta poco efectivo en climas templados, donde los estadios de vida libre permanecen vivos durante más tiempo en el medio que en los tropicales, en los que parece tener una mayor utilidad (**Torres-Acosta y Hoste, 2008**).

Una variante es un pastoreo intensivo en parcelas pequeñas "*intensive cell grazing*", que se diferencia del anterior en que el área se divide en muchas secciones pequeñas (menores de 0,5 hectáreas), albergando altas concentraciones de animales en cada una, realizando la rotación cada 2 días como máximo (en la rotación de pastos convencional se realizan, al menos, cada semana) y manteniendo de media periodos más largos de descanso en cada sección (**Ruiz-Huidobro *et al.*, 2019**). Aunque no siempre se consigue disminuir la cantidad de huevos excretados en el pasto respecto al sistema de rotación de pastos convencional, podría implementarse como una herramienta para mejorar la producción y sostenibilidad de las explotaciones (**Ruiz-Huidobro *et al.*, 2019**).

Otro método cuyo fin es reducir la cantidad de larvas infectantes en el pasto es el pastoreo mixto. Consiste en que dos especies de animales diferentes (excepto entre pequeños rumiantes) pastoreen en la misma zona o alternen su presencia en ella (**Torres-Acosta y Hoste, 2008; Kearney *et al.*, 2016**), basándose en la especificidad que presentan los nematodos por determinadas especies de animales. Sin embargo, existen parásitos que

infectan a diferentes especies, como *Trichostrongylus axei* (**Stear, Doligalska y Donskow-Schmelter, 2007**), y otros que, aun siendo específicos, han sido capaces de adaptarse a otros hospedadores en infecciones experimentales (**Torres-Acosta y Hoste, 2008**).

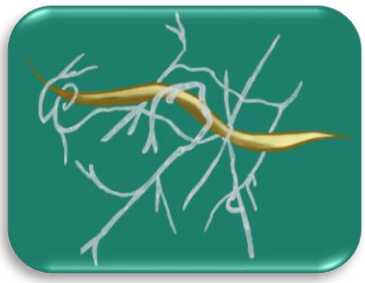
El movimiento del ganado más susceptible (hembras en periparto y animales jóvenes) hacia zonas de pastoreo libres de larvas infectantes es una opción aconsejable que se ha practicado en ganadería (**Stear, Doligalska y Donskow-Schmelter, 2007**). "Tradicionalmente" se tratan con antihelmínticos antes de movilizarlos, pero actualmente se recomienda tratarlos con posterioridad (**Torres-Acosta y Hoste, 2008**), es decir, es aún controvertido. Esto se debe a que los parásitos que sobrevivían al tratamiento se instauraban en el medio, creando una población resistente e impidiendo que se produjera el concepto de "refugio", del que hablamos anteriormente (**Torres-Acosta y Hoste, 2008; Kenyon *et al.,* 2009**). Otra consecuencia que podría darse es que, al mover sólo animales susceptibles, perdemos el efecto que generaban los animales resistentes al "diluir" el número de nematodos en el medio (**Bowman, 2011**).

No obstante, considerando lo anterior, sería necesario un estudio previo del clima y condiciones de la granja para implementar el método más adecuado (**Kearney *et al.,* 2016**).

3.3.3 Control biológico

Se ha investigado extensamente cómo eliminar las larvas infectantes del pasto mediante agentes biológicos, pero pocos métodos han conseguido ser aplicables en el medio (**Torres-Acosta y Hoste, 2008**).

Figura 3.12 Larva de NGI atrapada por *Duddingtonia flagrans*



La opción más estudiada y con resultados más prometedores ha sido la administración de hongos nematófagos como método profiláctico frente tricostrongílidos (**FAO, 2002; Rocha, Araújo y Amarante, 2007; Hoste y Torres-Acosta, 2011**). Estos consiguen disminuir la población de larvas infectantes en heces antes de que éstas migren a la vegetación (**Hoste y Torres-Acosta, 2011; Charlier *et al.,* 2018**). La especie *Duddingtonia flagrans* (**Figura 3.11**) destaca entre el resto por ser capaz de sobrevivir a su paso por el tracto digestivo de los animales en forma de esporas, permitiendo que sigan siendo funcionales una vez vuelven al medio a través de las heces de los animales (**Gómez-Rincón, Uriarte y Valderrábano, 2006; Torres-Acosta y Hoste,**

2008). Sin embargo, su aplicación en la granja ha arrojado resultados variables (**Gómez-Rincón, Uriarte y Valderrábano, 2006**), presentando ciertos inconvenientes como, por ejemplo, el hecho de que se necesite administrar esporas frecuentemente a los animales para que sea efectivo el tratamiento (**Stear, Doligalska y Donskow-Schmelter, 2007; Charlier *et al.,* 2018**), ya que durante la digestión ruminal del ganado se producen pérdidas importantes de éstas (**Ojeda-Robertos *et al.,* 2009**); y, también, por la posible vulnerabilidad que pueden presentar a las condiciones climáticas (**Epe *et al.,* 2009**). A pesar de ello, ya hay un producto comercializado, BioWorma® (https://www.bioworma.com), que ha obtenido resultados positivos, disminuyendo significativamente el número de larvas infectantes en équidos, vacuno y caprino (**Healey *et al.,* 2018**).

3.3.4 Control mediante la alimentación

El parasitismo puede afectar al crecimiento y a la producción de leche o lana debido al desvío de nutrientes hacia el mantenimiento de la respuesta inmune, entre otros (**Charlier *et al.,* 2018**). Por este motivo, la alimentación del ganado ha sido estudiada en busca de elementos que fueran capaces de disminuir la parasitación y/o sus síntomas (**Torres-Acosta y Hoste, 2008**).

Figura 3.13 Forraje

Una opción interesante para reducir estos efectos es el aporte proteico, con el que se ha observado una disminución de la contaminación larvaria de los pastos cuando se administraba a hembras en periparto (**Torres-Acosta y Hoste, 2008**). Además, hay estudios en ganado caprino que indican que el aporte proteico presenta mejores resultados en los animales más productivos (**Chartier *et al.,* 2000**). Asimismo, este método no representa una gran inversión económica ya que se puede suplementar con fuentes proteicas de bajo coste, como por ejemplo los bloques nutricionales de urea/melaza (**Torres-Acosta y Hoste, 2008**). Sin embargo, puede resultar difícil conocer cuánta cantidad es necesaria para cada individuo (**Torres-Acosta y Hoste, 2008**).

Por otro lado, existen plantas que aparte del aporte nutricional, presentan propiedades antihelmínticas. En general, su investigación se ha centrado en el forraje bioactivo que contenía polifenoles, destacando los taninos condensados (**Kearney *et al.,* 2016; Vercruysse *et al.,* 2018**), aunque en este caso, hay que tener en consideración que en altas concentraciones pueden ser tóxicos y también disminuir la digestibilidad de algunos nutrientes (**Kearney *et al.,* 2016**). El estudio de forrajes bioactivos ha obtenido resultados

variables, atribuyéndose a factores tecnológicos, del medioambiente y genéticos (**Charlier *et al.,* 2018**). A pesar de ello, se considera un método de control interesante y que necesita un mayor estudio para su uso en el futuro (**Charlier *et al.,* 2018**). Asimismo, estos compuestos se han asociado con una mejora de la inmunidad frente a GIN en pequeños rumiantes, aunque no está claro si se debe al aporte de proteína o si realmente son inmunoestimulantes (**Charlier *et al.,* 2018**).

Otra alternativa que se ha investigado es el aporte de oligoelementos. El más estudiado ha sido el uso de partículas de óxido de cobre (COWP - *Copper oxide wire particles*-), que comenzó con el objetivo de paliar la falta de este metal en rumiantes. Posteriormente, se descubriría que también tenía propiedades antihelmínticas (**Torres-Acosta y Hoste, 2008**). Sin embargo, es importante especificar que los resultados más consistentes se han producido frente a la especie *H. contortus*. Mientras que frente a otros nematodos como *T. circumcincta* y *Trichostrongylus spp.* han sido más variables (**Hoste y Torres-Acosta, 2011**).

3.3.5 Selección genética

En la naturaleza podemos encontrar tanto individuos como razas que presentan variabilidad genética (**Figura 3.14**) en resistencia, resiliencia o combinación de ambas, frente a la mayoría de las enfermedades estudiadas hasta ahora (**Gibson y Bishop, 2005**). Se considera que un animal es resistente a NGI cuando consigue regular la población de vermes, disminuyendo la carga parasitaria o la longitud y fecundidad de éstos (**Bishop, 2012; Meana y Rojo-Vázquez, 1999; Stear *et al.,* 1995**). Mientras que la resiliencia se relaciona con la tolerancia a la enfermedad, al describirse como la habilidad de los animales a no enfermar y mantener la producción durante la parasitación (**Bishop, 2012; Bisset y Morris, 1996**). La selección genética se basa en utilizar como herramienta la heredabilidad de ambos caracteres como método de control de NGI (**Bisset y Morris, 1996; McManus *et al.,* 2014; Piedrafita *et al.,* 2010**).

Figura 3.14 ADN

Dado que la resistencia frente a nematodos es poligénica y los vermes tienen que adaptarse a muchas "dianas" diferentes (**Bishop, 2012; McManus *et al.,* 2014**), una ventaja que presenta este método es el generar una menor presión de selección en el parásito para que éste mute, a diferencia de los antihelmínticos y, por ello, es más sostenible a largo plazo **(Bishop, 2012; Gibson y Bishop, 2005; Piedrafita *et al.,* 2010**). Por este motivo, hoy en día se establecen programas de selección genética en base a la resistencia y a la resiliencia

frente a NGI (**McManus *et al.,* 2014**). Si bien existen programas enfocados a cada uno por separado, la combinación de ambos es beneficiosa ya que además de influir en el impacto de la enfermedad en los animales, también afectaría a la epidemiología (**Bishop, 2012**).

Aunque la mejora genética puede basarse en la utilización de genes, no está claro cuál (es) podrían ser útiles para la selección y, por ello, se recurre a otras herramientas como las que se mencionan a continuación.

Por ejemplo, el uso de valores fenotípicos como indicadores de resistencia, para estimar la variabilidad que presentan entre y dentro de razas (**Dominik, 2005**). Se han estudiado diferentes caracteres fenotípicos en rumiantes como marcadores indirectos, tanto relacionados con la resistencia (recuento de huevos en heces o anticuerpos específicos frente al parásito -IgA, IgG e IgM-, como con la resiliencia y el impacto de la parasitación (tasa de crecimiento o indicadores de anemia) (**McManus *et al.,* 2014**). De ellos, el principal indicador que se ha utilizado hasta ahora es el recuento de huevos en heces (**Bishop, 2012; Stear y Wakelin, 1998**), presentando una heredabilidad de entre 0,2-0,4 (**Bishop, 2012; McManus *et al.,* 2014; Stear y Wakelin, 1998; Vagenas *et al.,* 2002**). La principal ventaja de la utilización de este indicador es que, además de conseguir disminuir el número de vermes en el hospedador, puede reducir la contaminación de los pastos y, por tanto, su transmisión al resto del ganado (**Torres-Acosta y Hoste, 2008**). En la actualidad existen programas de selección exitosos realizados en base a este indicador (**Moreno-Romieux *et al.,* 2017**) e incluso se promueve este método gracias a programas comerciales como *WormBoss* en Australia (**http://www.wormboss.com.au**). También hay que destacar otro marcador fenotípico que cada vez se usa con más frecuencia en Nueva Zelanda, el test CARLA (**https://www.agresearch.co.nz/doing-business/products-and-services/carla-saliva-test/**), basado en IgA específica de un antígeno de superficie de la L3 de *Trichostrongylus colubriformis*. Esta inmunoglobulina es capaz de impedir el establecimiento larvario al generar la expulsión rápida de las larvas. De esta manera, esta prueba mide la IgA específica en saliva de animales infectados utilizando el antígeno de superficie, donde una mayor reacción CARLA se relaciona con un menor recuento de huevos en heces y un mejor crecimiento de los animales infectados (**McManus *et al.,* 2014**). Además, se ha observado que las respuestas de IgA específica de antígeno presentan el doble de heredabilidad que el recuento de huevos en heces, por lo que podría suponer un mejor carácter de selección de resistencia (**Fairlie-Clarke *et al.,* 2019**).

Otra opción sería el uso de razas locales, que han evolucionado y adquirido naturalmente mecanismos de resistencia frente a estas infecciones al haberse adaptado a exposiciones elevadas de parásitos con un nivel de mantenimiento mínimo (**Gibson y Bishop, 2005**). Sin embargo, la demanda de productos de origen animal ha incentivado la tendencia a introducir razas más productivas, a costa de desplazar las razas locales existentes que se mantenían hasta ahora por su valor cultural y rusticidad (**Piedrafita *et al.,* 2010; Torres-Acosta y Hoste, 2008**). Estas razas, que consideramos como comerciales, han sobrevivido hasta ahora gracias al tratamiento con antihelmínticos, pasando genes no resistentes de generación en generación (**Piedrafita *et al.,* 2010**). Por este motivo, hoy en día se intenta evitar esta práctica y en muchos lugares se buscan otros métodos para mejorar la productividad, por ejemplo, seleccionando animales resistentes dentro del ganado/raza (**Torres-Acosta y Hoste, 2008**). A fin de preservar las razas locales y sus genes, la FAO ha destacado el papel que ejercen en ciertos ecosistemas y ha creado un banco de genes (embriones/semen) de algunas de ellas, además del banco de datos ya existente donde podemos consultar el estado de los recursos genéticos de estos animales a escala global (**FAO, 2007; Torres-Acosta y Hoste, 2008**). Con todo, es difícil evitar la dilución genética que se produce debido al cruce indiscriminado de razas, lo cual es considerado como una gran amenaza a la diversidad genética (**FAO, 2007**).

Algunas de las razas ovinas clasificadas como resistentes a nematodos gastrointestinales son la Barbados Black Belly (**Aumont, Gruner y Hostache, 2003**), Red Maasai (**Mugambi *et al.,* 1997**), Gulf Coast Native (**Li, Miller y Franke, 2001**), St Croix (**Stear y Murray, 1994**) y la oveja Canaria de Pelo (**González *et al.,* 2008**). Esta última corresponde a una de las dos razas autóctonas de las Islas Canarias (**Figura 3.15**) que hemos usado en el ensayo de la actual tesis. La oveja Canaria de Pelo es de origen africano y fue traída por los primeros habitantes de las islas, que la criaron durante años hasta ser posteriormente exportada al continente americano, justo después de su descubrimiento. Allí se adaptó y, hace relativamente pocos años, fue reintroducida en Canarias, donde se explota por su aptitud cárnica (**Ministerio de Agricultura, 2021**). Se caracteriza por la resistencia mostrada frente a *H. contortus* en inoculaciones experimentales (**González *et al.,* 2008**) y en infecciones mixtas naturales de este nematodo en conjunto con *T. circumcincta* y *Trichostrongylus* spp. (**Hernández, 2015**).

Figura 3.15 Imagen de corderos de la raza Canaria de Pelo

Una herramienta que nos sirve para identificar los mecanismos implicados en la resistencia frente a NGI es la transcriptómica, a través del estudio de los genes y del nivel de expresión que presentan en animales resistentes y susceptibles. Por ejemplo, se ha estudiado el transcriptoma de la mucosa abomasal de las dos razas ovinas utilizadas en esta tesis, raza Canaria de Pelo (resistente) y raza Canaria (susceptible), en un ensayo donde fueron infectadas con *H. contortus* (**Guo *et al.,* 2016**). En él se observó que la raza Canaria de Pelo (CAP) presentaba una mayor expresión de genes relacionados con las siguientes rutas: respuestas inflamatorias agudas de fácil inducción, activación del complemento, proliferación celular acelerada y subsiguiente reparación tisular, e inmunidad dirigida contra la fecundidad del parásito. Todas ellas parecen estar implicadas en el desarrollo de resistencia de esta raza (**Guo *et al.,* 2016**).

Tanto esta, como todas las razas mencionadas anteriormente se caracterizan por responder a la infección de forma efectiva. Aunque no se conocen exactamente los mecanismos mediante los cuales cada una consigue impedir o disminuir la infección por NGI, se ha estudiado la respuesta inmune que ocurre generalmente frente a estos.

Respuesta inmune

Los helmintos necesitan mantener una relación parásito-hospedador balanceada para poder sobrevivir, por lo que generalmente las enfermedades que producen cursan de forma subclínica (**Tizard, 2009**). Sin embargo, el que causen mayor o menor patología, va a depender, entre otras cosas, del grado de desarrollo de la respuesta inmune del hospedador. En él van a influir factores intrínsecos del animal que pueden alterar este equilibrio, como: edad, sexo, raza, genética, estado reproductivo, nutricional o comportamiento (**Guo *et al.,***

2016; Hayward, 2013; McRae *et al.,* 2015; Piedrafita *et al.,* 2010; Strain *et al.,* 2002) y factores externos a él, como por ejemplo: especie de nematodo, intensidad de la infección o suplementación nutricional (**Hayward, 2013; Stear *et al.,* 2003; Tizard, 2009**).

En ovino, el desarrollo de inmunocompetencia frente a los NGI comienza a manifestarse en los primeros meses de vida, aunque necesitan exponerse regularmente a ellos para poder conseguir una respuesta inmune protectora que suele alcanzarse a los 10-12 meses de edad (**McRae *et al.,* 2015**). Esta inmunocompetencia frente a NGI se ha relacionado tanto con la disminución de la carga parasitaria al prevenir el establecimiento de la mayor parte de larvas infectantes y favorecer la expulsión de vermes adultos, así como con el acortamiento y descenso de la fecundidad de los vermes (**McRae *et al.,* 2015; Stear *et al.,* 1995**).

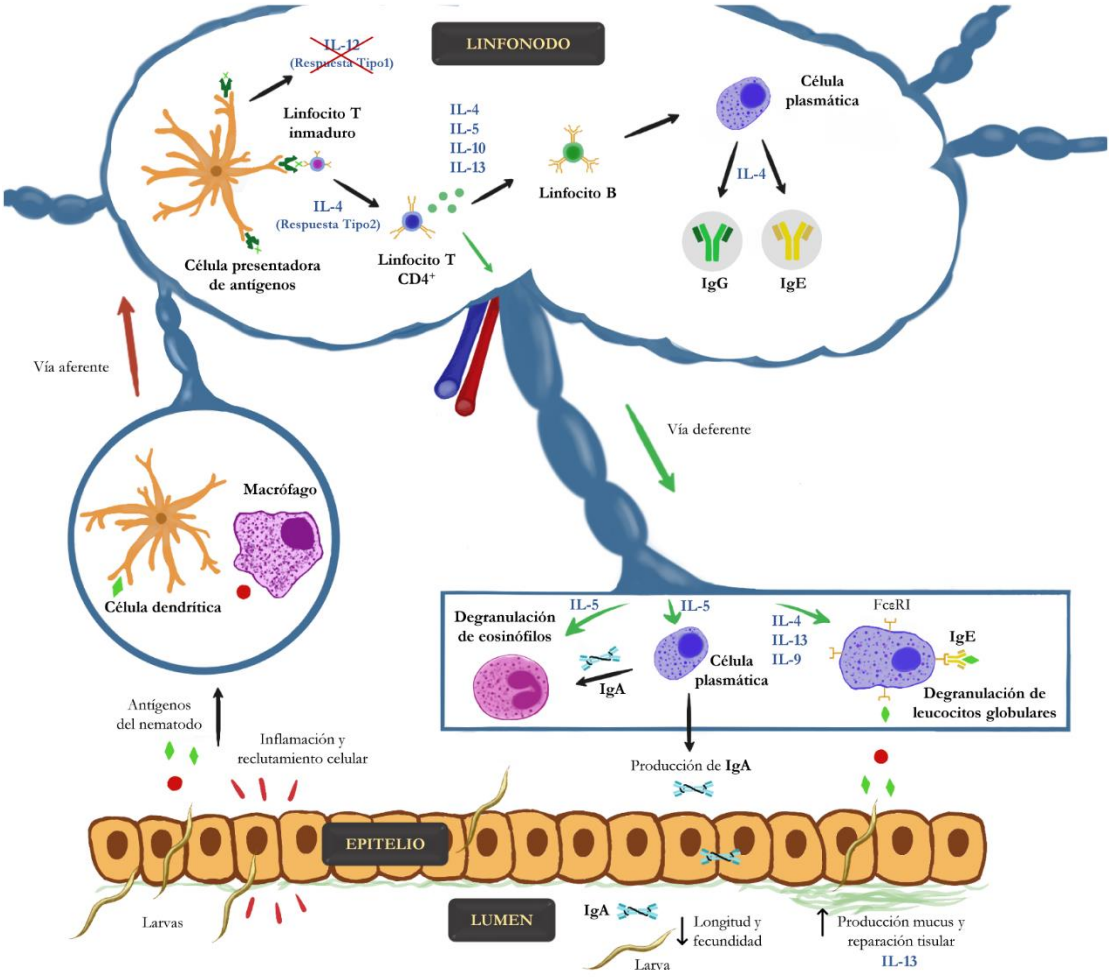
La respuesta inmune frente al nematodo *T. circumcincta* comienza cuando entra al organismo del hospedador y "expone" sus antígenos, generándose una respuesta inespecífica cuyo objetivo es el de eliminar al patógeno lo más rápidamente posible y reparar los daños que haya podido causar (**Tizard, 2009**). Sin embargo, para desarrollar un mecanismo de defensa frente al parásito, es necesaria la exposición repetida a él y requiere de días para que se pueda generar una respuesta inmune específica (**Balic, Bowles y Meeusen, 2000**). De esta forma, se producirá el reconocimiento antigénico por parte de los linfocitos T inmaduros, que derivará en una diferenciación y proliferación de linfocitos B y T. Ambos tipos de respuestas coexisten en el tiempo y en ellas se encuentran involucradas diversas células, citoquinas y anticuerpos (**McRae *et al.,* 2015; Tizard, 2009**).

La larva infectante al entrar causa daño en el epitelio de la mucosa abomasal, generando una reacción inflamatoria local, donde se reclutan mastocitos, eosinófilos, neutrófilos y células Natural Killer (NK) (**McRae *et al.,* 2015**) (**Figura 3.16**). Las células presentadoras de antígeno, como las dendríticas y los macrófagos, van a "captar" los antígenos parasitarios y migrar hacia los linfonodos regionales para presentárselos a los linfocitos T inmaduros. Debido a ello, se producen diferentes citoquinas que promueven la diferenciación de estas células en función de la respuesta que sea estimulada -Tipo1 o Tipo2- (**Aboshady *et al.,* 2020**). Estas respuestas se consideran antagonistas y, aunque normalmente el parasitismo por nematodos induce un mayor desarrollo de la respuesta Tipo2, en ocasiones también se observan respuestas Tipo1 (asociada a infecciones de parásitos intracelulares), generalmente en razas o individuos susceptibles que no responden adecuadamente a la infección (**Aboshady *et al.,* 2020; Hayward, 2013**), aunque otros estudios han detectado respuestas

típicas de tipo 1, por ejemplo con altos IFN-γ en animales resistentes y se ha sugerido que se trata de un balance más correcto entre ambas respuestas en ovino (**Colditz *et al.,* 1996; Venturina, Gossner y Hopkins, 2013**). La respuesta Tipo2 consiste en la activación de las células a través de la vía alternativa, generando interleuquina 4 (IL-4), IL-5, IL-10 e IL-13, que van a inducir la producción, activación y desplazamiento de mastocitos y eosinófilos hacia la zona de infección (**Mantovani *et al.,* 2013; McRae *et al.,* 2015**). También estimulan la diferenciación de células B inmaduras en células plasmáticas productoras de anticuerpos específicos frente al parásito (**McRae *et al.,* 2015**). En presencia de IL-5 se produce IgA y se activan los eosinófilos promoviendo su degranulación sobre las larvas, lo que podría contribuir al mecanismo de expulsión tardía de ellas (**Balic, Bowles y Meeusen, 2002; McRae *et al.,* 2015**). Por otra parte, la IgA está relacionada con el mecanismo de resistencia adquirida frente a L4 mediante la supresión mediada del crecimiento de estas, de manera que, indirectamente se estaría afectando la longitud del estadio adulto final y la reducción de la prolificidad, al disminuir el número de huevos debido al tamaño del verme (**Balic *et al.,* 2000; Henderson y Stear, 2006**).

Después de repetidas infecciones, la IL-4 estimula la producción de IgG (especialmente de la subclase IgG_1) e IgE específica frente al parásito por parte de las células plasmáticas (**Aboshady *et al.,* 2020; Tizard, 2009**). La IgE se va a unir a los mastocitos activándolos, considerándose entonces leucocitos globulares (**Balic *et al.,* 2000**). Por lo que, en las siguientes infecciones, las larvas van a estimular de esta manera una degranulación de ellos (**Aboshady *et al.,* 2020; Venturina *et al.,* 2013**). Esto hace que se liberen más mediadores proinflamatorios, que estimulan una respuesta de hipersensibilidad tipo 1, aumentando la producción del mucus del tracto gastrointestinal y el peristaltismo intestinal. Contribuyen, de esta forma, a la expulsión rápida de las larvas L3, al dificultar su establecimiento y supervivencia, así como de los vermes adultos (**Balic *et al.,* 2002; McRae *et al.,* 2015**).

Figura 3.16 Representación de la respuesta inmune frente a nematodos gastrointestinales (Adaptado de McRae *et al.,* 2015)



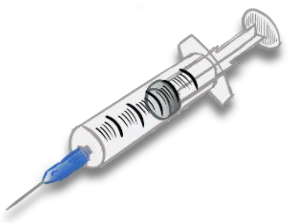
Aunque todo ello parece constituir el principal mecanismo de resistencia frente a este parásito en corderos, los eosinófilos no parecen jugar un rol importante en la respuesta, a diferencia de lo que ocurre con otro nematodo gastrointestinal, *Haemonchus contortus*. Es más, una marcada eosinofilia durante la infección con *T. circumcincta* se ha relacionado con una mayor susceptibilidad (**Henderson y Stear, 2006**). Por lo que, parece que la IgA (regulando longitud y el número de huevos en el útero de los vermes) y la IgG_1 e IgE específicas del parásito, junto a los leucocitos globulares (controlando la carga parasitaria), son las principales herramientas que tiene el ganado ovino para defenderse de las infecciones de *T. circumcincta* (**Aboshady *et al.,* 2020; Henderson y Stear, 2006; McRae *et al.,* 2015; Stear *et al.,* 1995**).

En relación con la respuesta inmune de la raza Canaria de Pelo (resistente) frente a GIN, en experiencias anteriores de nuestro grupo de investigación, se ha observado que la respuesta generada frente a infecciones experimentales de *H. contortus* está relacionada principalmente con la activación de eosinófilos, IgA y células Tγδ/WC1+, modulando la fecundidad del estadio adulto (**González *et al.,* 2008, 2011**). Aunque también se ha observado menores recuentos de huevos en heces en ovinos de esta raza expuestos a infecciones naturales mixtas en las que predominaban las especies/género *T. circumcincta* y *Trichostrongylus* spp. (**Hernández, 2015**), no se conocen los mecanismos responsables de esta protección. En esta tesis, al estudiar la respuesta inmune en infecciones experimentales de *T. circumcincta*, esperamos demostrar la resistencia a este verme y dilucidar alguno de los mecanismos implicados.

3.3.6 **Vacunas**

Las vacunas (**Figura 3.17**) frente a parásitos son una alternativa atractiva, con la que se trata de estimular una respuesta inmune eficaz en los animales, para aumentar la producción y mejorar el bienestar animal. Además, nos permite reducir la presencia de residuos químicos en el medioambiente, al requerir una menor frecuencia de tratamientos farmacológicos durante el año (**Vercruysse *et al.,* 2018**). Sin embargo, el desarrollo y comercialización de vacunas frente a parásitos se ha encontrado con diversos obstáculos a lo largo del tiempo (**Matthews *et al.,* 2016**). Por ello, es de destacar, que actualmente se comercialicen 3 vacunas frente a helmintos de rumiantes: "Barbervax®"/"Wirevax®" (de antígeno nativo del nematodo *H. contortus*, en Australia y Sudáfrica respectivamente); "Bovilis Huskvac®" (del nematodo *Dictyocaulus viviparus* vivo atenuado); y "Providean Hidatil EG 95®" (de antígeno recombinante del cestodo *Echinococcus granulosus*, en Sudamérica) (**Claerebout y Geldhof, 2020; Matthews *et al.,* 2016**).

Figura 3.17 Vacuna



Para que una vacuna se considere efectiva debe conseguir reducir los síntomas de la enfermedad y las pérdidas productivas, aunque existen vacunas cuyo efecto es poco duradero, así que su utilidad va a depender de las condiciones climáticas y de manejo de la explotación ganadera. De igual manera, se recomienda que el uso de vacunas vaya acompañado de otros métodos de control y se realice el diagnóstico para localizar y tratar, o apartar del rebaño, a los animales que no responden a la vacuna (**Vercruysse *et al.,* 2018**). Sin embargo, en la actualidad no se persigue una inmunidad total del rebaño y se le da mayor

importancia a buscar vacunas multivalentes, combinando antígenos o usando moléculas presentes en diferentes especies (**Matthews *et al.,* 2016**).

Las vacunas frente a GIN pueden componerse del parásito en su totalidad (debilitado/atenuado), como es el caso de vacunas de parásito irradiado (**Claerebout y Geldhof, 2020**). La primera vacuna comercializada frente helmintos de rumiantes era de este tipo y consistía en larvas vivas irradiadas de *Dictyocaulus viviparus*, nematodo pulmonar que afecta al ganado bovino. Ésta se desarrolló en la década de los 50 y se distribuyó bajo el nombre de Dictol®, cambiándolo posteriormente a Bovilis Huskvac® (MSD Animal Health), la cual continúa utilizándose en la actualidad (**Britton *et al.,* 2020; Jarrett *et al.,* 1959; Matthews *et al.,* 2016**). Si bien ha habido posteriores intentos de inmunización con diferentes especies de nematodos atenuados por irradiación, no se ha obtenido el éxito esperado (**Britton *et al.,* 2020**).

Por otro lado, existen vacunas basadas en antígenos, que pueden clasificarse atendiendo a su naturaleza. Se consideran nativas si los antígenos forman parte o son producidos por el parásito, y recombinantes cuando estos se sintetizan a partir de sistemas de expresión (**Nisbet, Meeusen, *et al.,* 2016**). La producción de antígenos nativos requiere infectar a animales para obtener los parásitos y, en algunos casos, sacrificarlos (si necesitamos vermes adultos), además de garantizar la cadena de frío, lo que, entre otras cosas, limita la distribución de la vacuna (**Matthews *et al.,* 2016**). Asimismo, es necesario que las vacunas sean de fácil producción y uniformes, para que sean comercialmente viables. Esto es difícil de conseguir cuando purificamos los vermes para extraer antígenos naturales, por ello se estudia como reproducirlos en su forma recombinante. De esta manera, se podría reducir el coste de producción y la variabilidad entre lotes, facilitando su comercialización (**Charlier *et al.,* 2018**). Sin embargo, conseguir recrear la conformación correcta de la molécula deseada es complicado y, hasta ahora, se ha observado que en la mayoría de los estudios no generan respuesta (**Matthews *et al.,* 2016**).

Además de esta clasificación, las vacunas se pueden catalogar según el origen del antígeno. Los denominados antígenos ocultos son aquellos que generalmente no se exponen al sistema inmune del hospedador. Es el caso de los que componen la vacuna "Barbervax®"/ "Wirevax®", producida a partir de proteínas nativas, de la membrana intestinal del nematodo *H. contortus* (aminopeptidasa H11 y complejo glicoproteico H-gal-GP) (**Claerebout y Geldhof, 2020; Matthews *et al.,* 2016**). Se cree que el éxito de esta vacuna reside en que *H. contortus*, al alimentarse de la sangre del animal, recibe los anticuerpos de éste, posiblemente

afectando al intestino del parásito alterando digestión (**Nisbet, Meeusen, *et al.,* 2016; Schallig, 2000**). Por esta razón, es capaz de proteger a los animales que presentan una inmunidad natural débil o poco efectiva. Sin embargo, las infecciones parasitarias no re-estimulan la respuesta inmune de los animales al no exponerse al antígeno, por lo que el efecto de la inmunización es poco duradero y son necesarias dosis constantes de ella (**Claerebout y Geldhof, 2020**). No obstante, el corto periodo de duración que presenta no supone un problema en países con pastoreo limitado a ciertas épocas del año o si se realiza manejo de pasto (**Vercruysse *et al.,* 2018**). Asimismo, se ha intentado desarrollar su forma recombinante, pero, hasta ahora, no ha conseguido estimular la respuesta inmune del ganado (**Matthews *et al.,* 2016**).

Otro tipo de antígenos son los de ES (excreción/secreción), proteínas liberadas por el parásito en el hospedador o en cultivos *in vitro* para la producción de vacunas (**Claerebout y Geldhof, 2020**) Un ejemplo es el prototipo de 8 proteínas recombinantes de *T. circumcincta* desarrollado por **Nisbet *et al.,* 2013**, que contiene diversos productos de ES del parásito, como Tci-ES20, Tci-MEP-1 y Tci-APY-1. Más adelante, se hablará en profundidad de esta vacuna, que ha sido utilizada en el ensayo que forma parte de esta tesis doctoral.

Por último, las vacunas de antígeno somático son aquellas desarrolladas a partir de proteínas que se encuentran en el cuerpo del parásito. Recientemente un grupo de investigación español dirigido por el Dr. Alunda (Universidad Complutense de Madrid) desarrolló una vacuna con la versión recombinante expresada en *E. coli* de una proteína somática del estadio adulto de *H. contortus* (Hc23), a la cual se le había atribuido un efecto protector con anterioridad (**Fawzi *et al.,* 2015**). La inmunización en corderos dio como resultado una reducción superior al 80% en el recuento de huevos en heces y de adultos en abomaso respecto al grupo control (no vacunado e infectado). Estos niveles de protección son similares a los alcanzados en experiencias que han utilizado antígenos nativos, por lo que se trata de un hallazgo prometedor en la producción de proteínas recombinantes (**Fawzi *et al.,* 2015**). Además, tanto las vacunas de antígeno somático como las de productos de ES, mantienen su efecto protector durante más tiempo que las de antígeno oculto, ya que son antígenos expuestos al sistema inmune del hospedador y la infección con el parásito produce un *boost* en la respuesta inmune. Aun así, la duración puede estar condicionada por el sistema inmune del animal y las características de la explotación ganadera (**Claerebout y Geldhof, 2020**).

La elección del adyuvante apropiado depende de los antígenos que componen la vacuna y se ha observado que éste puede ser el causante de que ésta sea o no eficaz (**Charlier *et al.,* 2018**). Normalmente, en las vacunas frente a nematodos se tiende a estimular una respuesta celular (Tipo2), al conocerse como la respuesta protectora frente a helmintos, con adyuvantes como el hidróxido de aluminio (**Fawzi *et al.,* 2015b**). Sin embargo, se ha observado eficacia en adyuvantes estimulantes de la respuesta humoral (Tipo1) como Quil A, en vacunaciones frente a *T. circumcincta* (**Matthews *et al.,* 2016; Nisbet *et al.,* 2013**).

Por último, es importante destacar que las vacunas frente a GIN deben presentar una relación coste/ efectividad que atraiga a los ganaderos, ya que, aunque se financian proyectos de vacunas frente a parásitos incluso con gran dotación presupuestaria (ej. PARAGONE: *vaccines for animal parasites*), las autoridades no consideran las lucha frente a éstos como un tema prioritario (**Claerebout y Geldhof, 2020; Vercruysse *et al.,* 2018**). Por este mismo motivo, sería ideal conseguir desarrollar vacunas capaces de proteger al ganado de múltiples especies a la vez (**Matthews *et al.,* 2016**). No obstante, antes de alcanzar estos objetivos, es vital seguir investigando la relación parásito/hospedador para ser capaces de orientar la respuesta inmune y elegir el adyuvante apropiado. Y, además, conseguir la conformación espacial adecuada de las proteínas recombinantes producidas (**Claerebout y Geldhof, 2020; Matthews *et al.,* 2016**).

Vacuna recombinante frente Teladorsagia circumcincta (Nisbet* et al.*, 2013)

Se trata de un prototipo vacunal que contiene 8 antígenos recombinantes, descritos en la **tabla 3.3.** Para testar su efectividad, inicialmente se realizaron dos ensayos en corderos de cruce de Texel. En ambos se siguió el mismo protocolo, que consistía en la administración subcutánea de la vacuna un total de 3 veces, cada 3 semanas junto con el adyuvante Quil A a los grupos vacunados, mientras que a los grupos controles se les administraba sólo el adyuvante. A continuación, se les sometía a infecciones experimentales mediante inoculaciones de larvas infectantes de *T. circumcincta*. El primer ensayo se llevó a cabo en corderos de 204-206 días de edad, observándose una reducción media del 70% de huevos en heces y del 75% de vermes adultos en abomaso en el grupo vacunado respecto al grupo control. En el segundo ensayo se utilizaron corderos de 172-178 días de edad, estableciéndose 4 grupos, dos vacunados y dos controles. Un grupo control y otro vacunado se sacrificaron 7 semanas después de la primera inoculación, sin observarse diferencias significativas entre ellos. Los restantes se sacrificaron 4 semanas después de estos, obteniéndose con la inmunización reducciones del 58% de huevos en heces y 57% de

nematodos totales en abomaso respecto al control (**Nisbet *et al.,* 2013**). Posteriormente se llevó a cabo otra experiencia con ovejas en periparto de la misma raza, donde se observó un 45% de reducción en la media del recuento de huevos acumulativo en heces en el grupo vacunado respecto al control (**Nisbet, McNeilly, *et al.,* 2016**).

Tabla 3.3 Antígenos recombinantes que componen la vacuna frente a *Teladorsagia circumcincta* (Adaptado de Nisbet *et al.,* 2013)

Antígeno	Función	Sistema de expresión
Tci-SAA-1 (Homólogo del antígeno protector de *Ancylostoma caninum* de *T. circumcincta*)	Antígeno de superficie asociado a L3 enriquecida	pET22b(+) *E. coli* BL21 (DE3)
Tci-MIF-1 (*Macrophage migration inhibitory factor-1*)	Factor de inhibición de la migración de macrófagos de L3 enriquecida	pET22b(+) *E. coli* BL21 (DE3)
Tci-ASP-1 (*Activation-associated secretory protein-1*)	Proteína secretora asociada a la activación de L4 enriquecida	pET SUMO *E. coli* BL21 (DE3)
Tci-TGH-2 (Homólogo de TGFβ)	Proteína homóloga del factor de crecimiento transformante beta	pET SUMO *E. coli* BL21 (DE3)
Tci-CF-1 (Cathepsin F-1)	Catepsina F secretada por L4 enriquecida	pPICZαC *Pichia pastoris* X33 strain
Tci-ES20 (Proteína de 20kDa con función desconocida)	Proteína de excreción/secreción (ES)	pPICZαC *Pichia pastoris* X33 strain
Tci-MEP-1 (*Astacin-like metalloproteinase-1*)	Metaloproteinasa de ES de tipo astacina	pET SUMO *E. coli* BL21 (DE3)
Tci-APY-1 (*Calcium-dependent apyrase-1*)	Apirasa activada por calcio de ES de L4 enriquecida	pSUMO *E. coli* BL21 (DE3)

Actualmente, la investigación de este prototipo se ha orientado principalmente a la simplificación de la vacuna, combinando una menor cantidad de proteínas y centrándose en las que hayan logrado estimular la respuesta inmune de los animales durante los ensayos (**Matthews *et al.,* 2016; Nisbet *et al.,* 2019**). Además, se han realizado diversos ensayos en corderos combinando 4 y 2 antígenos del cóctel de proteínas (**Britton *et al.,* 2020**). Si bien, en los primeros no hubo una reducción visible de las variables parasitológicas, en los ensayos con la vacuna de dos antígenos, Tci-APY-1/mTci-APY-1 (mutación de apirasa no funcional) y Tci-MEP-1) hubo cierta reducción, la cual mejoró cuando se utilizaba la mutación de Tci-APY -1 (**Nisbet *et al.,* 2019**).

No obstante, a pesar de que la inmunización con este prototipo vacunal ha generado resultados interesantes, presenta variabilidad en la respuesta entre animales y entre los diferentes ensayos que se han realizado (**Nisbet *et al.,* 2019**). Estos aspectos, sumados al número de proteínas que precisa el prototipo, son serios obstáculos para su comercialización (**Matthews *et al.,* 2016**).

Es por ello, que tiene lugar el ensayo desarrollado en esta tesis doctoral, donde se pretende estudiar la respuesta inmune desarrollada tras la vacunación y desafío con *T. circumcincta* a corderos mayores de 6 meses de dos razas ovinas canarias, con la intención de recabar información que permita optimizar dicho prototipo vacunal.

4. RESUMEN

1. INTRODUCCIÓN

El parasitismo causado por *Teladorsagia circumcincta* y otros nematodos gastrointestinales tiene un gran impacto en el sector ganadero de pequeños rumiantes, causando pérdidas económicas que se ven agravadas por la resistencia que han generado estos a los fármacos. Debido al aumento exponencial de la población mundial hay una mayor demanda de alimento y, por tanto, es imprescindible mejorar la producción de forma sostenible y disminuir la aparición de parásitos resistentes. Esto ha conllevado a la búsqueda de métodos alternativos/complementarios al uso de químicos, como pueden ser el desarrollo de vacunas o la selección de animales/razas resistentes.

En esta tesis se han combinado ambos métodos, contando con dos razas ovinas locales (Canaria -CAN- y Canaria de Pelo -CAP-) de las Islas Canarias (España), que han presentado diferente susceptibilidad a nematodos gastrointestinales con anterioridad, y con un prototipo vacunal frente a *T. circumcincta* que, aun presentando previamente cierta variabilidad individual, ha conferido protección.

2. OBJETIVOS

Comparar la eficacia del prototipo vacunal frente a *Teladorsagia circumcincta* y la respuesta generada tras la inmunización en corderos de 6 meses de las razas locales Canaria y Canaria de Pelo de las Islas Canarias (España) que han sido inoculados experimentalmente con 2000 L3 de este parásito de forma seriada.

Observar la resistencia a la infección por *T. circumcincta* en estas dos razas, comparando los datos parasitológicos de los grupos controles de ambas razas.

3. MATERIAL Y MÉTODOS

Se utilizaron 46 corderos de 6 meses de edad de las razas CAN y CAP, estableciéndose un grupo vacunado y otro control de cada raza. A los grupos vacunados se les administró el prototipo vacunal y a los controles sólo el adyuvante. Las inmunizaciones se llevaron a cabo cada 3 semanas (3 vacunaciones en total), seguidas de 3 inoculaciones orales semanales de 2000 L3 de *T. circumcincta* durante 4 semanas.

Se recogieron muestras de heces periódicamente para el recuento de huevos del parásito. Posteriormente, se sacrificaron los animales y se recolectó el contenido abomasal de cada uno para determinar la carga parasitaria, medir la longitud de los vermes hembra y realizar el recuento de huevos en útero.

Para analizar las IgA, IgG_1 e IgG_2 específicas frente a L3, L4 y adulto de *T. circumcinta* en suero mediante el ensayo por inmunoadsorción ligado a enzimas (E.L.I.S.A.) se obtuvieron muestras de sangre a los 77 días después de la primera inmunización.

Después del sacrificio, también se recogieron muestras de la región antropilórica del abomaso y linfonodos regionales. Las muestras de abomaso se usaron para su estudio histológico e inmunohistoquímico, consistiendo en el recuento de poblaciones celulares mediante técnicas de tinción (eosinófilos, mastocitos y leucocitos globulares) e inmunomarcaje (linfocitos $CD4^+$, $CD8^+$, $\gamma\delta^+$, $\gamma\delta$-$WC1^+$, $CD45RA^+$, MHC-II^+ y Galectina-14^+) respectivamente.

Los linfonodos abomasales, por su parte, se usaron para ensayos de estimulación linfocitaria. Esto se realizó con el objetivo de determinar las citoquinas IFN-γ, IL-6 e IL-17A específicas generadas frente a los antígenos parasitarios L3, L4 y adulto de *T. cicrcumcincta* mediante E.L.I.S.A. de captura; y, también, para realizar un fenotipado de las células producidas mediante citometría de flujo, todo esto realizado en los laboratorios del *Moredun Research Institute* (Edimburgo, Reino Unido).

Asimismo, una muestra adicional de tejido abomasal de cada animal se recolectó y mantuvo en *E.Z.N.A. RNA Lock Reagent* (Omega Bio-tek) para su conservación y posterior extracción de ARN. De esta manera, se pudo estudiar la expresión génica en los corderos, al recolectarse los datos utilizando las siguientes bases de datos y herramientas de búsqueda: "*Ovis aries* Kyoto Encyclopedia of Gene and Genomes" (KEGG), "Database for Annotation, Visualization and Integrated Discovery" (DAVID), "Ensembl", "Basic Local Alignment Search Tool" (BLAST), "Uniprot" y "GeneCards".

Por último, los análisis estadísticos se llevaron a cabo utilizando diferentes programas: *IBM SPSS Statistics version 24.0* para los datos parasitológicos, inmunológicos y recuento de poblaciones celulares en abomaso; *RStudio* con el paquete vers. 1.1.456 (*R Core Team* 2019) para el fenotipaje celular y determinación de citoquinas, además de para la realización de gráficas; y *R* vers. 3.4.4 (2018-03-15) con el paquete *Bioconductor lima* para observar las diferencias en la expresión génica de los animales.

4. RESULTADOS Y DISCUSIÓN

Parasitología (Capítulo 1)

La vacuna generó un efecto protector en la raza Canaria reduciendo de forma significativa la longitud de vermes hembra y huevos intraútero en el grupo vacunado cuando se comparaba con el grupo control. Por otra parte, parece que la raza Canaria de Pelo es más resistente a *T. circumcincta* que la raza Canaria, al contar, el grupo control, con parásitos de menor tamaño y con menor número de huevos en útero que los no vacunados de la raza Canaria, similar a lo observado anteriormente en inoculaciones simples experimentales con otro nematodo gastrointestinal, *Haemonchus contortus*.

Respuesta inmune (Capítulo 2)

La protección que generó la vacuna en la raza Canaria se asoció a dos inmunoglobulinas (IgA e IgG_2) específicas frente al parásito, además de a los leucocitos globulares, observándose correlaciones negativas entre ellos y distintas variables parasitológicas. Además, asociándose también a esta protección, se observó un mayor ratio de $CD4^+/CD8^+$.

Expresión génica (Capítulo 3)

Muchos de los genes que se encontraron sobre expresados en los animales inmunizados de la raza Canaria se han asociado al control de nematodos. Se observaron genes relacionados con la sensibilización y activación de mastocitos, y genes que participan en la formación del mucus. Asimismo, se encontraron genes relacionados con el reconocimiento y captura de antígenos, además de con la producción de citoquinas e inmunoglobulinas. Y otros genes que podrían ejercer una acción directa sobre el nematodo.

Los principales genes regulados a la baja en los vacunados estuvieron relacionados con algunos efectos negativos en caracteres productivos, aunque se desconoce su transcendencia al haberse observado en abomaso. Otros genes se relacionaron con la respuesta inmune.

5. CONCLUSIONES

El prototipo vacunal frente a *Teladorsagia circumcincta* confirió protección en la raza ovina Canaria, asociándose a una mayor expresión de genes relacionados con la respuesta inmune orientada al control de nematodos, y relacionada con IgA e IgG_2 específicas frente al parásito, leucocitos globulares y con un mayor ratio de $CD4^+/CD8^+$.

La raza ovina Canaria de Pelo demostró una mayor resistencia a infecciones experimentales seriadas de *Teladorsagia circumcincta* que la raza Canaria.

5. SUMMARY

1. INTRODUCTION

Parasitism produced by *Teladorsagia circumcincta* and other gastrointestinal nematodes (GINs) has a big impact on small ruminant livestock, generating economic losses that are aggravated by the resistance nematodes have developed against anthelmintics. Due to the world population growth, there is a greater demand for food and, therefore, it is essential to improve production in a sustainable way, reducing the occurrence of resistant parasites. This has led us to search for alternative/complementary methods to chemicals, such as developing vaccines or the use of resistant animals/breeds.

In this thesis, there is a combination of both methods, having two local sheep breeds (Canaria Sheep -CS- and Canaria Hair Breed -CHB-) of the Canary Islands (Spain), that had shown different levels of susceptibility against GIN previously, and a *T. circumcincta* prototype vaccine that had conferred protection but showed individual variability.

2. OBJECTIVES

To compare the efficacy of the prototype vaccine against *Teladorsagia circumcincta* and the response generated in 6-month-old lambs of Canaria Sheep and Canaria Hair Breed from the Canary Islands (Spain) following immunization and serial experimental inoculations with 2000 L3 of this parasite.

To study the resistance against *Teladorsagia circumcincta* in Canaria Sheep and Canaria Hair Breed, comparing parasitological data of control groups of both breeds.

3. MATERIAL AND METHODS

Forty-six CS and CHB lambs of 6 months old were separated in vaccinated and control groups of both breeds. Vaccinated groups were immunized with the prototype vaccine and control groups with adjuvant only. Immunizations were carried out every three weeks, following 3 weekly oral inoculations of 2000 L3 of *T. circumcincta* for 4 weeks.

Stool samples were periodically collected for parasite egg counting. Then, animals were euthanized and their abomasal content was collected to determine worm burden, measure female worm length and count eggs *in utero*.

To analyse specific IgA, IgG_1 and IgG_2 against *T. circumcincta* L3, L4 and adult stages, blood samples from 77 days after the first immunization were obtained.

Samples of the abomasum antropyloric mucosa were collected for histological and immunohistochemical studies. Several cell populations were counted, using staining techniques (eosinophils, mast cells and globule leukocytes) and immunomarking ($CD4^+$, $CD8^+$, $\gamma\delta^+$, $\gamma\delta$-$WC1^+$, $CD45RA^+$, MHC-II^+ and Galectin-14^+ cells) respectively.

Abomosal lymph nodes were collected, as well, for lymphocyte stimulation test to determine IFN-γ, IL-6 and IL-17A specific cytokines against L3, L4 and adult stages of *T. circumcincta* using capture E.L.I.S.A. Also, a phenotyping of the cells presented at local lymph nodes was performed using flow cytometry. All these analyses were carried out at Moredun Research Institute (Edinburgh, United Kingdom).

In addition, another sample of abomasal tissue was collected from each animal for RNA extraction and was preserved in *E.Z.N.A. RNA Lock Reagent* (Omega Bio-tek). Gene expression in CS lambs was studied, contrasting data with different databases and search tools: *Ovis aries* Kyoto Encyclopedia of Gene and Genomes (KEGG), Database for Annotation, Visualization and Integrated Discovery (DAVID), Ensembl, Basic Local Alignment Search Tool (BLAST), Uniprot and GeneCards.

Lastly, statistical analyses were carried out using different programmes: IBM SPSS Statistics version 24.0 for parasitological and immunological data, as well as cell population counting in abomasal tissue; RStudio package version 1.1.456 (R Core Team 2019) for immune cell phenotypes, cytokine data and graphs; and R version 3.4.4 (2018-03-15) with Bioconductor lima package to determine differences in gene expression between CS groups.

4. RESULTS AND DISCUSSION

Parasitology (1st Chapter)

Vaccine generated a protective effect in CS breed resulting in statistically significant reductions in female worm length and eggs *in utero* in vaccinated group compared to the control animals. On the other hand, CHB seems to be more resistant to *T. circumcincta* than CS, since the control group of this breed harboured shorter worms and with fewer eggs *in utero* than CS control group, similar to what has been observed previously in simple experimental inoculations with another GIN, *Haemonchus contortus*.

Immune response (2nd Chapter)

Protection conferred by the vaccine in CS was associated with two *T. circumcincta* specific immunoglobulins (IgA and IgG_2), as well as with globule leukocyte, establishing negative

correlations between them and different parasitological variables. Moreover, this group showed a higher $CD4^+/CD8^+$ ratio associated to this protection.

Gene expression (3rd Chapter)

Several up-regulated genes studied in immunized CS were associated with nematode control. There were some related to mast cell sensitization and activation, and others participating in mucus production. Genes associated with antigen recognition and capture, and cytokine and immunoglobulin production were observed upregulated as well. Also, there were genes that could have a direct effect on the parasite.

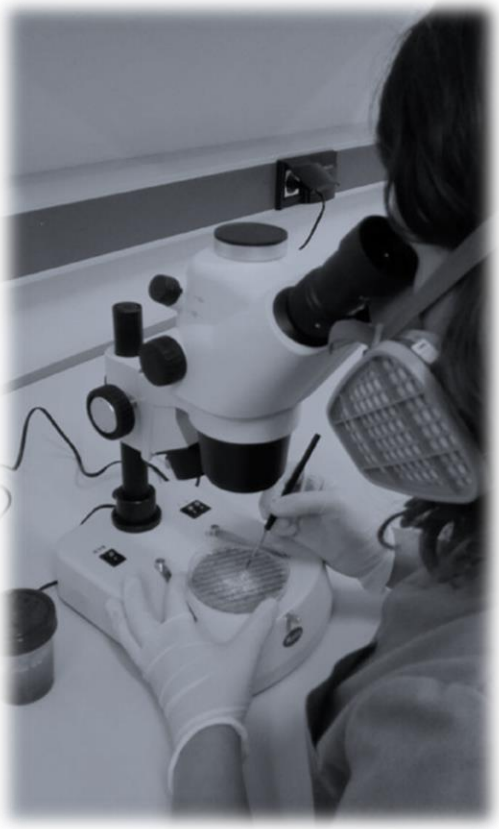
The main down-regulated genes detected in vaccinated animals were associated with a negative effect in some productive traits, although it is not clear if they could impair productivity since they are in the abomasum. Also, down-regulated genes were linked to the immune response.

5. CONCLUSIONS

The vaccine prototype against *Teladorsagia circumcincta* conferred protection to Canaria Sheep, showing a higher expression of genes that orientate the immune response towards nematode control, associated with specific IgA and IgG_2 against the parasite, globule leukocyte and a higher $CD4^+/CD8^+$ ratio.

Canaria Hair Breed showed a higher level of resistance against serial experimental infections with *Teladorsagia circumcincta* than Canaria Sheep.

6. CAPÍTULOS



Capítulo I

"Impacts of breed type and vaccination on *Teladorsagia circumcincta* infection in native sheep in Gran Canaria"

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VETERINARY RESEARCH

SHORT REPORT Open Access

Impacts of breed type and vaccination on *Teladorsagia circumcincta* infection in native sheep in Gran Canaria

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Abstract

Vaccines and genetic resistance offer potential future alternatives to the exclusive use of anthelmintics to control gastrointestinal nematodes (GIN). Here, a *Teladorsagia circumcincta* prototype vaccine was administered to two sheep breeds which differ in their relative levels of resistance to infection with GIN. Vaccination of the more susceptible Canaria Sheep (CS) breed induced significant reductions in worm length and numbers of worm eggs in utero (EIU) when compared to control CS sheep. In the more resistant Canaria Hair Breed (CHB), although vaccination induced a reduction in all parasitological parameters analysed, differences between vaccinated and control sheep were not statistically significant. Such interactions between sheep breed and vaccination may allow better integrated control of GIN in future.

Introduction, methods and results

One of the main limiting factors in sheep production worldwide is infection with gastrointestinal nematodes (GIN). In temperate regions, *Teladorsagia circumcincta* is amongst the most important of these parasites, both in terms of impact on animal health and welfare and in losses in productivity [1, 2]. Traditionally, these parasites have been controlled by regular administration of anthelmintics; however, the increasing prevalence of nematode resistance to these drugs requires alternative or complementary control methods [1, 3]. Sheep have been shown to develop protective immunity against a range of GIN following repeated exposure to the parasites [4, 5] and, amongst alternative control strategies being considered, those that exploit this phenomenon through selection of more genetically resistant animals [1] or by implementing effective vaccines [6] are attractive. Both strategies, vaccination and genetic resistance, are considered here.

Vaccines are considered an appealing alternative control measure for nematodes because they less likely to be subject to the development of parasite resistance and are environmentally friendly [7]. Although vaccination with parasite extracts has generated protection against GIN challenge in a number of trials, most recombinant versions of proteins identified in these fractions have failed to confer similar protection; this is a serious limitation for large-scale commercial vaccine production [5]. Recently, a vaccine based on eight recombinant antigens identified in *T. circumcincta* was shown to stimulate significant levels of protection in Texel-cross lambs [6] and also in ewes during the periparturient period [8] compared to matched challenged sheep. In both types of stock (lambs and ewes), significant reductions in faecal worm egg excretion were observed in vaccinates.

Several sheep breeds have been shown to be more resistant to GIN than other breeds [3]. The use of such resistant breeds offer a potential route to mitigate the effects of helminths in specific production systems. In the Canary Islands, for example, two local breeds of sheep are commonly farmed: the Canaria Hair Breed (CHB) and the Canaria Sheep (CS) breed. The CHB sheep have been shown to be more resistant than CS sheep when

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administered a single experimental infection of *Haemonchus contortus* [9]. Moreover, the former breed has been shown to be more resistant to a natural challenge infection comprising a mix of GIN [10]. Although both strategies are promising in terms of developing sustainable control methods for GIN with less reliance on the use of anthelmintics, neither is likely to entirely replace the use of anti-parasiticides [11]. Combining different alternative methods for worm control could be more effective than using either alone [12], and it would be of interest to explore, in reported resistant breeds, the additive, synergistic or antagonistic effect of vaccination to validate the combination of these control methods. This study tested this hypothesis by undertaking a comparative *T. circumcincta* vaccination and challenge study in the Canarian sheep breeds previously shown to be of different susceptibility to GIN.

Twenty-four CHB and CS lambs (4–5 months-old) were purchased, and, although no strongyle eggs were detected at purchase, they were dewormed with a subcutaneous application of ivermectin (Vectimax®, 0.2 mg/kg) and maintained in conditions designed to avoid helminth infection at the facilities of *Granja Experimental del Cabildo Insular de Gran Canaria* (Veterinary Faculty, Spain) until they were 6–7 months-old. Freedom from helminth infection was confirmed by further coprological testing just before the start of the trial. Animals were fed with a commercial pelleted sheep ration, with forage and water ad libitum throughout the experimental period. Animals were distributed randomly within breed in each experimental group (CS-vaccine; CS-control; CHB-vaccine; CHB-control). One lamb in the CHB-vaccine group died a few days after the start of the procedure from a post-traumatic renal haemorrhage.

The recombinant vaccine was produced exactly as described previously [6]. Sheep in the two vaccinated groups were each injected subcutaneously with 400 µg of vaccine antigens incorporating 50 µg of each protein: cathepsin F-1 (Tci-CF-1), astacin-like metalloproteinase-1 (Tci-MEP-1), a 20 kDa protein of unknown function (Tci-ES20), activation-associated secretory protein-1 (Tci-ASP-1), a homologue of a protective antigen from *Ancylostoma caninum* (Tci-SAA-1), macrophage migration inhibitory factor-1 (Tci-MIF-1), calcium-dependent apyrase-1 (Tci-APY-1) and a TGF homologue (Tci-TGH-2). These were administered in 10 mg of the adjuvant, Quil A (Vax Saponin, Guinness Chemical Products Ltd). Seven of the proteins were phosphate buffered saline (PBS)-soluble and administered in a single injection with 5 mg Quil A in PBS. Tci-MEP-1 is insoluble in PBS and was formulated with 2 M urea in PBS with 5 mg Quil A. The preparations were injected separately at two sites behind the shoulder of each sheep. Three immunizations were administered intervals of 3 weeks. Sheep in each control group received three immunizations with the same concentrations and volumes of urea/PBS/Quil A at the same time as the vaccinates. On the day of the final immunization, an oral trickle third stage larval (L3) challenge was initiated; each sheep was given 2000 *T. circumcincta* L3, three times per week for 4 weeks as described previously [6] (Figure 1). For these infections, a UK-derived *T. circumcincta* strain (MTci2, Weybridge, UK) was used, from which all vaccine antigens were originally derived [6].

Faecal egg counts (FEC) were performed three times per week from 12 days after the start of larval challenge until the end of the experiment 4 weeks later. Cumulative FEC values were estimated for each group using the trapezoidal method for calculation of area under the curve (AUC, [13]). FEC data patterns were analysed by fitting generalised additive mixed models (GAMM) as described previously [6]. Differences in cumulative FEC and total worm burden were analysed using negative binomial models accounting for data over-dispersion.

Vaccinated and control sheep of both breeds began to excrete *T. circumcincta* eggs 14–16 days after the start of challenge (Figure 2). GAMM analysis identified a statistically significant effect of sheep breed on mean FEC over the time-course of the experiment, with significantly higher FEC in non-vaccinated CS than observed in non-vaccinated CHB ($p=0.005$). In CS, FEC levels increased over time until 21 days after the start of challenge and, from 16 days post-challenge, vaccinated CS excreted substantially fewer eggs than CS control sheep at each

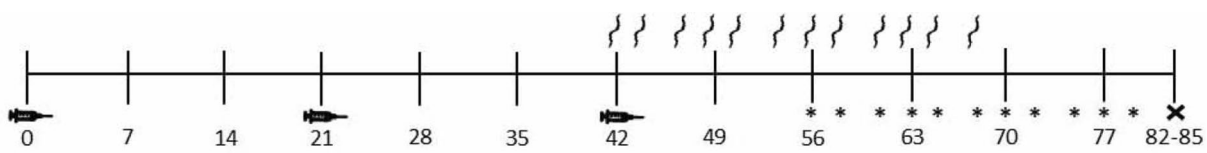

Figure 1 Experimental protocol scheme. The timeline represents days from the start of the experiment (first immunisation). The syringe icon represents each vaccine administration and the picture of larvae, the challenge inoculations. The "*" represents the collection of faeces sampled for faecal egg count analysis, and "x" denotes the time-point of euthanasia.

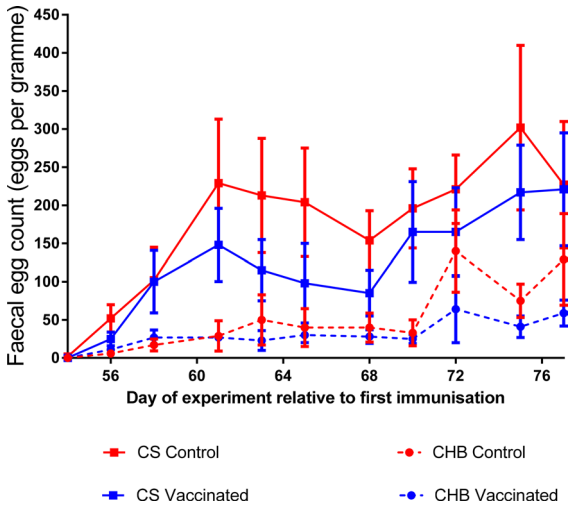

Figure 2 Faecal egg counts measured after challenge infection in two sheep breeds vaccinated against *T. circumcincta*. FEC are shown of sheep (CS, solid lines; CHB, dashed lines) challenged with 2000 *T. circumcincta* L3 three times per week for 4 weeks following immunization with an eight-protein cocktail in the context of Quil A (blue lines) or with Quil A only (red lines). Each data point represents the arithmetic mean FEC ± SEM ($n = 12$ for all groups except CHB vaccinated where $n = 11$).

time-point (Figure 2). GAMM analysis did not reveal a significant difference in mean FEC between vaccinated and unvaccinated CS ($p = 0.118$) or unvaccinated CHB sheep ($p = 0.478$) across the time-course. Mean cumulative FEC levels for CHB sheep for the duration of the challenge period were 1157 (± 504) eggs per gram (EPG) in controls and 720 (± 197) EPG in vaccinates, representing, overall, 38% lower cumulative FEC in the CHB vaccinates ($p = 0.385$; Figure 3A). Mean cumulative FEC for CS for the duration of the challenge period were 4181 (± 953) EPG in control sheep and 2860 (± 738) EPG in vaccinates, representing, overall, 32% lower mean cumulative FEC in CS vaccinates compared to the CS control lambs ($p = 0.427$; Figure 3B). Comparing the average cumulative FEC between control sheep of the two breeds, CS had, on average, 72% higher cumulative FEC levels than the CHB controls ($p = 0.038$).

Abomasal luminal and mucosal worm burdens (adult and larval stages) were enumerated following standard techniques [9]. The developmental stage (larva or adult) was determined based on length and reproductive structure development. Briefly, 30 adult female nematodes were randomly recovered from each abomasum and measured using a digital photo camera (ProgRes C12^{PLUS}) on an inverted microscope (Olympus CKX41) and their eggs in utero (EIU) counted [14]. Several lambs had insufficient worms in the aliquots so, in these cases, all worms were collected from the abomasum and enumerated. Mean worm lengths and numbers of EIU were analysed by one-way ANOVA and the differences between groups identified

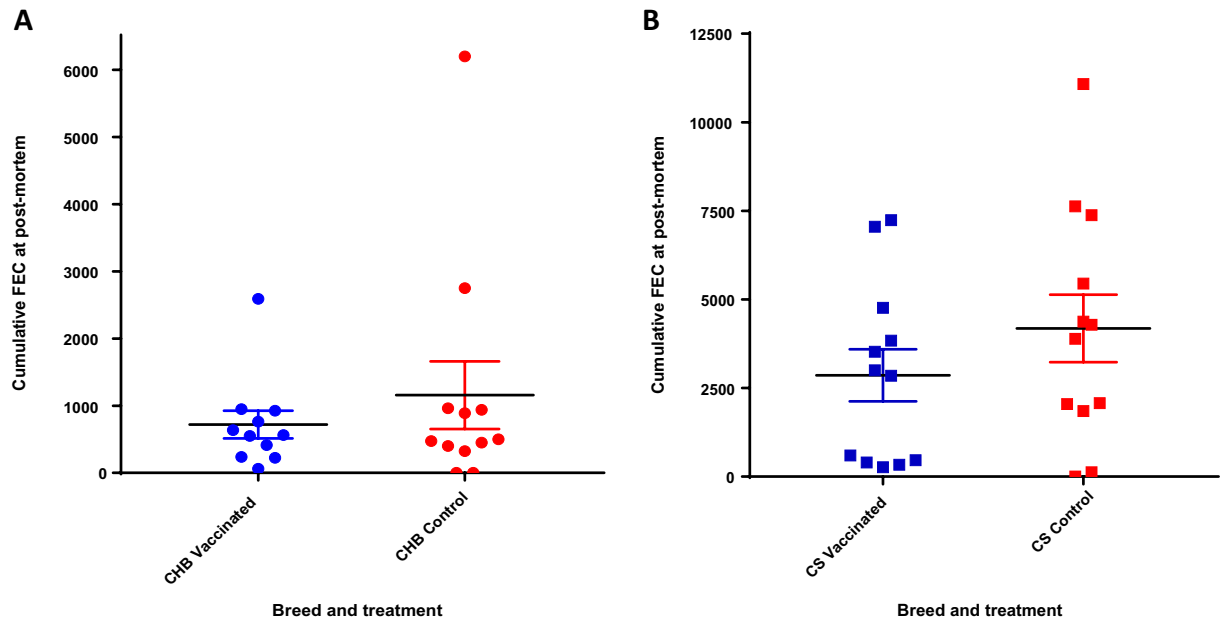

Figure 3 Cumulative faecal egg counts after challenge infection in two sheep breeds vaccinated against *T. circumcincta*. Cumulative FEC are shown of CHB sheep ("CHB" **A**) or CS ("CS" **B**) challenged with 2000 *T. circumcincta* L3 three times per week for 4 weeks following immunization with an eight-protein cocktail in the context of Quil A (CS or CHB vaccinated) or with Quil A only (CS or CHB Control). The mean of cumulative FEC is shown ± SEM ($n = 12$ for all groups except CHB vaccinated where $n = 11$).

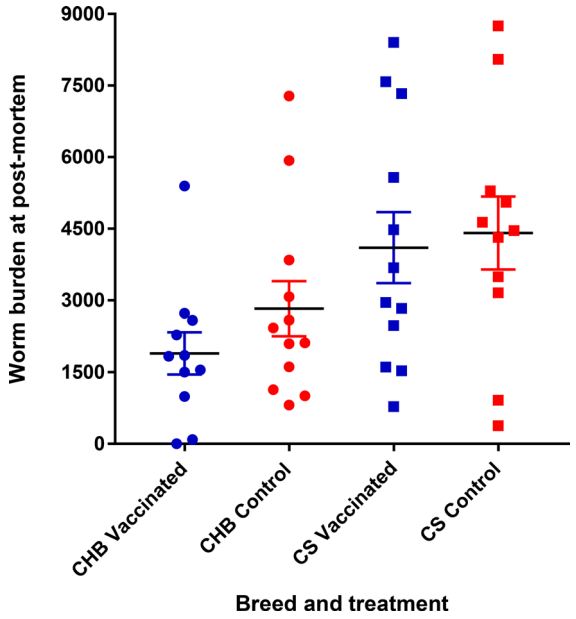

Figure 4 Total worm burdens after challenge infection in two sheep breeds vaccinated against *T. circumcincta*. Worm burdens are shown of sheep (CHB, circles; CS, squares) challenged with 2000 *T. circumcincta* L3 three times per week for 4 weeks following immunization with an eight-protein cocktail in the context of Quil A (blue symbols) or with Quil A only (red symbols). The mean worm burden is shown ± SEM ($n = 12$ for CS vaccinated and CHB control and in CS control and CHB vaccinated where $n = 11$).

using Tukey's multiple comparisons tests. CS vaccinates had similar average burdens at post-mortem (4103 ± 776) to control CS (4410 ± 732) ($p = 0.796$; Figure 4). CHB sheep vaccinates had 33% lower average worm burdens at post-mortem (1892 ± 424) compared to CHB controls (2827 ± 575). This difference was not statistically significant ($p = 0.329$; Figure 4). Comparing burdens between control lambs of the two breeds, CS had, on average, 36% higher worm burdens than CHB sheep; the difference was not statistically significant ($p = 0.109$). More immature worms were observed in the two CHB groups than in the CS groups, with a proportion of 38% and 27% of immature in total worm counts in the vaccinated and control groups of CHB sheep, and 12% and 6% of immatures in the vaccinated and control CS groups. The level of stunting in worms recovered from CHB controls was not significantly different from CHB vaccinates; however, worms from CHB controls were significantly shorter than those recovered from CS controls ($p < 0.0001$). Adult worms recovered from vaccinated CS lambs were significantly shorter than adult worms from the CS control animals ($p < 0.0001$) (Figure 5A). Similarly, CS vaccinates had significantly fewer EIU in female worms retrieved from their abomasa compared to control CS lambs ($p < 0.0001$). Female worms from CHB controls contained significantly fewer EIU than worms from CS controls ($p < 0.0001$) (Figure 5B).

Discussion

Here, the effect of a *T. circumcincta* prototype vaccine [6, 8] was tested in two breeds of sheep with known differences in their relatively susceptibility to experimental infection with *Haemonchus contortus* [9] and to natural GIN infection in which the predominant genera/species had been identified as *Trichostrongylus* spp., *T. circumcincta* and *H. contortus* [10]. There were two main objectives of the approach taken here: (1) to compare efficacy of the vaccine prototype in breeds of Spanish sheep with data obtained previously for British Texel-cross sheep [6, 8], and (2) to investigate whether the combination of genetic resistance and vaccination would have an additive effect in protection against *T. circumcincta* experimental challenge. Underpinning these objectives was the premise that CHB lambs would be more resistant than CS lambs to experimental infection with *T. circumcincta* larvae. Indeed, this was the case; when comparing the control groups of the two breeds, statistically-significant lower FEC levels over time, lower cumulative FEC, shorter worm length and fewer EIU were observed in CHB sheep when compared to CS sheep. In addition, sheep in the CHB control group harbored 36% fewer worms than the CS lambs, although the difference was not statistically significant. Genetic resistance to *T. circumcincta* in CHB lambs could be related to host mechanisms that cause a delay in larval development as a higher proportion of juvenile worms were enumerated in the CHB lambs than in the CS lambs at post-mortem. Although variability in *T. circumcincta* resistance has been described between individuals within a breed in several breeds [15–17], there have been few references of differences in resistance to this nematode between breeds [18].

In previous trials using this vaccine in Texel-cross lambs, significant differences between vaccinated and non-vaccinated control sheep were observed in both worm burden and FEC over time as well as in cumulative FEC [6]. In the work described here, FEC and worm burden parameters were reduced in vaccinated CS lambs, but the differences were not statistically significant, however, worm length and the number of eggs in female worm uteri were significantly lower in vaccinated CS lambs compared to non-immunised CS lambs. Worm length was not affected in vaccinated Texel-cross lambs [6], suggesting that mechanisms of protection induced by the vaccine, or timing of the response, may be different between breeds. Analogous to this observation, it has been reported that during GIN infection, some breeds of sheep are able to immunologically respond earlier than others [18] and different types of breed responses have been observed [14, 19]. These

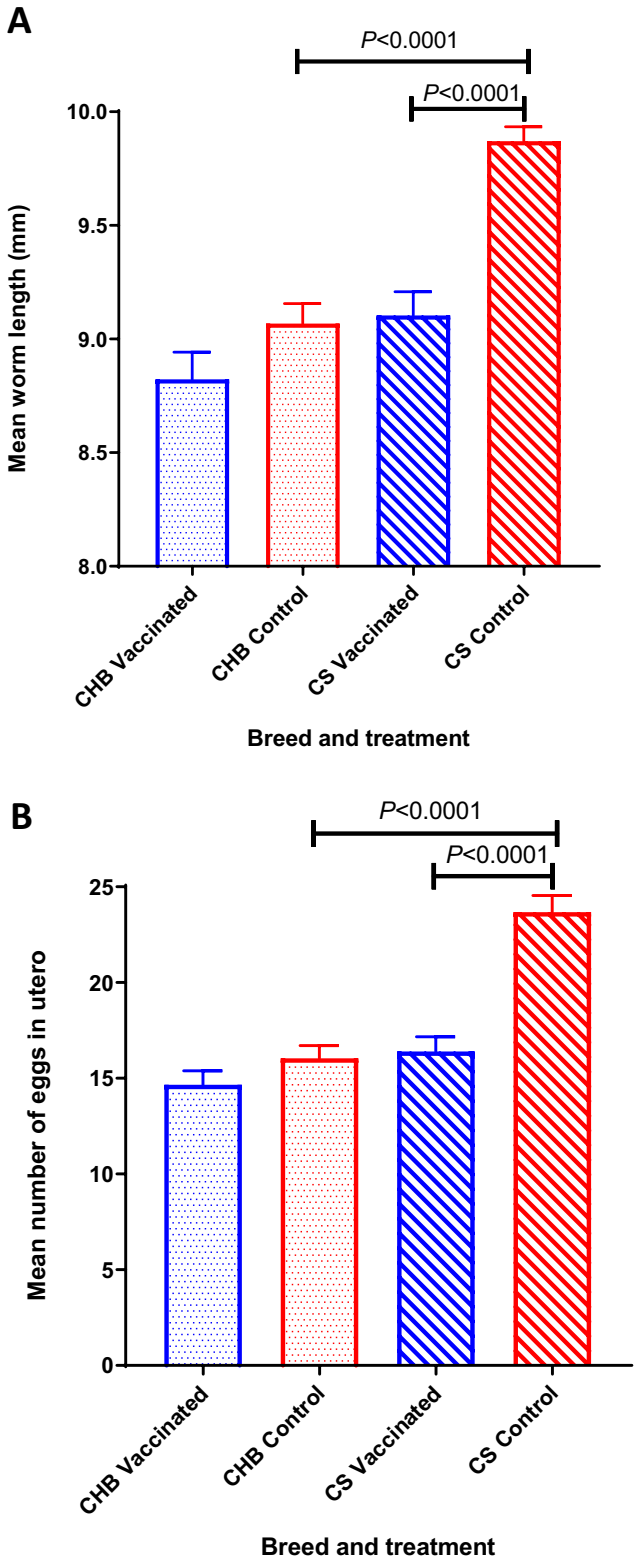

Figure 5 Effects of immunization of two native sheep breeds from Gran Canaria with recombinant antigens derived from *T. circumcincta* on worm length and egg production. Worm lengths (**A**) and the number of eggs in utero in female worms (**B**) are shown for sheep (CS = Canarian sheep; CHB = Canarian Hair Breed sheep) challenged with 2000 *T. circumcincta* L3 three times per week for 4 weeks following immunization with an 8-protein cocktail in the context of Quil A (CS-VAC; CHB-VAC) or with Quil A only (CS-Control; CHB-Control). The mean worm length or mean number of eggs in utero ± SEM is shown ($n = 193$, 284, 339 and 278 for CHB-VAC, CHB-Control, CS-VAC and CS-Control respectively).

differences during parasite exposure may be relevant in the vaccine-induced response in each breed of sheep.

In CHB lambs, although vaccinates had lower FEC over time and cumulative FEC, lower worm counts, and their nematodes were shorter, with fewer EIU than observed in the control CHB group, the differences were not statistically significant. Therefore, although there was some evidence that the vaccine may induce a protective effect in this breed, the high level of inherent resistance in CHB lambs of this age made demonstration of the additive or synergistic effects of vaccination less clear. When comparing data from the CHB vaccinates to the CS control sheep, significant differences in all parasitological parameters were observed; such interactions between breed and vaccination may allow better integrated control of GIN and suggest the potential for combining these approaches in an integrated strategy to helminth control [5, 12]. Identifying specific mechanisms of the effector response and discovering why each breed appears to behave differently using the same vaccine and challenge protocol may help inform formulation and delivery to improve the vaccine by stimulating more appropriate immune responses. Future studies will be designed to address this hypothesis.

Abbreviations

AUC: area under the curve; CHB: Canaria Hair Breed; CS: Canaria Sheep; EIU: eggs in utero; EPG: eggs per gram; FEC: faecal egg counts; GAMM: generalised additive mixed models; GIN: gastrointestinal nematodes; *H. contortus*: *Haemonchus contortus*; L3: third stage larval; M: molar; MTci2: UK derived *T. circumcincta* strain; PBS: phosphate buffered saline; SEM: standard error of the mean; *T. circumcincta*: *Teladorsagia circumcincta*; Tci-APY-1: calcium-dependent apyrase-1; Tci-ASP-1: activation-associated secretory protein-1; Tci-CF-1: cathepsin F-1; Tci-ES20: 20 kDa protein of unknown function; Tci-MEP-1: astacin-like metalloproteinase-1; Tci-MIF-1: macrophage migration inhibitory factor-1; Tci-SAA-1: an homologue of a protective antigen from *Ancylostoma caninum*; Tci-TGH-2: TGF homologue.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AJN, TMcN, JBM and JFG participated in the design of the study. JNH, CM, TPH, JFG carried out the experiments at ULPGC. HWW, YCM assisted JNH, CM, TPH and JFG with post-mortem procedures. DGP prepared the recombinant proteins. AJN assisted JNH in vaccine preparation and administration. JNH and JFG coordinated the experiment. AJN, TMcN, JBM, JNH, JFG participated in data analysis. AJN, JBM, TMcN, DGP, JNH and JFG drafted the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Experiments were approved by the Animal Welfare Ethics Committee of the Universidad de Las Palmas de Gran Canaria (OEBA_ULPGC_003_2014) and from the local authorities, following the rules of the Spanish Legislation (RD 53/2013). Several animals presented local granulomas in the injection area. They were followed by the researchers and all of them resolved and they had no systemic consequences. One lamb in the CHB-vaccine group died a few days after the start of the procedure from a post-traumatic renal haemorrhage. It was not related to animal handling. We followed the ARRIVE guidelines published in the online journal PLOS Biology in June 2010.

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Capítulo II

"Cellular and humoral immune responses associated with protection in sheep vaccinated against *Teladorsagia circumcincta*"

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VETERINARY RESEARCH

RESEARCH ARTICLE Open Access

Cellular and humoral immune responses associated with protection in sheep vaccinated against *Teladorsagia circumcincta*

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Abstract

Due to increased anthelmintic resistance, complementary methods to drugs are necessary to control gastrointestinal nematodes (GIN). Vaccines are an environmentally-friendly and promising option. In a previous study, a *Teladorsagia circumcincta* recombinant sub-unit vaccine was administered to two sheep breeds with different levels of resistance against GIN. In the susceptible Canaria Sheep (CS) breed, vaccinates harboured smaller worms with fewer eggs in utero than the control group. Here, we extend this work, by investigating the cellular and humoral immune responses of these two sheep breeds following vaccination and experimental infection with *T. circumcincta*. In the vaccinated CS group, negative associations between antigen-specific IgA, IgG_2 and Globule Leukocytes (GLs) with several parasitological parameters were established as well as a higher $CD4^+/CD8^+$ ratio than in control CS animals, suggesting a key role in the protection induced by the vaccine. In the more resistant Canaria Hair Breed (CHB) sheep the vaccine did not significantly impact on the parasitological parameters studied and none of these humoral associations were observed in vaccinated CHB lambs, although CHB had higher proportions of $CD4^+$ and $CD8^+$ T cells within the abomasal lymph nodes, suggesting higher mucosal T cell activation. Each of the component proteins in the vaccine induced an increase in immunoglobulin levels in vaccinated groups of each breed. However, levels of immunoglobulins to only three of the antigens (Tci-MEP-1, Tci-SAA-1, Tci-ASP-1) were negatively correlated with parasitological parameters in the CS breed and they may be, at least partially, responsible for the protective effect of the vaccine in this breed. These data could be useful for improving the current vaccine prototype.

Keywords: Nematode vaccine, Genetic resistance, Immune response, Immunoglobulins, Globule leukocytes, *Teladorsagia circumcincta*, Sheep, Cellular response

Introduction

The protective immune response in sheep against *Teladorsagia circumcincta* infection has been studied extensively and is associated with lower worm egg production and parasite burden, as well as shorter and less fecund female adult nematodes [1, 2]. A predominantly Type-2 response has been implicated in this immune response, recruiting eosinophils, mast cells, globule leukocytes (GL), with concomitant production of parasite-specific IgA, IgG and IgE as well as some cytokines such as IL-4 and IL-5 [3]. Associations between the nematode specific IgA levels and reduced worm length and worm eggs in utero (EIU) in *T. circumcincta* infections have been reported [1, 2, 4, 5].

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Until recently, control of *T. circumcincta* has been focused on the use of chemotherapeutics, but nematode resistance against anthelmintics has made this approach unsustainable [6]. Due to this issue, it is important to find alternative control measures, such as vaccination; understanding the mechanisms underlying the protective immune response is essential for the development of a successful vaccine [7]. Although finding a successful prototype recombinant vaccine against gastrointestinal nematodes (GINs) has been a difficult task, a recombinant sub-unit vaccine against *T. circumcincta* was recently developed and has shown repeated effectiveness [7–10]. Previous successful prototype vaccines against GIN were only effective in their native forms, preventing global distribution and commercialisation. This recombinant prototype has some features which could overcome this hurdle but, as with other prototype recombinant vaccines against GINs, repeated trials have shown variability in protective response [9]. In addition, the combination of 8 antigens in a single vaccine makes its production prohibitively expensive and simplifying the vaccine would be very desirable.

In a recent study [11], parasitological data was collected for evaluating the effects of this vaccine in two sheep breeds, the Canaria Sheep (CS) and the Canaria Hair Breed (CHB), which have different susceptibilities to *T. circumcincta*. Whereas in vaccinated CS animals there were significant reductions in worm length and numbers of worm intrauterine eggs, vaccination in CHB also induced reductions in parasitological variables, but these were not statistically significant. Here, as an extension of this study, we report the main immune responses that were found in vaccinated sheep. Data obtained would help increase the efficacy of the prototype vaccine and bring it nearer to market.

Materials and methods

Animals and experimental design

The experimental design was previously described in details in [11]. Briefly, 24 male lambs of CHB and another 24 male lambs of CS, all of them weaned, were dewormed and kept worm-free until they were six months-old. These animals were randomly selected, within each breed, to establish 4 groups (CHB-vaccine, CHB-control, CS-vaccine and CS-control) of 12 lambs in separated pens. One animal from the vaccinated group of CHB died for non-related causes before the experimental procedures began.

Vaccinated groups (CHB-vaccine and CS-vaccine) were injected subcutaneously (3 doses, on days 0, 21 and 42) with 400 μg of antigens [given as 2 injections: one with cathepsin F-1 (Tci-CF-1), a 20 kDa protein of unknown function (Tci-ES20), activation-associated secretory protein-1 (Tci-ASP-1), a homologue of a protective antigen from *Ancylostoma caninum* (Tci-SAA-1), macrophage migration inhibitory factor-1 (Tci-MIF-1), calcium-dependent apyrase-1 (Tci-APY-1) and a TGFβ homologue (Tci-TGH-2) in PBS; and the other with astacin-like metalloproteinase-1 (Tci-MEP-1) in PBS/Urea] plus 10 mg Quil A (*Vax Saponin, Guinness Chemical Products Ltd*) [8]. Control groups received the same volume of urea/PBS/10 mg Quil A each time. At the same time as the final immunisation (day 42), a "trickle infection" protocol was carried out where all animals were inoculated with 2000 third stage larvae (L3) of *T. circumcincta* three times per week for 4 weeks until day 68.

Faecal egg counts (FEC) were performed using modified McMaster technique [12] three times per week from day 56 after the start of immunisations, until the end of the experiment. At the end of the trial (days 82–85), lambs were euthanised, and adult and immature worms from aliquots of the abomasal content were obtained, counted and measured following standard techniques [11].

Enzyme-linked immunosorbent assay

Animals were bled from the jugular vein on day 77 following the first immunisation, four days prior to post-mortem. These samples were collected in silicone-coated tubes (Gel + Clot Act. VenoSafe™, TERUMO) for blood serum and were refrigerated at 4 °C for at least 30 min. Then, they were centrifugated at 1164 × *g* (Mixtasel, Selecta) and the serum obtained kept at -20 °C until use. ELISA was performed to determine levels of IgA, IgG_1 and IgG_2 in sera from individual sheep following a previously published protocol [13] with minor modifications. Microplates (*Corning*®) were coated overnight at 4 °C with 5 μg/mL of antigen (L4 and adult stage of *T. circumcincta*; or individual recombinant antigens from the vaccine) in carbonate buffer (50 μL per well). All incubations were done at 37 °C. After three washes with Phosphate Buffered Saline (PBS) + 0.05% Tween 20 and blocking for 1 h with 3% bovine serum albumin in PBS, samples diluted at 1:200 in Tris Buffered Saline containing 0.05% Tween®20 (TBST) were incubated for 1 h (each sample was performed in duplicate). Following a further wash, mouse anti-sheep IgA (1:8000 in TBST for parasite antigens and 1:4000 for vaccine antigens), IgG_1 or IgG_2 (clones McM1 and McM3 respectively, at 1:1000 in TBST) were added and incubated for 1 h. Plates were then washed and polyclonal rabbit anti-mouse immunoglobulins conjugated to HRP (*Dako*) added at 1:1000 and incubated for 1 h. After a final wash step, 100 μL of O-phenylene-diamine dihydrochloride substrate (*Sigma Fast OPD Tablets*) was added to each well (final concentrations of 0.4 mg/ml OPD, 0.4 mg/ml urea hydrogen

peroxide, and 0.05 M phosphate-citrate) and the reaction stopped by adding 25 µL of 2 M sulphuric acid. Finally, Optical Density (OD) values were read at 490 nm (Multiskan Ascent). A serum sample from a non-infected animal was included as a negative control, while the positive control sera was obtained from trickle infected vaccinated animals from this trial. The optical densities were transformed into an optical density index (ODI) for each animal using the formula: ODI = (mean OD—mean negative OD)/(mean positive OD—mean negative OD) [14], adding 1.0 to all values to avoid negative data for statistical analysis.

Lymphocyte stimulation assays

Abomasal lymph node (ALN) cells were collected aseptically at post-mortem (days 82–85) in cold transport wash media [TWM (HBSS w/o Ca^{2+} or Mg^{2+} supplemented with 2% heat inactivated foetal calf serum (FCS), 100U/mL penicillin, 100 µg/mL streptomycin and 2% gentamicin, all from Sigma)] and kept on ice until processing. In order to obtain single cell suspensions from the ALN, lymph nodes were rinsed with fresh cold TWM, placed in a petri dish and cleaned from excess adipose tissue. Around 3 mL of fresh TWM was added to the petri dish, and tissue was then dissociated using a sterile syringe plunger. Cell suspension was then filtered through 70 µm cell strainers to remove large clumps and tissue fragments. Samples were allowed to settle for 10 min and any debris/excess cells discarded. Following two washes with TWM, cell suspensions were frozen in freezing media (FBS + 10% cell culture grade DMSO (Sigma)) and stored in liquid nitrogen until processed. Prior to stimulations, frozen cells were gently defrosted in a water bath and warm complete media (RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 0.5% of gentamicin and 50 µM 2-mercaptoethanol (all from Sigma)) gently added to the vials. Cells were then washed twice with fresh media and re-suspended at 2×10^6 cells/mL. Lymphocyte stimulation assays (LSA) were carried out by incubating 2×10^5 ALN cells in triplicate with equal volumes of PBS (negative control), ConA (positive control), soluble L4 *T. circumcincta* or Adult *T. circumcincta* antigen (all stimulants at 5 µg/mL final concentration), in a total volume of 200 µL, at 37 °C with 5% CO_2 in air for 5 days. After 4 days, 50 µL of media from each replicate was collected and stored at −20 °C for cytokine measurements and replenished with fresh complete media containing methyl-^{3}H thymidine (0.5 µCi per well). Proliferation was measured by the incorporation of methyl-^{3}H thymidine during the final 18 h of culture and expressed as Stimulation Index (SI) by dividing the proliferation of samples incubated with *T. circumcinta* antigen by that from PBS controls.

Cytokine ELISA

Capture ELISAs were performed to examine the antigen specific secretion of interferon (IFN)-γ Interleukin (IL)-4 and IL-17A by ALN. All incubations were carried out at room temperature unless stated otherwise. Interleukin-4 and IFN-γ were quantified using commercial ELISA kits according to the manufacturer's instructions (MABTECH AB, Augustendalsvägen, SE, Sweden). For the quantification of IL-17A, polyclonal rabbit anti-bovine IL-17A antibodies were used alongside bovine recombinant protein (all from Kingfisher Biotech, Inc., St. Paul, MN). Washing steps for all ELISAs were performed 6 times with 350 µL washing buffer (Phosphate Buffered Saline (PBS) + 0.05% Tween®20) using a Thermo Scientific Wellwash™ Versa (ThermoFisher). Briefly, high-binding capacity ELISA plates (Immunolon™ 2HB 96-well microtiter plates, ThermoFisher) were incubated with coating antibodies overnight at 4 °C. Plates were then washed and blocked for 1 h with PBS containing 0.05% Tween®20 (Sigma, UK) and 0.1% BSA Bovine Serum Albumin (BSA, Sigma, UK). Following a further washing step, 50 µL of supernatants or standards were added in duplicate for 1 h. Subsequently, plates were washed, and detection antibodies added for 1 h. This was followed by washing and addition of Streptavidin-HRP (Dako, Agilent, Santa Clara, USA) for 45 min. After the final washing step, 50 µL of Sure-Blue TMB substrate (Insight Biotechnology, London, UK) was added and the reaction was stopped by the addition of equal volume of TMB stop solution (Insight Biotechnology, London, UK). Absorbance values were read at OD 450 nm using a Sunrise™ microplate reader (Tecan, Männedorf, CH, Switzerland). In order to quantify the cytokines of interest, samples were analysed 1:2 for IFN-γ or neat for IL-4 and IL-17A. All values were blanked corrected and concentrations determined from the standard curves included in all plates which were constructed using 7 serial dilutions of recombinant cytokines ranging from 400 to 6.25 pg/mL for IFN-γ (MABTECH AB); 2000 to 62.5 pg/mL for IL-4 (MABTECH AB) and 1500 to 23.43 pg/mL for IL-17A (Kingfisher).

Phenotyping of ALN cells by flow cytometry

Single colour flow cytometry was carried out in resuscitated ALN cells using the monoclonal antibodies detailed in Additional file 1 at pre-optimised concentrations. All incubations were carried out at room temperature and protected from light. Briefly, 5×10^5 ALN cells per antigen/no antibody control/secondary only control were blocked with 200 µL of 20% heat inactivated normal goat serum (BioRad) for 15 min. Following centrifugation,

supernatants were discarded and cells incubated with their corresponding antibody or FACS buffer (PBS + 5% heat inactivated FCS plus 0.02% sodium azide) for controls for 20 min. Cells were then washed twice with FACS buffer and incubated with anti-mouse IgG (H + L) conjugated to Alexa Fluor® 647 (Invitrogen, Life Technologies, USA) for 20 min. After two washes, cells were resuspended in dead cell stain Sytox Blue (Invitrogen, Life Technologies, USA) prior to acquisition. A minimum of 25 000 events were acquired using a MACSQuant® Analyzer 10 (Miltenyi Biotech, Germany) and analysed using FlowJo vX for Windows 7. Dead cell and doublet cell discrimination was performed during all analysis.

Histochemistry and morphometric analysis

Abomasal tissue samples (approximately 2 cm × 2 cm) were collected at post-mortem from the antropyloric region for histological and immunohistochemistry studies, performed as described previously [15]. Eosinophil and globule leukocyte (GL) counts were performed in abomasal tissue stained with haematoxylin and eosin (Additional file 2), while toluidine blue staining was used to determine mast cell numbers. For each animal sample, cells were counted in at least 20 randomly selected fields of 0.06 mm^2 (0.245 mm × 0.245 mm) at 400 × magnification with an optical microscope (Olympus CX31) and expressed as cell/mm^2. Eosinophils and mast cells were counted in selected fields in the lamina propria corresponding to the lower third of the abomasal mucosa. Globule leukocytes were counted in the upper third of the mucosa.

The tissue samples taken for immunohistochemistry were used to identify and count the following cell populations: $CD4^+$, $CD8^+$, $\gamma\delta TCR^+$, $MHCII^+$, $CD45RA^+$ and Galectin-14^+ (Additional file 1) [15]. Cells were counted in 20 fields located in upper and lower third of the mucosa and were expressed in cells/mm^2, as described above (Additional file 2).

Statistical analysis

IBM SPSS Statistics version 24.0 software was used for statistical analysis of parasitological, immunohistochemical and serum antibody data. Differences in FEC, cumulative FEC, worm counts, worm length and EIU were estimated as described in [11]. The data relative to specific immunoglobulin levels in serum were analysed using generalised linear models (GENLIN), using the estimation method Newton–Raphson and Pearson Chi-square scale. The general linear model (GLM) univariate was used for cellular counts, taking LSD (Least Significance Difference) test as reference.

Analyses for immune cell phenotypes were performed using RStudio package version 1.1.456 (R Core Team 2019) as follows: flow cytometry and proliferation data was analysed using two-way ANOVA to test for differences between breeds, vaccination groups (control, vaccinated), and their interaction. Cytokine data was analysed using a GLM as follows: IFN-γ and IL-4 data was log-transformed prior to analysis to satisfy normality of residuals and homoscedasticity. Both cytokines were fitted with effects of vaccine, breed and antigen-stimulation (treatment), as well as two-way interactions and the three-way interaction. Models were simplified by stepwise deletion based on Wald F-tests.

Spearman's rank correlation coefficient was used for studying correlations between immunological, cellular and parasitological variables.

For all analyses, probabilities of $p < 0.05$ were considered statistically significant. One animal from CS-control group was excluded from all statistical analyses because the abomasal wash sample was lost, meaning no worm burden data was available. Figures were produced with RStudio package version 1.1.456.

Results

Parasitological data

Parasitological data from this trial was previously presented in [11]. Briefly, vaccination induced a statistically significant reduction in worm length and EIU in the vaccinated CS group when compared to the control (adjuvant-only) CS group (Table 1).

Table 1 Parasitological data from Canaria Sheep (CS) and Canaria Hair Breed (CHB) sheep vaccinated with a prototype recombinant sub-unit *Teladorsagia circumcincta* vaccine and subsequently challenged with *T. circumcincta*

Group	Cumulative FEC	Worm burden	Worm length (mm)	EIU
CS Vac	2861 ± 737	3606 ± 803	9.10 ± 0.10**	16.39 ± 0.77**
CS Con	4372 ± 1021	4148 ± 761	9.87 ± 0.06**	23.67 ± 0.87**
CHB Vac	720 ± 205	1171 ± 459	8.82 ± 0.12	14.65 ± 0.73
CHB Con	1157 ± 503	2052 ± 548	9.07 ± 0.09	16.03 ± 0.67

Values shown are Means (± SEM) and values with statistically significant differences within breeds are represented with "**" at $p < 0.01$. Abbreviations: Vac: Vaccinated, Con: Control (adjuvant-only), FEC: Faecal egg count; EIU: Eggs in utero. Data obtained from González et al. [11].

Measurement of antibody responses to parasite and vaccine antigens in serum

In order to evaluate if the vaccine has induced some variations in the humoral immune response against this worm, the response to the L4 stage -target of the protective immunity in sheep- was firstly analysed in vaccinated and non-vaccinated infected groups of both breeds. Immunoglobulin A was the only isotype for which a statistically significant increase, associated with vaccination, against L4 antigen was observed, in the vaccinated CHB group ($p < 0.01$) compared to the CHB control group (Table 2). Negative correlations between levels of L4 extract-specific serum IgA and IgG_2 and worm length or EIU were observed in vaccinated groups, being only statistically significant in the CS vaccine recipients. Levels of L4 extract specific IgG_2 were also negatively associated with worm burden and cumulative FEC in CS vaccine recipients (Table 2).

Specific IgA, IgG_1 and IgG_2 levels in serum against all recombinant antigens were significantly higher in the vaccinated sheep than in control sheep (Table 3, Additional files 3, 4, 5, 6, 7 and 8). Specific serum IgA levels against all studied recombinant antigens were very similar within breeds, though levels of IgA specific for Tci-CF-1 were significantly lower than those against Tci-APY-1 in vaccinated CS animals (Additional files 3 and 4). Greater variability in vaccinated groups was observed in the levels of specific IgG_1 and IgG_2 against these recombinant proteins within breeds (Table 3, Additional files 3, 4, 5, 6, 7 and 8). In vaccinated CS, IgG_1 levels specific for Tci-CF-1 and Tci-SAA-1 were similar and were significantly higher than IgG_1 levels against the other recombinant proteins, although IgG_1 against Tci-MEP-1 was not significantly lower than Tci-SAA-1-specific IgG_1. IgG_1 levels against Tci-MEP-1 were significantly higher than IgG_1 levels specific for Tci-ES20 and Tci-APY-1. On the contrary, IgG_1 specific for Tci-MIF-1 was significantly lower than IgG_1 specific for Tci-ASP-1, Tci-ES20, Tci-MEP-1 and Tci-TGH-2 (Additional file 5). In vaccinated CHB animals, levels of IgG_1 specific for all recombinant antigens were similar, except for Tci-MIF-1 and Tci-SAA-1, which were not significantly different to each other, but were significantly lower than the other antigens (Additional file 6). Specific IgG_2 levels against all vaccine proteins were variable in both breeds; represented in Additional files 7 and 8. In vaccinated CS animals, levels of Tci-ASP-1 were significantly higher than IgG_2 levels against the other recombinant proteins, except Tci-SAA-1. Levels of IgG_2 specific for Tci-MIF-1 were lower than most of the other proteins. In general, specific levels of this immunoglobulin isotype against all proteins were variable and individual differences between antigens were recorded. In

Table 2 Levels of serum immunoglobulins against extracts of the fourth larval stage (L4) of *Teladorsagia circumcincta* in Canaria Sheep (CS) and Canaria Hair Breed (CHB) sheep vaccinated with a prototype recombinant sub-unit *T. circumcincta* vaccine and subsequently challenged with *T. circumcincta*

Isotype	Group	Mean ± SEM	Cumulative FEC	Worm burden	Worm length	EIU
Correlation						
IgA	CS	1.189 ± 0.054	−0.223	0.016	−0.333	−0.267
	CS Vac	1.160 ± 0.066	−0.371	−0.545	−0.776**	−0.615*
	CS Con	1.220 ± 0.089	0.027	0.542	−0.023	0.255
	CHB	1.335 ± 0.073	−0.077	−0.335	0.065	−0.007
	CHB Vac	1.509 ± 0.124**	− 0.545	−0.600	0.042	−0.212
	CHB Con	1.175 ± 0.055**	0.263	0.259	0.203	0.224
IgG1	CS	1.547 ± 0.057	−0.033	0.191	0.050	0.120
	CS Vac	1.508 ± 0.151	0.224	0.084	−0.238	−0.182
	CS Con	1.590 ± 0.145	−0.164	0.436	0.164	0.564
	CHB	1.305 ± 0.103	0.107	−0.179	0.259	0.234
	CHB Vac	1.312 ± 0.063	−0.227	−0.191	0.261	0.261
	CHB Con	1.300 ± 0.095	0.305	−0.042	0.196	0.245
IgG2	CS	1.241 ± 0.047	−0.158	0.037	−0.190	−0.239
	CS Vac	1.234 ± 0.070	−0.594*	−0.594*	−0.867**	−0.825**
	CS Con	1.249 ± 0.064	0.227	0.782**	0.291	0.473
	CHB	1.205 ± 0.050	0.345	0.012	0.448*	0.655**
	CHB Vac	1.255 ± 0.092	0.327	0.073	0.406	0.733*
	CHB Con	1.160 ± 0.046	0.261	0.042	0.483	0.578*

Antibody levels are expressed as optical density index (ODI) value + 1 (ODI + 1). Associations are expressed as Spearman's correlation coefficient. Statistically-significant differences within breeds for a specific isotype and antigen and significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Table 3 Levels of serum immunoglobulins against a subset of recombinant vaccine proteins in Canaria Sheep (CS) and Canaria Hair Breed (CHB) sheep vaccinated with a prototype recombinant sub-unit *Teladorsagia circumcincta* vaccine and subsequently challenged with *T. circumcincta*

Isotype	Antigen	Group	Mean ± SEM	Cumulative FEC	Worm burden	Worm length	EIU
Correlation							
IgA	Tci-MEP-1	CS Vac	1.656 ± 0.058**	−0.364	−0.650*	−0.497	−0.706*
		CS Con	1.188 ± 0.045**	0.473	0.727*	0.309	0.109
		CHB Vac	1.652 ± 0.071**	−0.073	−0.582	−0.321	−0.358
		CHB Con	1.246 ± 0.085**	0.109	0.399	0.385	−0.021
IgG1	Tci- ASP-1	CS Vac	1.877 ± 0.043**	−0.399	−0.196	−0.392	−0.636*
		CS Con	0.991 ± 0.004**	−0.405	0.223	−0.087	0.219
		CHB Vac	1.683 ± 0.067*	0.155	−0.036	−0.285	−0.358
		CHB Con	1.017 ± 0.013*	0.494	0.126	0.308	0.406
	Tci- SAA-1	CS Vac	2.104 ± 0.086**	−0.552	−0.252	−0.699*	−0.608*
		CS Con	0.995 ± 0.002**	0.018	−0.027	−0.391	−0.600
		CHB Vac	1.478 ± 0.089*	−0.191	−0.118	−0.152	−0.612
		CHB Con	0.999 ± 0.001*	0.182	0.280	−0.119	−0.098
IgG2	Tci- ASP-1	CS Vac	2.004 ± 0.049**	−0.399	−0.329	−0.392	−0.587*
		CS Con	0.998 ± 0.003**	−0.100	−0.073	−0.182	0.509
		CHB Vac	1.701 ± 0.059*	0.164	−0.118	−0.067	−0.164
		CHB Con	1.004 ± 0.001*	0.067	0.112	0.028	−0.021

Antibody levels are expressed as (ODI + 1). Only the antigens for which correlations with parasitology variables were established are shown in the table. Associations are expressed as Spearman's correlation coefficient. Statistically-significant differences within breeds for a specific isotype and antigen and significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

vaccinated CHB, the individual variability was also high: Specific IgG_2 levels against Tci-MEP-1 were higher than all the other proteins except Tci-CF-1. On the contrary, specific IgG_2 levels of Tci-MIF-1 were significantly lower than IgG_2 levels of all the other vaccine antigens. Detailed significances in IgG_2 levels specific for the other recombinant proteins in CHB are presented in Additional file 8.

Specific IgA levels against Tci-MEP-1 in the serum of vaccinated CS animals were negatively correlated with adult worm burden ($p < 0.05$) and EIU ($p < 0.05$) (Table 3). Levels of specific IgG_1 against Tci-SAA-1 in the serum of vaccinated CS animals were also negatively correlated with worm length and EIU ($p < 0.05$) and, in this group, levels of specific IgG_1 and IgG_2 against Tci-ASP-1 correlated negatively with EIU ($p < 0.05$) (Table 3). No other antigen-specific immunoglobulin isotype in any other vaccinated group was negatively correlated with any parasitological parameter (Additional files 3, 4, 5, 6, 7 and 8).

Cellular responses

Abomasal lymph node (ALN) cells from vaccinated animals from both breeds generally showed higher mean proliferative responses to *T. circumcincta* adult somatic antigen compared to L4 somatic antigen (Figure 1). No significant differences in antigen specific proliferation were observed between vaccinated or control animals for either breed, and no vaccine x breed interaction was observed.

Supernatant from antigen-stimulated ALN were harvested on day 4 post-stimulation and levels of IFN-γ and IL-4 quantified. Significantly higher levels of IFN-γ were present in ALN cells stimulated with both *T. circumcincta* adult and L4 antigens compare to unstimulated controls ($F = 3.72$, $DF = 2$, $p = 0.027$; Figure 2A). There was no evidence of an effect of breed or vaccination status, nor any interactions between vaccination and breed, although mean IFN-γ production was higher in antigen-stimulated ALN cells from CHB sheep. Similarly, IL-4 release was significantly higher in ALN cells stimulated with adult and L4 antigens compared to unstimulated controls ($F = 34.98$, $DF = 2$, $p < 0.001$; Figure 2B) but again there was no evidence of a main effect of breed or vaccination status, nor an interaction between vaccination and breed.

Antigen-specific IL-17A release was not detected in any of the ALN cultures (data not shown).

When the phenotype of the ALN cells was investigated by flow cytometry, significant effects of the vaccine and breed were identified. The proportions of $CD4^+$ T cells within ALN cell populations were higher in CHB sheep compared to CS sheep ($F = 4.42$, $p = 0.042$), which appeared largely driven by the control animals (Figure 3A). Similarly, the proportion of $CD8^+$ T cells

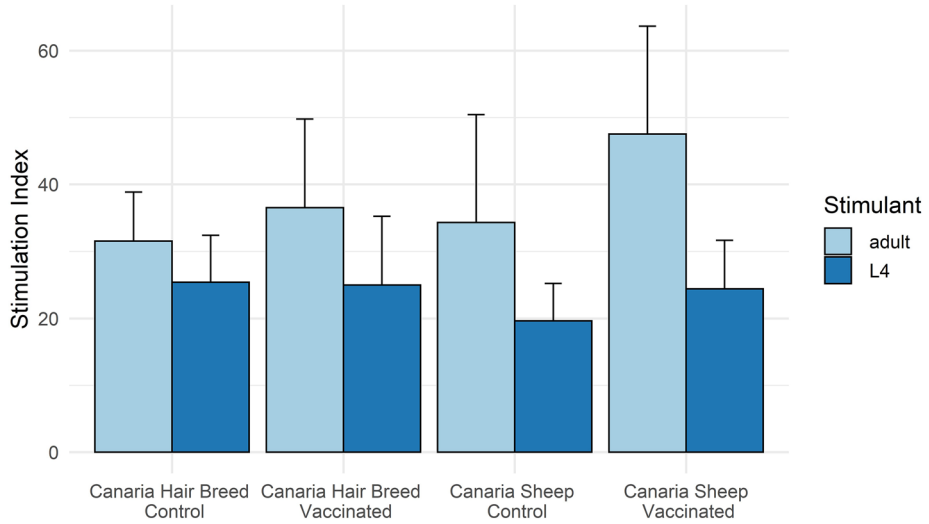

Figure 1 Antigen specific proliferation in Abomasal Lymph Nodes from Canaria Sheep and Canaria Hair Breed sheep vaccinated with a prototype recombinant sub-unit *Teladorsagia circumcincta* vaccine and subsequently challenged with *T. circumcincta*. Abomasal Lymph Nodes were collected at post-mortem and lymphocytes stimulated with 5 g/mL of *T. circumcincta* L4 or adult somatic antigen for 5 days. Proliferation was measured by the incorporation of methyl-^{3}H thymidine ([^{3}H]TdR; 0.5 µCi per well) for the final 18 h of culture. Data are presented as the stimulation index with error bars denoting ± SE.

was also significantly higher in CHB sheep (F = 8.72, $p = 0.005$; Figure 3B), and were also higher in controls compared to vaccinated groups (F = 10.74, $p < 0.002$). The proportion of CD14$^+$ cells were higher in CHB sheep (F = 5.72, $p = 0.022$; Figure 3G). Finally, the CD4$^+$:CD8$^+$ ratio was affected by both breed and vaccination, with higher CD4$^+$:CD8$^+$ ratios observed in the CS breed (H = 4.52, $p = 0.04$) and in vaccinated animals (F = 9.77, $p = 0.003$) (Figure 3H).

No differences in mean values of eosinophils, mast cell, GLs, CD4$^+$, CD8$^+$, CD45RA$^+$, MHCII$^+$, γδ$^+$ and galectin-14$^+$ populations in the abomasal mucosa were observed between vaccinated and non-vaccinated animals of either breed (Figures 4 and 5). Globule leukocyte (GL) levels showed negative correlations with several parasitological variables analysed in all groups, but particularly in vaccinated CS animals in which GL levels were negatively associated with all of the parasitology measures recorded (Table 4). No other cell population studied in vaccinated groups showed a negative association with parasitology in any group (Additional files 9 and 10).

Discussion

Naturally or experimentally-induced protection against *T. circumcincta* following infection is a complex phenomenon in which several immunoglobulins such as IgA, IgE, IgG, cells such as eosinophils, mast cells, globule leukocytes (GL), CD4$^+$, plasmatic cells and other factors, like IL-4 and/or 5 or galectins are implicated. Although the main "actors" of this response are well-known, there is great variability in the phenotype of immunity. Part of this variability is a consequence of host factors such as age, breed, genetic resistance, but also parasite factors such as stage of worm development and level of burden [3].

The fact that lambs naturally acquire protective immunity against GIN after continual field-infection, underpinned the concept of developing a vaccine against these types of pathogens (recently reviewed in Britton et al. [16]). But, because of the complexity of the protective mechanisms involved in natural immunity, it is a challenge to recreate these with a recombinant subunit vaccine. Certainly, several promising antigens have been identified, but most of them share similar limitations: (1) it may not be possible to produce them as effective recombinant antigens; (2) there is individual variability in protection conferred by vaccines [9]. Recently, a recombinant prototype against *T. circumcincta* was identified which successfully protected lambs and periparturient ewes [7, 8]. However, individual variability in responses was observed. Furthermore, a reduction in the number of immunogens would be desirable for decreasing vaccine production cost. This type of reductionist strategy needs to be led by an understanding of vaccine-induced immunity and, if possible, immunological correlates of protection.

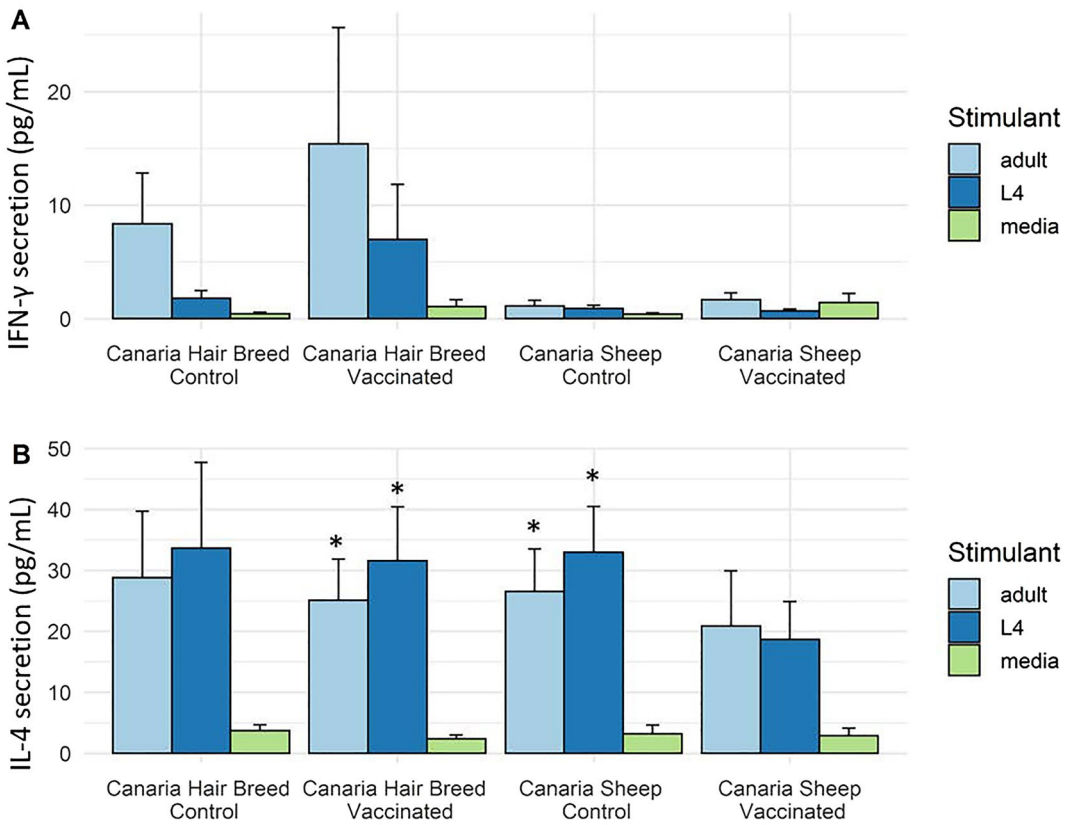

Figure 2 IFN-γ and IL-4 secretion by abomasal lymph node lymphocytes following stimulation with *Teladorsagia circumcincta* L4 or adult somatic antigen from Canaria Hair Breed and Canaria Sheep vaccinated with a prototype recombinant sub-unit *T. circumcincta* vaccine and subsequently challenged with *T. circumcincta*. IFN-γ (**A**) and IL-4 (**B**) secretion was examined in supernatants collected 4 days post-stimulation of abomasal lymph node lymphocytes with 5 g/mL of *T. circumcincta* L4 or adult somatic antigen. Data are expressed as the concentration of the cytokine release in picograms per mL (pg/mL). Results are shown as the mean values with error bars indicating ± SE. "*" denotes statistical significance for $p < 0.05$ when compared to media only secretion.

In the work presented here, the reduction of worm length and EIU in vaccinated CS groups was negatively correlated with levels of IgA and IgG_2 specific for extracts of L4 stage *T. circumcincta*. The role of IgA in protection of sheep against *T. circumcincta* has been proposed in previous studies of natural immunity, where it has been related to regulating worm growth targeting the L4 stage of this parasite [1, 17]. Several studies have associated IgA, IgG_1 and IgE with natural resistance against several GIN in sheep [18, 19]. IgG_2 has been also associated with natural protection against larval stages of *Trichostrongylus colubriformis* in genetically resistant sheep [20]. Interestingly, high levels of total IgG against L4 and excretory/secretory (ES) antigens of L4 were observed in Texel crossbred after vaccination [8]. Production of specific IgA against L4 *T. circumcincta* was also increased in vaccinated CHB. All the data suggest that a humoral response against L4 antigens is a consistent response possibly induced by this vaccine, which is logical as many of the antigens were selected based on their reactivity with IgA in the abomasal lymph or mucus from immune sheep [8], although abomasal lymph node cells were more strongly stimulated by adult *T. circumcincta* antigens than by L4 antigens, potentially due to immunomodulatory and/or suppressive molecules within L4 [21].

Globule leukocytes are considered to be activated mast cells [22]. Correlation studies also suggest a role for GL in the protection conferred by the vaccine in both breeds, and levels of this cell type were also negatively correlated with some elements of the parasitology in control groups. IL-4 is a key cytokine in mast cell maturation and, in agreement with histological data, it was also identified at high levels in ALN cells in all groups, independent of breed or vaccination status, following exposure to L4 and adult antigens. Globule leukocytes have been previously associated with resistance against GIN in animals naturally resistant to *T. circumcincta* [23, 24], generating a rapid larval rejection [25]. The data obtained here suggest that the vaccine confers protection in these breeds

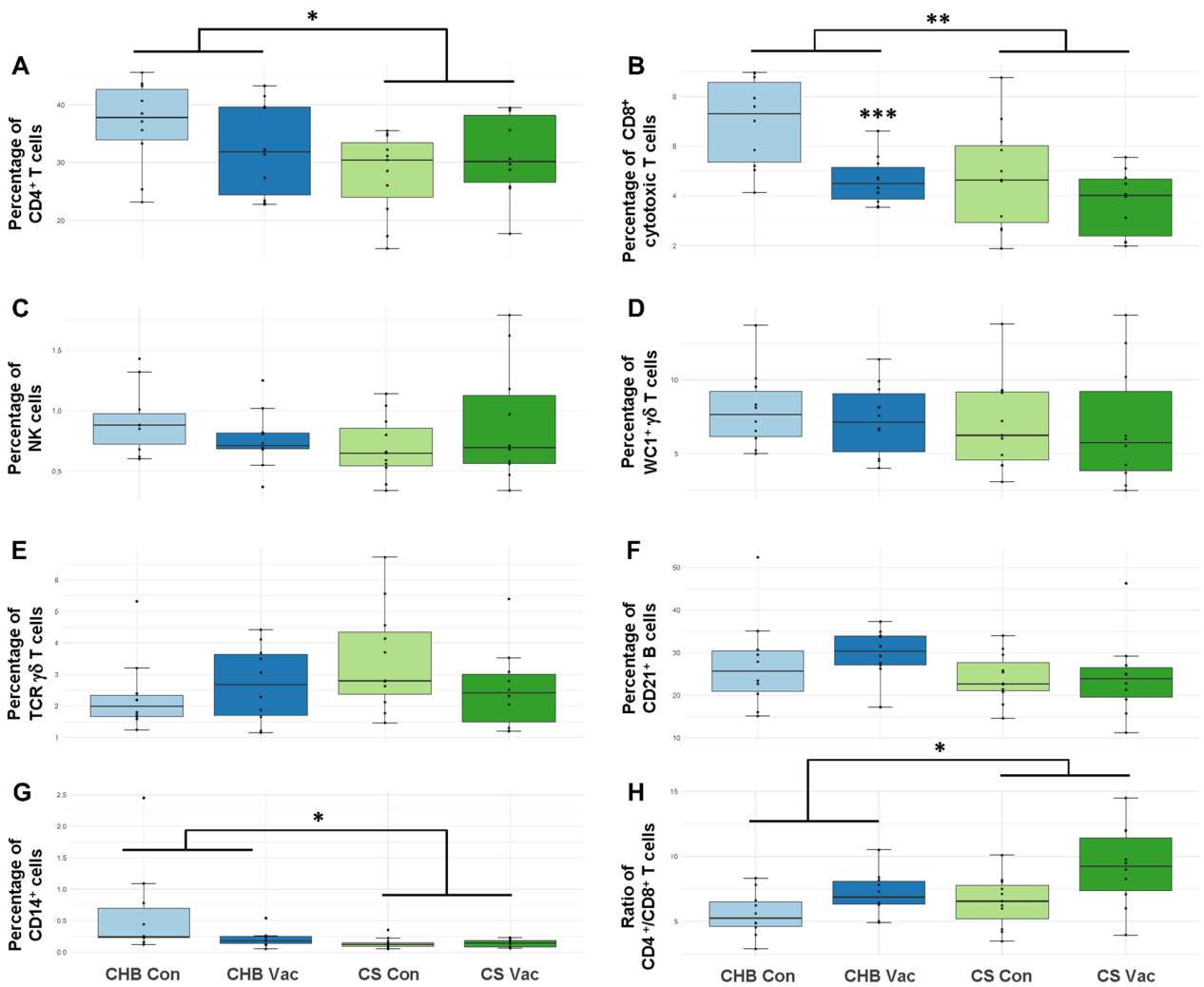

Figure 3 Phenotypic profile of Canaria Hair Breed (CHB) and Canaria Sheep (CS) abomasal lymph node (ALN) cells following vaccination with a prototype recombinant sub-unit *Teladorsagia circumcincta* vaccine and subsequent challenge with *T. circumcincta*. The expression of cell surface markers corresponding to main blood cell lineages: T helper $CD4^+$ cells, cytotoxic $CD8^+$ T cells and $\gamma\delta^+$ T cells, NK cells, $CD21^+$ B cells and $CD14^+$ myeloid cells (monocytes and macrophages) were examined by single colour flow cytometry. Cells were gated to eliminate dead cells and doublets. Threshold levels which determined positivity for the selected cell surface markers were set with secondary antibody only. Results are shown as boxplots with IQR and error bars indicating ± SE. "*" indicates statistical significance for $p < 0.05$, "**" $p < 0.01$ and "***" $p < 0.001$. Abbreviations: Vac Vaccinated, Con Control (adjuvant-only).

partly through improving mostly "natural mechanisms" of protection.

This vaccine prototype has now protected Texel crossbred lambs in five different trials [9] and, in this assay, also CS lambs were significantly protected. In CHB, all parasitological parameters were reduced in vaccinated group, although the reductions were not statistically significant compared to the control group. With the current data, it is not possible to confirm if the CHB lambs did not respond to the vaccine or if this breed is so naturally resistant to *T. circumcincta* that the vaccine could not add extra protection. In fact, the adjuvant-only control CHB group harboured fewer and shorter worms and excreted fewer eggs than either vaccinated or control CS lambs. Cellular and humoral response studied in this trial were, however, very similar between the breeds, with ALN cells from both breeds releasing IFN-γ and IL-4 following stimulation with larval and adult worm antigens. This indicates that in both breeds, infection with *T. circumcincta* induced a mixed T helper type 1 (Th1) / Th2 immune response, with possibly higher levels of IFN-γ produced by CHB lambs. While Th1 immunity, characterised by IFN-γ, has been linked with susceptibility to gastrointestinal

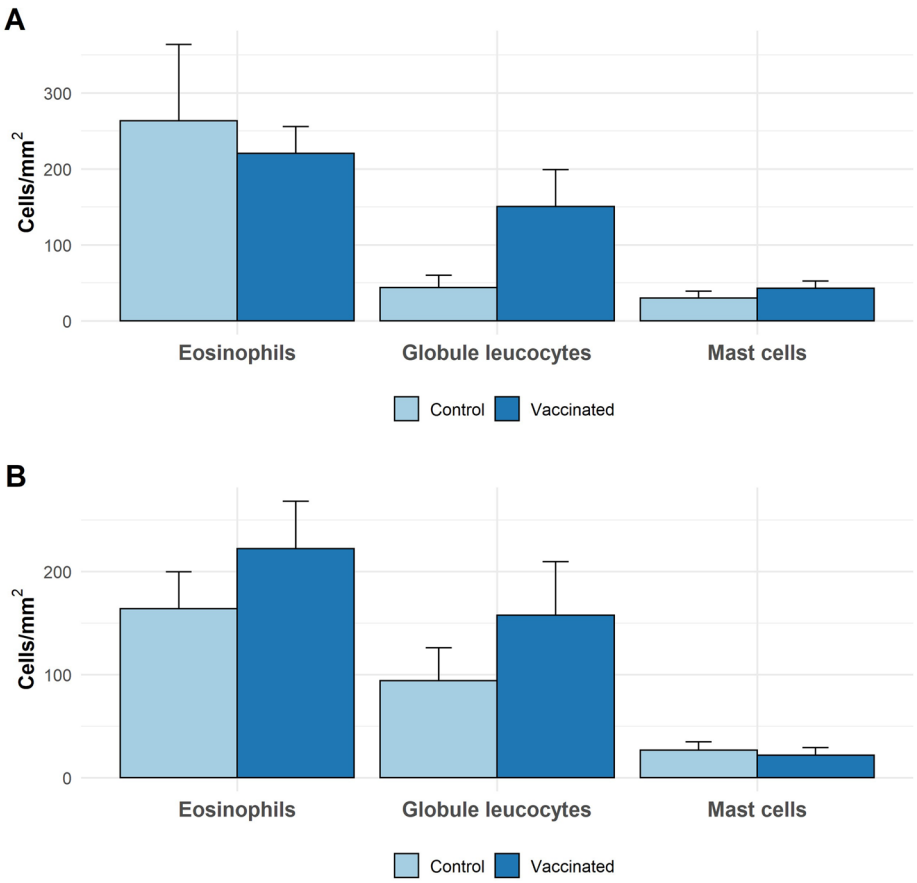

Figure 4 Eosinophils, globule leucocytes and mast cells in the abomasal wall in Canaria Sheep (A) and Canaria Hair Breed (B) following vaccination with a prototype recombinant sub-unit *Teladorsagia circumcincta* vaccine and subsequent *T. circumcincta* challenge. Values shown as means (cells/mm^2) ± SEM in abomasal tissue obtained at post-mortem following vaccination and parasite challenge.

nematodes [26], other studies indicate that Th1 immunity can be linked with resistance to *H. contortus*, for example, Caribbean hair sheep show higher IFN-γ production than susceptible sheep at 4 weeks post-infection [27]. More recently, a study in Creole goats has shown that resistance to *H. contortus* is associated with an upregulation of Th1 immune associated genes by 5 weeks post-infection, but that infection induces similar Th2 immune responses in resistant and susceptible goats [28]. These results provide growing evidence that a mixed Th1/Th2 immune response may be optimal for protection against ruminant GIN.

The main differences in cellular immune phenotypes between the two breeds were observed in ALN cell populations at post-mortem, whereby the proportion of $CD4^+$ and $CD8^+$ T cells within the ALN cells were higher in CHB lambs, and in particular the controls. $CD4^+$:$CD8^+$ ratios were also higher in vaccinated lambs of both breeds. These results suggest that the CHB induces stronger local adaptive immune responses, reflected by higher levels of recruitment to and expansion of T cells within the ALN. It also indicates that vaccination via the systemic route has had some influence on the balance of $CD4^+$ and $CD8^+$ T cells within the LN draining the site of infection, with a greater proportion of $CD4^+$ T cells possibly reflecting reactivation of memory $CD4^+$ T cells primed by the vaccine. While induction of mucosal immune responses via the systemic route is generally ineffective, this has previously been shown for *O. ostertagi*, a GIN of cattle closely related to *T. circumcincta*, where intra-muscular immunisation of cattle with native ASP-1 and Quil A induced antigen-specific proliferation of natural killer (NK) cells in the abomasal mucosa [29]. Interestingly, this NK response was lost when the antigen was formulated with $Al(OH)_3$, suggesting that the Quil A adjuvant, which was also used in this study, may be critical for systemically induced priming of the abomasal immune response.

As pointed out above, it would be desirable to reduce the number of recombinant proteins for commercial

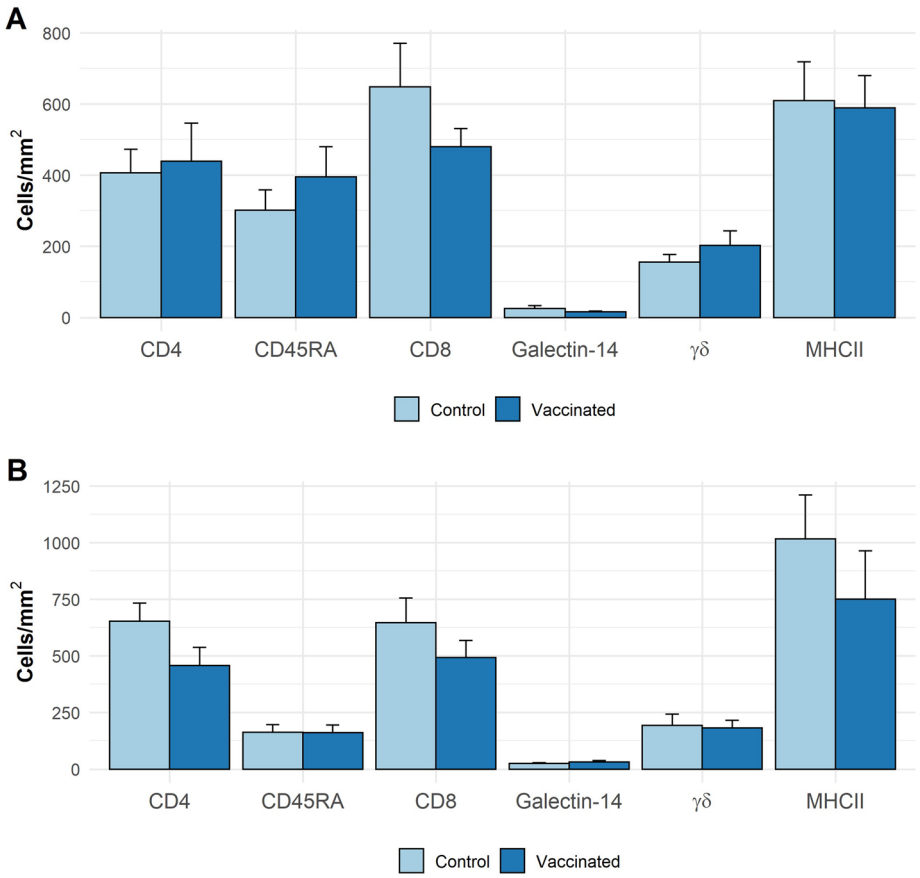

Figure 5 CD4, CD45RA, CD8, galectin-14, γδ and MHCII positive cells in the abomasal mucosa in Canaria Sheep (A) and Canaria Hair Breed (B) following vaccination with a prototype recombinant sub-unit *Teladorsagia circumcincta* vaccine and subsequent *T. circumcincta* challenge. Values shown are means (cells/mm^2) ± SEM in abomasal tissue obtained at post-mortem following vaccination and parasite challenge.

purposes [9]. Previous trials performed with Texel crossbred lambs have indicated Tci-APY-1 and Tci-MEP-1 as the most promising candidates for inclusion in a simplified vaccine for *T. circumcincta* [9]. Immunoglobulin levels and/or antibody:antigen avidity, relationship with parasitological parameters and recognition by adjuvant-only control lambs as they developed immunity following challenge, were the criteria for selecting these two proteins from the whole cocktail. The administration of Tci-MEP-1 and a mutated version of Tci-APY-1 performed as well as the whole vaccine prototype in Texel crossbred [9]. Tci-MEP-1 may also have a protective role in CS and CHB sheep in the trial presented here because in both breeds, specific serum IgA against this protein was negatively associated with parasitological parameters. The IgA response to Tci-APY-1 also showed negative associations with worm burden in CHB sheep. It seems that these two proteins could have a role in protection conferred by this prototype.

Table 4 Relationship between globule leukocytes and parasitological variables in Canaria Sheep (CS) and Canaria Hair Breed (CHB) sheep vaccinated with a prototype recombinant sub-unit *Teladorsagia circumcincta* vaccine and subsequently challenged with *T. circumcincta*

Group	Cumulative FEC	Worm burden	Worm length	EIU
CS Vac	−0.811**	−0.608*	−0.776**	−0.734**
CS Con	−0.409	−0.182	−0.764**	−0.545
CHB Vac	−0.545	−0.555	−0.309	−0.285
CHB Con	−0.664*	−0.630*	−0.340	−0.364

Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

However, data obtained with CS and CHB lambs showed some slightly different responses. In CS, following similar criteria, Tci-SAA-1 and Tci-ASP-1 would also be good candidates, because IgG_1 against both proteins and IgG_2 to Tci-ASP-1 was also negatively correlated with EIU. Interestingly, in Texel cross bred lambs,

in which no differences in worm length were detected [7], these associations were not observed. This could mean breed differences in vaccine protective mechanisms. Future studies must clarify why this response was apparently breed-dependent as it appears that the host breed is important in this response. Considering the humoral response, therefore, it is not clear which recombinant protein(s) should be selected to ensure protective immunity across several breeds, although Tci-MEP-1, Tci-APY-1 are promising candidates that have also been successfully tested [9].

Some of these proteins may not induce a strong humoral response but could trigger a critical cellular response. In fact, some successful vaccine prototypes against related GIN develop their protective mechanisms through cellular responses [30–32]. Noticeably, GLs were negatively associated with protection in both vaccinated breeds in this trial.

In conclusion, this recombinant vaccine protected six-month-old CS breed lambs and did not significantly reduce parasitological parameters in CHB sheep. Globule leukocytes and IgA and IgG_2 to L4 *T. circumcincta* antigens may be key mechanisms in protection conferred by the vaccine in the CS breed. Improving these responses at vaccination could reduce individual variability and enhance the response. Future studies considering sequential analysis of local immune response and/or depleting particular cell populations, including serially diluted samples at different times of infection for refining antibodies measurement accuracy. All these studies would be desirable for improving this vaccine prototype.

Abbreviations

γδTCR: Gamma Delta T Cell Receptor; ALN: Abomasal Lymph Node; BSA: Bovine Serum Albumin; CHB: Canaria Hair Breed; CS: Canaria Sheep; ConA: Concanavalin A; DMSO: Dimethyl Sulfoxide; EIU: Eggs in utero; ES: Excretory/Secretory; FACS: Fluorescence-Activated Cell Sorting; FBS: Fetal Bovine Serum; FCS: Fetal Calf Serum; FEC: Faecal egg counts; GENLIN: Generalised Linear Models; GIN: Gastrointestinal nematodes; GL: Globule Leukocyte; GLM: General Linear Models; HBSS: Hank's Balanced Salt Solution; *H. contortus*: *Haemonchus contortus*; Ig: Immunoglobulin; IL-4: Interleukin 4; IL-17A: Interleukin 17A; IFN-γ: Interferon gamma; L3: Third stage larval; L4: Fourth stage larval; LN: Lymph Node; LSA: Lymphocyte Stimulation Assays; LSD: Least Significance Difference; MHCII: Major Histocompatibility Complex II; M: Molar; NK: Natural Killer cells; OD: Optical Density; ODI: Optical Density Index; *O. ostertagi*: *Ostertagia ostertagi*; OPD: O-phenylene-diamine dihydrochloride; PBS: Phosphate buffered saline; RPMI: Roswell Park Memorial Institute medium; SEM: Standard error of the mean; SI: Stimulation Index; TBST: Tris Buffered Saline containing 0.1% Tween®20; *T. circumcincta*: *Teladorsagia circumcincta*; Tci-APY-1: Calcium-dependent apyrase-1; Tci-ASP-1: Activation-associated secretory protein-1; Tci-CF-1: Cathepsin F-1; Tci-ES20: 20 KDa protein of unknown function; Tci-MEP-1: Astacin-like metalloproteinase-1; Tci-MIF-1: Macrophage migration inhibitory factor-1; Tci-SAA-1: An homologue of a protective antigen from *Ancylostoma caninum*; Tci-TGH-2: TGF homologue; Th1: T helper type 1; Th2: T helper type 2; TWM: Transport Wash Media; TMB: 3,3′, 5, 5′ – Tetramethylbenzidine.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-021-00960-8.

Additional file 1. Antibody clones used for flow cytometry analysis and immunohistochemistry. "*" Antibody clone used for flow cytometry assays. "**" Antibody clone used for IHQ.

Additional file 2. Examples of abomasal mucosa sections from Canaria Hair Breed and Canaria Sheep experimentally infected with *Teladorsagia circumcincta* showing positive cells (x400). Haematoxylin and eosin staining for globule leukocyte in the upper layer (**A**) and eosinophils in the basal layer (**B**), both indicated with arrows. Immunohistochemical staining with CD4+ antibody (SBU T4 pool 44.38 + 44.97), showing CD4+ cells (arrows) in the apical (**C**) and basal area (**D**).

Additional file 3. IgA against vaccine proteins and their correlation with parasitology in Canaria Sheep lambs. Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 4. IgA against vaccine proteins and their correlation with parasitology in Canaria Hair Breed lambs. Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 5. IgG1 against vaccine proteins and their correlation with parasitology in Canaria Sheep lambs. Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 6. IgG1 against vaccine proteins and their correlation with parasitology in Canaria Hair Breed lambs. Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "**" at $p < 0.01$.

Additional file 7. IgG2 against vaccine proteins and their correlation with parasitology in Canaria Sheep lambs. Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 8. IgG2 against vaccine proteins and their correlation with parasitology in Canaria Hair Breed lambs. Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 9. Correlations between cells and parasitological variables in Canaria Sheep lambs. Associations are expressed as Spearman's correlation coefficient. Significant correlations are represented with "*" at $p < 0.05$.

Additional file 10. Correlations between cells and parasitological variables in Canaria Hair Breed lambs. Associations are expressed as Spearman's correlation coefficient.

Additional file 11: Interleukin-17A secretion by abomasal lymph node lymphocytes following stimulation with *Teladorsagia circumcincta* L4 or adult somatic antigen from Canaria Hair Breed and Canaria Sheep vaccinated with a prototype recombinant sub-unit *T. circumcincta* vaccine and subsequently challenged with *T. circumcincta*. IL-17A secretion was examined in supernatants collected 4 days post-stimulation of abomasal lymph node lymphocytes with 5 µg/mL of *T. circumcincta* L4 or adult somatic antigen. In general, antigen-specific IL-17A release was not detected in any of the ALN cultures. *N/D* IL-17A were not detected, *N/S* no sampled availability.

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Authors' contributions
AN, TMcN, JBM and JFG participated in the design of the study. JNH, CM, TPH, JFG carried out the experiments at ULPGC. YCM carried out LSA assays, cytokine ELISAs and flow cytometry at MRI. HWW, YCM assisted JNH, CM, TPH and JFG with post-mortem procedures. DGP prepared the recombinant proteins. AJN assisted JNH in vaccine preparation and administration. JNH and JFG coordinated the experiment. CM, JNH, ADH, YCM, JFG, TMcN, AJN and JBM participated in data analysis. CM, JNH and JFG drafted the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
Experiments were approved by the Animal Welfare Ethics Committee of the Universidad de Las Palmas de Gran Canaria (OEBA_ULPGC_003_2014) and from the local authorities, following the rules of the Spanish Legislation (RD 53/2013). Several animals presented local granulomas in the injection area. They were followed by the researchers and all of them resolved and they had no systemic consequences. One lamb in the CHB-vaccine group died a few days after the start of the procedure from a post-traumatic renal haemorrhage. It was not related to animal handling. We followed the ARRIVE guidelines published in the online journal PLoS Biology in June 2010.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Capítulo III

"Preliminary analysis of differential gene expression in 6-month-old Canaria Sheep after vaccination against *Teladorsagia circumcincta*"

Preliminary analysis of differential gene expression in 6-month-old Canaria sheep after vaccination against *Teladorsagia circumcincta*

Abstract

Gastrointestinal nematodes (GINs) have an important economic impact on small ruminant livestock. Moreover, resistance developed by these parasites highlights the importance of using complementary methods, such as vaccines. Recently, a recombinant *Teladorsagia circumcincta* prototype vaccine was tested in two local sheep breeds from the Canary Islands, Canaria Sheep (CS) and Canaria Hair Breed (CHB), that showed different levels of resistance against GIN. The vaccine conferred protection in the susceptible CS, significantly reducing worm length and number of eggs *in utero*, while in resistant CHB reductions were not significant. This protective effect in CS was associated with increased antigen-specific IgA, IgG_2 and Globule Leukocytes (GLs), as well as a higher $CD4^+/CD8^+$ ratio. Here, as an extension of the previous studies, we examined the Differentially Expressed Genes (DEGs) in abomasal tissue from the vaccinated and control groups of this breed following vaccination and experimental infection with *T. circumcincta*. Most of the up-regulated genes observed in vaccinated CS compared with controls were associated with immune response: mast cell sensitization, mucus production, antigen recognition, cytokine and immunoglobulin production or having a direct effect on nematode's viability. Down-regulated genes were associated with some negative effects on production traits, as well as different immune response functions. These results highlight the complexity of immune responses against GIN and the difficulties of finding suitable molecules to stimulate an appropriate response. Further studies will be needed to understand these mechanisms and refine the prototype vaccine.

Introduction

Resistance developed by parasites against every new commercialized anthelmintic has been constantly expanding (**Vercruysse *et al.*, 2018**). Therefore, searching for alternatives to anthelmintics is essential and vaccines are a promising option. Despite several attempts in developing effective vaccines, there are only a few which confer protection against gastrointestinal nematodes. However, most of the successful ones need to include native antigens, presenting certain difficulties when it comes to synthesizing recombinant ones, hence reducing their chances to be commercialized (**Britton, Emery, McNeilly, Nisbet, & Stear, 2020; Matthews, Geldhof, Tzelos, & Claerebout, 2016**).

In recent years, a recombinant prototype vaccine has been developed against the nematode *Teladorsagia circumcincta* consisting in a cocktail of 8 proteins (**Nisbet *et al.*, 2013**), conferring protection to lambs of different breeds and ages (**González *et al.*, 2019; Nisbet *et al.*, 2019, 2013**), as well as to periparturient ewes (**Nisbet, McNeilly, *et al.*, 2016**).

In spite of being a recombinant prototype, there are still some obstacles to overcome, such as breed and individual variability. Identifying and understanding the mechanisms underlying the protective immune response is the key to enhance the vaccine efficacy and reduce variability in response (**Nisbet, Meeusen, González, & Piedrafita, 2016; Piedrafita, Raadsma, Gonzalez, & Meeusen, 2010**).

For this purpose, recently, this recombinant vaccine was used in 6-month-old lambs of two local breeds from the Canary Islands with differences in their resistance to gastrointestinal nematodes (**González *et al.*, 2008**), Canaria Sheep (CS) and Canaria Hair Breed (CHB), followed by trickle infections of the parasite (**González *et al.*, 2019; Machín *et al.*, 2021**). Vaccination resulted in a protective effect on CS lambs, achieving statistically significant reductions in worm length and eggs in *utero* in this breed when comparing vaccinated and non-vaccinated lambs. This protection was mainly associated with the role played by IgA, IgG_2 and Globule Leukocytes (GLs), and, also, with higher ratios of $CD4^+/CD8^+$, suggesting these components could be decisive in successful outcomes to vaccination (**Machín *et al.*, 2021**). On the other hand, it was observed that immunization in CHB, although reducing several parasitological variables, did not achieve significant differences compared to the control group, probably, due to the, already high, natural resistance displayed by this breed at this age (**González *et al.*, 2019**).

As part of this experiment, to deepen our knowledge in the protection generated by the vaccine, transcriptomic

analyses of the abomasal tissue were carried out to explore its effect on gene expression. Here, we present the up and down-regulated genes and pathways affected by the vaccine in the CS breed, comparing vaccinated and control groups.

Material and Methods

Experimental design

Twenty-four CS male lambs were purchased and dewormed to be kept worm-free until they were six months-old at the facilities of *Granja Experimental del Cabildo Insular de Gran Canaria* (Veterinary Faculty, Universidad de Las Palmas de Gran Canaria, Spain). They were randomly selected to establish a vaccinated and a control group of 12 lambs in separated pens. Animals from the vaccinated group received subcutaneous injections (3 doses, on days 0, 21 and 42) with 400 µg of antigens as described previously (**Machín *et al.*, 2021**). Each dose was given as 2 separate injections: Tci-ASP-1, Tci-MIF-1, Tci-TGH-2, Tci-APY-1, Tci-SAA-1, Tci-CF-1 and Tci-ES20 PBS soluble proteins were administered as a single injection with 5mg Quil-A (Vax Saponin, Guinness Chemical Products Ltd); and Tci-MEP-1 was administered with 2M urea and 5mg Quil-A. The same volume of urea/PBS/10 mg Quil A was injected each time to the control group. These were administered at two sites behind the shoulder of each sheep. On the day of the final immunisation (day 42), a "trickle infection" protocol was initiated where all animals were orally inoculated with 2000 third stage larvae (L3) of *T. circumcincta* three times per week for 4 weeks until day 68. At the end of the trial (days 82-85), lambs were euthanised. Abomasal tissue samples were collected post-mortem from the antropyloric region and kept in E.Z.N.A. RNA Lock Reagent (Omega Bio-tek) at -80ºC for RNA extraction.

Experiments were approved by the Animal Welfare Ethics Committee of the Universidad de Las Palmas de Gran Canaria (OEBA_ULPGC_003_2014) and the local authorities, following the rules of the Spanish Legislation (RD 53/2013). Several animals presented local granulomas in the injection area. They were monitored by the researchers and all of them resolved, having no systemic consequences. We followed the ARRIVE guidelines (**Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010**) throughout the study.

RNA extraction and sequencing

Total RNA was extracted from the abomasal tissue (20mg) biopsies by first cutting the samples into smaller pieces with a sterile scalpel and then further homogenising each sample in RLT buffer (Qiagen Ltd, UK) using a Precellys bead basher (Bertin Instruments, UK) within a Precellys CK28 bead tube (Stretton Scientific, UK) for 3 x 23 second cycles at 5,800rpm with 2 minutes between cycles on ice. Samples were cleared by centrifugation at 14,000g at 4°C for 10 minutes and supernatants processed using a RNeasy mini-isolation kit (Qiagen Ltd, UK) according to the manufacturers' protocol and including an on-column DNase I digestion. RNA quantity and integrity were assessed using a Nanodrop spectrophotometer (Thermo Fisher, UK) and a Bioanalyser RNA Nanochip (Agilent Technologies Ltd, UK). Mean RNA integrity number (RIN) values across all samples was 8.1, indicating the successful extraction of high-quality RNA from the abomasal tissue biopsies. Prior to sequencing library preparation, the yield of total RNA was determined on a Qubit Fluorometer (ThermoFisher, UK) using the Broad Range RNA kit (Thermo Fisher, UK). The resulting RNA samples were sequenced on an Illumina HiSeq 4000 by The Centre for Genome Research (CGR) at the University of Liverpool, UK, generating 2x150 bp strand-specific, paired-end reads.

RNA-Seq Quality Control and alignment

R version 3.4.4 (2018-03-15) was used for the RNAseq data QC based on all 21 samples with the arrayQualityMetrics package. Base calls were made using the Illumina CASAVA 1.8 pipeline and cutadapt (v1.11) was used for adapter trimming. Processed sequences were then aligned to the *Ovis aries* genome assembly Oar_v3.1 (GCA_000298735.1) using the STAR aligner and the number of mapped read-pairs were counted based on the *Ovis aries* genome annotation (Ensembl release 91) also within STAR (**Dobin *et al.*, 2013**).

Statistical analysis

For the RNA-seq analysis, count data for the samples was normalized using TMM (Trimmed Mean of M-value) and transformed with VOOM (**Law, Chen, Shi, & Smyth, 2014**) to log2-counts per million with associated precision weights. A comparison between vaccinates versus controls in abomasal tissue was undertaken 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 <0.05 and a 2.5-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 and the resulting gene lists were 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 (DEGs) identified in the Vaccinated vs Control in CS abomasal tissue comparison were manually obtained using different

tools. DAVID v6.8 (**"DAVID: Database for Annotation, Visualization and Integrated Discovery," 2020**) was used in order to quickly obtain some gene symbols of *Ovis aries* species. The rests of gene symbols were studied through Ensembl Oar v3.1 (**"Ensembl Genome Browser," 2021**), Uniprot (**"Uniprot: The Universal Protein Resource," 2021**) and BLAST (**"BLAST: Basic Local Alignment Search Tool," 2021**); whilst aspects as biological process, cellular component and molecular function were investigated using GeneCards webpage (**"GeneCards: The Human Gene Database," 2021**).

Pathway analysis

CTD (**"CTD: Comparative Toxicogenomics Database," 2021**) was used for searching the enriched pathways of DEGs at p-value <0.05 with 2.5-fold differential expression, obtaining them from *Homo sapiens* annotations in KEGG (**"KEGG: Kyoto Encyclopedia of Genes and Genomes," 2021**) and REACT (**"REACT: Reactome Pathway Database," 2021**).

Results

Results about parasitology and cellular and humoral immune response of this experiment were previously published (**González *et al.*, 2019; Machín *et al.*, 2021**). Briefly, the vaccine induced a statistically significant reduction in worm length (Con= 9.87 ± 0.06; Vac=9.10 ± 0.10) and EIU (Con=23.67 ± 0.87; Vac=16.39 ± 0.77) in the vaccinated group when compared to the control (adjuvant-only) in CS (**Machín *et al.*, 2021**).

In relation to the immune response, levels of specific IgA and IgG_2 which bound extracts of L4 *T. circumcincta* and numbers of Globule Leukocytes (GLs) had negative associations with different parasitological parameters in vaccinated, when compared to non-vaccinated, CS lambs. Also, immunisation seemed to induce a higher $CD4^+/CD8^+$ ratio in these vaccinated animals (**Machín *et al.*, 2021**).

Transcriptomic analysis

Using the selected FDR corrected p-value cut-off of <0.05 and a FC cut-off of >2.5 we identified 96 differentially expressed genes (DEGs) between the Vaccinated vs Control abomasal tissue samples. Forty-five of these genes were up-regulated (**Table 1**) and 51 down-regulated (**Table 2**) in the vaccine group.

Up-regulated genes were mainly involved in the immune response: ITLN2, B3GNT8, MCP2, TRAV41, immunoglobulin V lambda chain (V lambda 18) and TRD associated with mucus production, cells or immunoglobulins; KRT18 and CSF with cytokine production; DFCR2, CLEC18A and CLDN3 with mechanisms of cell migration, involved in functions such as binding, cell or cell-cell adhesion respectively; TRAV41, TRD, DCSTAMP, GPR31 and SPSB2 (also linked to signalling) with antigen presentation; APLN, CAMPK2, SIRPB1, CCR10 had signalling functions; PF4, MPO, CL46 were involved in a direct killing effect on pathogens; and BHLHA15, PCSK1N, ZFP36L2, CL46 and CRB1 in innate immunity/inflammation.

Other up-regulated genes were involved in different roles, for instance: HMGN1, CDCA4 in the cell cycle; EPPK1, expressed in response to mechanical stress like wound healing, contributing to tissue architecture; or CCK gene, associated to digestive enzyme release and satiety effect.

Some of the down-regulated genes in the vaccinated group have been related to production traits. DKK1, FGF20, and KRT5 are associated to wool development and production. Others, such as IZUMO1R and RXFP2 are involved in fertilization and horn shape and size respectively. Moreover, SLC35F4 has a role in both traits, implicated in milk composition and wool staple length.

The rest of the significantly down-regulated genes have been associated to a variety of functions. Some of them are predominantly involved in the innate immune response: KLHL14, FBXL21P (also involved in Class I MHC mediated antigen processing and presentation), SLC2A5, GP2 and PLPP4, while others are associated with cell signalling (SCGB2A2) and cell-cell recognition (GPA33), cell adhesion molecules (LRRC4 and ADAM22 -the latter also linked to inhibition of cell proliferation-) and interferon gamma signalling (IRF6). Also, there were genes encoding receptors such as FCAMR (Fc fragment of IgA and IgM receptor) and FCRLA (Fc Receptor Like A); and another gene involved in suppressing nematode development, LGALS1.

In this study, 16 enriched KEGG and REACT pathways of the up-regulated genes (**Table 3**) and 11 pathways of the down-regulated genes (**Table 4**) were identified as being represented in the list of DEGs between vaccinated animals and controls. Among the up-regulated genes, we highlight signalling by GPCR (G-Protein-Coupled Receptors), signal transduction, GPCR ligand binding, GPCR downstream signalling and Class A/1 (Rhodopsin-like receptors) pathways for having a higher number of genes involved in them. The largest proportion of the down-regulated genes identified were participating in immune system and metabolism pathways.

Table 1. Up-regulated genes in Vaccinated vs Control abomasal tissue contrast at p-value < 0.05 with 2.5-fold threshold.

ID	Gene Symbol	Gene Description	Fold Change (FC)	P Value	Adjusted P Value
ENSOARG00000009191	ITLN2	Intelectin-2	15.64	$6.68E^{-04}$	$5.04E^{-01}$
ENSOARG00000009181	ITLN2	Intelectin-2	11.72	$6.36E^{-05}$	$2.10E^{-01}$
ENSOARG00000013777	CBR1	Carbonyl reductase [NADPH] 1-like	6.26	$8.98E^{-05}$	$2.10E^{-01}$
ENSOARG00000002472	MSMB	Microseminoprotein beta	5.54	$5.30E^{-03}$	$7.97E^{-01}$
ENSOARG00000003317	CCK	Cholecystokinin	5.49	$2.94E^{-02}$	$9.74E^{-01}$
ENSOARG00000009086	ITLN	Intelectin	5.10	$8.99E^{-03}$	$8.87E^{-01}$
ENSOARG00000009169	ITLN2	Intelectin-2	4.84	$1.94E^{-03}$	$6.98E^{-01}$
ENSOARG00000008679	TEX22	Testis-expressed protein 22	4.31	$9.60E^{-06}$	$1.12E^{-01}$
ENSOARG00000002945	DGCR2	Integral membrane protein DGCR2/IDD	3.78	$3.26E^{-03}$	$7.70E^{-01}$
ENSOARG00000004858	HMGN1	Non-histone chromosomal protein HMG-14	3.69	$2.68E^{-02}$	$9.74E^{-01}$
ENSOARG00000017946	BHLHA15	Class A basic helix-loop-helix protein 15	3.64	$2.85E^{-02}$	$9.74E^{-01}$
ENSOARG00000007191	ZFP36L2	Zinc finger protein 36, C3H1 type-like 2	3.63	$2.85E^{-02}$	$9.74E^{-01}$
ENSOARG00000011670	PCSK1N	Proprotein convertase subtilisin/kexin type 1 inhibitor	3.63	$6.12E^{-03}$	$8.08E^{-01}$
ENSOARG00000015586	NAT8L	N-acetylaspartate synthetase	3.50	$5.63E^{-04}$	$4.38E^{-01}$
ENSOARG00000007330	B3GNT8	UDP-glcnac:betagal beta-1,3-N-acetylglucosaminyltransferase 8	3.24	$4.04E^{-03}$	$7.72E^{-01}$
ENSOARG00000004520	MCP2	Mast cell protease 2	3.23	$1.52E^{-02}$	$9.59E^{-01}$
ENSOARG00000018581	LOC114110263	Uncharacterized LOC114110263	3.20	$1.62E^{-02}$	$9.67E^{-01}$
ENSOARG00000001067	ABCC4	Multidrug resistance-associated protein 4-like	3.18	$6.29E^{-03}$	$8.08E^{-01}$
ENSOARG00000004351	KRT18	Keratin, type I cytoskeletal 18	3.18	$2.09E^{-03}$	$6.98E^{-01}$
ENSOARG00000018410	RTN4R	Reticulon 4 receptor	3.08	$1.20E^{-02}$	$9.53E^{-01}$
ENSOARG00000014891	APLN	Apelin	3.00	$1.87E^{-04}$	$3.36E^{-01}$
ENSOARG00000005417	CLEC18A	C-type lectin domain family 18 member A-like	2.91	$1.00E^{-02}$	$9.05E^{-01}$
ENSOARG00000014766	PF4	Platelet factor 4	2.85	$1.19E^{-02}$	$9.53E^{-01}$
ENSOARG00000006951	CAMK2A	Calcium/calmodulin dependent protein kinase II alpha	2.84	$1.78E^{-03}$	$6.98E^{-01}$
ENSOARG00000019461	TRAV41	T-cell receptor alpha variable 41	2.82	$7.43E^{-03}$	$8.31E^{-01}$
ENSOARG00000015726	DCSTAMP	Dendrocyte expressed seven transmembrane protein	2.81	$5.27E^{-03}$	$7.97E^{-01}$
ENSOARG00000013958	CDCA4	Cell division cycle-associated 4	2.81	$1.13E^{-02}$	$9.48E^{-01}$
ENSOARG00000007467	SIRPB1	Signal-regulatory protein beta-1 isoform 3-like	2.81	$6.50E^{-03}$	$8.08E^{-01}$
ENSOARG00000009287	MPO	Myeloperoxidase	2.79	$6.73E^{-03}$	$8.08E^{-01}$
ENSOARG00000009762	DD3	Dihydrodiol dehydrogenase 3-like	2.72	$4.88E^{-04}$	$4.38E^{-01}$
ENSOARG00000004595	OXT	Oxytocin/neurophysin I prepropeptide	2.72	$1.44E^{-02}$	$9.57E^{-01}$
ENSOARG00000004795	-	*Ovis canadensis canadensis* isolate 43U chromosome 19 sequence	2.70	$3.22E^{-03}$	$7.70E^{-01}$
ENSOARG00000010845	CLDN3	Claudin 3	2.66	$2.33E^{-02}$	$9.74E^{-01}$
ENSOARG00000015430	CSF2	Colony stimulating factor 2	2.65	$3.24E^{-03}$	$7.70E^{-01}$
ENSOARG00000008914	TUSC1	Tumor suppressor candidate 1	2.61	$1.93E^{-02}$	$9.74E^{-01}$
ENSOARG00000000502	CL46	Collectin-46-like	2.61	$1.62E^{-02}$	$9.67E^{-01}$
ENSOARG00000002817	GPR31	G protein-coupled receptor 31	2.61	$1.39E^{-02}$	$9.57E^{-01}$
ENSOARG00000001912	-	Immunoglobulin V lambda chain (V lambda 18) gene, partial cds	2.59	$2.77E^{-02}$	$9.74E^{-01}$
ENSOARG00000014571	BBOX1	Gamma-butyrobetaine hydroxylase 1	2.57	$1.20E^{-02}$	$9.53E^{-01}$
ENSOARG00000017609	SPSB2	SplA/ryanodine receptor domain and SOCS box containing 2	2.57	$6.74E^{-03}$	$8.08E^{-01}$
ENSOARG00000002956	CCR10	C-C motif chemokine receptor 10	2.55	$3.03E^{-02}$	$9.74E^{-01}$
ENSOARG00000016951	KRT13	Keratin 13	2.54	$1.58E^{-03}$	$6.98E^{-01}$
ENSOARG00000000828	TRD	Rearranged T-cell receptor delta	2.53	$5.58E^{-03}$	$7.97E^{-01}$
ENSOARG00000019753	CDK5R2	Cyclin dependent kinase 5 regulatory subunit 2	2.51	$2.07E^{-03}$	$6.98E^{-01}$
ENSOARG00000002504	EPPK1	Epiplakin-like	2.50	$3.69E^{-03}$	$7.70E^{-01}$

Table 2. Down-regulated genes in Vaccinated vs Control abomasal tissue contrast at p-value < 0.05 with 2.5-fold threshold.

ID	Gene Symbol	Gene Description	Fold Change (FC)	P Value	Adjusted P Value
ENSOARG00000013619	DKK1	Dickkopf WNT signaling pathway inhibitor 1	-5.76	7.63E-05	2.10E-01
ENSOARG00000014064	MTHFS	5-formyltetrahydrofolate cyclo-ligase	-5.55	1.36E-02	9.57E-01
ENSOARG00000017027	ENTHD1	ENTH domain containing 1	-5.25	5.59E-04	4.38E-01
ENSOARG00000012434	FMO2	Flavin containing dimethylaniline monooxygenase 2	-4.81	2.85E-04	3.91E-01
ENSOARG00000006008	KLHL14	Kelch like family member 14	-4.74	1.50E-08	3.50E-04
ENSOARG00000018479	AQP8	Aquaporin 8	-4.18	8.28E-05	2.10E-01
ENSOARG00000010006	POU5F1	POU class 5 homeobox 1	-4.16	1.44E-02	9.57E-01
ENSOARG00000011969	GPA33	Glycoprotein A33	-4.05	5.16E-03	7.97E-01
ENSOARG00000021106	SLC35F4	Solute carrier family 35 member F4	-3.95	1.59E-04	3.10E-01
ENSOARG00000006864	FCAMR	Fc fragment of IgA and IgM receptor	-3.92	2.01E-03	6.98E-01
ENSOARG00000005904	CCDC178	Coiled-coil domain containing 178	-3.78	9.35E-04	6.24E-01
ENSOARG00000019995	CNR1	Cannabinoid receptor 1	-3.71	5.49E-04	4.38E-01
ENSOARG00000014282	SLC7A13	Solute carrier family 7 member 13-like	-3.67	3.88E-04	4.14E-01
ENSOARG00000011714	FMO5	Dimethylaniline monooxygenase [N-oxide-forming] 5-like	-3.60	5.60E-03	7.97E-01
ENSOARG00000005505	GBA3	Glucosylceramidase beta 3 (gene/pseudogene)	-3.44	3.44E-03	7.60E-01
ENSOARG00000002353	LRRC4	Leucine rich repeat containing 4	-3.40	7.50E-05	2.10E-01
ENSOARG00000012288	LOC114115244	MLV-related proviral Env polyprotein-like	-3.34	4.60E-03	7.72E-01
ENSOARG00000011416	ADAM22	ADAM metallopeptidase domain 22	-3.33	2.01E-03	6.98E-01
ENSOARG00000006764	LOC114111619	MLV-related proviral Env polyprotein-like	-3.29	3.86E-03	7.70E-01
ENSOARG00000001982	AICDA	Activation induced cytidine deaminase	-3.24	1.82E-03	6.98E-01
ENSOARG00000009585	FGF20	Fibroblast growth factor 20	-3.15	6.04E-05	2.10E-01
ENSOARG00000014847	FBXL21P	F-Box and Leucine Rich Repeat Protein 21, Pseudogene	-3.14	7.85E-05	2.10E-01
ENSOARG00000008942	SLC2A5	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5	-3.12	6.30E-03	8.08E-01
ENSOARG00000010166	FCRLA	Fc receptor like A	-3.00	3.96E-03	7.70E-01
ENSOARG00000000748	SMIM28	Small integral membrane protein 28	-2.95	2.28E-02	9.74E-01
ENSOARG00000018158	SYN3	Synapsin III	-2.95	2.43E-04	3.63E-01
ENSOARG00000019763	FAM19A3	Family with sequence similarity 19 member A3, C-C motif chemokine like	-2.95	1.72E-03	6.98E-01
ENSOARG00000017516	MROH8	Maestro heat like repeat family member 8	-2.92	1.95E-03	6.98E-01
ENSOARG00000013761	LGALS2	Galectin 2	-2.92	7.39E-03	8.31E-01
ENSOARG00000013930	IL17A	Interleukin 17A	-2.85	1.24E-03	6.98E-01
ENSOARG00000014805	SCGB2A2	Secretoglobin family 2A member 2	-2.83	2.43E-02	9.74E-01
ENSOARG00000014590	CRYM	Crystallin mu	-2.82	1.21E-02	9.53E-01
ENSOARG00000020934	ZBTB8B	Zinc finger and BTB domain containing 8B	-2.73	3.27E-03	7.70E-01
ENSOARG00000012472	GP2	Glycoprotein 2	-2.66	5.29E-03	7.97E-01
ENSOARG00000016974	KRT5	Keratin 5	-2.65	1.14E-02	9.48E-01
ENSOARG00000020141	RHBDL1	Rhomboid like 1	-2.63	1.03E-02	9.13E-01
ENSOARG00000001898	IZUMO1R	IZUMO1 receptor, JUNO	-2.62	7.27E-03	8.31E-01
ENSOARG00000014058	LPAR3	Lysophosphatidic acid receptor 3	-2.59	2.41E-04	3.63E-01
ENSOARG00000016094	EPHX4	Epoxide hydrolase 4	-2.59	2.56E-03	7.39E-01
ENSOARG00000004284	PLPP4	Phospholipid phosphatase 4	-2.58	2.31E-03	7.30E-01
ENSOARG00000011653	RXFP2	Relaxin Family Peptide Receptor 2	-2.57	2.44E-03	7.39E-01
ENSOARG00000010797	TEAD4	TEA domain transcription factor 4	-2.54	9.40E-03	9.00E-01
ENSOARG00000016296	HOXC6	Homeobox C6	-2.54	4.31E-03	7.72E-01
ENSOARG00000014295	MUC15	Mucin 15, cell surface associated	-2.53	1.77E-03	6.98E-01
ENSOARG00000015795	SLC7A3	Cationic amino acid transporter 3-like	-2.53	9.26E-03	9.00E-01
ENSOARG00000008699	BFSP2	Beaded filament structural protein 2	-2.52	1.29E-02	9.53E-01
ENSOARG00000012823	ST6GALNAC3	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	-2.52	6.82E-03	8.13E-01
ENSOARG00000006855	KCNA4	Potassium voltage-gated channel subfamily A member 4	-2.52	7.02E-04	5.13E-01
ENSOARG00000020948	IRF6	Interferon regulatory factor 6	-2.51	4.28E-05	2.10E-01
ENSOARG00000010377	ARL5C	ADP ribosylation factor like GTPase 5C	-2.50	1.25E-03	6.98E-01
ENSOARG00000008560	SLC23A1	Solute carrier family 23 member 1-like	-2.50	2.54E-02	9.74E-01

Table 3. Enriched pathways of the up-regulated genes of vaccinated compared to control lambs.

Pathways	Pathway ID	Annotated Genes Quantity	Annotated Genes
Signal Transduction	REACT:R-HSA-162582	8	APLN,CAMK2A,CCK,CCR10,CSF2,GPR31,OXT,PF4
Signalling by GPCR	REACT:R-HSA-372790	8	APLN,CAMK2A,CCK,CCR10,CSF2,GPR31,OXT,PF4
GPCR ligand binding	REACT:R-HSA-500792	6	APLN,CCK,CCR10,GPR31,OXT,PF4
GPCR downstream signalling	REACT:R-HSA-388396	6	APLN,CCK,CCR10,CSF2,OXT,PF4
Class A/1 (Rhodopsin-like receptors)	REACT:R-HSA-373076	6	APLN, CCK,CCR10,GPR31,OXT,PF4
Peptide ligand-binding receptors	REACT:R-HSA-375276	5	APLN,CCK,CCR10,OXT,PF4
Gastrin-CREB signalling pathway via PKC and MAPK	REACT:R-HSA-881907	4	CAMK2A,CCK,CSF2,OXT
G alpha (i) signalling events	REACT:R-HSA-418594	3	APLN,CCR10,PF4
DAP12 interactions	REACT:R-HSA-2172127	3	CAMK2A,CSF2,SIRPB1
Cytokine-cytokine receptor interaction	KEGG:hsa04060	3	CCR10,CSF2,PF4
Platelet activation, signalling and aggregation	REACT:R-HSA-76002	3	ABCC4,CSF2,PF4
Chemokine receptors bind chemokines	REACT:R-HSA-380108	2	CCR10,PF4
Cell-Cell communication	REACT:R-HSA-1500931	2	CLDN3,SIRPB1
Oxytocin signalling pathway	KEGG:hsa04921	2	CAMK2A,OXT
Platelet degranulation	REACT:R-HSA-114608	2	ABCC4,PF4
Response to elevated platelet cytosolic Ca^{2+}	REACT:R-HSA-76005	2	ABCC4,PF4

Table 4. Enriched pathways of the down-regulated genes of vaccinated compared to control lambs.

Pathways	Pathway ID	Annotated Genes Quantity	Annotated Genes
Immune System	REACT:R-HSA-168256	7	FBXL21P, FGF20, IL17A, IRF6, MUC15, PLPP4, SLC2A5
Metabolism	REACT:R-HSA-1430728	7	CRYM, FMO2, GBA3, MTHFS, SLC23A1, SLC2A5, TEAD4
Rap1 signalling pathway	KEGG:hsa04015	3	CNR1, FGF20, LPAR3
Neuroactive ligand-receptor interaction	KEGG:hsa04080	3	CNR1, LPAR3, RXFP2
Class A/1 (Rhodopsin-like receptors)	REACT:R-HSA-373076	3	CNR1, LPAR3, RXFP2
Termination of O-glycan biosynthesis	REACT:R-HSA-977068	2	MUC15, ST6GALNAC3
O-linked glycosylation of mucins	REACT:R-HSA-913709	2	MUC15, ST6GALNAC3
Drug metabolism - cytochrome P450	KEGG:hsa00982	2	FMO2, FMO5
Metabolism of water-soluble vitamins and cofactors	REACT:R-HSA-196849	2	MTHFS, SLC23A1
O-linked glycosylation	REACT:R-HSA-5173105	2	MUC15, ST6GALNAC3
Post-translational modification: synthesis of GPI-anchored proteins	REACT:R-HSA-163125	2	GP2, IZUMO1R

Discussion

Here, we have observed several up-regulated genes associated with the immune response. Interestingly, most of these DEGs observed between vaccinated and control groups of CS corresponded to genes linked to the immune response described in the previous study, especially to effector cells and immunoglobulins (**Machín *et al.*, 2021**).

Since the prototype vaccine used in this study needs refinement in terms of efficacy, vaccine antigen complexity and variability in response before it can be commercially exploited (**Nisbet *et al.*, 2019**), the search for new knowledge about the responses triggered by it in sheep is required. For this purpose, immune responses of vaccinated and non-vaccinated CS (the breed to which the vaccine conferred significant protection) serially infected with *T. circumcinta* were compared. The protection achieved was associated with levels of antigen-specific IgA and IgG_2, as well as GLs and higher ratios of $CD4^+/CD8^+$ (**Machín *et al.*, 2021**). Consequently, the next step was to analyse the gene expression in abomasal tissue to learn more about the mechanisms responsible for this protection at the site of infection.

One of the up-regulated genes, encoding immunoglobulin V lambda chain (V lambda 18), was 2.59 times more highly expressed in vaccinated than in control animals. This is not only considered a reflection of a greater production of immunoglobulins, which in this case was experienced by the vaccinated lambs (**Machín *et al.*, 2021**), but also, these immunoglobulin light chains (immunoglobulin free light chain κ y λ, FLCs) are believed to have a functional role *per se*. Within this functional role, we could highlight enzymatic (**Boivin *et al.*, 2004**) and proteolytic functions (**Paul *et al.*, 1995; Sun, Gao, Li, & Paul, 1994**); activation of prothrombinase, accelerating the conversion from fibrinogen to fibrin (**Thiagarajan *et al.*,**

2000); complement activation (**Jokiranta, Solomon, Pangburn, Zipfel, & Meri, 1999**); chemotactic action (**Edmundson & Ely, 1985**); and mast cell sensitization (**Redegeld & Nijkamp, 2003; Van Den Beucken *et al.*, 2001**), which could promote the release of various substances by these cells, including some proteases (**Boeckxstaens, 2015**), playing an important role in immediate hypersensitivity reactions (**Basile *et al.*, 2017; Redegeld *et al.*, 2002**).

Activated intraepithelial mast cells are considered as globule leukocytes in sheep and the immediate hypersensitivity responses associated with them have been described, in fact, as a key mechanism of early larval rejection in infections by gastrointestinal nematodes in sheep (**Nisbet, Meeusen, *et al.*, 2016**). Although higher mean globule leukocytes were found in vaccinated than in control animals in this experiment, this difference was not statistically significant. However, it is possible that globule leukocytes were more highly activated in the vaccinated group since there were significant negative correlations between their number and parasitological variables (**Machín *et al.*, 2021**). Evidence of greater activation of globule leukocyte/mast cells in vaccinated animals was provided by the transcriptomic data, with 3.23-fold higher expression of the gene that encodes mast cell protease 2 (MCP2) in the vaccinated than in the control group. This protein is specifically produced by mucosal mast cells (**Kasakura *et al.*, 2020**). Furthermore, colony stimulating factor 2 (CSF) was another up-regulated gene in vaccinated lambs and it could be responsible for both, immunoglobulin light chains and MCP2 increased productions (**Brown, Wright, & Miller, 2003; Ghildyal, Friend, Nicodemus, Austen, & Stevens, 1993; Kasakura *et al.*, 2020; Knight *et al.*, 2007; Miller, Wright, Knight, & Thornton, 1999; Wright *et al.*, 2002; Yamazaki *et al.*, 2015**). MCP2 production in vaccinated sheep is not surprising, since this molecule is released in response to parasites (**Kasakura *et al.*, 2020**) and, for instance, has been observed in the rapid rejection of *Trichinella spiralis* in mice (**Blum, Thrasher, Gagliardo, Fabre, & Appleton, 2009**) and in the mucus of sheep and goats infected with gastrointestinal nematodes (**French *et al.*, 2008; Huntley *et al.*, 1995; Macaldowie, Jackson, Huntley, Mackellar, & Jackson, 2003**). This sheep mast cell protease (SMCP) is even presented in the mucus of non-parasitized, but previously infected sheep (**Athanasiadou *et al.*, 2008**), unlike other proteases that could play an important role in the protection, such as intelectins (ITLN), which seem to require the presence of worms for its production, as it happens in sensitized animals (**Athanasiadou *et al.*, 2008**).

Genes encoding ITLN2 were the ones that showed the greatest differences in their expression between vaccinated and control groups in this study (**Table 1**). This is an interesting observation, since ITLN2 is considered as the most up-regulated expressed gene in genetically resistant mice experimentally infected with *Trichuris muris* (**Artis, 2006; Artis *et al.*, 2002; Artis, Villarino, *et al.*, 2004; Artis, Wang, *et al.*, 2004; Datta *et al.*, 2005**). A higher production of ITLN by epithelial cells in infected mice with *Trichinella spiralis* has been observed in proteomic studies (**Pemberton, Knight, Gamble, *et al.*, 2004**), also, being an up-regulated gene at the time of the expulsion of both nematodes in mice (**Artis, 2006; Pemberton, Knight, Wright, & Miller, 2004**), suggesting an important role in the control of these nematode infections. Evidence of this intelectin in mucus and epithelial cells of sheep rendered immune to infection with *T. circumcincta* by parasite exposure have been observed as well (**Athanasiadou *et al.*, 2008**).

ITLN2 in sheep is produced by mucous neck cells of the abomasal mucosa (**French *et al.*, 2008**). As with other lectins, they are highly conserved molecules that develop different functions, such as regulation of the cell proliferation and recognition of carbohydrates present in the pathogen cell wall (**Apostolopoulos & McKenzie, 2001; Artis, 2006; East & Isacke, 2002; Elola, Chiesa, Fernández-Alberti, Mordoh, & Fink, 2005; Iwanaga & Bok, 2005; Wang, Gray, Haudek, & Patterson, 2004**). It is known that they bind to galactofuranosyl residues of bacteria, and it is possible that they also bind carbohydrate molecules on or in nematodes, affecting their feeding or mechanisms of attachment of the parasite to the host. A different possibility is that ITLN2 could facilitate or induce the effector action of other host molecules (**Nishihara, Wyrick, Working, Chen, & Hedrick, 1986**), or it could have wider functions, such as facilitating tissue remodelling, inflammation, or having an immunoregulator role (**Artis, 2006**). Moreover, since ITLN2 seems to be activated by IL-4 (**French *et al.*, 2008**), which is produced by the mast cells, it could play an important role in regulating worm length and fecundity in the vaccinated sheep and this could agree with the data obtained in our previous studies, contributing also to mucus production.

The contribution of mucus to the control of GIN in vaccinated lambs is highlighted even more with 3.24-fold higher expression of B3GNT8, which regulates the expression of UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8, a protein participating in glycosylation of gel-forming mucin, contributing to giving a greater viscosity to the mucus (**Demouveaux, Gouyer, Gottrand, Narita, & Desseyn, 2018**) and it could impair parasite motility through gastrointestinal tract.

Probably, part of this enhanced immune response lays in a more suitable antigen recognition. For instance, some genes linked to the antigen recognition through T cell receptors such as regulating receptor alpha expression of T cells (TRAV41) and *O. aries* rearranged T-cell receptor delta

(TRD) respectively, were 2.82 and 2.53-fold up-regulated in vaccinated when compared to non-immunized lambs. Another up-regulated gene observed that was related to antigen recognition is dendrocyte expressed seven transmembrane proteins (DCSTAMP), which participates in the activation of dendritic cells. In addition, the capture of antigens could be enhanced by the G Protein-Coupled Receptor 31 (GPR31) gene, that encodes a protein associated with activation of C-X3-C motif chemokine receptor 1 (CX3CR1) macrophages of the digestive tract performing this function (**Morita *et al.*, 2019**). The fact that the splA/ryanodine receptor domain and SOCS box containing 2 (SPSB2) gene was also more highly expressed in vaccinated sheep highlights the importance of antigenic recognition stimulated by the vaccination. This gene has been associated with the presentation of antigens through MHC-I and the innate immune system (**https://www.genecards.org/cgi-bin/carddisp.pl?gene=SPSB2**).

Furthermore, there are genes participating in cell migration, such as integral membrane protein DGCR2/IDD (DGCR2), C-type lectin domain family 18 member A (CLEC18A) and claudin 3 (CLDN3), that were up-regulated in vaccinated animals, probably, to facilitate diapedesis. Since there were many up-regulated genes associated with immunity, it was foreseeable some genes related to intracellular signaling, required to trigger these responses, would be more present in vaccinated lambs, detecting increases of genes such as apelin (APLN), calcium/calmodulin dependent protein kinase II alpha (CAMK2A), signal-regulatory protein beta-1 isoform 3-like (SIRPB1) and C-C motif chemokine receptor 10 (CCR10) at the time of euthanizing.

Some of the up-regulated genes have been previously associated with direct effects on other parasites: in human, platelet factor 4 (PF4) gene, for instance, is able to selectively killing malaria parasites inside erythrocytes (**Love *et al.*, 2012**), apart from being involved in other functions like chemotactic activity of neutrophils/monocytes, inhibiting hematopoiesis and angiogenesis. Myeloperoxidase (MPO) was another up-regulated gene in the vaccine group, associated with the production of Neutrophil Extracellular Traps (NETs) (**Metzler *et al.*, 2011; Papayannopoulos, Metzler, Hakkim, & Zychlinsky, 2010**) which have been reported to be involved in the activity observed against helminths (**Bobardt, Dillman, & Nair, 2020**). Also, the collectin-46 (CL46) gene encodes a protein that is known for binding to bacteria surface carbohydrates (**Dec & Wernicki, 2006**) and likewise could also be binding to worms.

The gene regulating the production of cholecystokinin (CCK) was also up-regulated in vaccinated sheep. This protein is involved in the production of digestive enzymes and in the peristaltism of the gastrointestinal tract, responsible for inducing satiety (**El-Salhy, Gundersen, Gilja, Hatlebakk, & Hausken, 2014; Wan, Coleman, & Travagli, 2007**). This could represent a negative result associated with the vaccination, since, if vaccinated animals experience the feeling of satiety, they could eat less. It has been previously reported that sheep infected with *Trichostrongylus colubriformis* suffer from anorexia associated with increased levels of CCK, reducing reticular and duodenal motility (**Grovum, 1981**). However, there are multiple studies that show CCK, along with other neuroendocrine molecules, interacts with the immune system (**Alverdy, Stern, Poticha, Baunoch, & Adrian, 1997**). It has been demonstrated in *in vitro* studies that they induce cytokine release (**Cunningham *et al.*, 1995; Ferrara, McMillen, Schaefer, Zucker, & Modlin, 1990**), probably due to the existence of CCK receptors on lymphocytes (**Donabedian, Odum, Soljnard, Jackobsen, & Rehfeld, 1989; Sacerdote, Wiedermann, Wahl, Pert, & Ruff, 1991**), so they could have an important role in the mucosal immunity (**Alverdy *et al.*, 1997**). Here, in addition, it is possible that this protein could be especially relevant in stimulating IgA production (**Freier, Eran, & Faber, 1987**) and it has been documented how some GIN such as *Trichinella spiralis* and *Nippostrongylus braziliensis* can modify CCK activity increasing digestive tract motility (**Gay, Fioramonti, Garcia-Villar, & Buéno, 2001; Serna, Porras, & Vergara, 2006; Torrents, Torres, De Mora, & Vergara, 2002**) in a mechanism where mucosal mast cells could be involved (**Serna *et al.*, 2006**). This is another example of the responses triggered by parasites in their host's system, having crossed functions between homeostasis and immunity (**Alverdy *et al.*, 1997**).

Among the down-regulated genes in vaccinated animals some were related to wool production, such as Dickkopf WNT signaling pathway inhibitor 1 (DKK1), fibroblast growth factor 20 (FGF20) and keratin 5 (KRT5) (**Kang *et al.*, 2013; Shaomei Li *et al.*, 2020; Mu *et al.*, 2017**). It should be considered that these genes were observed in abomasum, and they could not have an effect on other tissues, such as skin. Another down-regulated gene was solute carrier family 35 member F4 (SLC35F4), also associated with wool production affecting fleece length and involved in milk composition (**Goyache *et al.*, 2021**). There were other genes related to reproductive traits, such as IZUMO1 receptor, JUNO (IZUMO1R) and relaxin family peptide receptor 2 (RXFP2), implicated in fertilization and horn shape and size respectively (**Hu *et al.*, 2019**). These data suggest vaccine trials could be complemented with production studies in order to commercialize the prototype.

The other down-regulated genes were associated with innate immune responses, such as kelch like family member 14 (KLHL14) (**S. Li *et al.*, 2018**), F-box and leucine rich

repeat protein 21 (FBXL21P), solute carrier family 2 member 5 (SLC2A5), glycoprotein 2 (GP2) and phospholipid phosphatase 4 (PLPP4), along with genes related to cell signalling (secretoglobin family 2A member 2 -SCGB2A2-) and cell-cell recognition (glycoprotein A33 -GPA33-), cell adhesion molecules (leucine rich repeat containing 4 -LRRC4- and ADAM metallopeptidase domain 22 -ADAM22-), interferon gamma signalling (IRF6) or genes that encode receptors such as FCAMR (Fc fragment of IgA and IgM receptor) and FCRLA (Fc receptor like A), which may play a role in the immune response to microbes mediated by IgA and IgM and in B-cell differentiation respectively. Another gene, commonly known for having a role in suppressing nematode development, the galectin 1 (LGALS1) gene (**Takeuchi *et al.*, 2019**), was also downregulated in vaccinated group.

All these results highlight the complexity of immune responses against GIN. Either way, in both groups, animals were infected for more than 40 days, and it is foreseeable that both groups developed immune responses that would lead to parasite control. Although the differences observed in parasitology were only significant in worm length and eggs *in utero*, here, there were several genes related to their control. Based on the results of this study, the role of mast cell/GLs and mucus in protection induced by the vaccine against the parasite appears to be critical. Vaccine-induced protection is probably further favoured by enhanced antigen presentation and cell communication due to the vaccine effect, in addition to the expression of some genes which seem to have a direct effect on worms. Whilst IgA is probably a determinant in protection mechanisms, its role is slightly more contradictory after vaccination since there are also down-regulated genes associated with the immunoglobulin.

Future studies comparing CS and other breeds such as crossbred Texel, as well as carrying out samplings at different time points of the vaccine schedule and infection, could help to improve the identification of these mechanisms, hence refining the prototype vaccine. For instance, having a non-infected and non-vaccinated group would be desirable for comparing the gene expression, besides between vaccinated and control groups of different breeds and at different time points after infection.

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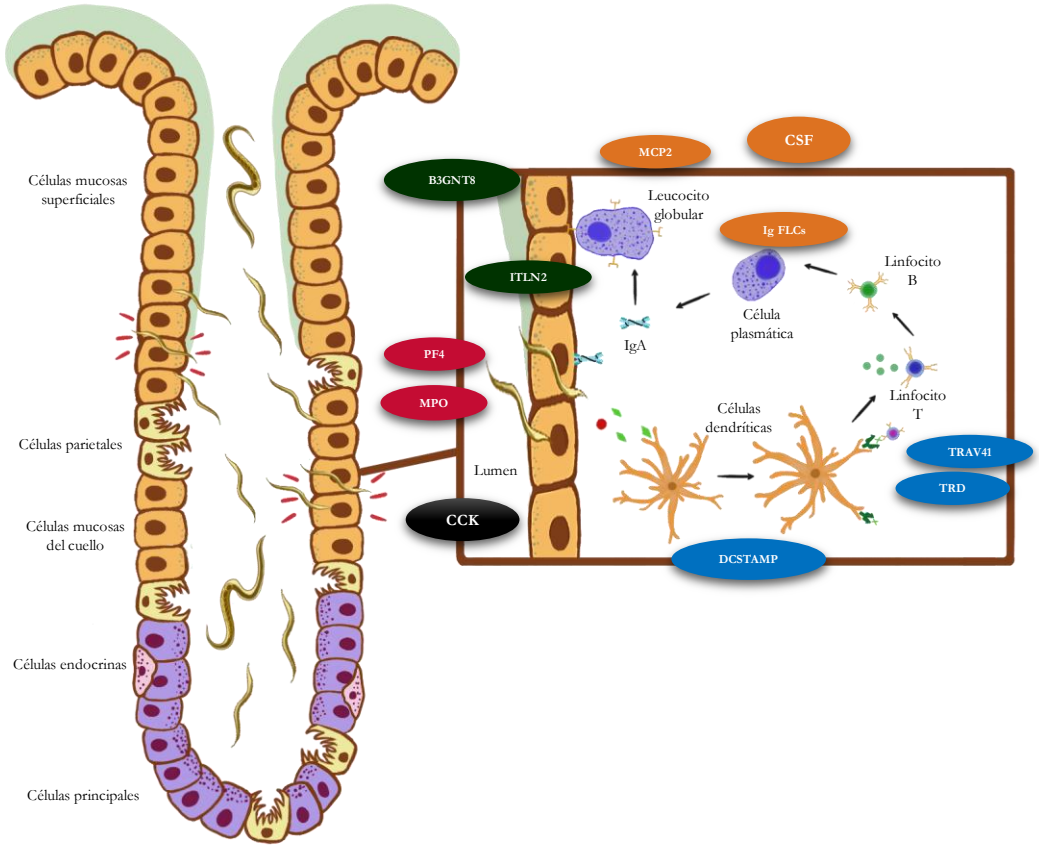
7. DISCUSIÓN GENERAL

Figura 7.1 Representación de los mecanismos de la respuesta inmune generada en los animales vacunados de la raza Canaria.



En esta figura se representan los principales mecanismos descritos a lo largo de la tesis que podrían estar asociados al efecto protector de la vacuna, reduciendo longitud y fecundidad del parásito *Teladorsagia circumcincta*, en corderos mayores de 6 meses de la raza Canaria. Los mecanismos de la respuesta inmune que podrían estar implicados son: las inmunoglobulinas A y G_2 específicas frente al parásito, los leucocitos globulares y un mayor ratio de $CD4^+/CD8^+$. Por otro lado, al estudiar la expresión génica se observaron genes relacionados con esos mecanismos, estando más expresados en los animales vacunados respecto a los controles.

Por tanto, para facilitar la interpretación de esta figura, los genes más destacados que se han asociado a la respuesta inmune inducida en los animales vacunados se han diferenciado en 5 colores, clasificándolos según su aportación a esta respuesta. Entre los genes más expresados encontramos en color azul a los que se han relacionado con una mejora en la captación y reconocimiento de los antígenos; TRAV41 y TRD podrían estar regulando la expresión de los receptores de las células T, y DCSTAMP activando a las células dendríticas. Por otro lado, los genes de color naranja, Ig FLCs, MCP2 y CSF, representan a los que se

han asociado con una mayor sensibilización y activación de mastocitos, célula que se asoció a la protección conferida por la vacuna. Respecto al aumento de la producción y viscosidad del mucus para impedir el avance de los nematodos, observamos dos genes en color verde (ITLN2 y B3GNT8). Además, en color magenta, también encontramos otros genes más expresados en los animales vacunados que podrían afectar de forma directa al parásito (PF4 y MPO). Y, por último, se ha observado que el gen CCK (color negro) podría tener diversas funciones, aumentando el peristaltismo, la liberación de citoquinas, estimulando la producción de IgA e, incluso, involucrando a los mastocitos/LG.

Debido al papel clave de los leucocitos globulares en esta respuesta, sería interesante estudiar los niveles de IgE específica frente al parásito. Además, cabe destacar que esta tesis analiza un punto concreto de la respuesta y convendría realizar estudios que permitan analizar cronológicamente los mecanismos que explican la protección conferida por la vacuna para mejorar el prototipo.

8. CONCLUSIONES

1. El prototipo vacunal recombinante frente a *Teladorsagia circumcincta* consiguió reducir significativamente la longitud y número de huevos en útero de los vermes en corderos de la raza ovina local Canaria experimentalmente infestadas de forma seriada con 2000 L3 de este parásito. Esto contrasta con lo observado en un cruce de la raza ovina Texel, donde la protección se asoció al control de la carga parasitaria y recuentos de huevos en heces.

2. Las inmunoglobulinas A y G_2 específicas frente a L4 de *Teladorsagia circumcincta*, los leucocitos globulares y un mayor ratio de $CD4^+/CD8^+$, parecen participar en los mecanismos protectores de este prototipo vacunal en la raza Canaria.

3. Las asociaciones negativas observadas entre las variables parasitológicas y la IgA específica frente a la proteína recombinante Tci-MEP-1, la IgG_1 frente a Tci-SAA-1 y Tci-ASP-1, y la IgG_2 frente a Tci-ASP-1 en la raza en la que la vacuna confirió protección (Canaria) sugieren que los antígenos vacunales Tci-MEP-1, Tci-SAA-1 y Tci-ASP-1 podrían estar implicados en la protección conferida por el prototipo vacunal.

4. La mayor expresión de genes vinculados con la respuesta inmune como los asociados a la activación de mastocitos, reconocimiento antigénico, producción de citoquinas y de inmunoglobulinas, así como genes que parecen tener una acción directa sobre nematodos observada en los animales del grupo vacunado de la raza Canaria con respecto a los animales del grupo control sugiere, que esta mayor expresión génica está relacionada con el efecto protector de la vacuna.

5. Este efecto protector no se observó en la raza ovina Canaria de Pelo, probablemente debido a la resistencia racial inherente que poseen, aunque hubo reducciones no significativas en las variables parasitológicas tras la vacunación. Sin embargo, al comparar el grupo vacunado de esta raza con el control y vacunado de la Canaria, se observaron reducciones significativas en carga parasitaria, producción y excreción de huevos, lo que puede sugerir un efecto aditivo de la vacuna a la resistencia racial en la raza Canaria de Pelo.

6. La raza ovina Canaria de Pelo presentó vermes más cortos y menos prolíficos que la raza ovina Canaria tras haber sido sometidos a inoculaciones seriadas de 2000 L3 de este parásito, lo cual sugiere que la raza ovina Canaria de Pelo presenta una mayor resistencia frente a *Teladorsagia circumcincta*.

9. CONCLUSIONS

1. The prototype vaccine against *Teladorsagia circumcincta* significantly reduced worm length and number of eggs *in utero* in Canaria Sheep lambs that has been experimentally infected with trickle infections of 2000 L3 of this parasite. This differ from what has been previously observed in Texel crossbred, where protection was associated with the control of worm burden and faecal egg counts.

2. Specific immunoglobulins A and G against *Teladorsagia circumcincta* L4 stage, globule leukocytes and a higher ratio of $CD4^+/CD8^+$ may be involved in the protective mechanisms of this prototype vaccine in Canaria Sheep.

3. The negative associations between parasitological variables and specific IgA against the recombinant protein Tci-MEP-1, specific IgG_1 against Tci-SAA-1 and Tci-ASP-1, and specific IgG_2 against Tci-ASP-1 observed in Canaria Sheep, suggest the vaccine antigens Tci-MEP-1, Tci-SAA-1 and Tci-ASP-1 could be involved in the protection conferred by the prototype vaccine.

4. Most of the up-regulated genes observed in vaccinated CS lambs in comparison with CS control group were involved in the immune response, such as genes associated with mast cell activation, antigen recognition, cytokine and immunoglobulin production, as well as genes that seem to have a direct effect on nematodes. All these data suggest that these up-regulated genes could be related to the protection conferred by the vaccine.

5. This protection was not observed in Canaria Hair Breed, probably, due to their innate resistance of this breed against this parasite, although there were non-significant reductions in parasitological variables in immunized animals. However, comparing vaccinated group of this breed with vaccinated group of Canaria Sheep, significant reductions in worm burden, and egg production and excretion were observed, suggesting a possible additive effect of the vaccine to the breed resistance in Canaria Hair Breed lambs.

6. Canaria Hair Breed lambs harboured shorter and less prolific worms and lower faecal egg counts than Canaria Sheep after being experimentally trickle infected with 2000 L3 of this parasite, suggesting a higher resistance of Canaria Hair Breed against *Teladorsagia circumcincta* than Canaria Sheep.

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AGRADECIMIENTOS

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13. ANEXOS

Documentos adicionales del artículo científico:

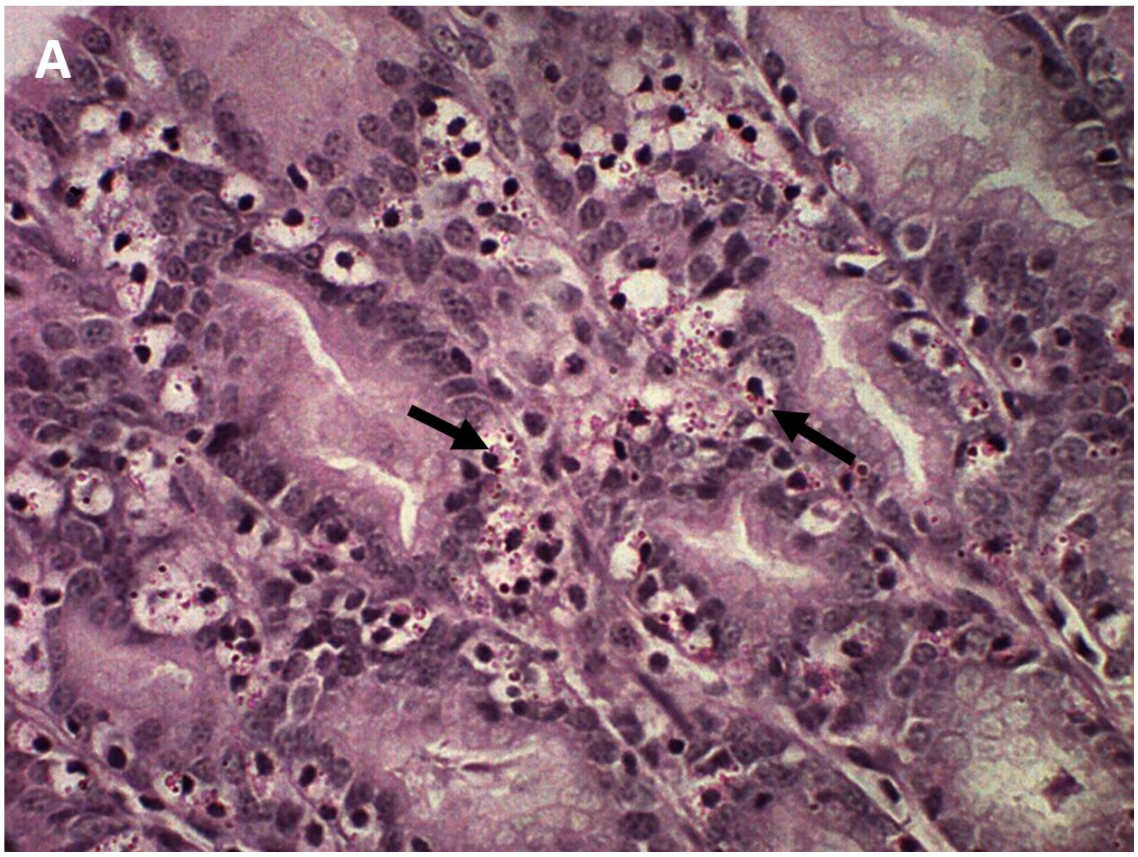
"Cellular and humoral immune responses associated with protection in sheep vaccinated against *Teladorsagia circumcincta*"

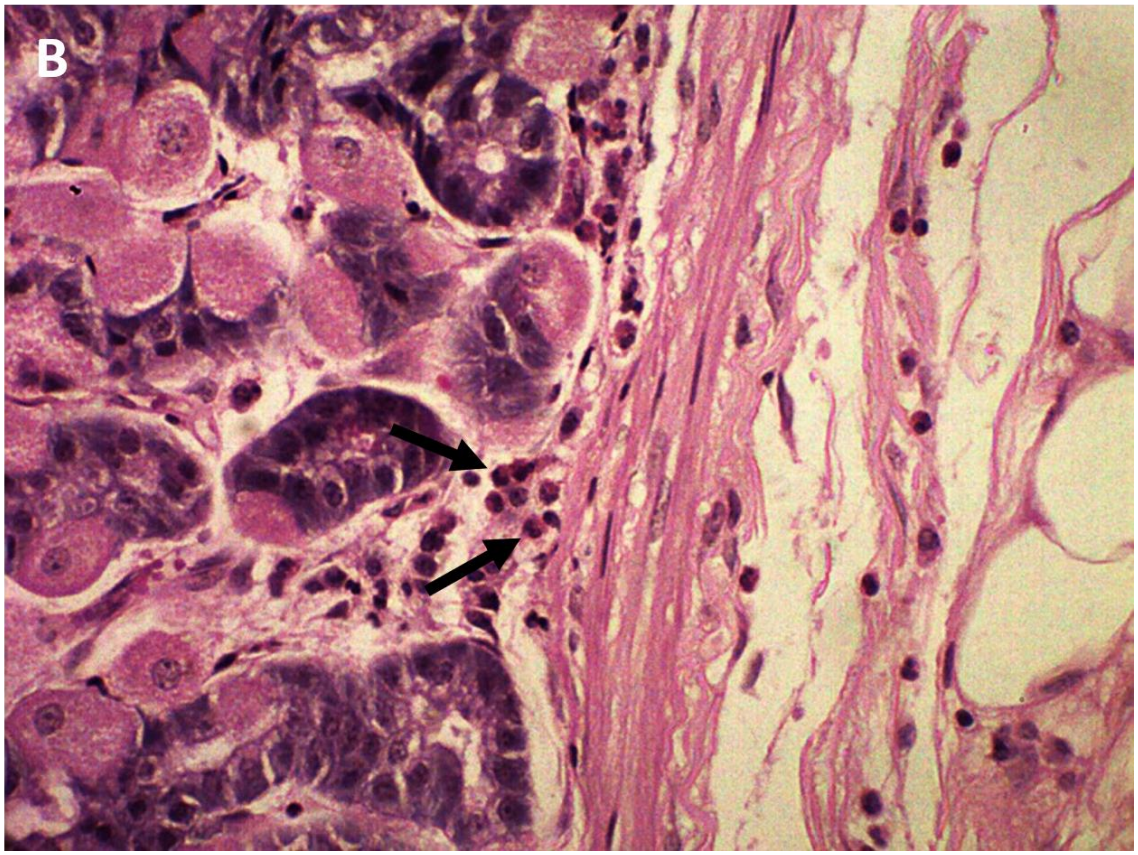
Additional file 1. Antibody clones used for flow cytometry analysis and immunohistochemistry.

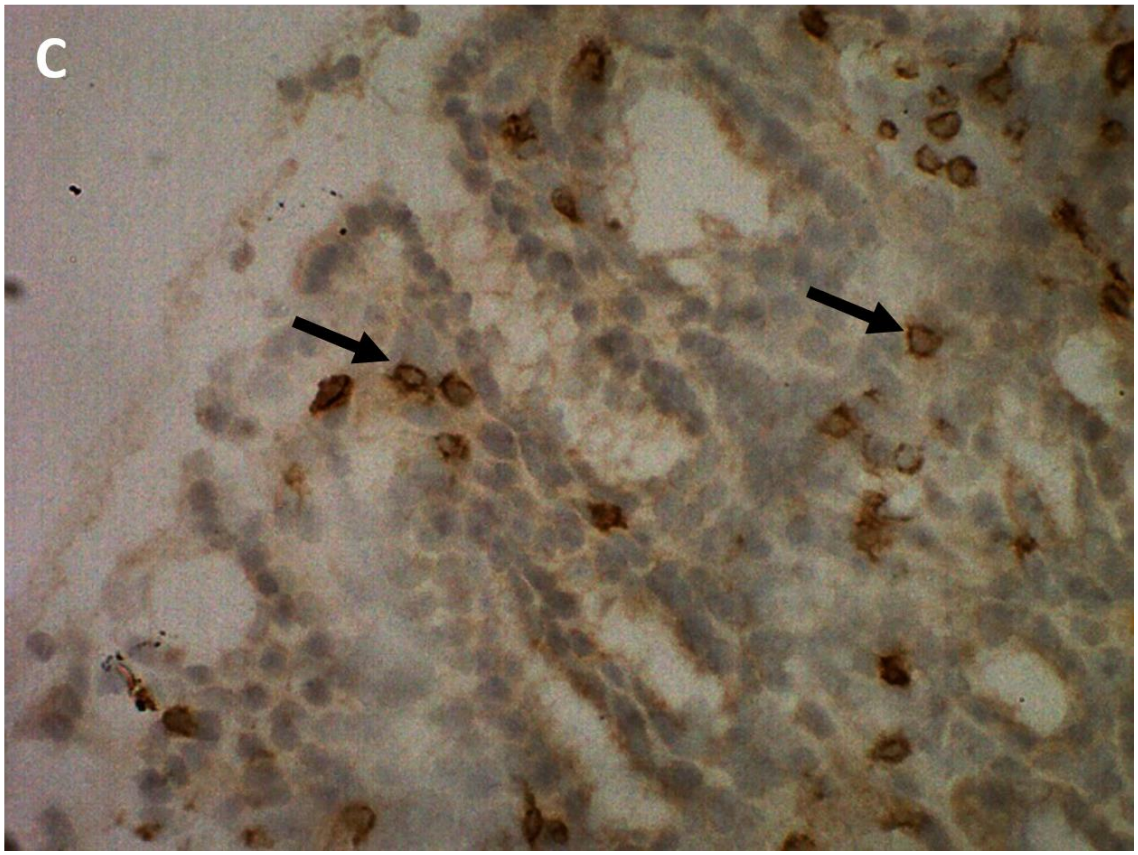
Antigen	Marker for	Antibody clone	Isotype	Reference
CD4	T helper cells	SBU T4 pool 44.38+44.97	IgG_2a+IgG_1	[33]
CD8	Cytotoxic T cells	SBU T8 pool 33-65	IgG_2a	[33]
NKp46	Natural killer cells	EC1.1	IgG_1	BioRad
WC1	$WC1^+$ γδ T cells	SBU T19 19.19*	IgG_2	[34]
γδTCR	γδ T	86D**	IgG_1	[34]
CD21	B cells	CC21	IgG_1	BioRad
CD14	Myeloid cells	TUK4	IgG_1	BioRad
MHCII	Antigen presentation	28.1	IgG_1	[35]
CD45RA	Naive T cells	SBU-P220	IgG_1	[15]
Galectin-14	Released by eosinophils	EL 1-2	IgG_1	[15]

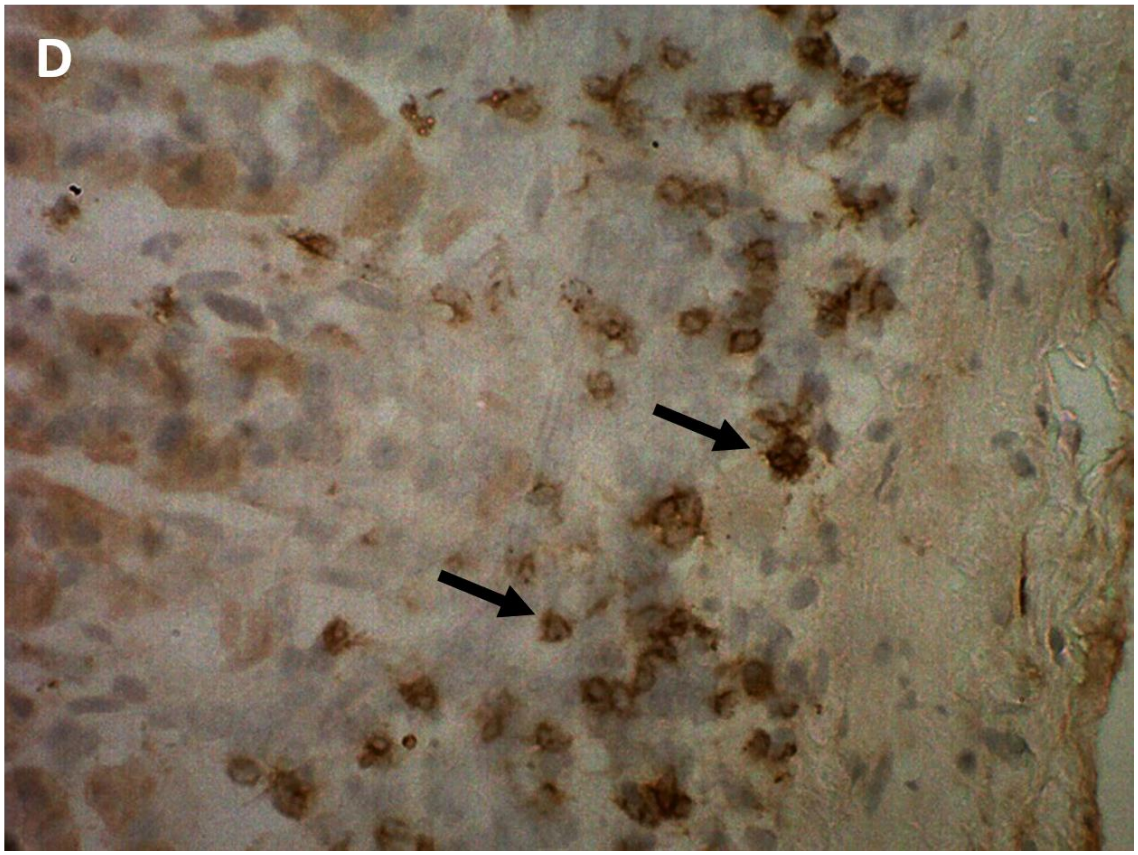
"*" Antibody clone used for flow cytometry assays. "**" Antibody clone used for IHQ.

Additional file 2. Examples of abomasal mucosa sections from Canaria Hair Breed and Canaria Sheep experimentally infected with *Teladorsagia circumcincta* showing positive cells (x400).




B


C



Haematoxylin and eosin staining for globule leukocyte in the upper layer (A) and eosinophils in the basal layer (B), both indicated with arrows. Immunohistochemical staining with CD4+ antibody (SBU T4 pool 44.38 + 44.97), showing CD4+ cells (arrows) in the apical (C) and basal area (D).

Additional file 3. IgA against vaccine proteins and their correlation with parasitology in Canaria Sheep lambs.

				Correlation			
Isotype	**Antigen**	**Group**	**Mean ± SEM**	**Cumulative FEC**	**Worm burden**	**Worm length**	**EIU**
IgA	**Tci-APY-1**	**CS Vac**	1.773 ± 0.109[a]	-0.231	-0.510	-0.392	-0.175
		CS Con	1.170 ± 0.036[b]	0.224	0.430	-0.006	0.079
	Tci-ASP-1	**CS Vac**	1.622 ± 0.123[a,c]	-0.203	-0.301	-0.329	-0.371
		CS Con	0.937 ± 0.037	-0.232	0.460	-0.260	0.027
	Tci-CF-1	**CS Vac**	1.452 ± 0.056[c]	0.049	-0.427	-0.056	-0.203
		CS Con	1.239 ± 0.068[b]	-0.335	0.255	-0.236	-0.518
	Tci-MEP-1	**CS Vac**	1.656 ± 0.058[a,c]	-0.364	-0.650*	-0.497	-0.706*
		CS Con	1.188 ± 0.045[b]	0.473	0.727*	0.309	0.109

Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 4. IgA against vaccine proteins and their correlation with parasitology in Canaria Hair Breed lambs.

				Correlation			
Isotype	**Antigen**	**Group**	**Mean ± SEM**	**Cumulative FEC**	**Worm burden**	**Worm length**	**EIU**
IgA	**Tci-APY-1**	**CHB Vac**	1.710 ± 0.111[a]	-0.282	-0.536	-0.552	-0.467
		CHB Con	1.154 ± 0.032[b,c]	0.364	0.673*	0.700*	0.336
	Tci-ASP-1	**CHB Vac**	1.581 ± 0.096[a]	-0.200	-0.473	-0.309	-0.576
		CHB Con	1.031 ± 0.026[b]	0.261	0.564	0.179	0.305
	Tci-CF-1	**CHB Vac**	1.778 ± 0.150[a]	0.132	0.027	-0.383	-0.024
		CHB Con	1.292 ± 0.098[c]	0.409	0.656*	0.196	-0.021
	Tci-MEP-1	**CHB Vac**	1.652 ± 0.071[a]	-0.073	-0.582	-0.321	-0.358
		CHB Con	1.246 ± 0.085[c]	0.109	0.399	0.385	-0.021

Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 5. IgG_1 against vaccine proteins and their correlation with parasitology in Canaria Sheep lambs.

				Correlation			
Isotype	**Antigen**	**Group**	**Mean ± SEM**	**Cumulative FEC**	**Worm burden**	**Worm length**	**EIU**
IgG_1	**Tci-APY-1**	**CS Vac**	$1.777 \pm 0.091^{a,g}$	-0.119	-0.238	-0.294	-0.350
		CS Con	1.008 ± 0.021^{b}	-0.382	0.264	-0.300	0.127
	Tci-ASP-1	**CS Vac**	$1.877 \pm 0.043^{a,c}$	-0.399	-0.196	-0.392	-0.636*
		CS Con	0.991 ± 0.004^{b}	-0.405	0.223	-0.087	0.219
	Tci-CF-1	**CS Vac**	2.193 ± 0.073^{d}	0.517	-0.217	0.147	-0.007
		CS Con	1.020 ± 0.012^{b}	-0.410	0.237	-0.410	-0.009
	Tci-ES20	**CS Vac**	1.854 ± 0.034^{a}	-0.091	0.133	0.224	0.070
		CS Con	1.009 ± 0.004^{b}	-0.427	0.173	-0.409	-0.064
	Tci-MEP-1	**CS Vac**	$2.008 \pm 0.012^{c,e}$	-0.399	-0.217	-0.294	-0.434
		CS Con	1.051 ± 0.017^{b}	0.591	0.927**	0.418	0.118
	Tci-MIF-1	**CS Vac**	1.691 ± 0.095^{g}	0.042	-0.224	0.280	-0.070
		CS Con	1.004 ± 0.002^{b}	0.119	0.027	0.237	0.023
	Tci-SAA-1	**CS Vac**	$2.104 \pm 0.086^{d,e}$	-0.552	-0.252	-0.699*	-0.608*
		CS Con	0.995 ± 0.002^{b}	0.018	-0.027	-0.391	-0.600
	Tci-TGH-2	**CS Vac**	$1.892 \pm 0.045^{a,c}$	-0.483	-0.308	-0.497	-0.510
		CS Con	0.992 ± 0.002^{b}	0.027	0.027	0.100	-0.018

Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 6. IgG_1 against vaccine proteins and their correlation with parasitology in Canaria Hair Breed lambs.

				Correlation			
Isotype	**Antigen**	**Group**	**Mean ± SEM**	**Cumulative FEC**	**Worm burden**	**Worm length**	**EIU**
IgG_1	**Tci-APY-1**	**CHB Vac**	1.747 ± 0.058[a]	0.309	0.000	-0.164	-0.224
		CHB Con	1.005 ± 0.008[b]	-0.382	-0.049	-0.455	-0.720**
	Tci-ASP-1	**CHB Vac**	1.683 ± 0.067[a]	0.155	-0.036	-0.285	-0.358
		CHB Con	1.017 ± 0.013[b]	0.494	0.126	0.308	0.406
	Tci-CF-1	**CHB Vac**	1.753 ± 0.042[a]	0.782**	0.409	-0.224	0.442
		CHB Con	1.001 ± 0.001[b]	-0.007	0.102	-0.067	-0.423
	Tci-ES20	**CHB Vac**	1.752 ± 0.043[a]	0.000	-0.118	-0.333	0.200
		CHB Con	1.004 ± 0.001[b]	-0.058	0.028	-0.267	-0.102
	Tci-MEP-1	**CHB Vac**	1.774 ± 0.049[a]	0.555	0.382	-0.285	0.042
		CHB Con	1.029 ± 0.017[b]	0.154	0.287	0.063	-0.021
	Tci-MIF-1	**CHB Vac**	1.441 ± 0.108[c]	0.118	-0.009	-0.261	-0.103
		CHB Con	1.028 ± 0.008[b]	0.056	0.336	0.545	0.182
	Tci-SAA-1	**CHB Vac**	1.478 ± 0.089[c]	-0.191	-0.118	-0.152	-0.612
		CHB Con	0.999 ± 0.001[b]	0.182	0.280	-0.119	-0.098
	Tci-TGH-2	**CHB Vac**	1.712 ± 0.069[a]	-0.009	-0.055	-0.358	-0.527
		CHB Con	0.996 ± 0.001[b]	0.102	0.359	0.366	0.380

Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "**" at $p < 0.01$.

Additional file 7. IgG_2 against vaccine proteins and their correlation with parasitology in Canaria Sheep lambs.

				Correlation			
Isotype	**Antigen**	**Group**	**Mean ± SEM**	**Cumulative FEC**	**Worm burden**	**Worm length**	**EIU**
IgG_2	**Tci-APY-1**	**CS Vac**	1.755 ± 0.081[a]	-0.021	-0.042	-0.189	-0.056
		CS Con	1.036 ± 0.013[b,e]	0.245	0.191	0.427	0.118
	Tci-ASP-1	**CS Vac**	2.004 ± 0.049[c]	-0.399	-0.329	-0.392	-0.587*
		CS Con	0.995 ± 0.003[b,e]	-0.100	-0.073	-0.182	0.509
	Tci-CF-1	**CS Vac**	1.780 ± 0.069[a,d]	0.336	-0.182	-0.070	-0.203
		CS Con	1.078 ± 0.039[b]	-0.342	0.415	-0.260	-0.027
	Tci-ES20	**CS Vac**	1.359 ± 0.062[f]	-0.476	-0.294	0.000	-0.147
		CS Con	1.027 ± 0.017[b,e]	-0.845**	-0.427	-0.573	-0.091
	Tci-MEP-1	**CS Vac**	1.796 ± 0.120[a,d]	-0.147	-0.203	-0.301	-0.105
		CS Con	0.970 ± 0.008[e]	0.141	0.059	-0.260	-0.273
	Tci-MIF-1	**CS Vac**	1.343 ± 0.074[f]	-0.266	-0.308	0.077	-0.077
		CS Con	1.005 ± 0.008[b,e]	-0.382	0.036	-0.382	0.100
	Tci-SAA-1	**CS Vac**	1.952 ± 0.097[c,d]	-0.427	-0.364	-0.545	-0.336
		CS Con	1.007 ± 0.006[b,e]	-0.464	-0.200	-0.527	0.145
	Tci-TGH-2	**CS Vac**	1.567 ± 0.065	-0.098	-0.077	-0.203	-0.098
		CS Con	0.991 ± 0.003[b,e]	-0.227	0.009	-0.100	0.464

Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 8. IgG_2 against vaccine proteins and their correlation with parasitology in Canaria Hair Breed lambs.

				Correlation			
Isotype	**Antigen**	**Group**	**Mean ± SEM**	**Cumulative FEC**	**Worm burden**	**Worm length**	**EIU**
IgG_2	**Tci-APY-1**	**CHB Vac**	$1.733 \pm 0.050^{a,c}$	-0.191	-0.364	-0.200	-0.212
		CHB Con	0.989 ± 0.012^{b}	0.060	-0.063	0.000	0.140
	Tci-ASP-1	**CHB Vac**	1.701 ± 0.059^{a}	0.164	-0.118	-0.067	-0.164
		CHB Con	1.004 ± 0.001^{b}	0.067	0.112	0.028	-0.021
	Tci-CF-1	**CHB Vac**	$1.844 \pm 0.048^{c,d}$	0.791**	0.409	-0.103	0.600
		CHB Con	1.048 ± 0.014^{b}	-0.063	-0.098	-0.056	-0.280
	Tci-ES20	**CHB Vac**	$1.754 \pm 0.048^{a,c}$	0.555	0.227	-0.503	0.212
		CHB Con	1.019 ± 0.004^{b}	-0.388	-0.081	-0.312	-0.632*
	Tci-MEP-1	**CHB Vac**	1.891 ± 0.046^{d}	-0.100	-0.209	-0.467	-0.600
		CHB Con	1.036 ± 0.009^{b}	-0.301	0.021	-0.287	-0.406
	Tci-MIF-1	**CHB Vac**	1.569 ± 0.078	-0.282	-0.191	-0.212	-0.030
		CHB Con	1.022 ± 0.005^{b}	-0.312	-0.084	-0.242	-0.389
	Tci-SAA-1	**CHB Vac**	1.692 ± 0.067^{a}	0.264	0.282	0.321	-0.006
		CHB Con	1.016 ± 0.002^{b}	0.079	0.186	0.155	0.014
	Tci-TGH-2	**CHB Vac**	1.709 ± 0.061^{a}	0.327	0.009	-0.042	-0.091
		CHB Con	1.028 ± 0.005^{b}	0.187	-0.109	0.282	0.278

Associations are expressed as Spearman's correlation coefficient. Same letters mean no significant differences between groups. Significant correlations are represented with "*" at $p < 0.05$ and "**" at $p < 0.01$.

Additional file 9. Correlations between cells and parasitological variables in Canaria Sheep lambs.

Cell	Group	Cumulative FEC	Worm burden	Worm length	EIU
Eosinophils	**CS Vac**	-0.056	0.273	0.021	0.021
	CS Con	0.018	-0.236	-0.291	-0.555
Mast cells	**CS Vac**	-0.406	-0.378	-0.224	-0.308
	CS Con	0.045	0.609*	-0.082	0.045
CD4$^+$	**CS Vac**	0.392	-0.105	0.245	0.140
	CS Con	-0.273	0.139	0.067	0.527
CD8$^+$	**CS Vac**	-0.119	-0.007	-0.217	-0.238
	CS Con	-0.473	-0.200	-0.709*	-0.491
γδ$^+$	**CS Vac**	0.175	0.469	0.084	0.112
	CS Con	-0.300	-0.409	-0.300	0.009
CD45RA$^+$	**CS Vac**	0.126	0.119	0.098	0.000
	CS Con	-0.218	-0.127	-0.109	0.300
MHCII$^+$	**CS Vac**	-0.014	0.336	0.126	-0.056
	CS Con	0.036	0.327	0.218	0.418
Galectin-14$^+$	**CS Vac**	-0.119	-0.056	-0.259	-0.483
	CS Con	0.018	-0.250	-0.152	-0.171

Associations are expressed as Spearman's correlation coefficient. Significant correlations are represented with "*" at $p < 0.05$.

Additional file 10. Correlations between cells and parasitological variables in Canaria Hair Breed lambs.

Cell	Group	Cumulative FEC	Worm burden	Worm length	EIU
Eosinophils	**CHB Vac**	-0.527	-0.436	-0.067	-0.236
	CHB Con	-0.347	-0.524	-0.056	-0.203
Mast cells	**CHB Vac**	-0.564	-0.255	-0.406	-0.382
	CHB Con	-0.403	-0.517	0.070	-0.336
CD4$^+$	**CHB Vac**	0.345	-0.042	0.167	0.250
	CHB Con	-0.056	-0.196	-0.119	-0.007
CD8$^+$	**CHB Vac**	-0.427	-0.145	0.333	-0.103
	CHB Con	0.200	0.049	-0.098	0.434
γδ$^+$	**CHB Vac**	-0.418	-0.427	0.079	-0.406
	CHB Con	-0.357	-0.559	-0.182	-0.357
CD45RA$^+$	**CHB Vac**	0.009	-0.455	0.321	0.248
	CHB Con	0.056	0.217	0.126	0.287
MHCII$^+$	**CHB Vac**	0.209	0.173	0.515	0.333
	CHB Con	0.441	0.476	0.490	0.524
Galectin-14$^+$	**CHB Vac**	-0.005	-0.424	-0.608	-0.462
	CHB Con	-0.438	-0.503	-0.266	-0.203

Associations are expressed as Spearman’s correlation coefficient.

Additional file 11: Interleukin-17A secretion by abomasal lymph node lymphocytes following stimulation with *Teladorsagia circumcincta* L4 or adult somatic antigen from Canaria Hair Breed and Canaria Sheep vaccinated with a prototype recombinant sub-unit *T. circumcincta* vaccine and subsequently challenged with *T. circumcincta*.

Group	Animal number	Media only	ConA	L4	Adult
CS Vac	93327	N/D	45,2416451	N/D	N/D
CS Vac	93331	N/S	N/S	N/S	N/S
CS Vac	93333	N/D	76,2874025	N/D	N/D
CS Vac	93335	N/D	N/D	N/D	N/D
CS Vac	93336	N/D	85,1242285	N/D	N/D
CS Vac	93338	N/D	74,2732136	N/D	N/D
CS Vac	93340	N/D	N/D	N/D	N/D
CS Vac	93341	N/D	180,065352	N/D	N/D
CS Vac	93342	N/D	58,6632501	N/D	N/D
CS Vac	93344	N/D	N/D	N/D	N/D
CS Vac	93347	N/D	27,5775765	N/D	N/D
CS Vac	93348	38,5814849	28,170608	N/D	N/D
CS Con	93324	N/D	52,0909778	N/D	N/D
CS Con	93325	N/S	N/S	N/S	N/S
CS Con	93326	4,28015129	286,770137	N/D	55,6419668
CS Con	93328	N/D	N/D	N/D	N/D
CS Con	93329	0,25177361	280,72757	N/D	N/D
CS Con	93330	N/S	N/S	N/S	N/S
CS Con	93332	N/D	N/D	N/D	N/D
CS Con	93334	N/D	20,0072614	N/D	N/D
CS Con	93337	6,79788735	36,0036256	N/D	N/D
CS Con	93339	N/D	N/D	N/D	N/D
CS Con	93343	N/D	197,36893	N/D	N/D
CS Con	93346	251,698258	4,89923617	N/D	N/D
CHB Vac	93352	N/D	169,611108	N/D	N/D
CHB Vac	93353	N/S	N/S	N/S	N/S
CHB Vac	93356	N/D	860,31041	N/D	N/D
CHB Vac	93359	N/D	N/D	N/D	N/D
CHB Vac	93362	N/S	N/S	N/S	N/S
CHB Vac	93365	N/D	4,7836985	3,27305687	N/D

CHB Vac	93368	N/D	38,6602812	N/D	N/D
CHB Vac	93369	N/D	43,0786979	N/D	N/D
CHB Vac	93370	N/D	215,266433	N/D	N/D
CHB Vac	93372	N/D	N/D	N/D	N/D
CHB Vac	93373	N/D	106,164657	N/D	N/D
CHB Con	93349	N/D	22,8911909	N/D	N/D
CHB Con	93350	N/D	86,3490375	N/D	N/D
CHB Con	93351	N/D	60,1738917	N/D	N/D
CHB Con	93354	N/D	53,7472202	N/D	N/D
CHB Con	93355	N/S	N/S	N/S	N/S
CHB Con	93357	N/D	64,7058166	N/D	N/D
CHB Con	93358	N/D	N/D	N/D	N/D
CHB Con	93361	N/D	38,0318212	N/D	N/D
CHB Con	93363	N/D	125,131482	N/D	N/D
CHB Con	93364	N/D	24,5162759	N/D	N/D
CHB Con	93367	N/D	62,5452225	N/D	N/D
CHB Con	93371	N/D	118,194073	N/D	N/D

IL-17A secretion was examined in supernatants collected 4 days post-stimulation of abomasal lymph node lymphocytes with 5 µg/mL of T. circumcincta L4 or adult somatic antigen. In general, antigen-specific IL-17A release was not detected in any of the ALN cultures. N/D IL-17A were not detected, N/S no sampled availability.