TITULO: *INTERACCIÓN PARÁSITO-HOSPEDADOR EN FASCIOLOSIS OVINA: MECANISMOS DE RESPUESTA EN FASES TEMPRANAS Y TARDÍAS*

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TÍTULO DE LA TESIS: INTERACCIÓN PARÁSITO-HOSPEDADOR EN FASCIOLOSIS OVINA: MECANISMOS DE RESPUESTA EN FASES TEMPRANAS Y TARDÍAS.

DOCTORANDO/A: RAÚL PÉREZ CABALLERO

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El estudio realizado por el doctorando Raúl Pérez Caballero para la realización de su tesis doctoral, ha tenido como objeto el analizar la respuesta inmunitaria del hospedador ovino durante las fases iniciales y tardías de la infección por *Fasciola hepatica*, considerando tanto la respuesta innata y como la adaptativa, e identificando los posibles mecanismos efectores, reguladores e inmunopatológicos, inducidas por el parásito o resultantes de mecanismos patogénicos. Se ha llevado a cabo en el marco de los proyectos de investigación desarrollados en el grupo AGR-133: AGL2015-67023-C2-1-R-INTERFAS (Interacción parásito-hospedador en la fasciolosis: bases moleculares y celulares para el desarrollo de vacunas), FP7-PARAVAC-KBBE-2010-4-265862 (Vaccines against helminth infection), H2020-SFS-2014-2-635408-PARAGONE (Vaccines for animal parasites), todos ellos enfocados al desarrollo de vacunas frente a parásitos.

Para su desarrollo se realizaron varias infecciones experimentales con *F. hepatica* en ovinos, todas ellas con el mismo protocolo: lotes de 5-10 animales, infecciones vía oral con 150 metacercarias, seguimiento clínico, parasitológico e inmunológico, sacrificios seriados. Se abordó un primer ensayo de inmunización con la molécula rFh14-3-3z, que permitió realizar un análisis global de las respuestas inmunitarias y de los mecanismos adaptativos humorales y celulares. Un segundo ensayo se centró tanto en fases tempranas con sacrificios seriados de los animales a los días 1, 3, 9 y 18 post-infección, como en fases tardías, a las 14 semanas post-infección y tercer ensayo incluyó el estudio de fases tempranas siguiendo el mismo protocolo.

En estos ensayos se realizaron análisis patológicos, parasitológicos y fundamentalmente inmunológicos, a partir de muestras de sangre y de líquido peritoneal, para estudiar las poblaciones celulares de respuesta innata (macrófagos, células dendríticas y células presentadoras de antígeno) y de respuesta adquirida (linfocitos T CD4, CD8, $WC1^{+}\gamma\delta$), así como anticuerpos específicos IgG1 e IgG2.

Los resultados han puesto de manifiesto el predomino de respuestas Th2 no protectoras en los ensayos realizados; la existencia de una importante activación de las poblaciones leucocitarias durante la fase peritoneal, con modificaciones en su capacidad oxidativa; y las modificaciones de las poblaciones leucocitarias peritoneales durante las fases iniciales y tardías de la infección. Estos resultados se han publicado en tres artículos científicos, que son los que constituyen el cuerpo de la tesis doctoral, todos ellos en el primer cuartil (Q1), tanto del área de Ciencias Veterinarias como del área de Parasitología del JCR:

> Pérez-Caballero, R., Siles-Lucas, M., González-Miguel, J., Martínez-Moreno, F.J., Escamilla, A., Pérez, J., Martínez-Moreno, A., Buffoni-Perazzo, L., 2018. Pathological, immunological and parasitological study of sheep vaccinated with the recombinant protein 14-3-3z and experimentally infected with *Fasciola hepatica*. **Vet. Immunol. Immunopathol**. 202, 115–121. (10.1016/j.vetimm.2018.07.006).

Perez-Caballero, R., Martínez-Moreno, F.J., Zafra, R., Molina-Hernández, V., Pacheco, I., Ruiz-Campillo, M.T., Escamilla, A., Pérez, J., Martínez-Moreno, A., Buffoni, L., 2018. Comparative dynamics of peritoneal cell immunophenotypes in sheep during the early and late stages of the infection with *Fasciola hepatica* by flow cytometric analysis. **Parasites & Vectors**. 11:640 (doi.org/10.1186/s13071-018-3250-5).

Pérez-Caballero, R., Buffoni, L., Martínez-Moreno, F.J., Zafra, R., Molina-Hernández, V., 2018. Expression of free radicals by peritoneal cells of sheep during the early stages of *Fasciola hepatica* infection. **Parasites & Vectors**. 11:500. (doi.org/10.1186/s13071-018-3072-5).

Además de estas publicaciones, el doctorando, en el desarrollo de su proyecto de investigación, ha presentado resultados de su trabajo como comunicaciones orales y poster en varios Congresos Internacionales y ha participado en la publicación de otros artículos.

También cabe reseñar que, como parte de su plan formativo e investigador, el doctorando ha realizado estancias de formación en el University College de Dublín, bajo la dirección de la Dra. Grace Mulcahy y en el Moredun Research Institute (Edimburgo), con el Dr. Tom McNeilly, e igualmente ha participado en varios Workshops organizados en el proyecto PARAGONE.

Por todo ello se considera que el estudio conducido por el doctorando Raúl Pérez Caballero, reúne todos los requisitos necesarios para ser presentado y defendido ante el tribunal para optar al Grado de Doctor en Veterinaria por la Universidad de Córdoba y **se autoriza la presentación de la tesis doctoral**.

Córdoba, 8 de noviembre de 2018

Firma de los directores

Fdo.: Leandro Buffoni Perazzo Fdo.: Álvaro Martínez Moreno

ÍNDICE

AGRADECIMIENTOS

Después de tan largo recorrido y un laborioso e intenso trabajo a mis espaldas no puedo comenzar de otro modo que con las líneas que suponen la parte más personal y emotiva de este libro, agradeciendo a todas aquellas personas que se subieron a este tren y compartieron conmigo esta aventura.

Como no podría ser de otra forma y en primerísimo lugar, a vosotros, Rafa y Marisol, mis dos grandes pilares en todo este camino llamado vida. Cuando tienes la inmensa fortuna de que la vida te regale unos maravillosos padres como los que a mí me otorgó cuesta imaginar que un trabajo tan bien hecho como el que ellos han realizado pueda repetirse. A pesar de que al principio de la andanza se cuestione, como todo padre supongo, si se va a poder hacer de la manera en que se desea, doy fe y garantizo que lo vuestro ha sido *Cum Laudem*. Por todo vuestro esfuerzo, por todo vuestro cariño, por vuestro tesón, por vuestra dedicación, por vuestros ánimos y por un infinito etcétera os devuelvo un cachito de esa parte con el fin de este ciclo. Todo este recorrido ha sido de los tres, hemos vivido lo positivo y lo negativo, y ahora toca cerrar el círculo y abrir otro nuevo.

Hay veces que el universo te brinda la suerte de contar con otras dos personas a las que, con orgullo, puedes considerar tus segundos padres y no puedes dejar de agradecerles, de la misma manera, el

esfuerzo y atención que te ofrecieron durante tantos años. Por eso, vosotros, Conchi y Antonio, os merecéis tener un preciado hueco en estas páginas.

Si desde pequeño tienes una ilusión y un objetivo tan claro como el que tuve yo con esta preciosa carrera nunca sabes qué personas van a estar al final del camino para verte alcanzarlo. A pesar de que en mi caso sólo cuento con un As de la baraja, mi abuela Antonia, que ha podido estar presente hasta final de la partida, sé que, en algún lugar en lo más alto de aquel Reino, las otras tres grandes estrellas: Francisco, José y Dolores, estarán orgullosos de lo que están viendo.

Y ahora te toca a ti, Flora, mi pequeña flor del desierto. No podías faltar. Aunque no has podido participar en toda la martón, sí has compartido quizás el relevo más duro pero a la misma vez ansiado del final de toda esta etapa. Gracias por ser mi confidente, por todos esos momentos y por tu aguante y tenacidad. Gracias por ser la guinda del pastel de esta travesía.

Quisiera agradecer a mi director de Tesis, Álvaro Martínez Moreno, por haberme dado la oportunidad de comenzar esta gran carrera de fondo: la investigación. No sólo por acogerme en tu departamento, sino por haber sido esa clase de director que siempre tiene un sí para

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Y a ti, Belén Huerta, gracias por haber sido la primera persona en ofrecerme la llave a este mundo investigador y por la confianza depositada en mí cuando aún quedaban unos escasos dos años para terminar mi carrera. Y a pesar de que no pudimos trabajar juntos, nunca olvidaré ese detalle y quizás, en un futuro, podamos aunar fuerzas en un laboratorio.

Una de las mayores satisfacciones que me ha dado este viaje ha sido poder conocer a tantísima gente que ha aportado su enorme granito de arena en todos los niveles. Por ello merecen su reconocimiento en estas líneas, por ser mis mentores internacionales.

En primer lugar, agradecer a la Dra. Christina Strube, del Institut für Parasitologie de Hanóver, haberme abierto las puertas de su casa parasitológica y contribuido a crear la primera de muchas magníficas experiencias en estos años. Y por haber invertido su tiempo y dedicación, no sólo en el laboratorio sino por haberme hecho sentir uno más entre compañeros y amigos, muchas gracias a Debby Joeckel.

La aventura continúo en Dublín, en el University College of Dublin y Conway Institute. Agradecer enormemente a la Dra. Grace Mulcahy por toda predisposición y ayuda concedida durante esos meses. Su calidad como persona va mucho más allá de su grandeza profesional. Quisiera hacer una doble mención especial a dos personas que fueron mi mano derecha y un inmenso apoyo. A ti, Laura Garza, porque honestamente sin tu ayuda y esfuerzo, que mucho de tu tiempo te robé, nada de lo productivo que fueron esas estancias hubiera sido posible. Gracias por hacerme sentir un miembro más de ese grupo dublinés.

Y a mi gran Maestro citometrista, Alfonso Blanco. Por tu grandísima ayuda, sabiduría y por esos ratos de citómetro y risas, no puedo menos que agradecerte absolutamente todo lo que hiciste por mí.

A vosotros, mis compañeros del Moredun Research Institute (Edimburgo), gracias por todo. Al Dr. Tom McNeilly, por su doble sí y haberme dado la oportunidad de compartir esa experiencia en un lugar tan prestigioso. Y a Yolanda Corripio-Miyar, porque a pesar de haber sido en muchos momentos una tortura para ti, siempre tuviste un segundo de tu tiempo para dedicarme y ofrecerme ayuda, conocimiento y brindarme un lugar en el que sentirme como en casa. Mil gracias a ambos.

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"De que me sirve saber geometría para dividir el campo si no puedo compartirlo con mi hermano".

Lucio Séneca.

"Aquel que tiene un porqué para vivir se puede enfrentar a todos los cómos".

F. W. Nietzsche.

ABREVIATURAS

ADN/DNA: ácido desoxirribunocleico (*deoxyribonucleic acid*)

APC: células presentadoras de antígeno (*antigen presenting cells*)

Arg-1: arginasa 1

BMGY: medio de glicerol (*Buffered Glycerol-complex Medium*)

Bp: pares de bases (*base pairs*)

BSA: albúmina sérica bovina (*Bovine Serum Albumin*)

CAT: catalasa

CD: cúmulo de diferenciación (*Cluster of Differentiation*)

CL1: catepsina L1

DAF-2DA: diacetato de 4,5-diaminofluoresceína (4,5-*diaminofluorescein diacetate*)

DC: células dendríticas (*Dendritic Cells*)

DCFH-DA: diacetato de dicloro-dihidro-fluoresceína (*Dichloro-dihydro-fluorescein diacetate*)

DPBS: búfer fosfatado salino de Dulbecco (*Dulbecco's Phosphate-Buffered Saline*)

dpi: días post-infección (*days post-infection*)

E. coli: *Escherichia coli*

ELISA: ensayo inmunoenzimático (*Enzyme-Linked ImmunoSorbent Assay*)

F. gigantica: *Fasciola gigantica*

F. hepatica: *Fasciola hepatica*

FABP: proteína de unión a ácidos grados (*Fatty Acid Binding Protein*)

FEC: contaje de huevos en heces (*Faecal Egg Count*)

FhES: productos de excreción-secreción de *F. hepatica*

FSC-SSC: dispersión frontal-dispersión lateral (*Forward Scatter-Side Scatter*)

g: gravedad (*gravity*)

GPx: glutatión peroxidasa

GST: glutatión S-transferasa

h: horas

H_2O_2: peróxido de hidrógeno

IFN-γ: interferón gamma (*Interferon Gamma*)

IgG1: inmunoglobulina G1

IgG2: imunoglobulina G2

IL-10: interleucina 10 (*Interleukine 10*)

IL-4: interleucina 4 (*Interleukine 4*)

iNOS: sintetasa inducible de óxido nítrico

ITT: indonesia de cola delgada (*Indonesian thin-tail*)

kDa: kilodalton

LAP: leucinaminopeptidasa

log: logaritmo en base 10

M: molar

MHCII: complejo mayor de histocompatibilidad clase II (*Major Histocompatibility Complex Clase II*)

min: minutos

ml: mililitro

mM: micromolar

mm: milímetros

MPX: peroxidasa mitocondrial (*Mitocondrial Peroxidase*)

MRLC: cadena liviana reguladora de miosina (*Myosin Regulatory Light Chain*)

NEJ: formas juveniles desenquistadas (*Newly Excysted Juveniles*)

nm: nanómetros

NO: óxido nítrico

OD: densidad óptica (*Optical Density*)

P. pastoris: *Pichia pastoris*

PBMC: células polimorfonucleares periféricas (*Peripheral Blood Mononuclear Cells*)

PBS: búfer fosfato salino (*Phosphate Buffer Saline*)

PC: cavidad peritoneal (*Peritoneal Cavity*)

PCP: poblaciones celulares peritoneales (*Peritoneal Cell Populations*)

PCR: reacción en cadena de la polimerasa (*Polymerase Chain Reaction*)

PD-L1: ligando 1 de muerte programada (*Programmed Death-Ligand 1*)

PGK: proteína fosfoglicerato quinasa (*Phosphoglycerate Kinase*)

pi: post-infección

pMΦ: macrófagos peritoneales (*peritoneal macrophages*)

Prx: peroxirredoxina

rFh: proteína recombinante de *F. hepatica*

RNS: especies de nitrógeno reactivo (*Reactive Nitrogen Species*)

ROS: especies de oxígeno reactivo (*Reactive Oxigen Species*)

rpm: revoluciones por minuto

SAP: saponina

Sb14ζ: proteína recombinante Sb14-3-3ζ de *Schistosoma bovis*

Sm14: antígeno recombinante de *Schistosoma mansoni*.

SOD: superóxido dismutasa

spi: semanas post-infección

spp.: especies

syn.: sinónimo/s (*synonym/s*)

TCBZ: triclabendazol

TCBZ-R: resistencia al triclabendazol

TcCPX: peroxidasa tryparedoxina citoplasmática

TGF-β: factor de crecimiento tumoral beta (*Tumoral Grow Factor Beta*)

TGR: tiorredoxina glutatión reductasa (*thioredoxin glutathione reductase*)

Th1: linfocitos T colaboradores 1 (*lymphocytes T helper 1*)

Th2: linfocitos T colaboradores 2 (*lymphocytes T helper 2*)

TMB: tretrametilbenzidina

TNF-α: factor de necrosis tumoral alfa (*Tumor Necrosis Factor Alfa*)

Treg: linfocitos T reguladores

UC: control negativo (*Uninfected Control*)

UI: unidades internacionales

UV: ultravioleta

WHO: Organización Mundial de la Salud (*World Health Organization*)

wpi: semanas post-infección (*weeks post-infection*)

µg: microgramos

µl: microlitros

ºC: grados centígrados

INTRODUCCIÓN Y OBJETIVOS

1. Introducción.

1.1. El parásito.

Fasciola hepatica (*F. hepatica*) es el agente etiológico de la fasciolosis, una enfermedad parasitaria que afecta a un amplio rango de mamíferos, entre los que se encuentran rumiantes, caballos, animales silvestres como ciervos, conejos y liebres, así como el ser humano. Los ejemplares adultos de *F. hepatica* se localizan a nivel hepático ocasionando severas alteraciones tanto a nivel orgánico como sistémico. La importancia de esta enfermedad radica en las importantes pérdidas económicas que ocasiona al sector ganadero estimadas en 3 billones € por año a nivel mundial (Mehmood y cols., 2017). Los costes económicos derivados de la misma se deben principalmente la disminución en la producción láctea, la composición de la canal y el incremento del tiempo necesario para alcanzar el peso oportuno para llegar a matadero (Charlier y cols., 2014; Howell y cols., 2015). El ser humano puede verse igualmente afectado por esta parasitosis, la cual ha sido descrita por la OMS como una zoonosis de carácter re-emergente en varias partes del mundo.

Este helminto es un trematodo hermafrodita perteneciente al phylum Platelmintos (Gegenbaur, 1859), clase Trematoda (Rudolphi, 1808), subclase Digenea, orden Equinostomida (LaRue, 1957), familia

Fasciolidae (Railliet, 1895), género *Fasciola* (Linnaeus, 1758), especie *F. hepatica*. Los adultos de esta especie (Figura 1) presentan una forma corporal foliácea y aplanada dorso-ventralmente, característica anatómica que le da nombre al género. Su forma adulta mide entre 25-35 mm de largo y 10 mm de ancho, es de color pardo-grisáceo, su tegumento está cubierto por un conjunto de espinas orientadas caudalmente, lo que le permite mantener la posición en el interior de los conductos biliares y erosionar el parénquima hepático y los vasos sanguíneos (Bennett, 1975). Presenta una ventosa oral, que se continúa con una faringe, esófago y los ciegos intestinales, y una ventosa ventral de mayor tamaño.

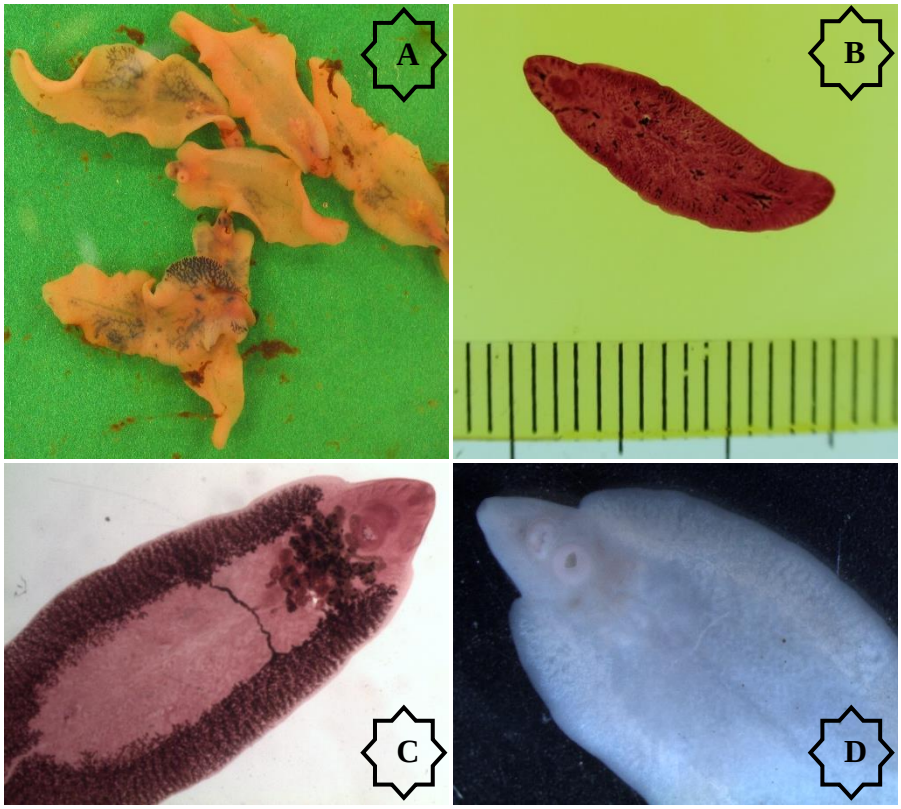

Fig. 1. Especímenes adultos de *Fasciola hepatica* (A, B), teñidos (B, C) y observados bajo la lupa (B-D).

1.2. Ciclo biológico.

Fasciola hepatica cuenta con un complejo ciclo de tipo indirecto (Figura 2) en el que intervienen moluscos gasterópodos del género *Galba* como hospedadores intermediarios.

El nicho ecológico de los ejemplares adultos lo constituyen los conductos biliares y la vesícula biliar de una amplia variedad de especies animales,

aunque se consideran más susceptibles los rumiantes, principalmente ovinos (Manga y cols., 1990; Konopka, 1993; Mitchell, 1995), caprinos (Khallaayoune y cols., 1991) y bovinos (González-Lanza y cols., 1989; Genicot y cols., 1991; Poglayen y cols., 1995).

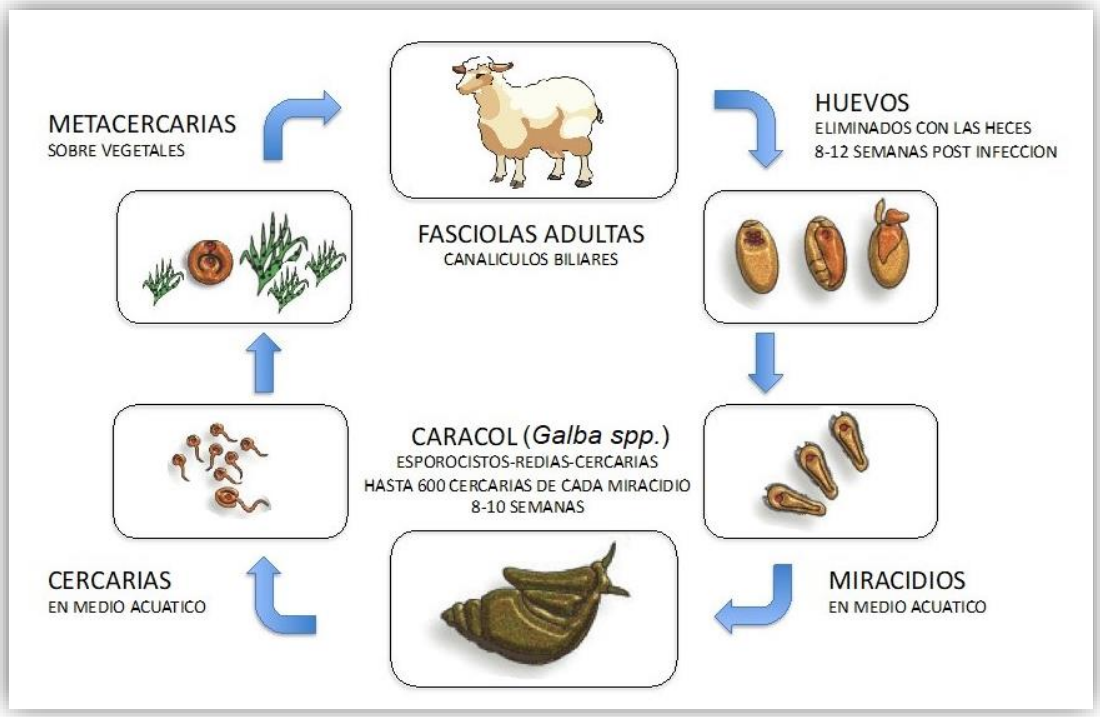

Fig. 2. Ciclo biológico de *Fasciola hepatica.*

El ciclo comienza cuando el parásito adulto elimina huevos no embrionados que alcanzan el intestino delgado a través del conducto colédoco y salen al medio ambiente con las heces del hospedador

definitivo. Los huevos (Figura 3) contienen un óvulo fertilizado rodeado de un gran número de yemas granulares. Son de color amarillo marronáceo u ocráceo, forma ovalada y con un tamaño de 130–145 μm de largo por 70–90 μm de ancho, y presentan un opérculo poco definido (Andrews, 1999).

Una vez en el medio ambiente, comienza en el interior del huevo el desarrollo de una larva móvil (Figura 3B), que presenta una cubierta ciliada, denominada miracidio. Esta fase del ciclo presenta una duración variable y depende significativamente de factores medioambientales, principalmente de la temperatura y humedad. En condiciones óptimas de humedad constante y 22-26ºC, el desarrollo larvario suele suceder en 9-10 días. Una vez se produce la eclosión y se libera el miracidio (Figura 3C), éste comienza a desplazarse activamente para así contactar un hospedador intermediario adecuado. Para ello, precisa de un medio ambiente con abundante humedad, siendo las aguas encharcadas y lodazales ambientes propicios para esto y donde se localiza habitualmente el caracol. Al ser una larva que no se alimenta, su supervivencia en el medio ambiente es limitada y depende de sus reservas energéticas, siendo aproximadamente de 24 horas.

El hospedador intermediario corresponde a un pequeño caracol pulmonado de 4-11 mm perteneciente al género *Galba* (syn. *Lymnaea*),

siendo *Galba truncatula* (Figura 3D) la especie mayoritariamente involucrada en la transmision de *F. hepatica* en Europa. Este pequeño molusco suele localizarse en zonas con escaso drenaje o pantanosas, lodazales, o a orillas de pequeños arroyos (Euzeby, 1971).

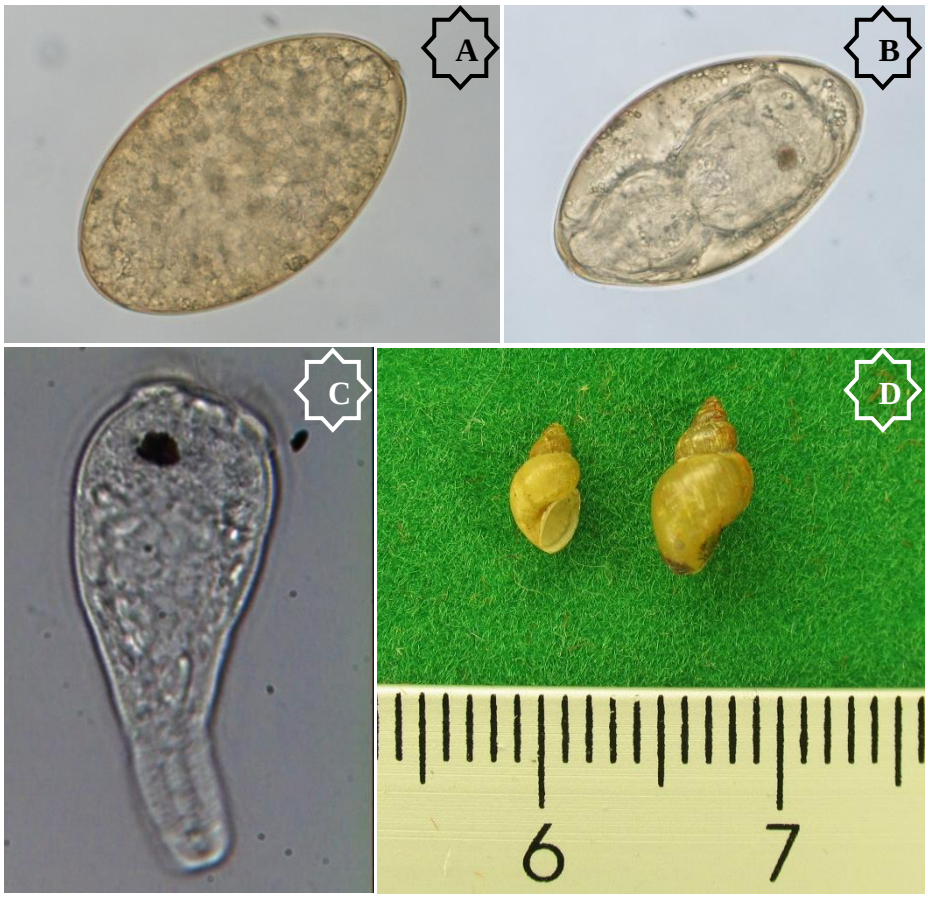

Fig. 3. Huevo no embrionado (A) y embrionado con el miracidio en su interior (B) de *Fasciola hepatica*. Miracio libre en el medio (C). Concha de dos ejemplares de *Galba truncatula*, el hospedador intermediario de *F. hepatica*.

Una vez que el miracidio contacta con el hospedador intermediario, penetra en el mismo a través de su pie, pierde su cubierta ciliada y se localiza en la región periesofágica del caracol para evolucionar a la siguiente fase larvaria denominada esporocisto. El esporocisto se desplaza hacia el hepatopáncreas donde continúa su crecimiento y desarrollo. En el interior del mismo comienzan a desarrollarse las siguientes formas larvarias denominadas redias, que tras su crecimiento, rompen el esporocisto y son liberadas en el interior del caracol. Cuando las condiciones medioambientales son adversas para el molusco, se suele producir una nueva y segunda generación de redias, pero en condiciones normales las redias dan origen al siguiente estadio larvario: la cercaria.

Tras un período aproximado de 4-7 semanas, las cercarias abandonan el caracol y pasan al medio ambiente, donde se desplazan activamente utilizando su larga cola, para posteriormente adherirse a través de su ventosa ventral a superficies firmes como hojas de hierbas y plantas. Una vez adheridas, pierden dicha cola y se enquistan rodeándose de una cubierta resistente. Esta estructura se denomina metacercaria (Figura 4 A-B), y corresponde a la forma infectante para los hospedadores definitivos. Se considera que las metacercarias podrían sobrevivir durante un período de hasta un año en el medio ambiente debido a su gran capacidad de resistencia. Este largo tiempo de supervivencia

depende de una humedad suficiente y temperaturas moderadas. Estas formas infectantes podrían mantener su capacidad infectiva en un rango térmico que se encontraría entre los -2ºC durante un período de 8 semanas (Taylor, 1949) y los 25ºC, donde no sobrevivirían más de 6 semanas (Boray, 1963).

Fig. 4. Metacercaria con la doble cubierta (A) y una vez desprendida de la cubierta externa (B). Forma juvenil desenquistada (C).

Una vez que los animales ingieren las metacercarias durante el pastoreo, éstas pasan al tracto gastrointestinal donde se producirá su

desenquistamiento. Una primera fase de activación tiene lugar en el rumen y es iniciada por una alta concentración de dióxido de carbono, ambiente reductor y 39ºC de temperatura, mientras que la segunda fase de desenquistamiento o "emergencia" acontece en el intestino delgado, en la zona inferior a la desembocadura del conducto colédoco y es desencadenada por la bilis y por el propio parásito. Una vez desenquistadas, las formas larvarias juveniles (Figura 4C) atraviesan la pared intestinal y penetran en la cavidad abdominal en las primeras 2 horas tras la infección. Posteriormente, migran por el espacio peritoneal y alcanzan el hígado a los 4-6 días post-infección. Es importante señalar que no todas las metacercarias ingeridas consiguen implantarse en el hígado y dar origen a un ejemplar adulto, tan sólo el 25-60% tiene éxito (Boray, 1969; Sexton y cols., 1990; Wijffels y cols., 1994; Creaney y cols., 1996; Spithill y cols., 1997).

Una vez que han atravesado la cápsula de Glisson y penetran en el parénquima hepático, comienza una fase de migración por el mismo que dura aproximadamente 5-6 semanas y durante la cual las formas juveniles inmaduras van formando túneles o galerías en su desplazamiento al mismo tiempo que se alimentan y destruyen la arquitectura tisular. Tras esta fase de migración, los vermes ingresan en los conductos biliares donde alcanzan la madurez sexual o comenzará la

eliminación de huevos, para así completar el ciclo biológico. Los primeros huevos aparecen en las heces del hospedador a partir de 55-60 días (Martínez-Moreno y cols., 1996; Pérez-Écija y cols., 2010) desde la ingestión de las metacercarias. El período de prepatencia es de 10-12 semanas y el período mínimo para completar el ciclo es, por lo tanto, de 17-18 semanas.

1.3. Patogenia.

La patogenia originada por este trematodo depende de la fase del ciclo en el que se encuentre. Por un lado, las formas juveniles realizan migraciones por el parénquima hepático que originarán graves hemorragias debido a la rotura de vasos sanguíneos. Por su parte, las formas adultas, cuya localización son los conductos biliares, realizan su acción patógena debido al proceso erosivo que ocasionan las espinas del tegumento sobre la mucosa del conducto biliar.

El cuadro clínico está condicionado por varios factores dependientes del animal como son la edad, especie animal y el estado inmunológico, y dependientes del parásito como son la dosis infectante, la pauta de reinfección y la carga parasitaria. Aunque la forma crónica es la más común, básicamente se describen tres formas clínicas: aguda, con una

alta mortalidad, subaguda y crónica, con importante repercusión en las distintas producciones y la capacidad reproductiva (Behm y Sangster, 1999; Mulcahy y cols., 1999; Maqbool y cols., 2000; Schweizer y cols., 2005).

La forma aguda, aunque poco común, suele suceder en la especie ovina y se debe a una ingesta masiva de metacercarias en un breve período de tiempo (aproximadamente 1 semana). La acción traumática originada por la migración de un gran número de formas juveniles desenquistadas provoca rotura de vasos sanguíneos que origina pequeños focos hemorrágicos, donde el parásito queda temporalmente adherido (Kelly, 1993). Como consecuencia de ello, los animales presentan un cuadro de anemia hemorrágica aguda de tipo normocítica-normocrómica. Los signos más habituales son palidez de las mucosas, disnea, hepatomegalia con dolor abdominal y ascitis.

La forma subaguda se debe a la ingesta de un número elevado de metacercarias en un espacio de tiempo más prolongado, suficiente para no provocar una fasciolosis aguda. En el hígado se pueden encontrar tanto formas inmaduras como adultos. Los animales presentan, en este caso, anemia hipocrómica y macrocítica, con reticulocitosis. Los signos más habituales son: palidez en las mucosas, dolor a la palpación, letargia

y pérdida de peso. En algunos animales se observa edema submandibular y ascitis.

La forma crónica es la más frecuente y suele ser evidente a partir de 3-4 meses posterior a la primoinfección, aunque esto último puede variar en función de la especie y edad del animal, como así también de la carga parasitaria (Urquhart, 1956; Dawes, 1961; Rusthon, 1977; Chen y Mott, 1990; De Paulaa y cols., 2010). Esta forma es consecuencia de la acción patógena que generan las formas adultas en los conductos biliares. Los signos más frecuentes son pérdida de peso, palidez de las mucosas, ascitis y edema submandibular, si bien en vacuno estos dos últimos signos no son constantes. Se pone en evidencia una anemia hipocrómica y macrocítica con reticulocitosis acompañada de hipoalbuminemia.

Las lesiones que se pueden observar a nivel orgánico atienden a los dos momentos principales que se describen durante la infección: la fase de migración de las formas juveniles y la fase de asentamiento biliar de los adultos (Dow y cols., 1968).

Durante la fase de migración realizada por las formas juveniles desenquistadas es frecuente observar congestión hepática y numerosos trayectos hemorrágicos, lo que constituye un cuadro de hepatitis hemorrágica multifocal (Zafra y cols., 2013a,b). También se pueden

evidenciar por la acción de las formas juveniles, e incluso adultos, hepatomegalia con presencia de trayectos hemorrágicos y fibrosos tanto en la superficie hepática como en el parénquima, siendo frecuentes las hemorragias subcapsulares. De la misma forma se describe fibrosis hepática como consecuencia de la cicatrización de los trayectos causados durante la migración de las formas juveniles

Cuando las fasciolas han colonizado el hígado, migrado y asentado en los conductos biliares, podemos observar un hígado con los contornos irregulares, pálido y consolidado. El lóbulo izquierdo suele ser el más afectado ya que, debido a su localización ventral, suele ser el primero en ser invadido por las metacercarias tras su migración abdominal, mientras que el resto de lóbulos menos dañados sufren una hipertrofia compensatoria (Sinclair, 1967). Entre las lesiones observadas se encuentra colangitis y colangiectasia como resultado de la acción de las fasciolas adultas en los conductos biliares, con engrosamiento de las paredes de los conductos y ensanchamiento de su diámetro (Meeusen y cols., 1995; Pérez y cols., 1999). Los nódulos linfáticos regionales están aumentados de tamaño y aparecen de color grisáceo. Histológicamente, se aprecia una hiperplasia severa del epitelio de los conductos biliares, con áreas multifocales de ulceración y necrosis. La lámina propia está expandida por un infiltrado inflamatorio compuesto de eosinófilos,

neutrófilos, linfocitos y células plasmáticas (Rushton y Murray, 1977; Kelly, 1993; Zafra y cols., 2013a,b) y, externamente, se observa una gruesa banda de tejido conjuntivo denso.

1.4. Respuesta inmunitaria en animales infectados.

La dinámica de la respuesta inmunitaria desencadenada en la infección por *F. hepatica* tiene una marcada diferencia en los momentos iniciales y tardíos de la infección. La acción inmunomoduladora que ejerce el parásito y que ha sido ampliamente demostrada en diversos estudios (Hamilton y cols., 2009; Dowling y cols., 2010; Vukman y cols., 2013; McNeilly y Nisbet, 2014; Rodríguez y cols., 2015 y 2017; Motran y cols., 2018), conlleva una polarización de la respuesta inmunitaria de tipo celular. Es así, que al inicio de la infección aparecen elementos (IFN-γ) de una respuesta inmunitaria celular y a medida que la infección progresa se produce una respuesta inmunitaria de tipo Th2, que resulta ineficaz para controlar el desarrollo parasitario. En este sentido, en bovinos, se observó que, durante las fases iniciales, se desarrolla una respuesta de carácter mixto con elevados niveles de IL-10, TGF-β, IL-4 y IFN-γ. Sin embargo, a medida que progresa la infección se establecía una respuesta inmunitaria Th2/Treg (Flynn y Mulcahy, 2008).

Tanto en modelos murinos como en rumiantes, inmediatamente después de la infección, la respuesta inmunitaria es de carácter proinflamatoria, durante aproximadamente unas 4-6 semanas hasta que los adultos comienzan a entrar en los conductos biliares (Espino y cols., 2010). La supresión de esta respuesta Th1 es crucial para la supervivencia de *F. hepatica* en su hospedador debido a que la inmunidad frente a la infección por *Fasciola* requiere una fuerte respuesta inflamatoria Th1 (Gironès y cols., 2007; Moreau y Chauvin, 2010).

En relación a la intervención de los leucocitos, se ha observado que los eosinófilos están involucrados en procesos de citotoxicidad celular mediada por anticuerpos y participan en la destrucción mediada por anticuerpos de las formas juveniles de *F. hepatica* en una variedad de especies hospedadoras (Piedrafita y cols., 2001). En las infecciones experimentales en ovejas, la eosinofilia se ha descrito que alcanza unos valores máximos en la fase aguda (Escamilla y cols., 2017; Ruiz-Campillo y cols., 2018).

Durante los estadios crónicos, las células Treg liberan citoquinas que inhiben ciertas citoquinas inflamatorias Th1/Th2, mientras que en bovinos infectados se ha detectado que las células mononucleares sanguíneas circulantes (PBMC) producen *in vitro* altos niveles de IL-4 e IFN-γ en presencia de TGF-β y IL-10 (Flynn y Mulcahy, 2008). Este

perfil inmunológico descrito es similar al observado en ovinos infectados con *F. hepatica*, habiéndose también observado un perfil mixto de Th1/Th2 en la tercera semana post-infección para posteriormente detectarse un incremento de la expresión génica de citoquinas del patrón Th2 (Alvarez Rojas y cols., 2015).

La respuesta inmunitaria humoral durante la infección por *F. hepatica* se caracteriza por la producción de altos niveles de inmunoglobulina G específica (O'Neill y cols., 2000; Buffoni y cols., 2012; Wesołowska y cols., 2016), siendo la IgG1 el isotipo predominante, lo que es característico de una respuesta de tipo Th2.

1.5. Acción inmunomoduladora de *F. hepatica*.

Como se ha mencionado anteriormente, en la actualidad es bien sabido que durante la infección por *F. hepatica* en rumiantes se produce una respuesta inmunitaria de carácter celular y humoral que varía a lo largo del curso de la infección tanto en su intensidad como su tipología.

Se considera que el tipo de respuesta inmunitaria que se desarrolla durante la infección está condicionado en gran medida por la producción y liberación de diversas moléculas del parasito que ejercen una actividad

biológica sobre los mecanismos efectores de la respuesta inmunitaria del hospedador.

Se ha demostrado que *Fasciola hepatica* induce la activación prioritaria de los linfocitos T colaboradores (Th2, anti-inflamatorio) (Mulcahy y cols., 1998), mientras que la administración de productos de excreción-secreción de formas adultas de *F. hepatica* (FhES) inhibe el desarrollo de las respuestas Th1 e induce respuestas inmunitarias de tipo Th2 *in vivo* (O'Neill y cols., 2001 , Donnelly y cols., 2005, 2008 y 2010). En bovinos infectados, se ha observado que la infección induce una supresión de la proliferación leucocitaria (Graham-Brown y cols., 2017) mientras que suprime la respuesta de tipo proinflamatoria (Garza-Cuartero y cols., 2016).

En 2009, Hamilton y cols., observaron que antígenos del tegumento mostraban capacidad para suprimir la maduración e inhibir ciertas funciones de células dendríticas, mientras que antígenos glycanos de *F. hepatica* disminuyen la expresión de MHCII (Rodríguez y cols., 2015). Similares observaciones fueron realizadas a partir de otras moléculas derivadas de productos de excreción-secreción respecto de su efecto modulador sobre la actividad de las células dendríticas (Ruiz-Jiménez y cols., 2017).

Estos datos indican claramente que existen diversas moléculas del parásito que ejercen un efecto inmunomodulador sobre la respuesta inmunitaria del hospedador, lo que posiblemente condicione la polarización a un tipo de respuesta inmunitaria Th2 no protectora, permitiendo así su desarrollo y viabilidad dentro del hospedador.

1.6. Mecanismos celulares efectores de la respuesta inmunitaria.

Otro aspecto de gran importancia en el desarrollo de una respuesta protectora eficaz frente a la invasión parasitaria es la producción de moléculas oxidantes (radicales libres) por parte de las células del sistema inmunitario.

Un radical libre puede ser definido como cualquier especie de molécula capaz de existir de forma independiente y que contiene un electrón desapareado en un orbital atómico. Estas moléculas se producen continuamente en las células bien de forma accidental como productos del metabolismo celular o deliberadamente durante, por ejemplo, la fagocitosis (Cheeseman y Slater, 1993). Los radicales libres son moléculas altamente reactivas capaces de dañar estructuras moleculares biológicas como el ácido desoxirribonucleico (ADN), proteínas, hidratos de carbono y lípidos (Young y Woodside, 2001).

Con el objeto de analizar en mayor profundidad los mecanismos efectores celulares durante la infección por *F. hepatica*, en las dos últimas décadas ha habido un creciente interés en determinar de qué forma intervienen las especies de oxígeno (ROS) y nitrógeno (RNS) reactivos (Halliwell y Cutteridge, 1999). Los ROS y RNS, cuya acción tóxica es debida a la presencia de electrones desapareados en su orbital externo (Davies, 1995; Cadenas y Davies, 2000; Dröge, 2002; Valko y cols., 2007), se producen por un amplio rango de mecanismos, entre los que se incluyen: (a) metabolismo aeróbico generado por la cadena respiratoria mitocondrial, (b) luz ultravioleta (UV), radiación X o γ, o (c) reacciones catalizadas por metales. También se generan por contaminantes aéreos o durante el desarrollo de procesos inflamatorios en los cuales intervienen macrófagos y neutrófilos frente a diversos organismos patógenos (Valko y cols., 2006). Cuando estas especies reactivas se presentan en una alta concentración ejercen efectos dañinos en macromoléculas celulares esenciales como proteínas, lípidos y ácidos nucleicos (Valko y cols., 2006). Por lo tanto, durante el desarrollo de la respuesta inmunitaria, estos efectos producidos por dichas moléculas constituyen una acción "beneficiosa", ya que determinadas células fagocíticas producen una gran cantidad de ROS/RNS para luchar contra diversos organismos patógenos.

Uno de los radicales libres de mayor relevancia es el óxido nítrico (NO). Su papel ha sido ampliamente estudiado y se ha propuesto que media en importantes y diversas funciones como molécula efectora en la resistencia del hospedador a una variedad de patógenos (Nathan y Kie, 1994). Dada la multitud de funciones, entre las que se incluyen regulación y señalización, la modificación de los niveles de NO pueden desencadenar importantes efectos. Las propiedades citotóxicas de esta molécula dependen de la producción de aniones peroxinitrito, resultado de la reacción entre óxido nítrico y anión superóxido (Kolodziejczyk y cols., 2006; Ushio-Fukai, 2006; Miller y cols., 2009).

Se sabe que durante la infección por *F. hepatica*, el hospedador responde mediante la estimulación celular (Kolodziejczyk y cols., 2006; Baska y cols., 2013) y activando mecanismos asociados a la generación de especies reactivas de oxígeno (ROS). El daño celular oxidativo puede ocurrir en el curso de la enfermedad como consecuencia de la destrucción tisular causada por los componentes tóxicos producidos por las metacercarias (Galtier y cols. 1987; Fiss y cols., 2013). Se ha observado que, durante la fase inicial de la infección, las formas juveniles originan reacciones tóxicas e inmunológicas tanto localizadas como generalizadas que resultan en estrés oxidativo y mecanismos de destrucción de tejido hepático (Behm y Sangster, 1999). Se ha descrito

que el estrés oxidativo es una característica importante de la fasciolosis crónica en la especie ovina (Saleh y cols., 2005). Bottari y cols. (2015) sugieren que los altos niveles de los radicales nitrogenados en el hígado de animales infectados podrían originar citotoxicidad debido a la capacidad del NO de generar peroxinitrito e iniciar una variedad de reacciones oxidativas como la modificación de ácidos nucleicos, lípidos y proteínas (Keita y cols., 2000).

Otro de los radicales libres que está siendo objeto de estudio durante el desarrollo de diversos procesos patógenos, incluida la infección por *F. hepatica*, lo constituye el peróxido de hidrógeno (H_2O_2). Se ha demostrado que el H_2O_2 es producido a nivel mitocondrial en ejemplares adultos de *F. hepatica* y constituye un compuesto formado como un producto final de procesos de respiración en dicho verme (Prichard y Scholfield, 1971). Se ha observado que el H_2O_2 puede originar daños en multitud de componentes celulares, destacando, entre sus efectos, la afectación del ácido desoxirribonucleico (Mello-Filho y cols., 1984). El H_2O_2, que puede originarse también a través de aniones supéroxido que son producidos por las células fagocíticas (Robinson y Badwey, 1994), puede estar presente en mayor concentración en los sitios donde se produce reacción inflamatoria.

1.7. Métodos de control

En la actualidad la lucha contra la fasciolosis está basada casi exclusivamente en el empleo profiláctico y terapéutico de antihelmínticos. Dentro del espectro farmacológico que se muestra como eficaz frente a las trematodosis en rumiantes se incluye el triclabendazol (TCBZ), albendazol, closantel y clorsulón, entre otros. El triclabendazol ha demostrado su alta eficacia contra las formas adultas e inmaduras (Barrera y cols., 2012) desde los dos primeros días posteriores a la infección (Boray y cols., 1983), mientras que otros fármacos sólo son eficaces desde la sexta a la decimocuarta semana después de la infección (Kelley y cols., 2016). Como consecuencia de ello, se considera que el triclabendazol es el fármaco de elección para el control de la enfermedad, particularmente en el tratamiento de la fasciolosis aguda en el ganado ovino. El uso de estas sustancias químicas en el tratamiento de las infecciones parasitarias en animales destinados al consumo humano no se hace sostenible a largo plazo debido a la presencia de los mismos o residuos derivados de ellos en tejidos animales que podrían pasar a la cadena de producción siendo dañinos cuando son consumidos por el ser humano. Por ello se hace necesario el uso de programas que monitoricen la existencia de estos compuestos y controlen su exposición al consumidor (Dalton y Mulcahy, 2001; Cooper y cols., 2012). Otras de

las desventajas que presenta el uso de fármacos es la pérdida de su eficacia con la consiguiente aparición de resistencias. El principal exponente de este caso es, precisamente, el triclabendazol, cuyo primer caso de resistencia fue descrito en Australia en 1995 (Overend y Bowen, 1995) y se ha hecho extensible a otros países a nivel mundial (Fairweather, 2009; Kelley y cols., 2016).

Asimismo, el aumento de la resistencia a estos fármacos plantea la necesidad de buscar métodos alternativos de control, como son la selección genética de animales resistentes (Roberts y cols., 1997a,b) y sobre todo métodos de control inmunológico como el desarrollo de vacunas, que se ha mostrado como una alternativa viable y prometedora (Mulcahy y Dalton, 1998; Spithill y Dalton, 1998; Dalton y Mucahy, 2001; Dalton y cols., 2003a,b; Meeusen y Piedrafita, 2003; Hillyer, 2005).

1.8. Desarrollo de vacunas – Identificación de candidatos vacunales.

Una de las principales aplicaciones que se puede derivar del conocimiento de los mecanismos inmunomoduladores de *Fasciola* y de los procesos inmunogénicos desarrollados durante la infección es el

desarrollo de vacunas para el control inmunológico de la enfermedad. En el caso de *Fasciola hepatica* se han identificado hasta la actualidad numerosas moléculas que forman parte bien de su tegumento externo o bien son productos de excreción-secreción liberados durante su metabolismo que, expuesto al sistema inmunitario del hospedador, desencadenan una respuesta inmunitaria tanto humoral como celular (Martínez-Moreno y cols., 1996).

Un aspecto a considerar en la identificación de posibles candidatos vacunales es el empleo de la proteína nativa, procedente directamente del propio parásito, frente a su homólogo de tipo recombinante. De forma general, las proteínas nativas de *F. hepatica* se obtienen mediante procesos que incluyen un laborioso tratamiento de los trematodos adultos recolectados del hígado de animales infectados y que implican el desarrollo de diversas técnicas como, por ejemplo, cromatografía de afinidad (Timanova-Atanasova y cols., 2004; Buffoni y cols., 2010; Teofanova y cols., 2012).

Por otro lado, las proteínas de tipo recombinante se obtienen a través de estudios genéticos en los cuales se identifica previamente la secuencia de genes que codifican para las proteínas que se desean producir (Mokhtarian y cols., 2016a,b), y dichos genes son posteriormente introducidos mediante el empleo de plásmidos en distintos

microorganismos (bacterias como *E. coli* o levaduras como *P. pastoris*) (Mokhtarian y cols., 2018), lo que posibilita su producción en cadena. A pesar de que su obtención requiere un menor tiempo respecto de aquellas de tipo nativo y se obtienen proteínas purificadas sin residuos que puedan interferir en su identificación, existe cierta controversia sobre la eficiencia de las proteínas recombinantes respecto de su homóloga nativa debido a la falta de eficacia demostrada en gran diversidad de estudios experimentales de vacunación (Morrison y cols., 1996).

Además, como candidato vacunal se ha valorado recientemente el empleo de vacunas ADN que codifiquen para determinados componentes del parásito, como proteínas que intervengan en la vía glucolítica. Esta nueva alternativa, que se está considerando actualmente como una estrategia prometedora para proteger tanto a seres humanos como a animales frente a diversas enfermedades, incluyendo la fasciolosis, presenta ciertos inconvenientes aún no esclarecidos como, por ejemplo, la pobre capacidad inmunogénica que se ha detectado mediante el uso de dichas vacunas, por lo que esta alternativa requiere aún de un mayor conocimiento (Wesołowska y cols., 2016).

Aunque los primeros ensayos sobre inmunización experimental frente a *Fasciola hepatica* fueron llevados a cabo aproximadamente hace 50 años atrás (Haroun y Hillyer, 1986), es en las últimas dos décadas cuando se

comenzó a explorar el potencial inmunogénico de determinadas proteínas (cisteínas, glutatión S-transferasa, hemoglobina, proteínas de unión a ácidos grasos, etc.) (Hillyer, 2005). Hasta la fecha se han identificado y empleado en ensayos experimentales de inmunización gran variedad de candidatos vacunales, tanto en animales de laboratorio como en sus hospedadores habituales (bovinos, ovinos y caprinos).

De entre las distintas proteínas de *Fasciola hepatica* con potencial inmunogénico identificadas para los mencionados ensayos de inmunización caben descatar: catepsinas B y L (Villa-Mancera y cols., 2008; Golden y cols., 2010; Wesołowska y cols., 2018a), moléculas de defensa contra helmintos (HDM) (Orbegoza-Medina y cols., 2018), peroxirredoxina (Prx) (Sekiya y cols., 2006; Mendes y cols., 2010a; Buffoni y cols., 2012), leucinaminopeptidasa (LAP) (Piacenza y cols., 1999; Acosta y cols., 2008; Maggioli y cols., 2011a), glutatión S-transferasa (GST) (Sexton y cols., 1994), tiorredoxina glutatión reductasa (TGR) (Acosta y cols., 1998; Maggioli y cols., 2004, 2011b), proteínas de unión a ácidos grasos (FABP) (Casanueva y cols., 2001; Buffoni y cols., 2012), saponinas (SAP) (Espino y cols., 2010; Rivera y Espino, 2016), hemoglobina (Dalton y cols., 1996; Dewilde y cols., 2008), cadena liviana reguladora de miosina (MRLC) (Henker y cols., 2017) y paramiosina y kunitz (Spithill y cols., 1999; Cancela y cols.,

2004), entre otras. Asimismo, recientemente se han realizado formulaciones antigénicas que incluyen el desarrollo de vacunas ADN que contienen determinados genes codificantes para ciertas proteínas de *F. hepatica*. En este sentido, se han llevado a cabo estudios con vacunas ADN codificantes para la proteína fosfoglicerato quinasa (PGK) tanto en modelos murinos como ovinos (Wesołowska y cols., 2016; Wesołowska y cols., 2018b).

Esta diversidad en la identificación y producción de proteínas de *F. hepatica* ha permitido elaborar diferentes formulaciones antigénicas que, combinadas con ciertos adyuvantes, han sido empleadas para el desarrollo de vacunas y administradas en varios modelos animales con el objeto determinar su potencial inmunogénico y su capacidad protectora frente a la infección por *F. hepatica*, con resultados variables. Por ejemplo, el uso de catepsina L1 ha generado niveles de protección que varían entre 38’2 al 69’4 % en la especie bovina (Dalton y cols., 1996; Golden y cols., 2010), 33% en la especie ovina (Piacenza y cols., 1999) y 51% en modelos murinos (Jayaraj y cols., 2009). En estos últimos hospedadores se ha conseguido alcanzar tasas del 60% de protección mediante el uso de catepsina B, cifras que ascienden al 83% cuando se realiza una vacunación conjunta con dos formas distintas de captesinas (Jayaraj y cols., 2009). El empleo de otras proteínas como

hemoglobina ha inducido una significativa protección del 43% en los bovinos inmunizados (Dalton y cols., 1996), mientras que la leucinaminopeptidasa incrementa en un 89% el porcentaje de protección frente a la infección por *Fasciola hepatica* en ovinos (Piacenza y cols., 1999).

Por el contario, existen estudios en los cuales dichos antígenos no han demostrado capacidad de inducción de respuestas protectoras significativas frente a la infección. En 2010 Pérez-Écija y cols., emplearon catepsina L1 en la especie caprina no obteniendo reducción de la carga parasitaria mientras que el uso de FABP en la especie ovina tampoco generó resultados positivos respecto de la reducción de dicho parámetro (Ramajo y cols., 2001). Maggioli y cols. (2016) utilizaron TGR como antígeno vacunal en bovinos no observando niveles significativos de resistencia, mientras que Dewilde y cols. (2008) y Buffoni y cols. (2012) no detectaron disminución de la carga parasitaria mediante el uso de peroxirredoxina como candidato vacunal en la especie bovina y caprina, respectivamente.

Los estudios relativos a ensayos vacunales en rumiantes se han centrado en su gran mayoría en los análisis de la respuesta inmunitaria durante las fases tardías de la infección (Buffoni y cols., 2010; Kesik-Brodacka, 2017), cuando los ejemplares adultos se encuentran asentados en su

localización definitiva. Aunque, como se ha mencionado anteriormente, se han obtenido resultados prometedores, no se dispone aún de una formulación vacunal que se considere óptima, especialmente para su empleo en pequeños rumiantes.

Es por ello que se requiere un conocimiento más extenso y profundo sobre los acontecimientos que ocurren no solo durante la fase tardía sino también durante las fases iniciales de la infección, con el objeto de dilucidar qué parámetros y cuáles son los mecanismos efectores celulares de la respuesta inmunitaria que se desencadenan cuyo propósito es comprender de mejor manera la interacción parásito-hospedador.

1.9. Nuevos candidatos vacunales: proteína recombinante 14-3-3.

Entre las moléculas identificadas con potencial inmunogénico se encuentran las proteínas 14-3-3. Éstas representan una familia de proteínas ampliamente distribuidas en diferentes taxones parasitarios (Siles-Lucas y cols., 2000; van Hemmert y cols., 2001; Koyama y cols., 2001; Gadahi y cols., 2016) que actúan espontáneamente como dímeros de fosfoserina-treonina. Presentan dos regiones bien definidas dentro de su secuencia (llamadas firmas) (Obenauer y cols., 2003) y se encuentran

asociadas con numerosas moléculas de interés biológico en tejidos tanto de mamíferos como no mamíferos (Yaffe, 2002). Esta familia de proteínas presenta un papel crucial en los mecanismos de señalización de células eucariotas que están involucrados en el control del ciclo celular, organización del citoesqueleto, alteraciones en la transcripción en respuesta a estímulos ambientales y en la muerte celular programada (van Hemmert y cols., 2001; Sluchanko y Gusev, 2010; Weidner y cols., 2016).

Las proteínas 14-3-3 se han aislado y caracterizado en diversos organismos parásitos (Siles-Lucas y cols., 1998; McGonigle y cols., 2002): en *Schistosoma* spp. y *Echinococcus* spp., las funciones biológicas de varias isoformas de la citada proteína se han estudiado ampliamente, destacando las relacionadas con el ciclo celular y la regulación de la fisiología. Otras funciones incluyen la actuación como molécula adaptadora para estimular las interacciones proteína-proteína, la regulación de la localización subcelular de las proteínas y la inhibición de enzimas activadoras (van Hemmert y cols., 2001). Por todo ello, se consideró la posibilidad de usar tales moléculas como herramientas vacunales contra la infección por determinadas especies parásitas, analizando su capacidad inmunoprotectora en ensayos experimentales con polipéptidos de tipo recombinantes de la proteína 14-3-3

(Schechtman y cols., 2001; Zhang y cols., 2001; Siles-Lucas y cols., 2003). Con dicha finalidad, Siles-Lucas y cols. (2003) demostraron que ratones inmunizados con una proteína recombinante de *Echinococcus multilocularis* 14-3-3 (E14t) adquirían protección frente a la infección primaria, aunque no secundaria, de la forma alveolar de esta parasitosis. En la misma línea, Uribe y cols. (2007) demostraron que la proteína recombinante Sb14ζ de *Schistosoma bovis* otorgaba protección en ratones infectados por este trematodo.

Asimismo, hasta la actualidad, se desconoce el potencial inmunógeno de esta proteína y su capacidad inmunoprotectora en rumiantes frente a la infección por *F. hepatica*.

2. Objetivos.

Por consiguiente, teniendo en cuenta la necesidad de aportar nuevos conocimientos relativos al desarrollo de la inducción experimental de respuestas inmunes de carácter protector frente a la infección por *Fasciola hepatica*, como así también analizar los mecanismos celulares de la respuesta inmunitaria, se ha planteado la realización del presente trabajo de investigación con el que se opta a la obtención del Grado de Doctor, y cuyo objetivo principal es estudiar la interacción parásito-

hospedador y los mecanismos de respuesta desarrollados en fases tempranas y tardías de la infección por *Fasciola hepatica* en ovinos vacunados y no vacunados.

Este objetivo general se concreta a partir de los objetivos específicos detallados a continuación:

1. Analizar la respuesta inmunitaria inducida por la molécula recombinante 14-3-3z de *Fasciola hepatica*, procedente de formas juveniles, en ensayos vacunales en la especie ovina. Este objetivo se estructura a su vez en los siguientes apartados:

1.1. Determinar la capacidad protectora de la proteína rFh14-3-3z en combinación con el adyuvante Montanide ISA 71 VG, considerando como parámetros de protección su influencia sobre la población parasitaria (carga parasitaria y dinámica de eliminación de huevos) y la reducción del daño hepático (en base al examen anatomopatológico cuantitativo).

1.2. Caracterizar la respuesta inmunitaria adquirida en base a la producción de anticuerpos específicos anti-Fh 14-

3-3z de los isotipos IgG1 (respuesta tipo Th2) e IgG2 (respuesta tipo Th1).

2. Investigar la respuesta inmunitaria celular desarrollada en la cavidad peritoneal durante infecciones experimentales, comparando fases tempranas y tardías de la infección. Este objetivo igualmente se concreta en los siguientes apartados:

2.1. Determinar la dinámica de reclutamiento de las células peritoneales implicadas en la respuesta inmunitaria durante la infección, tanto en fases tempranas como tardías.

2.2. Analizar la producción de radicales libres (óxido nítrico y peróxido de hidrógeno) de las células peritoneales durante la fase temprana de la infección en ovinos infectados, no infectados y vacunados con la proteína recombinante catepsina L1.

El desarrollo de los objetivos generales y específicos ha permitido la elaboración de tres trabajos de investigación que han sido publicados en prestigiosas revistas científicas con revisión anónima por pares, y que motivan la elaboración del presente trabajo de Tesis Doctoral en el formato de compendio por artículos (Art. 24. Tesis como compendio de

publicaciones, Propuesta por la Comisión de Másteres y Doctorado de 14 de diciembre de 2011 y aprobada por Consejo de Gobierno de 21 de diciembre de 2011 de la Universidad de Córdoba):

1. **R. Pérez-Caballero**, M. Siles-Lucas, J. González-Miguel, F.J. Martínez-Moreno, A. Escamilla, J. Pérez, A. Martínez-Moreno, L. Buffoni. Pathological, immunological and parasitological study of sheep vaccinated with the recombinant protein 14-3-3z and experimentally infected with *Fasciola hepatica*. Veterinary Immunology and Immunopathology. 2018, 202:115-121.
   doi: 10.1016/j.vetimm.2018.07.006
2. **Raúl Pérez-Caballero**, F. Javier Martínez-Moreno, Rafael Zafra, Verónica Molina-Hernández, Isabel L. Pacheco, M. Teresa Ruiz-Campillo, Alejandro Escamilla, José Pérez, Álvaro Martínez-Moreno and Leandro Buffoni. Comparative dynamics of peritoneal cell immunophenotypes in sheep during the early and late stages of the infection with *Fasciola hepatica* by flow cytometric analysis. Parasites & Vectors. 2018, 11:640 doi.org/10.1186/s13071-018-3250-5.

3. **Raúl Pérez-Caballero**, Leandro Buffoni, F. Javier Martínez-Moreno, Rafael Zafra, Verónica Molina-Hernández, José Pérez and Álvaro Martínez-Moreno. Expression of free radicals by peritoneal cells of sheep during the early stages of *Fasciola hepatica* infection. Parasites & Vectors. 2018, 11:500 doi:10.1186/s13071-018-3072-5.

RESUMEN

Fasciola hepatica es el agente etiológico de la fasciolosis, enfermedad descrita como la principal causa de pérdidas económicas en el sector agrícola y ganadero en países desarrollados y en vías de desarrollo como consecuencia de las tasas de morbilidad y mortalidad que origina en los animales de abasto. La OMS la describe como una enfermedad parasitaria re-emergente con un especial impacto en humanos alrededor de todo el mundo.

Entre los distintos principios activos disponibles para hacer frente a esta enfermedad, el triclabendazol se erigió como el fármaco de elección en el tratamiento de la fasciolosis. Debido al uso de antihelmínticos como único medio eficaz para el control de la infección, se hace necesario el estudio e implementación de vías alternativas que permitan el control de esta enfermedad, como la obtención de líneas genéticas resistentes o el desarrollo de vacunas.

La infección por *F. hepatica* desencadena la actuación de la respuesta inmunitaria como consecuencia del reconocimiento de numerosos antígenos de excreción-secreción (FhESPs) o de la superficie del parásito. Estas moléculas han sido identificadas y purificadas con el fin de realizar ensayos vacunales con los que determinar su capacidad inmunógena y qué antígeno pudiera constituir el candidato idóneo de una vacuna eficaz. La mayoría de los estudios vacunales realizados en

rumiantes se han centrado en el empleo de antígenos obtenidos a partir de la fase adulta del parásito, si bien se torna de vital importancia identificar áquellos que juegan un papel relevante durante todas las fases de la infección.

Con este motivo, se empleó en un **primer estudio** un antígeno (rFh14-3-3z) de la familia de proteínas 14-3-3, con el que poder determinar el perfil inmunológico y el componente parasitológico, haciendo hincapié en el número de fasciolas asentadas en hígado y la dinámica de eliminación de huevos en heces.

Se usaron 24 ovejas de raza merina de seis meses de edad divididas aleatoriamente en tres grupos (*n*=8) con el siguiente modelo: el grupo 1 fue inmunizado con la proteína recombinante Fh14-3-3z y el adyuvante Montanide™ ISA 71 VG, el grupo 2 se inmunizó únicamente con Montanide™ ISA 71 VG (control adyuvante) y el grupo 3 fue el grupo control de infección (infectado y no vacunado). Ocho semanas posteriores a la primera inmunización los animales fueron infectados oralmente con una única dosis de 150 metacercarias. A las 6 semanas de la infección se realizó un muestreo de heces semanalmente a todos los animales para analizar la dinámica de eliminación de huevos mediante McMaster con sulfato de zinc. La presencia de huevos en heces se detectó a las 8 semanas post-infección en el grupo 1 y las 9 semanas en

los grupos 2 y 3. De la misma manera, una vez sacrificados se recogieron los adultos de *F. hepatica* de los hígados para realizar su recuento, así como la medición de los vermes. El grupo 1 presentó una carga de 52’25 ± 16’71 fasciolas, similar al grupo 3 con una carga media de 51’13 ± 12’84 y el grupo 2 evidenció una mayor carga parasitaria con 68’13 ± 23’79 adultos. La respuesta inmunitaria humoral, para los isotipos IgG1 e IgG2, analizada mediante ELISA indirecto mostró un incremento significativo en IgG1 tres semanas después de la inmunización, mientras que IgG2 aumenta su producción significativamente únicamente después de la inmunización en el grupo 2.

Debido a los ensayos que han demostrado la falta de protección en animales inmunizados con diferentes antígenos de *F. hepatica* y a la capacidad inmunomoduladora del parásito se hace necesario conocer la respuesta inmunitaria que se desencadena en las primeras fases de la infección. Por ello, el **segundo estudio** tuvo como objetivo el análisis poblacional de linfocitos (TCD4, TCD8 y $WC1^{+}\gamma\delta$), macrófagos (CD14), MHCII como célula presentadora de antígenos y células dendríticas (CD83) de cavidad peritoneal de animales infectados y sacrificados en fases iniciales y tardías. En este ensayo se emplearon 37 ovejas de raza merina de seis meses de edad, las cuales fueron divididas aleatoriamente en tres grupos atendiendo al momento del sacrificio: el

grupo 1 (*n*=20) (estadios tempranos) y grupo 2 (*n*=10) (estadios tardíos) fueron infectados con una dosis de 150 metacercarias. Los animales del grupo 1 fueron sacrificados a los 1, 3, 9 y 18 días post-infección (dpi), así mismo, el grupo 2 fueron sacrificados a las 14 semanas post-infección (spi). El grupo 3 no se infectó y se describió como grupo control negativo. Los parámetros parasitológicos del grupo 2 mostraron una carga parasitaria de 67'78 ± 13'85 adultos con una tasa de implantación con niveles de 45'19% ± 9'24. La presencia de huevos en heces se detectó a las 8 semanas de la infección, alcanzando su máximo a la semana 13. El análisis de las distintas poblaciones leucocitarias se determinó mediante citometría de flujo con distintos anticuerpos monoclonales. Los animales sacrificados en las fases tempranas únicamente mostraron un descenso significativo de los TCD4 a los 1 y 18 dpi. Asimismo, la población de CD14 peritoneal evidenció un descenso a las 9 y 18 dpi, mientras que MHCII y CD83 describen un patrón similar con una significativa disminución a los 3 y 9 dpi. Los animales sacrificados a las 14 semanas aumentaron significativamente su población de $WC1^{+}\gamma\delta$ pero sufrieron un descenso significativo en las subpoblaciones de macrófagos y células dendríticas peritoneales.

Continuando con una mejor compresión de los mecanismos defensivos del hospedador frente a esta enfermedad, el **tercer trabajo** realizado

tuvo como objetivo analizar la dinámica poblacional en líquido peritoneal y la producción de radicales libres en animales infectados y vacunados. Para este estudio se emplearon 45 ovejas de raza merina de seis meses de edad agrupadas aleatoriamente como se describe: el grupo 1 (*n*=5) fueron animales no infectados ni vacunados y fue descrito como grupo control negativo, el grupo 2 (*n*=20) fue experimentalmente infectado con 150 metacercarias y el grupo 3 (*n*=20) fue inmunizado con rFhCL1 e infectado con 150 metacercarias. El grupo 1 fue sacrificado a las 12 spi mientras que los grupos 2 y 3 fueron sacrificados a los 1, 3, 9 y 18 dpi. La respuesta inmunitaria humoral evidenció diferencias significativas en IgG1 desde las 6ª semana post-vacunación en el grupo 3 y únicamente a los 1 dpi para IgG2. El número total de leucocitos reveló un aumento significativo a los 9 y 18 dpi para el grupo 2 y 3. Más específicamente, el grupo 2 evidenció un aumento significativo de granulocitos a los 9 dpi y de las tres poblaciones peritoneales a los 18 dpi. Por su parte, el grupo 3 aumentó el número de linfocitos a los 3 y 18 dpi, así como la población de macrófagos y granulocitos a los 9 y 18 dpi. La medición de radicales libres por parte de los leucocitos se determinó mediante citometría de flujo usando 2 reactivos diferentes: DAF-2DA para el óxido nítrico y DCFH-DA para el peróxido de hidrógeno. En relación a la producción H_2O_2, el grupo 2 incrementó su producción por

parte de los macrófagos a los 3 y 9 dpi, y a los 18 dpi por parte de los granulocitos. Asimismo, los granulocitos en el grupo 3 mostraron un aumento de este radical libre a los 9 y 18dpi. Finalmente, la producción de NO en el grupo 2 sufrió un incremento significativo por parte de los granulocitos a los 9 y 18 dpi y en los macrófagos a los 18 dpi. Sin embargo, el grupo 3 aumentó la producción de NO a los 3 dpi por parte de los macrófagos y a los 3, 9 y 18 dpi en los granulocitos.

ABSTRACT

Fasciola hepatica is the ethyological agent of fasciolosis, a disease described as the major cause of economic losses to the agricultural industry in developed and developing countries due to the high morbility and mortility rates in livestock. It has been described as a tropical re-emerging disease by the WHO.

Triclabendazole is the drug of choice against fasciolosis, among different drugs available for the control of the disease. Due to the use of anthelmintic as the only effective mean for the control of the infection, alternative ways for disease control are required, as genetically resistant species or the development of vaccines.

Fasciola hepatica infection triggers the immune response as a consequence of the recognition of numerous excretory and secretory products (FhESPs) or tegumental proteins. These molecules have been identified and purified in order to perform vaccine trials aimed at determining their immunogenic capacity so as to develop a vaccine. Most of the vaccine trials carried out in ruminants had been conducted using the adult fluke antigens that are exposed to the immune system. Therefore, it is essential to identify the molecules that play a key role during the course of infection.

On this reason, an antigen from the 14-3-3 protein family (14-3-3z) has been used in a first study. With the use of rFh14-3-3z antigen we determined the immunological and parasitological parameters since the first vaccination, with special attention to the number of adult flukes in the liver and the dynamic of faecal egg count.

Twenty-four female Merino-breed sheep 6 month old were used for the study. Sheep were randomly allocated into three groups (*n*=8). Animals from group 1 were immunised with the recombinant antigen rFh14-3-3z in Montanide adjuvant (Montanide™ISA 71 VG -Seppic®). Group 2 was immunised with Montanide adjuvant (adjuvant control group) and animals from group 3 were not immunised and remained as positive infection control. Eight weeks after first immunisation all animals were orally challenged with a single dose of 150 metacercariae of *F. hepatica*.

To assess egg output, faecal samples were individually collected weekly from week six after challenge until the end of the trial, and were analysed by a zinc sulphate-based flotation method using the McMaster chamber. Eggs were first detected at 8 wpi in group 1 and 9 wpi in group 2 and 3. Once sheep were slaughtered the livers were collected and the adult flukes were recovered for measurement and weighing. Fluke burden of group 1 was 52’25 ± 16’71, similar to the group 3 with a mean of 51’13 ± 12’84 adult flukes. The group 2 showed a higher fluke burden of 68’13

± 23’79 adults. On regard to the humoral immune system, indirect ELISA showed a significant increase of IgG1 at week 3 after immunisation, while IgG2 increased significantly its values only after immunisation in group 2.

Due to the failure of many vaccination trials using broad variety of *F. hepatica* antigens and to the immunomodulatory capacity of the liver flukes, the study of the immune response during the early stages of the infection is vital for a better understanding of the immunological pathways elicited during the host-parasite interaction.

Thus, the second study was aimed at analysing the peritoneal lymphocyte (TCD4, TCD8 and $WC1^{+}\gamma\delta$), macrophage (CD14), MHCII as an antigen presenting cell, and dendritic cells (CD83) populations of infected animals and slaughtered at the early and late stages of infection.

Thirty-seven female Merino-breed sheep, aged 6 months, were used for the study. Animals were randomly allocated into 3 groups according to the slaughtering time: group 1 (n=20) (early stage) and group 2 (n=10) (late stage) were infected with a single dose of 150 metacercariae. Sheep from group 1 were sacrified at 1, 3, 9 and 18 days post-infection (dpi). In the same way, animals from group 2 were slaughtered at 14 weeks post-infection (spi). The group 3 was not infected and remained as a

negative control group. Parasitological parameters from group 2 showed a fluke burden of 67'78 ± 13'85 adults with a implantation rate of 45'19% ± 9'24. Eggs in faecal samples were first detected at week 8 post-infection, reaching the highest figures at 13 wpi. The analysis of the different leukocyte populations was determined by flow cytometry with diverse monoclonal antibodies. Animals sacrified at the early stages of infection only showed a significant decrease of TCD4 at 1 and 18 dpi. Furthermore, CD14 peritoneal population showed a decrease at 9 and 18 dpi, while MHCII and CD83 described a similar pattern with a significant decrease at 3 and 9 dpi. Animals slaughtered at 14 wpi increase significantly $WC1^{+}\gamma\delta$ subpopulation but macrophage and dendritic cells peritoneal populations showed a significant decrease.

Continuing with a better understanding of host immunological mechanisms against the disease, our third study aimed to analyse the dynamic of peritoneal populations and the production of free radicals in immunised and infected animals. For this study, Forty-five six-month-old male merino sheep were used and randomly allocated as described: group 1 (*n*=5) was not infected and not vaccinated and remained as negative control group, group 2 (*n*=20) was experimentally infected with 150 metacercariae and group 3 (*n*=20) was immunised with rFhCL1 and infected with 150 metacercariae. Group 1 was slaughtered at 12 wpi,

while groups 2 and 3 were sacrified at 1, 3, 9 and 18 dpi. Humoral immune response showed significant differences in IgG1 at 6 post-immunisation in group 3 and only at 1 dpi for IgG2. Total number of leukocytes revealed a significant increase at 9 and 18 dpi for group 2 and 3. Granulocytes from group 2 increased significantly at 9 dpi and the three peritoneal populations at 18 dpi. Group 3 increased the number of lymphocytes at 3 and 18 dpi, as well as macrophage and granulocyte populations at 9 and 18 dpi. Free radical measurement was determined with flow cytometry analysis using two different reagent: DAF-2DA for nitric oxide and DCFH-DA for hydrogen peroxide. In relation to H_2O_2 production, macrophages from group 2 increased the production at 3 and 9 dpi, and granulocytes at 18 dpi. Finally, NO production in group 2 increased significantly in granulocytes at 9 and 18 dpi and in macrophages at 18 dpi. However, group 3 increased NO production at 3 dpi in macrophages and at 3, 9 and 18 dpi in granulocytes.

PRIMER ESTUDIO

Pathological, immunological and parasitological study of sheep vaccinated with the recombinant protein 14-3-3z and experimentally infected with *Fasciola hepatica*

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Abstract

In this study, the immunogenicity and protective capacity of a new recombinant vaccine candidate, the rFh14-3-3z protein was analysed in sheep experimentally challenged with *Fasciola hepatica*, in terms of fluke burden, faecal egg counts, hepatic damage and humoral immune response. Three groups of 8 animals each were used for study, group 1 was immunised with the rFh14-3-3z in Montanide adjuvant, whereas group 2 and 3 remained as adjuvant control and infection control groups, respectively. The parasitological analysis showed that no significant reduction in fluke burden, fluke size and faecal egg counts was detected. The extent of hepatic damage was very similar between groups. Nonetheless, animals immunised with the rFh14-3-3z protein induced the development of specific IgG1 and IgG2, being the IgG1 the predominant antibody; which confirms the immunogenicity of this protein in sheep. This is the first report of the 14-3-3z proteins as vaccine against the infection with *F. hepatica*.

Key Words

rFh14-3-3z; Vaccine; Montanide; *Fasciola hepatica*; immunisation.

Introduction

The trematode *Fasciola hepatica* is the causative agent of fasciolosis, a major parasitic disease of livestock and responsible for large economic losses to the agricultural industry. The parasite localises in the bile ducts and gallbladder of the animals and produces a severe traumatic hepatitis during the migratory and biliary stage that may lead to loss of the hepatic function. The life cycle of the parasite is complex and involves small pond freshwater snails of the genus *Galba* spp. as intermediate hosts. The disease mainly affects ruminants but other mammals such as pigs, horses, deer or wild animals may also get infected, as well as humans, particularly in endemic areas.

The use of anthelmintics is the most effective method for controlling the disease but resistance to different drugs has been recorded and is increasing worldwide (Moll et al., 2000; Alvarez-Sanchez et al., 2006; Olaechea et al., 2011; Novobilský and Höglund, 2015; Venturina et al., 2015), hence new control strategies such as immunological control may be a feasible option and the use of vaccines is still under development with many promising results (Dalton and Mulcahy, 2001; Almeida et al., 2003; Golden et al., 2010; Villa Mancera et al., 2014).

The 14-3-3 is a family of 24–33 kDa proteins expressed by unicelular and multicellular organisms which are known to play a key role in cell

signaling pathways, activation of dendritic cells, cell division and apoptosis (van Hemert et al., 2001; Weidner et al., 2016). Within parasites, they have been identified and isolated from protozoans and helminths and recognised at the host-parasite interface (Victor et al., 2012). In a previous study, the 14-3-3 proteins were localized at the gut, tegument, reproductive organs and excretory-secretory products in the juvenile and adult stage of *F. gigantica*, and showed cross-reactivity with 14-3-3 proteins of other trematodes (Chaithirayanon et al., 2006). Later on, these proteins were seen to be associated with the surface and outer structures and oral sucker, secretion extracts and cell cycle control of newly excysted juveniles (NEJs) of *F. hepatica* (Robinson et al., 2009; Hernández-González et al., 2010; De la Torre-Escudero et al., 2011). These data suggest that the parasite 14-3-3 proteins might play an important role at the early stage of *F. hepatica* infection.

The involvement of the 14-3-3z during the early and late stage of *F. hepatica* infection still needs to be fully elucidate. There are closelyrelated proteins which have shown immunomodulation capacity in ruminants infected with *Fasciola* sp. A recent study (Tian et al., 2018) reported that a recombinant 14-3-3e from *Fasciola gigantica* with 100% identity to 14-3-3 from *F. hepatica* stimulated IL-10 and TGF-β production by PBMCs and was also related to nitric oxide release and

cell apoptosis. Moreover, it has been shown that immature flukes induce apoptosis of peritoneal leucocytes and eosinophils during the early stage of infection in sheep (Escamilla et al., 2016, 2017). In addition, there is further evidence that during the acute stage of infection with *F. hepatica* in sheep, when immature liver flukes burrow and migrate through the liver parenchyma, a polarised Th2 local immune response occurs in liver and hepatic lymph nodes (Pacheco et al., 2017).

The 14-3-3 protein family has been tested as prospective diagnostic or vaccine candidates (Lally et al., 1996; Schechtman et al., 2001; Siles-Lucas et al., 2007; Yang et al., 2016), though few reports are available about their use as vaccines against parasitic infections. Meng et al. (2012) used 14-3-3 proteins against the infection with Toxoplasma gondii and observed a protective immune response and the induction of a Th1 pathway profile in vaccinated mice, whereas Yang et al. (2016) reported high levels of Th1 cytokines and a significant protection in animals infected with *Trichinella spiralis*. In addition, Siles-Lucas et al. (2003) also obtained a significant protection against the infection with *Echinococcus multilocularis* in primarily infected mice, after vaccination with the protein 14-3-3 zeta (14-3-3z) isoform of the parasite. In trematode infections, similar results were recorded by Zhang et al. (2001); Siles-Lucas et al., (2007) and Uribe et al. (2007) in mice

vaccinated with the 14-3-3z isoform of *S. japonicum* and of *Schistosoma bovis* and infected with *S. japonicum*, *S. mansoni* and *S. bovis*, respectively, in terms of reduction of parasite burden in vaccinated animals. Therefore, there is evidence to support that vaccination with 14-3-3z proteins may elicit significant protection against parasite plathyhelminths.

The aim of this study was to evaluate protection against *F. hepatica* infection in sheep immunised with the 14-3-3z of *F. hepatica*, by assessing the fluke burden, the hepatic damage and the humoral immune response. This is the first report of the use of 14-3-3z protein as vaccine candidate against the infection with *F. hepatica* in sheep.

Material and Methods

Isolation and sequencing of the Fasciola hepatica 14-3-3z protein

Total RNA was extracted from a *F. hepatica* adult worm collected from a naturally infected cow at the Coreses slaughterhouse (Zamora, Spain) with the RNeasy Minikit (Quiagen, Spain), following the manufacturer's instructions. Total RNA was then subjected to reverse transcription with the First Strand cDNA Synthesis Kit (Roche, Spain). cDNA was then subjected to PCR with the following primers: Fh1433zFwd (5´-gg*gaattc*ccATGTCGCCGTGCTGGTTGAC) and Fh1433zRev (5´-

gg*ctcgac*TTACTTGTCACCAGCATCAAC), containing the specific 14-3-3z sequence (GenBanck accession number MG518623) and the adaptors including restriction sites for *Eco*RI and *Xho*I. Primers were designed on the previously reported *F. gigantica* 14-3-3z sequence (GenBank accession number AY878648.1; Chaithirayanon et al., 2006), to cover the full length coding sequence, including the start and stop codons. PCR was performed in 35 cycles at 94°C for 30 sec, 48°C for 30 sec and 72°C for 40 sec, followed by a final extension step at 72°C for 5 min. PCR product was electrophoresed in a 1% agarose gel in TBE together with the molecular weight markers (50 bp DNA Ladder; Thermo Fischer Scientific, Spain) and stained with ethidium bromide. The band at the expected molecular weight (777 base pairs) was excised and purified with the PureLink® Quick Gel Extraction Kit (Thermo Fischer Scientific, Spain). The purified band and the expression vector pGEX4T2 (GE Healthcare, Spain) were doubled digested with *Eco*RI and *Xho*I (Thermo Fischer Scientific, Spain), purified from an agarose gel as above-mentioned, and ligated with T4 DNA ligase (Thermo Fischer Scientific, Spain). The ligation reaction was used to transform competent *Escherichia coli* BL21 cells (GE Healthcare, Spain). Cells were grown overnight in agar plates containing ampicillin, transformants were selected and used for protein expression, as described in Siles-

Lucas et al. (2000). Protein purity and integrity after thrombin cleavage was checked in a 12% acrylamide gel stained with Coomassie blue, were a band of the expected molecular weight (25.3 kDa) was found (data not shown).

Vaccine Preparation

The 14-3-3z recombinant protein of *F. hepatica* (rFh14-3-3z) was diluted in Montanide™ ISA 71VG adjuvant (Seppic®). Each immunisation dose was prepared as follows: 100µg of rFh14-3-3z was mixed with 100µg of Montanide reaching a final volume of 1.5 ml per dose.

Animals

Twenty-four female Merino-breed sheep 6 months old were used for the study. Prior to commencing the experimental trial, animals were individually identified using Avid Friendchip® microchips (Avid Identification Systems Inc, CA, USA), orally administered Diclazuril (Rumicox®, Esteve) and Ivermectin (Ivomec®, Merial) and confirmed to be free of liver fluke infection by faecal analyses and ELISA for *F. hepatica* specific antibodies. Sheep were housed in covered pens and fed daily with hay and commercial pelleted ration.

Experimental design

Sheep were randomly allocated into three groups of eight animals each. Animals from group 1 were immunised with the recombinant antigen rFh14-3-3z in Montanide adjuvant (Montanide™ ISA 71VG -Seppic®). Group 2 was immunised with Montanide adjuvant (adjuvant control group) and animals from group 3 were not immunised and remained as positive infection control. Immunisation was carried out subcutaneously on two occasions at intervals of four weeks.

Eight weeks after first immunisation all animals were orally challenged with a single dose of 150 metacercariae of *F. hepatica* (Ridgeway Research Ltd., St Briavels, UK) within a gelatine capsule using a bolus dosing gun. Fifteen weeks post-infection, sheep were euthanised by an intravenous injection of T61® (Intervet, Barcelona, Spain). This experiment was performed in accordance to the University of Cordoba Bioethics Committee (N. 7119) and European (86/609/CEE) and Spanish Directives (RD 223/1988).

Pathological methods

At necropsy, the liver was photographed on the visceral and diaphragmatic surface for gross evaluation, and liver pathology was assessed according to the following score (Table 1). Tissue samples from

the left and right hepatic lobe were collected and fixed in 10% buffered formalin, embedded in paraffin wax and sections were stained with the haematoxylin eosin method for histopathological evaluation.

Table 1.

Score for gross pathology assessment of hepatic damage in sheep.

Score	Pathology
0	Absolutely no pathology evident- liver normal colour, consistency and no visible signs of fluke lesions
1	Small areas of scar tissue and lesions, <5% of the liver affected.
2	Moderate areas of scar tissue and lesions, occurring in 5-10% of the liver.
3	Moderate areas of scar tissue, thickening of bile ducts, small to moderate areas of necrosis, pus, 10-20% of liver affected
4	Moderate to large areas of scar tissue, thickened bile ducts evident. Moderate areas of necrosis, pus, haemorrhage. 20-30% of liver affected.
5	Large areas of scar tissue, thickened bile ducts. Multiple necrotic foci, pus, haemorrhage, severe degeneration and >30% of the total liver affected.

Parasitological methods

To assess egg output, faecal samples were individually collected weekly from week six after challenge until the end of the trial, and were analysed by a zinc sulphate-based flotation method using the McMaster chamber. During necropsy, the gallbladder was opened and liver was dissected, bile ducts were cut and opened and all flukes were collected, counted and

measured. The liver was cut into small pieces and placed into warm water (40 ºC) for 30 min to collect remaining flukes.

Antibody detection

Blood samples were taken and plasma was collected to detect specific antibodies against rFh14-3-3z by ELISA. Briefly, 96 wells ELISA plates were coated (100 µl/well) with 5µg/ml of rFh14-3-3z for IgG1 or 10µg/ml for IgG2, diluted in 0.05 M carbonate–bicarbonate buffer pH 9.6 and incubated at 37 °C overnight. After 5 washes with phosphate buffer saline (PBS) 0.05% Tween 20, plates were blocked with 100µl/well of blocking buffer containing 1% BSA diluted in PBS and incubated at 37 °C for 30 min. To detect IgG1, wells were washed and 100 µl/well of plasma diluted in blocking buffer was added and incubated at 37 °C for 30 min. Triple serial dilutions were performed to determine endpoint titre. Similarly, IgG2 was detected by adding 100 µl/well of plasma diluted at 1:10 and at 1:25. After washing, 100 µl/well of primary antibody diluted 1:5000 (mouse anti-bovine IgG1 and anti-bovine IgG2; 7500820 - 7500830 Cedi-Diagnostics), in blocking buffer was added and incubated at 37 °C for 30 min. After incubation, wells were washed and anti-mouse IgG-HRP was added at 37 °C for 30 min (AbD-Serotec, STAR13B). Plate was washed and 100 µl/well of Tetramethylbenzidine

(TMB-Sigma) were added and incubated for 10 minutes at room temperature. The reaction was stopped by adding of 100 µl/well of 1 M sulfuric acid and optical density was measured at 450 nm using a microplate photometer (Multiskan^{TM} FC, Thermo Scientific). Results are shown as antibody titre – $\log_{10}$ – for IgG1, and as optical density for IgG2.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). When significant differences were found, comparisons between groups were made using the Mann–Whitney *U* test for non-parametric distributions. Weekly data from each group was analysed by comparing each time-point values to pre-immunisation values. To assess the correlation between fluke burden and faecal egg counts, a Spearman correlation coefficient was performed. *P* values of 0.05 or lower were considered statistically significant.

Results

Parasitological results

The analysis of faecal egg output did not show significant differences between groups. (Fig. 1). Eggs were first detected at 8 wpi in group 1 and 9 wpi in group 2 and 3, and all infected animals of the trial were positive with egg detection by the eleventh week post-infection.

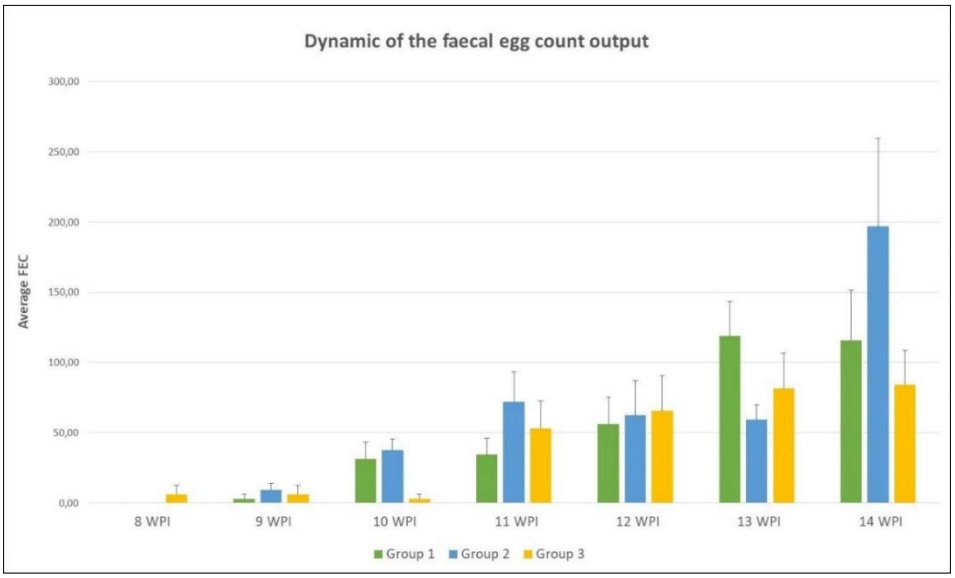

Fig. 1. Faecal egg output during the course of infection. Columns show the mean values of egg counts per group. Bars represent standard error. WPI, Weeks post-infection.

The mean fluke burden and mean length of flukes are shown in Table 2. Fluke burden was 52.25±16.71, 68.13±23.79 and 51.13±12.84 for group

one, two and three, respectively, this means an implantation rate of 34.83±11.14, 45.42±15.86 and 34.08±8.56.

Table 2.

Fluke burden of immunised and control groups 15 weeks after challenge with F. hepatica metacercariae. Weight is expressed in grams and represents the total weight of the fluke burden per group. ± expresses standard deviation.

Group	Fluke burden	Implantation rate	Length/Width (cm)	Weight (grams)
Group 1 (rFh14-3-3z + Adjuvant)	52.25±16.71	34.83±11.14	2.11±0.20/0.87±0.18	7.04±2.96
Group 2 (Adjuvant control)	68.13±23.79	45.42±15.86	2.11±0.28/0.79±0.16	5.58±4.33
Group 3 (Infection control)	51.13±12.84	34.08±8.56	1.89±0.32/0.86±0.15	6.00±1.40

Although individual variation was high in all groups, statistical analyses showed no significant difference between groups. This result indicates that no reduction of fluke burden was observed in any of the vaccinated groups. Faecal egg count is shown in Fig. 1. The correlation coefficient between fluke burden and faecal egg count from each group in week 14 post-infection was of 0.09 for group 1 and of 0.3 for groups 2 and 3 and showed no significant differences.

Antibody response to rFh14-3-3z

Dynamics of plasma levels of specific anti-rFh14-3-3z IgG1 and IgG2 is presented in Fig. 2 and 3, respectively. All animals immunised with the recombinant antigen (group 1) developed an IgG1-IgG2 antibody response following immunisation. A sharp and significant increase in IgG1 was detected three weeks after immunisation, reaching the maximun value and showing a gradual decrease from that onwards ($P < 0.05$) (Fig. 2). For IgG2, a limited but significant production ($P < 0.05$) was observed only after vaccination in group 1 (Fig. 3).

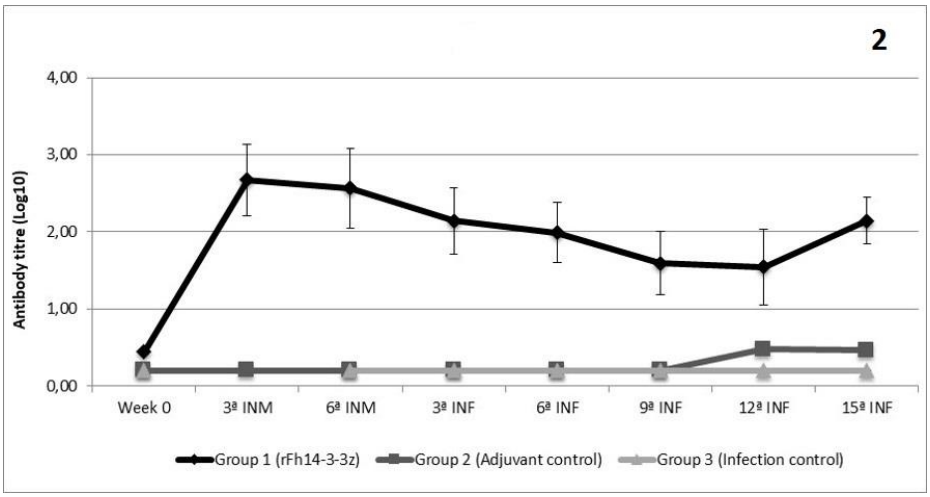

Fig. 2. Plasmatic levels of specific anti-rFh14-3-3z IgG1. Each point represents mean values of antibody titre log10. Bars at each point shows standard error.

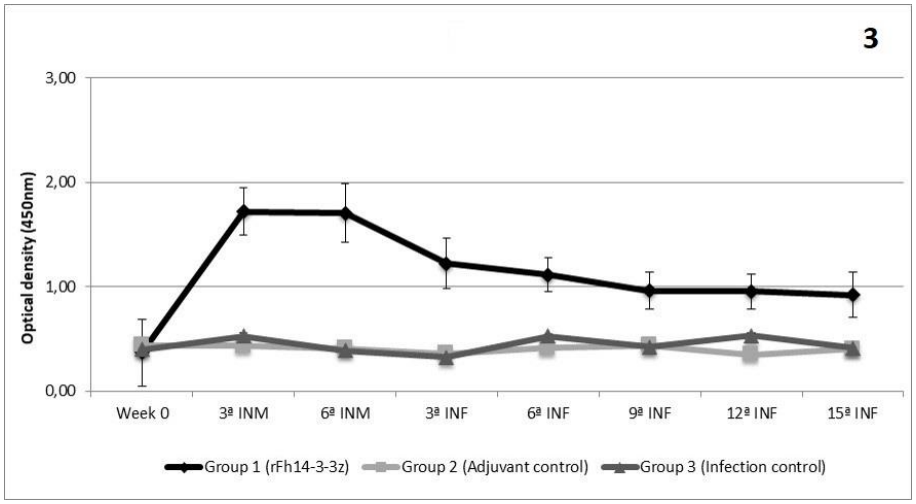

Fig. 3. Plasmatic levels of specific anti-rFh14-3-3z IgG2. Each point represents mean values of optical density measured at 450 nm. Bars at each point shows standard error.

In contrast, no statistically significant rise in the specific antibody level of neither IgG1 nor IgG2 was detected in the immunised animals (group 1) after the experimental challenge. Comparison between groups showed that no production of specific anti-rFh14-3-3z antibodies was detected in animals from groups 2 and 3 during the trial.

Pathological results

Gross hepatic lesions were similar in all groups and consisted of fibrous scars, tortuous whitish tracts and patches over the liver surface, mainly located in the left hepatic lobe (Fig. 4). Bile ducts were whitish and enlarged. Individual analysis of the liver pathology within each group

showed a mean score of 4 for groups 1 and 2, and a mean score of 4.5 for group 3. No statistical differences were detected between groups. Histopathological changes were similar in the three infected groups and consisted of bile duct hyperplasia, fibrosis in inflammatory infiltration of eosinophils, lymphocytes and plasma cells in portal areas, granulomas with necrotic center surrounded by macrophages, lymphocytes and eosinophils, fibrotic areas in the hepatic parenchyma with variable amount of inflammatory infiltrate composed of eosinophils, lymphocytes, plasma cells and macrophages, some of them containing haemosiderin pigment.

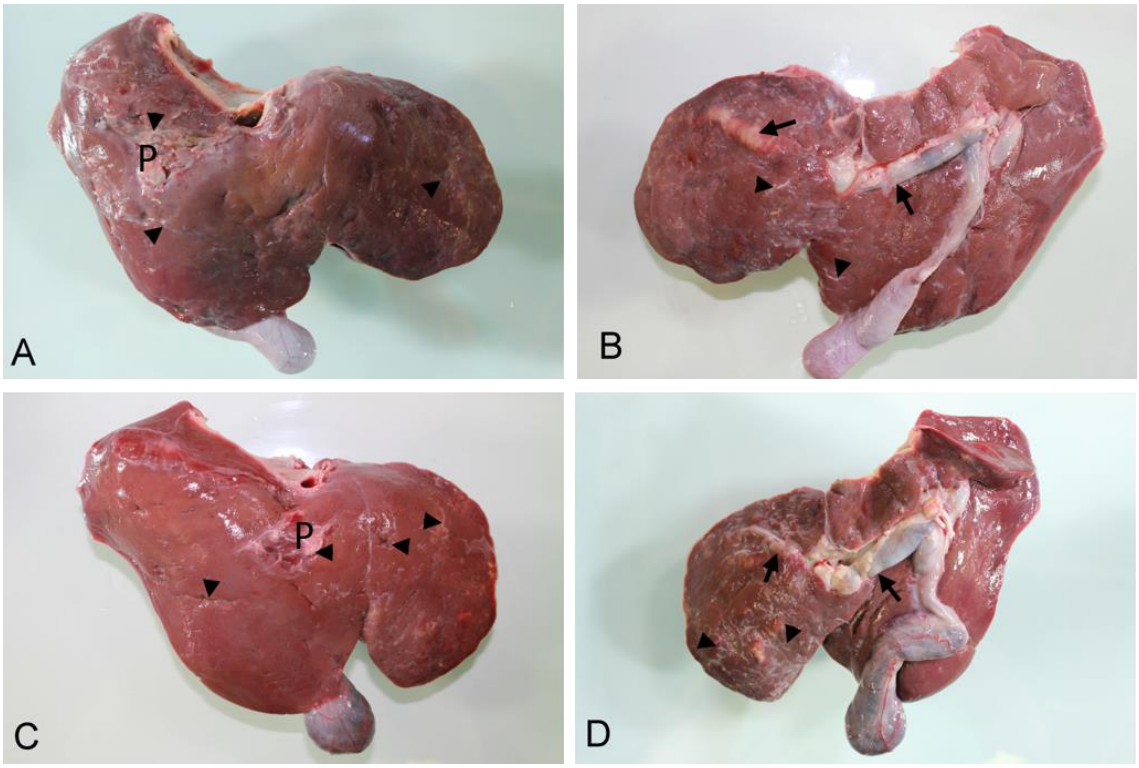

Fig. 4. Severe tortuous whitish tracts and scars (arrowheads) and patches (P) on the diaphragmatic and visceral surface of livers from group 1 (A–B) and group 3 (C–D). Bile ducts are enlarged and whitish (arrows).

Discussion

It is well known that sheep is the most susceptible animal species among ruminants to liver fluke infection, and many studies aimed at stimulating a protective immune response have been conducted in this host (Piacenza et al., 1999; Almeida et al., 2003; Maggioli et al., 2011; Wesołowska et al., 2016; Pacheco et al., 2017). We here report the results of the first attempt to use recombinant 14-3-3z protein as vaccine against the

infection with *F. hepatica* in sheep. This protein family has previously been assessed in vaccination trials against helminth infections with promising results (Siles-Lucas et al., 2003, 2007; Uribe et al., 2007; Zhang et al., 2001), though no data is available about the use of the 14-3-3z against the liver fluke infection in ruminants. In this study, the analyses of the parasitological and pathological results, in terms of fluke burden and hepatic damage, indicate that sheep immunised with rFh14-3-4z protein in Montanide™ ISA 71 V G adjuvant (Seppic®) developed no significant protection against the experimental challenge. Vaccinated animals showed a similar implantation rate and fluke burden when compared with the control groups.

Consistent with these results, no statistical differences between groups were observed in neither faecal egg output nor fluke size. The analyses of the parasitological parameters showed a weak positive correlation between fluke burden and faecal egg counts in all groups, which lies along the same line of previous studies carried out in sheep (Duménigo et al., 1999) and cattle (Radfar et al., 2015) in which these two variables were seen to show a positive linear correlation.

On the other hand, vaccination produced a strong humoral immune response which consisted of high level of rFh14-3-3z-specific IgG1-IgG2, being IgG1 the predominant isotype. This observation confirms

the immunogenicity of the 14-3-3 proteins in sheep and is in line with the results of previous studies. Schechtman et al. (2001) and Siles-Lucas et al. (2003) observed a high production of specific IgG1 and IgG2 after vaccination with 14-3-3 in mice, whereas recent studies showed that anti-14-3-3 antibodies were detectable as early as one week after vaccination (Yang et al., 2016) and for up to eight months post-vaccination (Lampe et al., 2017). Interestingly, no boosting effect on the antibody level was observed in the vaccinated animals after the experimental infection, likewise experimental challenge did not elicit antibody production in the unvaccinated animals. This lack on the production of a humoral immune response against 14-3-3 proteins in only-infected animals observed in our study was previously described by Siles-Lucas et al. (2003) and Uribe et al. (2007) in primarily infected mice with *E. multilocularis* and *S. bovis*, respectively; in which the specific antibodies were detected at a very low level or not detected. On the contrary, other studies reported a significant production of specific anti-14-3-3 antibodies in unvaccinated and infected animals with *S. mansoni* and *S. japonicum* (Schechtman et al., 2001; Qian et al., 2012). These contrasting findings suggest that the production of specific 14-3-3 immunoglobulins may vary not only between the different helminths infections but also among parasite species belonging to the same genus, which is bewildering as

14-3-3 proteins are highly conserved among different species (Schechtman et al., 2001). Another reason for the absence of specific antibody production might be due to a difference in the nature of the native and the recombinant protein or in the conformational epitopes which are being or not being recognised by the different animals of the trial, since antibodies against the recombinant protein were detected in vaccinated sheep but not in infected-control animals. To date, no studies are available about the use of the native form of *F. hepatica* 14-3-3 protein hence, there is a lack of knowledge about the immunogenic capacity of the native Fh14-3-3; and few epitope mapping studies have been conducted in *F. hepatica* infections using immunodominant proteins. Sexton et al., (1994) observed a different level in the humoral immune response to peptides of the FhGST protein in vaccinated and infected sheep with *F. hepatica*, whereas a recent study showed that vaccinated cattle developing significant resistance against the infection produced antibodies against different peptides of the whole FhCL1 protein in protected and non-protected animals (Garza-Cuartero et al., 2018).

One of the key indicators of vaccine protection is the extent of hepatic damage produced by the liver flukes (Mendes et al., 2010; Pérez-Ecija et al., 2010). Our results indicate that the majority of animals showed a

severely extensive damage and no significant differences were detected between vaccinated and control groups. This finding, together with the lack of fluke burden reduction observed in the vaccinated animals suggest that vaccination with rFh14-3-3z, which elicited significant levels of protection against other helminth infections in murine models (Zhang et al., 2001; Siles-Lucas et al., 2003; Uribe et al., 2007; Yang et al., 2016) is not an appropriate vaccine candidate against *F. hepatica* infection in sheep.

The failure in producing a protective immune response observed in our study is consistent with some previous vaccination trials reported by our group in which the immunisation with single antigens in goats did not induce reduction in the number of recovered flukes (Buffoni et al., 2010; Zafra et al., 2010; Buffoni et al., 2012). Similarly, Maggioli et al. (2016) also observed no significant reduction in fluke burden when using single antigens against the infection with *F. hepatica* in cattle. In contrast, some previous studies reported the development of significant protection when single peptides or proteins were used as vaccines in cattle (Golden et al., 2010) and sheep (Piacenza et al., 1999; Villa-Mancera and Méndez-Mendoza, 2012). It is possible that the protection failure in the vaccinated animals observed in this study might be attributed to several factors such as: the 14-3-3z proteins do not behave as immunodominant

antigens or do not play a key role at the host-parasite interface in sheep fasciolosis, as seen by the absence of antibody production in the infected animals; the humoral immune response produced in the vaccinated animals was not strong enough or the antibodies lacked sufficient avidity and, hence were not able to neutralise the 14-3-3z-specific epitopes. It is worth to acknowledge that studies on the cellular immune response are also required to further explain this lack of protection.

Conclusions

Administration of a double dose of the rFh14-3-3z in Montanide adjuvant did not developed reduction in liver fluke burden nor hepatic damage in sheep challenged with 150 metacercariae of *F. hepatica*. The rFh14-3-3z was highly immunogenic as was demonstrated by the significant production of high level of anti-rFh14-3-3z IgG-IgG2, being IgG1 the predominant subclass. Non vaccinated and infected animals failed at producing specific antibodies, indicating a possible minor role of the Fh14-3-3z proteins during host-parasite interface. The vaccination protocol used in this study is not suitable for developing significant protection against the infection with *F. hepatica* in sheep. Since this is the first report of this protein as vaccine candidate against the infection with liver fluke in large animals, further studies using other animal

models or following different immunisation protocols are still required to finally conclude the potencial capacity of the rFh14-3-3z proteins as vaccine.

Conflict of interest statement

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

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References

Almeida, M.S., Torloni, H., Lee-Hoo, P., Vilar, M.M., Thaumaturgo, N., Simpson, A.J., Tendler, M., 2003. Vaccination against *Fasciola hepatica* infection using a *Schistosoma mansoni* defined recombinant antigen, Sm14. Parasite Immunol. 25, 135–137.

Alvarez-Sanchez, M.A., Mainar-Jaime, R.C., Perez-Garcia, J., Rojo-Vazquez, F.A., 2006. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet. Rec. 159, 424–425.

Buffoni, L., Zafra, R., Pérez-Écija, A., Martínez-Moreno, F.J., Martínez-Galisteo, E., Moreno, T., Pérez, J., Martínez-Moreno, A., 2010. Immune response in goats immunised with glutathione S-transferase and experimentally challenged with *Fasciola hepatica*. Parasitol. Int. 59, 147–153.

Buffoni, L., Martínez-Moreno, F.J., Zafra, R., Mendes, R.E., Pérez-Écija, A., Sekiya, M., Mulcahy, G., Pérez, J., Martínez-Moreno, A., 2012. Humoral immune response in goats immunised with cathepsin L1, peroxiredoxin and Sm14 antigen and experimentally challenged with *Fasciola hepatica*. Vet. Parasitol. 185, 315–321.

Chaithirayanon, K., Grams, R., Vichasri-Grams, S., Hoffman, A., Korge, G., Viyanant, V., Upatham, E.S., Sobhon, P.O., 2006. Molecular and immunological characterization of encoding gene and 14-3-3 protein in *Fasciola gigantica*. Parasitol. 133, 763–775.

Dalton, J.P., Mulcahy, G., 2001. Parasite vaccines–a reality? Vet. Parasitol. 98, 149–167.

De la Torre-Escudero, E., Manzano-Román, R., Valero, L., Oleaga, A., Pérez-Sánchez, R., Hernández-González, A., Siles-Lucas, M., 2011. Comparative proteomic analysis of *Fasciola hepatica* juveniles and *Schistosoma bovis* schistosomula. J. Proteomics 74, 1534–1544.

Duménigo, B.E., Espino, A.M., Finlay, C.M., Mezo, M., 1999. Kinetics of antibody-based antigen detection in serum and faeces of sheep experimentally infected with *Fasciola hepatica*. Vet. Parasitol. 86, 23–31.

Escamilla, A., Bautista, M.J., Zafra, R., Pacheco, I.L., Ruiz, M.T., Martínez-Cruz, S., Méndez, A., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2016. *Fasciola hepatica* induces eosinophil apoptosis in the migratory and biliary stages of infection in sheep. Vet. Parasitol. 216, 84–88.

Escamilla, A., Pérez-Caballero, R., Zafra, R., Bautista, M.J., Pacheco, I.L., Ruiz, M.T., Martínez-Cruz, S., Martínez-Moreno, A., Molina-

Hernández, V., Pérez, J., 2017. Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infection in sheep. Vet. Parasitol. 238, 49–53.

Garza-Cuartero, L., Geurden, T., Mahan, S.M., Hardham, J.M., Dalton, J.P., Mulcahy, G., 2018. Antibody recognition of cathepsin L1-derived peptides in *Fasciola hepatica*-infected and/or vaccinated cattle and identification of protective linear B-cell epitopes. Vaccine 36, 958–968.

Golden, O., Flynn, R.J., Read, C., Sekiya, M., Donnelly, S.M., Stack, C., Dalton, J.P., Mulcahy, G., 2010. Protection of cattle against a natural infection of *Fasciola hepática* by vaccination with recombinant cathepsin L1 (rFhCL1). Vaccine 28, 5551–5557.

Hernández-González, A., Valero, M.L., Sánchez del Pino, M., Oleaga, A., Siles-Lucas, M., 2010. Proteomic analysis of in vitro newly excysted juveniles form *Fasciola hepatica*. Mol. Biochem. Parasitol. 172, 121–128.

Lally, N.C., Jenkins, M.C., Dubey, J.P., 1996. Development of a polymerase chain reaction assay for the diagnosis of neosporosis using

the *Neospora caninum* 14-3-3 gene. Mol. Biochem. Parasitol. 75, 169–178.

Lampe, K., Gottstein, B., Becker, T., Stahl-Hennig, C., Kaup, F.J., Mätz-Rensing, K., 2017. Immunization of rhesus macaques with *Echinococcus multilocularis* recombinant 14-3-3 antigen leads to specific antibody response. Parasitol. Res. 116, 435–439.

Maggioli, G., Acosta, D., Silveira, F., Rossi, S., Giacaman, S., Basika, T., Gayo, V., Rosadilla, D., Roche, L., Tort, J., Carmona, C., 2011. The recombinant gut-associated M17 leucine aminopeptidase in combination with different adjuvants confers a high level of protection against *Fasciola hepatica* infection in sheep. Vaccine 29, 9057–9063.

Maggioli, G., Bottini, G., Basika, T., Alonzo, P., Salinas, G., Carmona, C., 2016. Immunization with *Fasciola hepatica* thioredoxin glutathione reductase failed to confer protection against fasciolosis in cattle. Vet. Parasitol. 224, 13–19.

Mendes, R.E., Pérez-Ecija, R.A., Zafra, R., Buffoni, L., Martínez-Moreno, A., Dalton, J.P., Mulcahy, G., Pérez, J., 2010. Evaluation of

hepatic changes and local and systemic immune responses in goats immunized with recombinant Peroxiredoxin (Prx) and challenged with *Fasciola hepatica*. Vaccine 28 (16), 2832–2840.

Meng, M., He, S., Zhao, G., Bai, Y., Zhou, H., Cong, H., Lu, G., Zhao, Q., Zhu, X.Q., 2012. Evaluation of protective immune responses induced by DNA vaccines encoding *Toxoplasma gondii* surface antigen 1 (SAG1) and 14-3-3 protein in BALB/c mice. Parasit. Vectors 5, 273.

Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in the Netherlands. Vet. Parasitol. 91, 153–158.

Novobilský, A., Höglund, J., 2015. First report of closantel treatment failure against *Fasciola hepatica* in cattle. Int. J. Parasitol. Drugs Drug Resist. 5, 172–177.

Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Vet. Parasitol. 178, 364–366.

Pacheco, I.L., Abril, N., Morales-Prieto, N., Bautista, M.J., Zafra, R., Escamilla, A., Ruiz, M.T., Martínez-Moreno, A., Pérez, J., 2017. Th1/Th2 balance in the liver and hepatic lymph nodes of vaccinated and unvaccinated sheep during acute stages of infection with *Fasciola hepatica*. Vet. Parasitol. 238, 61–65.

Pérez-Ecija, R.A., Mendes, R.E., Zafra, R., Buffonni, L., Martínez-Moreno, A., Pérez, J., 2010. Pathological and parasitological protection in goats immunised with recombinant cathepsin L1 and challenged with *Fasciola hepatica*. Vet. J. 185, 351–353.

Piacenza, L., Acosta, D., Basmadjian, I., Dalton, J.P., Carmona, C., 1999. Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. Infect. Immun. 67, 1954–1961.

Qian, C.Y., Wang, J., Yu, C.X., Yin, X.R., Song, L.J., Zhang, W., Jin, Y., Ke, X.D., 2012. Characterization of IgG responses of rabbits to Sj14-3-3 protein after experimental infection with *Schistosoma japonicum*. Parasitol. Res. 111, 2209–2211.

Radfar, M.H., Nourollahi-Fard, S.R., Mohammadyari, N., 2015. Bovine fasciolosis: prevalence, relationship between faecal egg count and worm burden and its economic impact due to liver condemnation at Rudstar abattoir, Northern Iran. J. Parasit. Dis. 39, 522–525.

Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P., Ranganathan, S., 2009. An integrated transcriptomic and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*. Mol. Cell. Proteomics 8, 1891–1907.

Schechtman, D., Tarrab-Hazdai, R., Arnon, R., 2001. The 14-3-3 protein as a vaccine candidate against schistosomiasis. Parasite Immunol. 23, 213–217.

Sexton, J.L., Wilce, M.C., Colin, T., Wijffels, G.L., Salvatore, L., Feil, S., Parker, M.W., Spithill, T.W., Morrison, C.A., 1994. Vaccination of sheep against *Fasciola hepatica* with glutathione S-transferase. Identification and mapping of antibody epitopes on a three-dimesional model of the antigen. J. Immunol. 152, 1861–1872.

Siles-Lucas, M., Nunes, C.P., Zaha, A., Breijo, M., 2000. The 14-3-3 protein is secreted by the adult worm of *Echinococcus granulosus*. Parasite Immunol. 22, 521–528.

Siles-Lucas, M., Merli, M., Mackenstedt, U., Gottstein, B., 2003. The *Echinococcus multilocularis* 14-3-3 protein protects mice against primary but not secondary alveolar echinococcosis. Vaccine 21, 431–439.

Siles-Lucas, M., Uribe, N., López-Abán, J., Vicente, B., Orfao, A., Nogal-Ruiz, J.J., San Feliciano, A., Muro, A., 2007. The *Schistosoma bovis* Sb14-3-3ζ recombinant protein cross-protects against Schistosoma mansoni in BALB/c mice. Vaccine 25, 7217–7223.

Tian, A.L., Lu, M.M., Calderón-Mantilla, G., Petsalaki, E., Dottorini, T., Tian, X.W., Wang, Y., Huang, S.Y., Hou, J.L., Li, X., Elsheikha, H.M., Zhu, X.Q., 2018. A recombinant *Fasciola gigantica* 14-3-3 epsilon protein (rFg14-3-3e) modulates various functions of goat peripheral blood mononuclear cells. Parasit. Vectors 11:152, 1–14.

Uribe, N., Siles-Lucas, M., López-Abán, J., Esteban, A., Suarez, L., Martínez-Fernández, A., del Olmo, E., Muro, A., 2007. The Sb14-3-3ζ recombinant protein protects against *Schistosoma bovis* in BALB/c mice. Vaccine 25, 4533–4539.

Van Hemmert, M.J., Steensma, H.Y., Van Heusden, G.P., 2001. 14-3-3 proteins: key regulators of cell division, signaling and apoptosis. Bioessays 23, 936–946.

Venturina, V.M., Alejandro, M.A., Baltazar, C.P., Abes, N.S., Mingala, C.N., 2015. Evidence of *Fasciola* spp. resistance to albendazole, triclabendazole and bromofenofos in water buffaloes (*Bubalus bubalis*). Ann. Parasitol. 61, 283–289.

Victor, B., Kanobana, K., Gabriël, S., Polman, K., Deckers, N., Dorny, P., Deelder, A.M., Palmblad, M., 2012. Proteomic analysis of *Taenia solium* metacestode excretion−secretion proteins. Proteomics 12, 1860–1869.

Villa-Mancera, A., Méndez-Mendoza, M., 2012. Protection and antibody isotype responses against *Fasciola hepatica* with specific

antibody to pIII-displayed peptide mimotopes of cathepsin L1 in sheep. Vet. J. 194, 108–112.

Villa-Mancera, A., Reynoso-Palomar, A., Utrera-Quintana, F., Carreón-Luna, L., 2014. Cathepsin L1 mimotopes with adjuvant Quil A induces a Th1/Th2 immune response and confers significant protection against *Fasciola hepatica* infection in goats. Parasitol. Res. 113, 243–250.

Weidner, J.M., Kanatani, S., Uchtenhagen, H., Varas-Godoy, M., Schulte, T., Engelberg, K., Gubbels, M.J., Sun, H.S., Harrison, R.E., Archour, A., Barragan, A., 2016. Migratory activation of parasitized dendritic cells by the protozoan *Toxoplasma gondii* 14-3-3 protein. Cell. Microbiol. 18, 1537–1550.

Wesołowska, A., Zawistowska-Denisiak, A., Norbury, L.J., Wilkowski, P., Januszkiewicz, K., Pyziel, A.M., Zygner, W., Wedrychowicz, H., 2016. Immune responses in rats and sheep induced by a DNA vaccine containing the phosphoglycerate kinase gene of *Fasciola hepatica* and liver fluke infection. Acta Parasitol. 61, 212–220.

Yang, J., Zhu, W., Huang, J., Wang, X., Sun, X., Zhan, B., Zhu, X., 2016. Partially protective immunity induced by the 14-3-3 protein form *Trichinella spiralis*. Vet. Parasitol. 231, 63–68.

Zafra, R., Pérez-Écija, R.A., Buffoni, L., Mendes, R.E., Martínez-Moreno, A., Martínez-Moreno, F.J., Pérez, J., 2010. Evaluation of hepatic damage and local immune response in goats immunized with native glutathione S-transferase of *Fasciola hepatica*. J. Comp. Pathol. 143, 1101–1119.

Zhang, Y.B., Taylor, M.G., Johansen, M.V., Bickle, Q.D., 2001. Vaccination of mice with a cocktail DNA vaccine induces a Th1-type immune response and partial protection against *Schistosoma japonicum* infection. Vaccine 20, 724–730.

SEGUNDO ESTUDIO

Comparative dynamics of peritoneal cell immunophenotypes in sheep during the early and late stages of the infection with *Fasciola hepatica* by flow cytometric analysis

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Abstract

Background: The peritoneal cell populations (PCP) are thought to play a crucial role during the early immune response in Fasciola hepatica infection while newly excysted juveniles (NEJ) are migrating in the peritoneal cavity (PC) towards the liver. In this study, we aimed to determine the immunophenotypes of the PCP and to analyse the dynamics of the recruitment of the PCP during the early and late stage of the infection in sheep infected with F. hepatica.

Methods: Thirty-seven sheep were divided into three groups: Group 1 (n = 20) and 2 (n = 10) were challenged with F. hepatica, Group 3 (n = 7) was not infected and remained as uninfected control (UC). After the slaughtering, peritoneal lavages were carried out to isolate peritoneal cell populations at 1, 3, 9 and 18 days post-infection (dpi) for Group 1 and at 14 weeks post-infection (wpi) for Group 2 and 3. Flow cytometry was conducted to assess the dynamics of peritoneal cavity cell populations.

Results: TCD4 cells showed a significant decrease at 1 and 18 dpi when compared to UC; no statistical differences were detected for TCD8 and $WC1^{+}\gamma\delta$ during the early stage of the infection with respect to the UC. CD14 cells exhibited a decreasing trend, with a significant decrease at 9 and 18 dpi when compared to the UC. The dynamics of MHCII and CD83 cells showed a similar increasing pattern from 3 to 18 dpi. During

the chronic stage, both TCD4 and TCD8 cells showed no significant differences when compared to the UC, although a slight but statistically significant higher level of $WC1^{+}\gamma\delta$ cells was observed. A lower percentage of antigen-presenting cells (APCs) was detected with respect to the UC.

Conclusions: The recruitment of the lymphocytes subsets did not show a significant increase during the course of the infection and only $WC1^{+}\gamma\delta$ cells displayed a significant increase at the chronic stage. For the CD14, a decreasing trend was observed during the early stage, which was statistically significant at the chronic stage of the infection. Peritoneal CD83 and MHCII cells developed an increasing trend during the early stage of infection, and showed a significant decrease at the late stage of the infection.

Keywords: Flow cytometry, NEJ, *Fasciola hepatica*, Peritoneal cells, Recruitment.

Background

Fasciola hepatica is a globally spread highly pathogenic trematode which mainly occurs in domestic ruminants as a chronic disease and produces major economic losses in terms of production loss and liver condemnation. Fasciolosis has been recognised by the WHO as a re-emerging neglected tropical disease and it is also of public health interest since it causes human infection as a food-borne parasitic disease; it is estimated that 2.4 million people are infected worldwide in over 70 countries [1].

It is well known that natural hosts do not develop an effective acquired resistance against the infection [2] and that anthelmintic treatment is the best means to control the infection. However, chemical residues in food and their impact on the environment [3, 4], as well as drug resistance reported in various countries [5–8], foster the study of new control methods such as vaccine development, although no vaccine formulation is commercially available to date.

The life-cycle of the parasite inside the animal host is complex: after the infection, the newly excysted juveniles (NEJ) penetrate the intestinal wall within the first two hours post-infection, enter the PC and migrate towards the liver, a process that usually takes about four to six days [9]. By the time *F. hepatica* reaches the mature stage inside its final

location in the bile ducts, the disease has become a chronic infection and the immune system of the host has already been affected by the parasite: there is supporting evidence that F. hepatica has the capacity to immunomodulate the host's immune response [10–14].

At the early and late stage of the infection, NEJ and adults worms release a broad variety of antigenic molecules. Some of them include excretory-secretory products which mainly consist of proteins [15], exosome-like vesicles and tegument glycoproteins [16, 17] that may trigger local and systemic immune responses, hence the role of the peritoneal cell population is key for understanding the initial stage of the host-parasite interaction.

In *F. hepatica* infected sheep at the initial stages of the infection, the peritoneal cavity fluid was recently reported to primarily consist of lymphocytes, macrophages and eosinophils, with lymphocytes and macrophages being the predominant cell populations and displaying a varying ratio along the course of infection [18]. Therefore, these two cell populations are considered as the main phenotypes of interest since they are thought to be one of the first immunocompetent cells involved in the early immune response once NEJ reach the PC. In this regard, it has been shown in a murine model that liver fluke NEJ are killed inside the peritoneal cavity [19], and in a previous study it has been observed that

peritoneal macrophages (pMΦ) from rats developed cytotoxic mechanisms against *F. hepatica* NEJ [20]. Recently, it has been confirmed in an in vivo model that migrating *F. hepatica* NEJ causes alternative activation of pMΦ [18] and apoptosis in peritoneal leukocytes in sheep [21].

The aim of this study was to determine the immunophenotype of the PCP using flow cytometry to better comprehend its dynamics during the early and late stage of infection in sheep experimentally challenged with F. *hepatica*.

Methods

Animals

Thirty-seven female Merino-breed sheep, aged 6 months, were used for the study. Before commencing the study, all animals were confirmed to be free of *F. hepatica* infection by faecal analysis and by an in-house developed ELISA using microplates coated with recombinant *F. hepatica* cathepsin L1 (FhCL1) for detection of specific antibodies. Furthermore, all sheep were given Ivermectin (Noromectin®, Karizoo, Barcelona, Spain) and Diclazuril (Rumicox®, Esteve, Barcelona, Spain) in order to exclude potential presence of parasites. An individual clinical monitoring of the animals was conducted during the

trial, which included a weekly clinical examination, and blood sampling for assessment of complete blood counts (data not shown). Simultaneously, faecal samples were taken weekly (from week 0 to week 14 of the trial) for detection of eggs belonging to gastrointestinal worms or *Eimeria* spp. oocysts. In addition, animals were housed in covered pens in clean and healthy conditions in order to avoid the appearance of other pathogens, and were fed daily with hay and commercial pelleted ration.

Experimental design

The study was designed to allow a comparative analysis of animals between the early and late stage of infection with *F. hepatica*, hence sheep were randomly allocated into three groups according to the slaughtering time. Group 1, 2 and 3 consisted of 20, 10 and 7 animals, respectively. At day 0 of the trial, animals from Group 1 (early stage) and 2 (late stage) were experimentally challenged with a single dose of 150 metacercariae of *F. hepatica* of bovine origin (Ridgeway Research Ltd., St Briavels, UK) administered in gelatine capsules, using a dosing gun. The twenty animals from Group 1 were sacrificed in batches of five sheep at each one of the following time points: 1, 3, 9 and 18 days post-infection (dpi). Animals from Group 2 and 3 were slaughtered 14 weeks

post-infection (wpi). Animals from Group 3 were not infected and remained as uninfected control group (UC). All animals were humanly euthanised by an intravenous injection of T61® (Intervet, Barcelona, Spain).

Parasitological methods: egg output and liver fluke burden

Sheep of the late stage of infection study (Group 2) were sampled for detection of egg output and liver fluke burden. From week six post-infection (wpi) onwards, faecal samples were collected weekly from each animal and faecal examinations were performed using a flotation method for detection of *F. hepatica* eggs. In brief, 3 g of faeces were thoroughly mixed with 42 ml of saturated $ZnSO_4$ solution and eggs were counted using a modified McMaster method [22]. Each sample was analysed in duplicate and results were expressed as mean of eggs per gram of faeces (EPG).

During necropsy, the gallbladder and the main bile ducts were opened and the liver was dissected and carefully examined for the presence of liver flukes. The liver was then cut into small pieces and placed into warm water (45 °C) for 30 min to collect remaining flukes which were not observed during bile duct opening. Finally, all liver flukes were counted.

Isolation of peritoneal cavity cells

A peritoneal lavage was conducted immediately after the slaughtering of each of the animals to obtain the peritoneal fluid. First, the ventral area of the abdomen was shaved and disinfected with polyvinylpyrrolidone iodine 10% (AGB, Madrid, Spain). A small incision (1–2 cm) was made in the skin over the midline and subcutaneous tissue was dissected. The *linea alba* and peritoneum were sectioned with blunt scissors to avoid haemorrhage. A 40-cm-long cannula connected to a syringe was inserted into the abdominal cavity and 60 ml sterile DPBS containing 9500 UI of heparin (warmed to 37 °C; Eurotubo®, Deltalab, Madrid, Spain) was injected into the abdominal cavity. After gently massaging the abdomen for 1 min, 40 ml of peritoneal fluid were withdrawn. Then, peritoneal fluid was centrifuged at 2300× *g* for 5 min and the supernatant was discarded. Cell pellets were resuspended again in DPBS and incubated for 15 min in an erythrolysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM disodium EDTA, dH_2O). A second centrifugation step (2300× *g* for 5 min) was performed to eliminate lysed erythrocyte membranes and the pellet was resuspended in 1 ml of medium. After that, the concentration of peritoneal cells was quantified using a Trypan Blue exclusion technique.

The final concentration of the cells was then adjusted to 1×10^6 cells/ml in order to carry out a flow cytometry assay.

Cell population identification and isolation

Different monoclonal antibodies were used to determine the immunophenotype of the peritoneal cavity cell populations in uninfected and infected sheep at 1, 3, 9 and 18 dpi and at 14 wpi by flow cytometry analysis. Main lymphocyte subpopulations were identified separately as TCD4 and TCD8. WC1 was used to select the γδTCR cells. MHC class II was used for identification of APC, and CD14 was used as a marker for macrophages. CD83 was used to identify dendritic cells.

Flow cytometry acquisition was performed with a CyFlow Cube 6® cytometer (4 colours + FSC + SSC; Sysmex-Partec, Barcelona, Spain). Briefly, 200 µl of peritoneal fluid was diluted with 200 µl of phosphate buffered saline (PBS, pH = 7.2) and gently stirred. Afterwards, samples were incubated at 4 °C for 30 min in darkness with the different conjugated antibodies (Table 1) diluted at 1:400, according to the manufacturer's instructions. All antibodies, with the exception of the mouse anti-human CD83, have been previously used for flow cytometry assay in peripheral blood and lymphoid organs of ovines and goats [23, 24]. As negative control antibodies, mouse isotype control

IgG1 and IgG2a [Bio-Rad (formerly AbD-Serotec) Kidlington, UK] were used for FITC and RPE labels, respectively.

Table 1

Monoclonal antibodies used in flow cytometry analysis of peritoneal cavity cell populations.

Peritoneal cell immunophenotypes	Antibodies	Fluorochromes	Clones	References
Isotype controls				
Control FITC	mouse control IgG1	FITC	-	MCA928F
Control RPE	mouse control IgG2a	RPE	-	MCA929PE
Cell populations				
TCD4	mouse anti-sheep CD4	RPE	clone 44.38	MCA2213PE
TCD8	mouse anti-bovine CD8	FITC	clone CC63	MCA837F
WC1$^{+}\gamma\delta$	mouse anti-bovine WC1	FITC	clone CC15	MCA838F
CD14	mouse anti-bovine CD14	FITC	clone CC-G33	MCA2678F
CD83	mouse anti-human CD83	FITC	clone HB15e	MCA1582F
MHCII	mouse anti-bovine MHC class II	FITC	clone IL-A21	MCA2445F

Then, the samples were centrifuged at 2300× *g* for 5 min and the supernatant was discarded. After that, two washes with PBS (centrifuged at 2300× *g* for 5 min) were carried out. Finally, cells were resuspended in PBS before acquisition.

Cells were identified by their morphological features gated as defined by their forward and side-scatter profiles (FSC *vs* SSC dot plot) in order to exclude cellular debris. Briefly, cellular debris were excluded by gating the leukocyte populations of interest based on the FSC-SSC (see panel 2.1 in Additional file 1: Figure S1). Once each of the leukocyte

populations were defined as mentioned (i.e. lymphocytes, macrophages or dendritic cells), 10,000 events were counted and cell identification was then performed. The immunophenotype of interest was identified using the specific fluorescence channel vs SSC dot plots (see panels 3.1 and 3.2 in Additional file 1: Figure S1). For FITC-labelled antibodies, the FL1 channel (green detector, 488 nm laser, 536/40 nm) was used; for CD4-RPE antibody, the FL2 channel (orange detector, 488 nm laser, 590/50 nm) was used. The different peritoneal cell populations from all animals and from each of the slaughtering time points were determined separatedly, in different tubes. Having obtained the number of each immunophenotype, the percentage of the cell subset from the total leukocyte subpopulations was determined (on the basis of 10,000 events).

Results were analysed for changes in fluorescence and expressed as the mean of the percentage of each slaughtering time point. Data was analysed using Infinicyt™ Software v.1.8 (Cytognos, Salamanca, Spain).

Statistical analysis

Statistical analysis was performed with GraphPad Prism v.6.0 (GraphPad Software, Inc., San Diego, CA, USA). The Kolmogorov-

Smirnov test was applied to evaluate if distributions were parametric. Comparison between pairs of groups were made using the two-tailed Mann-Whitney U-test for non-parametric distributions. Data from each stage of the infection (Groups 1 and 2) were statistically compared to data from control animals (Group 3). *P*-values of 0.05 or lower were considered statistically significant.

Results

Parasitological results

The number of parasites in animals from Group 2 is expressed as mean ± standard deviation (SD). The mean liver fluke burden was 67.78 ± 13.85 which represents an implantation rate of 45.19% ± 9.24. The dynamics of egg output is shown in Fig. 1. Eggs were first detected at 8 wpi and all animals were positive to egg detection at 11 wpi. The egg output showed a progressive increasing trend from 10 wpi onwards, reaching a maximum level at 13 wpi.

In regard to the clinical monitoring of the animals during the trial, no clinical manifestations of disease, eggs or coccidian oocysts (apart from *F. hepatica* eggs in G2) were detected along the course of the trial.

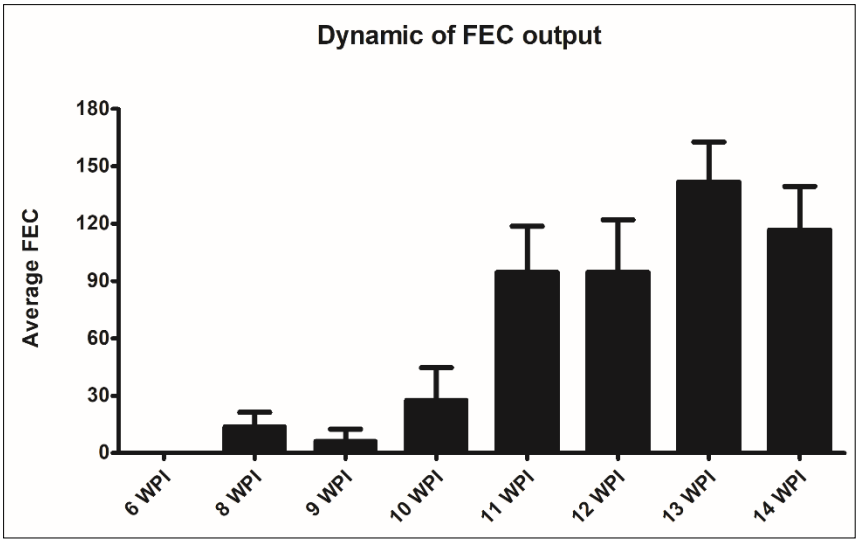

Fig. 1 Faecal egg output during the course of infection from animals of Group 2 (*n* = 10). Columns show the mean values of egg counts per week in infected animals from 6 until 14 wpi. Bars represent standard error.

Peritoneal leukocyte populations

Results of peritoneal leukocyte populations during the early and late stage of infection are expressed as the percentage (mean ± range) of each cell subpopulation (i.e., for TCD4, TCD8 and $WC1^{+}\gamma\delta$ results are expressed as the percentage of these three cell subsets of the total lymphocytes). The APC are represented as the percentage of MHCII. For macrophages and dendritic cells CD14 and CD83 were used, respectively. Values are shown in Table 2.

Table 2 Percentage of peritoneal cells. Cell immunonophenotypes are expressed as the mean of the percentage of each time point of challenged sheep from Group 1 (early stage) and from Group 2 (late stage) and UC animals (Neg. Ctl). Ranges of each immunophenotype are shown between brackets. Total peritoneal cell counts and concentration of cells are expressed as the mean of each Group at the different time points.

Cell Populations	Percentage (%)					
	Group 1			Group 2		Group 3
	1dpi (*n*=5)	3dpi (*n*=5)	9dpi (*n*=5)	18dpi (*n*=5)	14wpi (*n*=10)	NegCtl (*n*=7)
TCD4	12.42 (8.14–18.29)	27.24 (19.57–33.73)	37.00 (34.18–40.11)	18.68 (14.00–23.45)	33.68 (25.08–45.21)	30.98 (22.09–38.56)
TCD8	13.47 (10.13–15.65)	25.04 (18.98–28.92)	9.24 (7.52–12.42)	22.86 (17.73–34.22)	29.93 (13.25–41.55)	18.40 (2.59–22.91)
WC1$^{+}\gamma\delta$	5.28(4.30–6.40)	1.71 (1.13–2.44)	5.22 (3.40–7.69)	7.41 (4.85–10.93)	7.30 (4.57– 10.33)	2.28 (1.13–2.44)
CD14	60.15 (39.82–78.17)	63.48 (49.86–79.07)	41.42 (38.76–43.31)	31.84 (15.50–62.19)	19.18 (11.19–29.85)	69.44 (53.68–86.9)
CD83	22.78 (8.58–47.50)	2.17 (0.52–7.70)	11.89 (9.48–14.06)	23.21 (18.16–33.71)	6.17 (1.62– 8.00)	23.21 (20.75–27.43)
MHCII	48.68 (27.55–63.02)	9.04 (1.84–16.53)	22.54 (20.91–24.62)	40.23 (14.98–93.13)	6.66 (3.44–14.59)	62.00 (65.14–84.24)
Total cell count (40 ml)	598.16x10^6	298.32x10^6	3,771.60x10^6	19,918.00x10^6	1,002.40x10^6	418.20x10^6
Concentration (cells/ml)	14.95x10^6	7.46x10^6	94.29x10^6	497.95x10^6	25.06x10^6	10.46x10^6

Early stage of the infection

The total number of cells obtained from 40 ml of peritoneal fluid at 1, 3, 9 and 18 dpi was 598.16 × 10^6, 298.32 × 10^6, 3771.60 × 10^6 and 19,918.00 × 10^6, respectively (Table 2). The dynamics of the percentage of the peritoneal lymphocytes and APC in the negative and infected animals during the early stage of infection is shown in Fig. 2a-f. In the infected animals, flow cytometry analyses showed that lymphocyte cell subpopulation dynamics was different for TCD4, TCD8 and $WC1^+\gamma\delta$ cells. No significant differences with respect to the UC Group were detected for TCD8 and $WC1^+\gamma\delta$ cells, although slight non-significant variations were observed at different time points. The dynamics of TCD4 cells was irregular, with an initial decrease at 1 dpi (U = 0, df = 8, P = 0.0357), then an increase at 3 and 9 dpi and again a significant decrease at 18 dpi (U = 1, df = 8, P = 0.0159) when compared to animals of the UC.

Regarding the dynamics of APC, a different tendency was observed during the early stage of infection for CD14, MHCII and CD83 cells (Fig. 2d-f). CD14 cells exhibited a decreasing trend, with a significant decrease at 9 (U = 0, df = 8, P = 0.0079) and at 18 dpi (U = 2, df = 8, P = 0.0317) when compared to the UC. The dynamics of MHCII and CD83 cells was quite similar with no significant modifications at 1

dpi and a significant decrease at 3 and 9 dpi with respect to the UC sheep. The decrease of MHCII cells was statistically significant at 3 dpi ($U = 0$, $df = 8$, $P = 0.0079$) and 9 dpi ($U = 0$, $df = 8$, $P = 0.0079$); CD83 cells were significantly reduced at 3 and 9 dpi ($U = 0$, $df = 8$, $P = 0.0079$) with respect to the uninfected sheep. When compared, both MHCII and CD83 cells showed the same increasing pattern from 3 to 18 dpi.

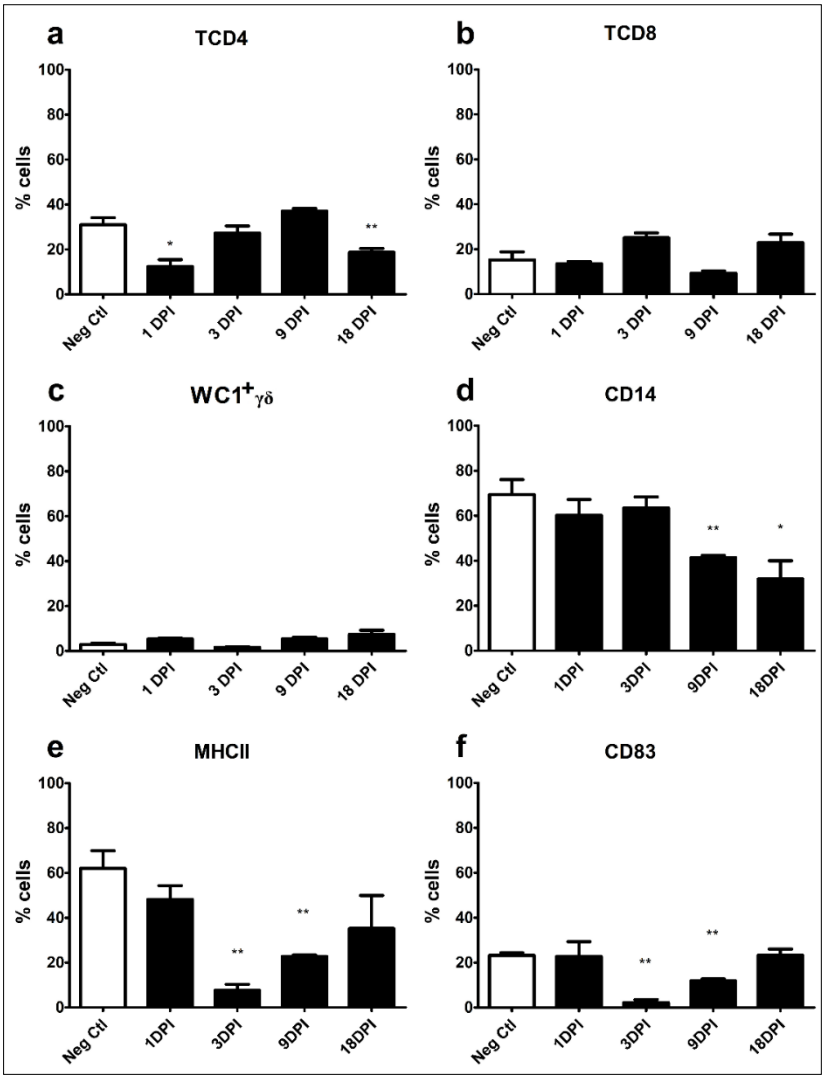

Fig. 2 Percentage of cell populations in the peritoneal fluid during the early stages of infection. The specific cell populations are shown in the following figure: (a) TCD4; (b) TCD8; (c) $WC1^{+}\gamma\delta$; (d) CD14; (e) MHCII; (f) CD83. Cell subsets of uninfected (Neg. Ctl) and infected sheep (Group 1) are shown in columns. Mann-Whitney U-test was used to compare data from Groups 1 and 3. Values represent the mean ± standard deviation (SD). Asterisks indicate different levels of significance between groups: $*P \leq 0.05$, $**P \leq 0.01$.

Late stage of the infection

The total number of cells obtained from animals of the Group 2 and 3 was 1002.40×10^6 and 418.20×10^6, respectively (Table 2).

At 14 wpi, the percentage of both TCD4 and TCD8 cells showed no significant differences compared to the uninfected group, although a slight but statistically significant higher level of $WC1^+\gamma\delta$ cells ($U = 0$, $df = 15$, $P = 0.0079$) was detected in the infected sheep (Fig. 3).

A noticeably lower percentage of CD14, MHCII and CD83 cells in infected animals was detected when compared to the uninfected sheep. Statistical analysis showed a significant decrease for the three cell populations ($U = 0$, $df = 15$, $P = 0.0079$).

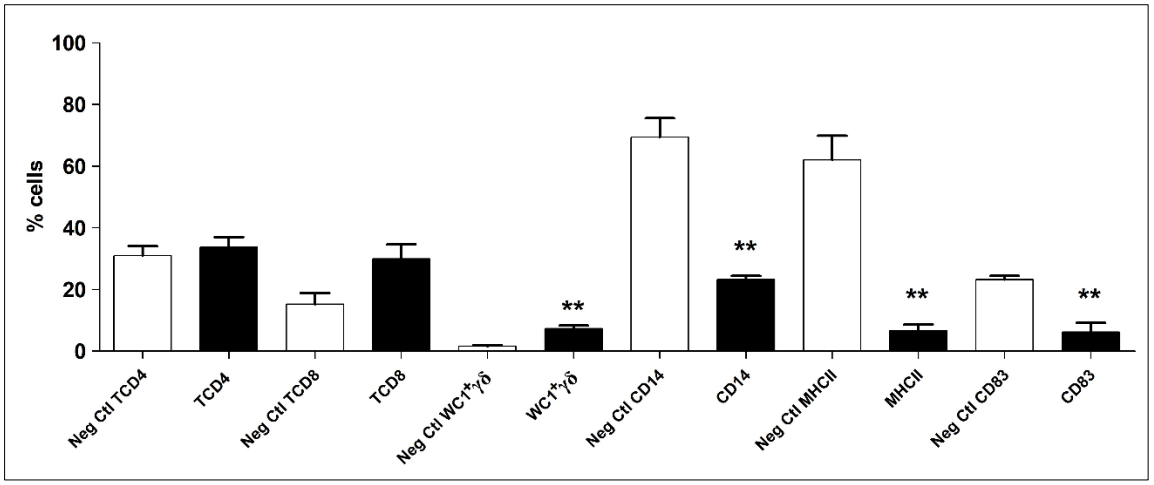

Fig. 3 Percentage of peritoneal cell populations during the late stages of infection. Each cell subset of uninfected (Neg. Ctl) and challenged animals (Group 2) are shown in columns. Mann-Whitney U-test was used to compare data from Groups 2 and 3. Values represent the mean ± standard deviation (SD). Asterisks indicate different levels of significance between groups: $*P \leq 0.05$, $**P \leq 0.01$.

Discussion

Peritoneal leukocyte population in sheep includes mainly lymphocytes and macrophages (and DC), with a minor proportion of eosinophils and neutrophils [21]. Within these cells, macrophages and DC are considered APC which play a crucial role in the innate and local immune response. Meanwhile, T lymphocytes are known to be of key importance as they immunoregulate the host immune response through various mechanisms such as cytokine production. Currently, many *F. hepatica* molecules have been shown to produce severe effects on these

cell populations [13, 25–30], hence many studies are focusing on these particular cell populations. The host immune response against *F. hepatica* is believed to be more effective during the initial peritoneal or early hepatic migratory stages [20]. Therefore, we aimed to (i) determine the immunophenotype and the dynamics of the PCP, which are believed to be one of the main immunocompetent cells involved in the early immune response once NEJ have entered the PC, and to (ii) carry out a comparative study between the acute and chronic stage of the infection.

In our study we observed that the dynamics of the lymphocyte subsets did not show major variations in the peritoneal cavity during the early and late stage of the infection. Peritoneal macrophages showed a decreasing trend whereas MHCII and CD83 cells tended to develop an increasing trend along the infection. Indeed, at the beginning of the infection, when NEJ are migrating through the peritoneal cavity (3–9 dpi), the MHCII and CD83 cells were significantly reduced; later on, at 9–18 dpi, when the peritoneal migratory stage of the parasite is supposed to have finished, there was a significant reduction of CD14 cells. Subsequently, all three populations were again significantly reduced in the late stage of the infection in comparison to the UC.

TCD4 cells were the more prominent subpopulation both at the early and late stage of the infection and it was the only one that was

modified during the early stage, with a significant decrease at 1 and 18 dpi. TCD8 cells, the second population, did not vary significantly along the infection, but $WC1^{+}\gamma\delta$ cells, the minority subpopulation, was slightly increased in the chronic stage. To our knowledge, there are no previous in vivo studies in *F. hepatica*-infected ruminants regarding the dynamics of peritoneal lymphocyte phenotypes during the early and late stage of the infection. In *F. hepatica* infected goats, our group previously studied the dynamics of circulating TCD4, TCD8 and $WC1^{+}\gamma\delta$ cells from peripheral blood during chronic stages of infection, and a significant reduction of TCD4 cells was found at 5 and 12 wpi in respect to the UC, whereas no significant differences were noted for TCD8 and $WC1^{+}\gamma\delta$ cells [31]. The different dynamics of TCD4 and $WC1^{+}\gamma\delta$ cells in chronic stages of the present study compared to the previous study in goats suggests a different behaviour of peritoneal and peripheral TCD4 and $WC1^{+}\gamma\delta$ cell subsets. Alternatively, this may be due to different cell subset recruitment in *F. hepatica* infected sheep and goats. Recently it was reported that total peritoneal lymphocyte population was only increased at 9 dpi in *F. hepatica* infected sheep in respect to UC, and a decrease was observed at 18 dpi, probably due to the increment in the recruitment of eosinophils [18]. In this regard, in some animal models experimentally infected with enteric helminths, there is supporting

evidence that once infection was overcome, a persistent TCD4 population, in a significantly high frequency, occurs at the peritoneal cavity [32], something we partly saw in our study at the chronic stage, when all liver flukes were thought to be already established in the liver. Due to their influence on the polarisation of the immune response in subsequent reinfections, TCD4 cells have been described as the major population for the development of resistance to helminth infection, as occurs with gastrointestinal nematodes [33, 34]. In addition, this cell population is also known to control immunoregulatory mechanisms during helminth infections by means of cytokine secretion [28].

An unexpected result of our study was the observation of a lack of significant increase in the peritoneal T cell population during the early stages, as it has been described in *F. hepatica* infected rats, in which peritoneal TCD4 and TCD8 were significantly augmented at early stages [28]. In fact, the recruitment of these T cell subpopulations (TCD4, TCD8 and $WC1^{+}\gamma\delta$) in the organ in which the infection was established has been reported for ruminants in helminth infections [35]. In infected goats, we previously detected that the early hepatic lesions due to the penetration of the NEJ in the liver occurred in between 7 and 9 dpi, with a noticeable cellular infiltrate surrounding the initial hepatic lesions, mainly composed of TCD4 and TCD8 [31]. Therefore, it may be

hypothesised that as *F. hepatica* NEJ migration through the PC only occurs during the first 4–6 dpi [9], this period might be insufficient to develop a significant increment in the recruitment of peritoneal T cells, which might not affect the recruitment of the lymphocyte subsets in the liver during the early stage.

With respect to the $WC1^{+}\gamma\delta$ T cells, an increase in the percentage was only detected at the late stage of the infection. The influence of the variation of the dynamics of $WC1^{+}\gamma\delta$ observed on the immune response during the early and late stage of the infection still remains to be elucidated. There is supporting evidence that γδ T cells may play an important role in the immune response as they can mediate effector activities such as production of INF-ɣ or TNF-α [36]. Moreover, in cattle the $WC1^{+}\gamma\delta$ T cells were shown to act as APC for αβ T cells [37]. This might condition some immunoregulatory processess which can have an effect on the outcome of the infection in reinfected animals, since this cell population is known to develop memory activity against different pathogens [38, 39].

One of the most noteworthy findings of our study was the influence of the infection on peritoneal CD14, CD83 and MHCII cells over time. A significant decrease could be observed in the early stage in the three studied subpopulations: during the period of peritoneal

migration of juvenile flukes (3–9 dpi) for CD83 and MHCII cell populations, and at the time of penetration in the liver parenchyma for the CD14 cell population. Similarly, a marked and significant reduction of the three subpopulations was demonstrated at the chronic stage when compared to UC. Although there are very few studies focused on the dynamics of the peritoneal macrophages (CD14), dendritic cells (CD83) and MHCII cells during infection in ruminants, and the results of these studies are somehow dissimilar [18, 21], there is strong evidence of the modulation of APC functions by *F. hepatica* antigens. In previous *in vitro* studies conducted in mice, tegumental antigens were shown to suppress DC maturation and function [27, 30], *F. hepatica* glycans showed to downregulate the expression of MCHII [25] and some other ES-derived proteins and peptides modulated DC activity by different mechanisms [14, 40]. We have also demonstrated that *F. hepatica* also modulates the oxidative response of pMΦ, inducing an increase in nitric oxide and hydrogen peroxide production during the early stage of infection in sheep [41]. Moreover, we previously described a specific induction of apoptosis in peritoneal leukocyte populations in the early stage of *F. hepatica* infection in sheep [21], which probably affects the subpopulations of CD14, MCHII and CD83 analysed in this study.

In the chronic stage of the infection, the percentages of the three APC subpopulations were also significantly reduced in comparison to UC. At this time, adult parasites have been located in the bile ducts for several weeks and the activity of some peritoneal macrophages and dendritic cells remains uncertain. It has been suggested that the peritoneum acts as an important lymphoid organ where presentation of antigen to the immune cells takes place and is followed by their migration to the inflammatory site [35] and the role of some particular peritoneal APC could be related to the recruitment of macrophages and lymphocytes in the inflammatory infiltrate surrounding the large bile ducts [21]. However, recent transcriptomic analysis in *F. hepatica* infected sheep have revealed a complex and different pattern of response in early [42] and late stages of infection [43] and the recruitment and functionality of different cell populations can be modulated in different ways in the different tissues of the host [44].

In summary, we have analysed the cellular immune response elicited in the peritoneal cavity in sheep infected with *F. hepatica* and provided novel data regarding immune cell recruitment over the course of the infection. To our knowledge, this is the first report of the use of flow cytometry for the assessment of the dynamics of local

immunocompetent cells at the abdominal cavity in the *F. hepatica* natural host.

Conclusions

We have identified the immunophenotype and the recruitment of the peritoneal cell population in sheep uninfected and infected with *F. hepatica* during the early and late stage of the infection. There is no statistically significant increment in the recruitment of the peritoneal TCD4, TCD8 and $WC1^{+}\gamma\delta$ cells along the course of infection, with the exception of $WC1^{+}\gamma\delta$ cells at the chronic stage. The dynamics of the CD14 cells recruitment at the peritoneal cavity displayed a decreasing trend during the early stage and was significantly decreased at the chronic stage of the infection. CD83 and MHCII cells developed an increasing trend during the early stage of infection, and were significantly decreased at the chronic stage of the infection.

Additional file 1: Figure S1. Gating strategy for the identification of cell immunophenotypes by flow cytometric analysis.

1. Dot-plot samples (1): FSC and SSC in log scale are faced in order to exclude debris. Red square shows the leukocyte populations of interest.

2. Dot-plot (2.1) and histogram (2.2): once leukocyte populations are gatted, lineal FSC is faced to log SSC so that white cells can be shown and identified properly. The histogram is an extra support for the correct gating in leukocyte subsets.

3. Dot-plots for fluorochromes (3.1 and 3.2): according to the fluorochrome in each antibody, RPE or FITC channels are faced to log SSC, and in each dot-plot it is only shown the subset of interest which was gated in dot-plot 2.

Abbreviations

dpi: days post-infection; **wpi**: weeks post-infection; **WHO**: World Health Organisation; **NEJ**: newly excysted juveniles; **APC**: antigen presenting cells; **DC**: dendritic cells; **PC**: peritoneal cavity; **pMΦ**: peritoneal macrophages; **UC**: uninfected control; **PCP**: peritoneal cell populations

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Declarations

Ethics approval

This experiment was performed in accordance with the University of Córdoba Bioethics Committee (code no. 1118) and European (2010/63/UE) and Spanish Directives (RD 1201/2005) on animal experimentation.

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this study are included within the article. Raw data are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RPC, JP, AMM and LB conceived and designed the study. RPC, FJMM, ILP, VMH, RZ, MTRC, AE and LB collected the samples and RPC analysed the samples. RPC and LB performed the statistical analysis. RPC, AMM and LB wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol. 2005; 35:1255–78.

2. Molina-Hernández V, Mulcahy G, Pérez J, Martínez-Moreno Á, Donnelly S, O'Neill SM, et al. *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. Vet Parasitol. 2015; 208:101–11.

3. Dalton JP, Mulcahy G. Parasite vaccines—a reality? Vet Parasitol. 2001; 98:149–67

4. Cooper KM, Kennedy DG, Danaher M. *ProSafeBeef* and anthelmintic drug residues—a case study in collaborative application of multi-analyte mass spectrometry to enhance consumer safety. Anal Bioanal Chem. 2012; 404:1623–30.

5. Ortiz P, Scarcella S, Cerna C, Rosales C, Cabrera M, Guzmán M, et al. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Cajamarca (Peru): a clinical trial and in vivo efficacy test in sheep. Vet Parasitol. 2013; 195:118–21.

6. Brockwell YM, Elliott TP, Anderson GR, Stanton R, Spithill TW, Sangster NC. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int J Parasitol Drugs Drug Res. 2014; 4:48–54.

7. Fairweather I. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet Parasitol. 2011; 180:133–43.

8. Álvarez-Sánchez MA, Mainar-Jaime RC, Pérez-García J, Rojo-Vázquez FA. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet Rec. 2006; 159:424–25.

9. Moazeni M, Ahmadi A. Controversial aspects of the life cycle of *Fasciola hepatica*. Exp Parasitol. 2016; 169:81–9.

10. Dalton JP, Robinson MW, Mulcahy G, O'Neill SM, Donelly S. Immunomodulatory molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic development. Vet Parasitol. 2013; 195:272–5.

11. Alvarado R, To J, Lund ME, Pinar A, Mansell A, Robinson MW, et al. The immune modulatory peptide FhHDM-1 secreted by the helminth *Fasciola hepatica* prevents NLRP3 inflammasome activation by inhibiting endolysosomal acidification in macrophages. FASEB J. 2016; 31:85-95.

12. Finlay CM, Stefanska AM, Coleman MM, Jahns H, Cassidy JP, McLoughlin RM, et al. Secreted products of *Fasciola hepatica* inhibit the induction of T cell responses that mediate allergy. Parasite Immunol. 2017; 39:1–6.

13. Rodríguez E, Carasi P, Frigerio S, da Costa V, van Vliet S, Noya V, et al. *Fasciola hepatica* immunoregulates CD11c+ cells by interacting with the macrophage gal/GalNAc lectin. Front Immunol. 2017; 8:6–8.

14. Noya V, Brossard N, Berasaín P, Rodríguez E, Chiale C, Mazal D, et al. A mucin-like peptide from *Fasciola hepatica* induces parasite-specific Th1-type cell immunity. Parasitol Res. 2016; 115:1053–63.

15. Hernández-González A, Valero ML, Pino MS del, Oleaga A, Siles-Lucas M. Proteomic analysis of in vitro newly excysted juveniles from *Fasciola hepatica*. Mol Biochem Parasitol. 2010; 172:121–8.

16. Cwiklinski K, de la Torre-Escudero E, Trelis M, Bernal D, Dufresne PJ, Brennan GP, et al. The extracellular vesicles of the helminth patogen,

Fasciola hepatica: biogenesis pathways and cargo mollecules involved in parasite pathogenesis. Moll Cell Proteomics. 2015; 14:3258–73.

17. García-Campos A, Ravidà A, Nguyen DL, Cwiklinski K, Dalton JP, Hokke CH, et al. Tegument glycoproteins and cathepsins of newly excysted juvenile *Fasciola hepatica* carry mannosidic and paucimannosidic N-glycans. Plos Negl Trop Dis. 2016;10(5): e0004688.

18. Ruiz-Campillo MT, Molina-Hernández V, Pérez J, Pacheco IL, Pérez R, Escamilla A, et al. Study of peritoneal macrophage immunophenotype in sheep experimentally infected with *Fasciola hepatica*. Vet Parasitol. 2018; 257:34–9.

19. Davies C, Goose J. Killing of newly excysted juveniles of *Fasciola hepatica* in sensitized rats. Parasite Immunol. 1981; 3:81-96.

20. Piedrafita D, Parsons JC, Sandeman RM, Wood PR, Estuningsih SE, Partoutomo S, et al. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile *Fasciola hepatica in vitro* is mediated by reactive nitrogen intermediates. Parasite Immunol. 2001; 23:473–82.

21. Escamilla A, Pérez-Caballero R, Zafra R, Bautista MJ, Pacheco IL, Ruiz MT, et al. Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infections in sheep. Vet Parasitol. 2017; 238:49–53.

22. Manual of Veterinary Parasitological Laboratory Techniques. Ministry of Agriculture, Fisgeries and Food. Reference Book 418 (Third edition, 1986). Her Majesty's Stationery Office, London.

23. Navarro JA, Caro MR, Seva J, Rosillo MC, Gomez MA, Gallego MC. Study of lymphocyte subpopulations in peripheral blood and secondary lymphoid organs in the goat using monoclonal antibodies to surface markers of bovine lymphocytes. Vet Immunol Immunopathol. 1996;51:147–56.

24. Chan SSM, McConnell I, Blacklaws BA. Generation and characterization of ovine dendritic cells derived from peripheral blood monocytes. Immunology. 2002; 107:366–72.

25. Rodríguez E, Noya V, Cervi L, Chiribao ML, Brossard N, Chiale C, et al. Glycans from *Fasciola hepatica* Modulate the Host Immune Response and TLR-Induced Maturation of Dendritic Cells. PLoS Negl Trop Dis. 2015; 9(12): e0004234.

26. Dowling DJ, Hamilton CM, Donnelly S, La Course J, Brophy PM, Dalton J, et al. Major secretory antigens of the Helminth *Fasciola hepatica* activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses. Infect Immun. 2010; 78:793–801.

27. Hamilton CM, Dowling DJ, Loscher CE, Morphew RM, Brophy PM, O'Neill SM. The *Fasciola hepatica* tegumental antigen suppresses dendritic cell maturation and function. Infect Immun. 2009; 77:2488–98.

28. Jedlina L, Kozak-Ljunggren M, Wedrychowicz H. In vivo studies of the early, peritoneal, cellular and free radical response in rats infected with *Fasciola hepatica* by flow cytometric analysis. Exp Parasitol. 2011; 128:291–7.

29. Vukman KV, Adams PN, Metz M, Maurer M, O'Neill SM. *Fasciola hepatica* Tegumental Coat Impairs Mast Cells' Ability To Drive Th1 Immune Responses. J Immunol. 2013; 190:2873–9.

30. Aldridge A, O'Neill SM. *Fasciola hepatica* tegumental antigens induce anergic-like T cells via dendritic cells in a mannose receptor-dependent manner. Europ J Immunol. 2016;46:1180-92.

31. Zafra R, Pérez J, Buffoni L, Martínez-Moreno FJ, Acosta I, Mozos E, et al. Peripheral blood lymphocyte subsets in *Fasciola hepatica* infected and immunised goats. Vet Immunol Immunopathol. 2013;155:135-138.

32. Steinfelder S, Rausch S, Michael D, Kühl AA, Hartmann S. Intestinal helminth infection induces highly functional resident memory CD4 + T cells in mice. Eur J Immunol. 2017;47:353–63.

33. Gill HS, Watson DL, Brandon, MR. Monoclonal antibody to CD4+ T-cells abrogates genetic resistance to Haemonchus contortus in sheep. Immunol. 1993;78:43-9.

34. Balic A, Bowles VM, Meeusen ENT. Cellular profiles in the abomasal mucosa and lymph node during primary infection with *Haemonchus contortus* in sheep. Vet Immunol Immunopathol. 2000;75:109–20.

35. Mitra R, Dharajiya N, Kumari L, Varalakshmi C, Khar A. Migration of antigen presenting cells from periphery to the peritoneum during an inflammatory response: role of chemokines and cytokines. FASEB J. 2004;18:1764-66.

36. Hsu H, Chen C, Nenninger A, Holz L, Baldwin CL, Telfer JC. WC1 is a hybrid γδ TCR coreceptor and pattern recognition receptor for pathogenic bacteria. J Immunol. 2015;194:2280-88.

37. Lahmers K K, Norimine J, Abrahamsen M S, Palmer G H, Brown WC. The CD4+ T cell immunodominant Anaplasma marginale major surface protein 2 stimulates γδ T cell clones that express unique T cell receptors. J Leukoc Biol. 2005;77:199-208.

38. Blumerman SL, Herzig CT, Baldwin CL. WC1+ γδ T cell memory population is inducen by killed bacterial vaccine. Eur J Immunol. 2007;37:1204-16.

39. Chien, Y. H., and Y. Konigshofer. 2007. Antigen recognition by γδ T cells. Immunol Rev. 2007;215:46–58.

40. Ruiz-Jimenez C, Celias D, Silvane L, Cervi L, Espino AM. *Fasciola hepatica* Fatty Acid Binding Protein (Fh12) inhibits the activation of murine dendritic cells. J Immunol. 2017;198:1.

41. Pérez-Caballero R, Buffoni L, Martínez-Moreno FJ, Zafra R, Molina-Hernández V. Expression of free radicals by peritoneal cells of sheep during the early stages of *Fasciola hepatica* infection. Parasit Vectors. 2018;11:500 (doi:10.1186/s13071-018-3072-5).

42. Rojas CAA, Scheerlinck JP, Ansell BRE, Hall RS, Gasser RB, Jex AR. Time-Course Study of the Transcriptome of Peripheral Blood Mononuclear Cells (PBMCs) from Sheep Infected with *Fasciola hepatica*. PLoS One. 2016;11(7): e0159194.

43. Fu Y, Browne JA, Killick K, Mulcahy G. Network Analysis of the Systemic Response to *Fasciola hepatica* Infection in Sheep Reveals Changes in Fibrosis, Apoptosis, Toll-Like Receptors 3/4, and B Cell Function. Front Immunol. 2017; 8:485.

44. Sachdev D, Gough KC, Flynn RJ. The Chronic Stages of Bovine *Fasciola hepatica* Are Dominated by CD4 T-Cell Exhaustion. Front Immunol. 2017;8:1002.

TERCER ESTUDIO

Expression of free radicals by peritoneal cells of sheep during the early stages of *Fasciola hepatica* infection

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Abstract

Background: The majority of vaccination studies against infection with *F. hepatica* in a natural host have been conducted at the late stage of the infection when the host's immune response is already immunomodulated by the parasite towards a Th2 non-protective response. This study was aimed at analysing the dynamic of the cell population present in peritoneal liquid and the production of free radicals by the peritoneal leukocytes in infected and vaccinated sheep with recombinant cathepsin L1 of *F. hepatica* (rFhCL1) in early stages of the infection.

Methods: Forty-five sheep were divided into three groups: Group 1 remained as negative control (*n*=5), Group 2 (*n*=20) was challenged with *F. hepatica* and Group 3 (*n*=20) was vaccinated with rFhCL1 and challenged with *F. hepatica*. After the slaughtering, peritoneal lavages were carried out at 1, 3, 9 and 18 days post-infection (dpi) to isolate peritoneal cell populations. Flow cytometry was conducted to assess levels of hydrogen peroxide (H_2O_2) and nitric oxide (NO).

Results: There was a significant increase in the total number of leukocytes at 9 and 18 dpi in infected and vaccinated groups. Production of H_2O_2 was significantly increased in peritoneal granulocytes in both infected and vaccinated groups. Production of nitric oxide showed a significant rise in the granulocytes and monocytes/macrophages in

infected and vaccinated sheep. The NO production by granulocytes at 3 and 9 dpi was significantly higher in the vaccinated than in the infected animals.

Conclusions: Experimental infection induced an increase in the total number of leukocytes within the abdominal cavity at 9 and 18 dpi, being more noticeable in vaccinated animals. Production of H_2O_2 occurred mainly in granulocytes of vaccinated and infected animals. Production of NO was incremented in vaccinated and non-vaccinated animals in all peritoneal cells. Vaccinated animals produced significant higher level of H_2O_2 and NO than infected animals.

Keywords: *Fasciola hepatica*, Nitric oxide, ROS, Peritoneal cells, Vaccines, Sheep

Background

Fasciolosis, caused by *Fasciola hepatica*, is a globally distributed parasitic disease that mainly affects ruminant livestock and causes great impact in terms of economic losses to the agricultural industry [1–2]. The World Health Organization (WHO) recognises it as a food-borne trematode infection and as an important zoonotic disease. Immature and mature forms of the parasite inhabits the liver of the host and produces a hepatitis which may alter the liver function [3].

There are few effective strategies to control the disease though it is widely accepted that the use of anthelmintics is the best means to control the infection. Specific drugs differ in their efficacy as some of them may not affect early immature stages of the parasite, hence triclabendazole (TCBZ) has become the drug of choice in many countries. A key drawback lies on the anthelmintic resistance to various drugs including TCBZ which has been globally reported [4–7]. Consequently, in the past two decades, many attempts have been conducted to develop a viable vaccine showing diverse results in cattle and sheep. The majority of the vaccination studies have been focussed on the late stage of the infection when the immune response of the host is known to be already immunomodulated by the parasite towards a non-protective Th2 response and the inhibition of protective pro-

inflammatory products [8–9]. Therefore, studies in infected and vaccinated animals during the early stage of infection, when the parasite is migrating and establishing in the liver may shed light on the initial immunological pathways of the disease.

Previous studies have shown that some parasites can trigger free radical production by leukocytes including superoxide radical, nitric oxide, and hydrogen peroxide [10–11]. In *F. hepatica* infections, resident peritoneal leukocytes such as macrophages might be involved in the killing of newly excysted juvenile liver flukes (NEJ). This may be accomplished by a parasite-specific antibody dependent mechanisms since most juvenile parasites are killed in the gut or the abdominal cavity, before reaching the liver [12]. In this way, some studies have shown an increase in the production of reactive oxygen species (ROS) produced by peritoneal leukocytes in rats infected with *Fasciola hepatica* [13, 14], which might be involved in the killing of migrating immature liver flukes during the early host-parasite interface. Moreover, free radical-induced cytotoxicity against NEJ of *Fasciola* sp. has been previously reported in infected sheep [15].

The aim of this work was to develop an in vivo study using flow cytometry in order to investigate the dynamic of the cell population present in peritoneal liquid and the production of free radicals by

leukocytes (macrophages and granulocytes) present in peritoneal liquid in infected animals (vaccinated and non-vaccinated) with recombinant cathepsin L1 of *F. hepatica* (rFhCL1) in early stages of the disease.

Methods

Animals and experimental design

Forty-five six-month-old male Merino sheep obtained from a liver fluke-free farm were used for the experimental trial. Before beginning the study, animals were confirmed to be free of liver fluke infection by faecal analyses and ELISA for *F. hepatica* specific antibodies. Sheep were housed in covered pens and fed daily with hay and commercial pelleted ration.

Sheep were randomly divided into three groups: Group 1 consisted of 5 animals which were neither immunised nor experimentally challenged (n=5), hence remained as negative control group, Group 2 consisted of 20 animals (n=20) which were experimentally infected with *F. hepatica* (positive control group) and Group 3 consisted of 20 sheep which were immunised with rFhCL1 and experimentally challenged with *F. hepatica* (n=20). In addition, animals from Groups 2 and 3 were subdivided into smaller groups of five animals each according to slaughtering day: 1, 3, 9 and 18 days post-infection (dpi).

Animals from Group 3 received the vaccine twice by subcutaneous inoculation on weeks 0 and 4 of the trial. At week 8 of the experiment, animals from Groups 2 and 3 were orally challenged with one single dose of 150 metacercariae of *F. hepatica* (Ridgeway Research Ltd., St Briavels, UK) administered in gelatine capsules using a dosing gun. As previously mentioned, five animals from Groups 2 and 3 were euthanised by an intravenous injection of T61® (Intervet, Barcelona, Spain) at each time-point; animals from the negative control group (Group 1) were euthanised on the 12th week of the trial.

Purification of recombinant *F. hepatica* cathepsin L1

Recombinant *F. hepatica* cathepsin L1 (rFhCL1) was expressed in the yeast Pichia pastoris and purified as described elsewhere [16]. Yeast transformants were cultured in 250ml BMGY broth, buffered to pH 6.0, in 1 l baffled flasks at 30°C until an OD600 of 2–6 was reached. Cells were harvested by centrifugation at 2000 ×*g* for 5min and protein expression was induced by resuspending the cells in 50ml BMMY broth, buffered at pH 6.0, 7.0 or 8.0, containing 1% methanol. The cultures were grown at 30°C with shaking at 225 ×rpm for 3 days, and filter-sterilized methanol was added daily to maintain a final concentration of 1%. Recombinant proteins were purified from the yeast medium by

affinity chromatography using Ni-NTA-agarose (Qiagen, Montreal, Canada). Briefly, a column prepared with 1ml of resin was equilibrated by passing through 10ml 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole. Ten millilitres of yeast media supernatant was mixed with 40 ml of the same buffer and applied to the column. The column was washed with 15ml of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole, and bound protein was eluted using 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 250 mM imidazole. Purified recombinant proteases were dialysed against phosphate buffered saline (PBS) and stored at -20°C. Before immunisation, electrophoresis in polyacrylamide gel was carried out to check protein purity.

Vaccine preparation

The recombinant protein cathepsin L1 of *F. hepatica* (rFhCL1) was diluted in ISA 70 Montanide adjuvant. Each immunisation dose was prepared as follows: 100µg of rFhCL1was diluted in PBS containing 1mg/ml of the adjuvant, reaching a final volume of 1ml per dose.

Liver pathology

Necropsy was performed and the liver was removed and photographed on both visceral and diaphragmatic surface for gross evaluation. Liver tissue samples showing hepatic lesions were collected and fixed in 10% neutral buffered formalin for 24h, then routinely processed and embedded in paraffin wax. Four-micron-thick tissue sections were stained with hematoxylin and eosin (H&E) for histopathology. Gross hepatic lesions during the early stages of infection in challenged animals (Groups 2 and 3) were counted using the Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

Antibody detection

Blood samples were taken at weeks 0 and 6 of the trial and at 1, 3, 9 and 18 days post-infection (dpi), and plasma was collected to detect specific IgG1 and IgG2 antibodies against rFhCL1 by ELISA. Briefly, 96-well ELISA plates were coated with 5µg/ml of rFhCL1 (100µl/well) diluted in 0.05M carbonate-bicarbonate buffer pH 9.6 and incubated at 37°C overnight. After 5 washes with phosphate buffer saline (PBS) 0.05% Tween 20, plates were blocked with 100µl/well of blocking buffer containing 1% BSA diluted in PBS and incubated at 37°C for 30min. To detect IgG1, wells were washed and 100µl/well of plasma diluted in

blocking buffer was added and incubated at 37°C for 30min. Triple serial dilutions were performed to determine endpoint titre. Similarly, IgG2 was detected by adding 100µl/well of plasma diluted at 1:25. After washing, 100µl/well of primary antibody diluted 1:5000 (mouse anti-bovine IgG1 and anti-bovine IgG2; 7500820–7500830 Cedi-Diagnostics, Lelystad, the Netherlands), in blocking buffer was added and incubated at 37°C for 30min. After incubation, wells were washed and anti-mouse IgG-HRP (STAR13B, BIO-RAD – Formerly AbD-Serotec-, Kidlington, UK) was added at 37°C for 30min. Plates were washed and 100µl/well of tetramethylbenzidine (TMB; Sigma-Aldrich, Madrid, Spain) were added and incubated for 10min at room temperature. The reaction was stopped by adding of 100µl/well of 1M sulphuric acid and optical density was measured at 450 nm using a microplate photometer (MultiskanTM FC, Thermo Fisher Scientific, Madrid, Spain). Results are shown as antibody titre -log10-for IgG1, and as optical density for IgG2.

Isolation of peritoneal cell population

To collect peritoneal cell population, abdominal lavage of each sheep was immediately conducted after the slaughtering as previously described [17]. Briefly, the ventral region of the abdomen was sheared,

shaved and disinfected using 10% polyvinylpyrrolidone iodine (AGB, Madrid, Spain). A 2cm incision was made in the skin over the midline and subcutaneous tissue was dissected. The white line and peritoneum were sectioned with blunt scissors to avoid haemorrhage. A 40cm long cannula connected to a syringe was inserted into the abdominal cavity and 40ml sterile DPBS containing 9500 UI of heparin (Eurotubo®, Company, Madrid, Spain) (warmed at 37°C) was injected into the abdominal cavity. After softly massaging the abdominal cavity for 1min, 40ml of peritoneal fluid were withdrawn. Peritoneal fluid was centrifuged at 1500 ×rpm for 5min and the supernatant was discarded. Cell pellets were resuspended again in DPBS and incubated for 15min in an erythrolysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM disodium EDTA, dH2O). A second centrifugation step (1500 ×rpm for 5min) was performed to eliminate lysed erythrocyte membranes and the pellet was resuspended in 1ml of medium. After that, the concentration of peritoneal cells was quantified using the Trypan Blue exclusion technique. The final concentration was adjusted to 1 × 106cells/ml for analysis by flow cytometry assay.

Flow cytometry assay

Flow cytometry acquisition was performed with a CyFlow Cube 6® (Sysmex-Partec, Barcelona, Spain) cytometer. Leukocytes were identified by their characteristic appearance on a FSC-SSC dot plot and gated in order to exclude cellular debris. Ten thousand events were analysed for changes in fluorescence intensity (enzymatic activity) of macrophages and granulocytes. For oxidative metabolism, hydrogen peroxide (H_2O_2) and nitric oxide (NO) production, filtered light at green was used (488nmFL1 channel). The results were analysed using FCS Express 4.0 (DeNovo Software, Los Angeles, USA) and Flowing Software 2.0 (Centre for Biotechnology, Turku, Finland).

In order to measure intracellular H_2O_2, the cell-permeable dye DCFH-DA (Dichloro-dihydro-fluorescein diacetate) was used. The non-fluorescence reduced form is converted into the fluorescent form when is oxidised and, in this way, the fluorescence can be detected by flow cytometry. A 200µl sample of peritoneal cells were incubated in dark and 37°C for 20min with 1ml of DCFH-DA 10µM. After incubation, samples were centrifuged at 1500 ×rpm for 5 min and the supernatant was discarded. Two washes of PBS centrifuged at 1500 ×rpm for 5min were carried out and the pellets are resuspended in DPBS.

Intracellular NO was measured using the cell-permeable dye DAF-2DA (4,5-diaminofluorescein diacetate), because the non-fluorescent reduced form is converted into the fluorescent form when oxidised, thus allowing the detection by flow cytometry. In the same way, a 200µl sample of peritoneal cells was incubated in dark and 37°C for 180min. From this point onwards the methodology was similar to that described for DCFH-DA.

Statistical analysis

Statistical analysis was carried out with GraphPad Prism v.6.0 (GraphPad Software Inc., San Diego, CA, USA). The Kolmogorov-Smirnov test was applied to evaluate whether distributions were parametric. Comparison between pairs of groups was made using a two-tailed Mann-Whitney U-test for non-parametric distributions. $P<0.05$ was considered statistically significant.

Results

Progression of infection: liver pathology and antibody production

No hepatic changes were observed in the negative control animals (Group 1). In infected and vaccinated animals (Groups 2 and 3), gross and histopathological hepatic changes were absent at 1 and 3 dpi, though

tortuous whitish tracts and haemorrhagic spots, which mainly occurred along the left hepatic lobe, were detected at 9 and 18 dpi. Further description of the gross hepatic lesions as well as the histopathological changes have been previously described in our recent study [18].

The levels of plasmatic specific anti-rFhCL1 IgG1 and IgG2 are presented in Fig. 1a, b. All vaccinated animals with the recombinant antigen developed an IgG1-IgG2 antibody response following immunisation. In vaccinated animals, a statistically significant production of IgG1 was detected after immunisation at each slaughtering time-point (U=0, df=8, P=0.0006 for 6 wpv; U=0, df=8, P=0.0097 for 1, 9 and 18 dpi and U=3, df=8, P=0.0449 for 3 dpi) and showing an increasing trend at the end of the trial (Fig. 1a). Production of IgG2 showed a similar pattern to that observed for IgG1 but was overall limited and only statistically significant after immunisation (U = 2, df = 8, P = 0.0062; Fig. 1b). No production of specific anti-rFhCL1 IgG1 and IgG2 was detected in the negative and positive control groups (Groups 1 and 2).

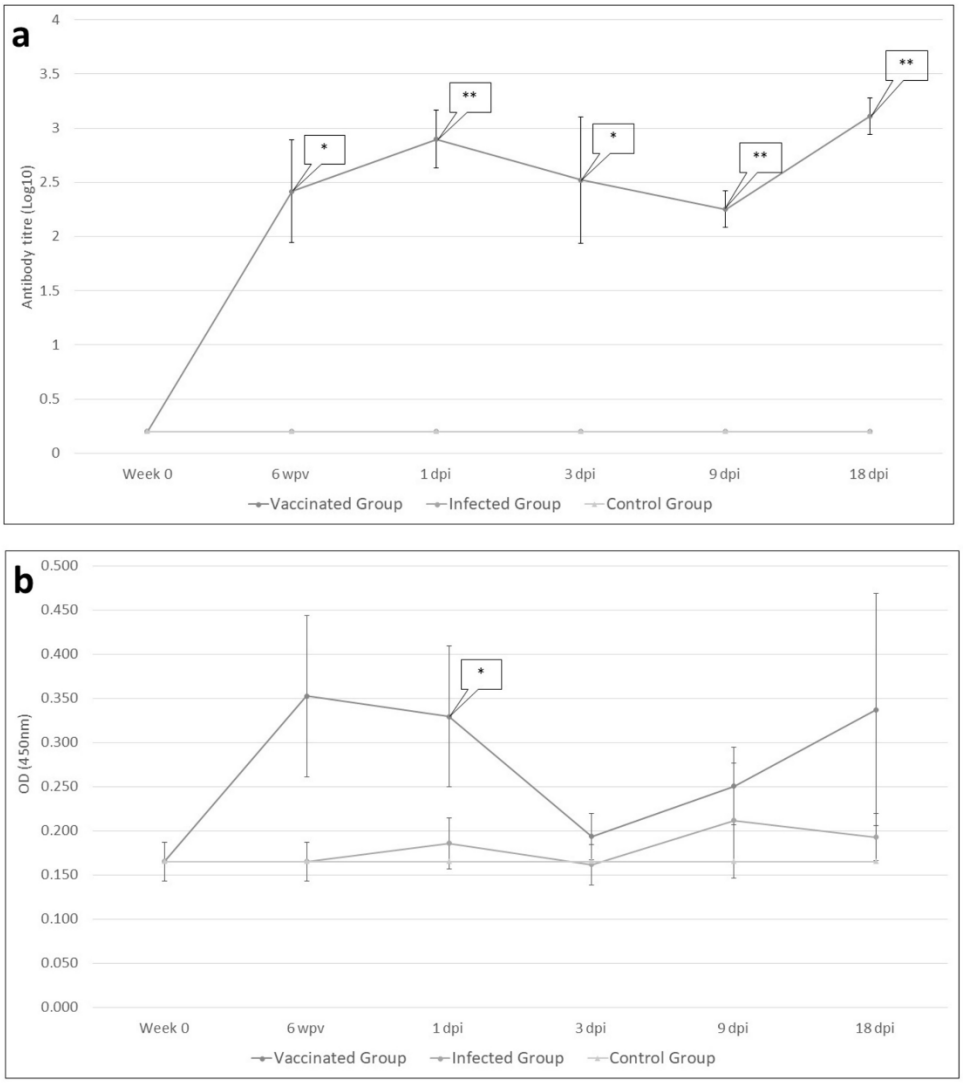


Fig. 1 Plasma levels of specific rFhCL1 IgG1 (a) and IgG2 (b). Each point represents mean values of antibody titre - log10 - (IgG1) and of optical density (IgG2) measured at 450 nm. Bars at each point represents tandard error. Immunisation with rFhCL1 developed a significant rise in the level of IgG1 isotype; dynamics of IgG1 in uninfected and infected sheep (Groups 1 and 2) shows a similar pattern, hence it is overlapped in the figure. Significant IgG2 production was detected only at 1 day post-infection (dpi) during the trial.

Dynamics of peritoneal leukocytes during infection

The total number of leukocytes population from peritoneal liquid was assessed by flow cytometry and expressed as 1×10^6cells/ml. There was a significant increase in the number of leukocytes at 9 and 18 dpi, in both infected ($U = 3$, $df = 8$, $P = 0.0439$ and $U = 0$, $df = 8$, $P<0.0001$, respectively) and vaccinated ($U = 0$, $df = 8$, $P = 0.0009$ and $U = 0$, $df = 8$, $P<0.0001$, respectively) groups in comparison with the negative control group (Fig. 2).

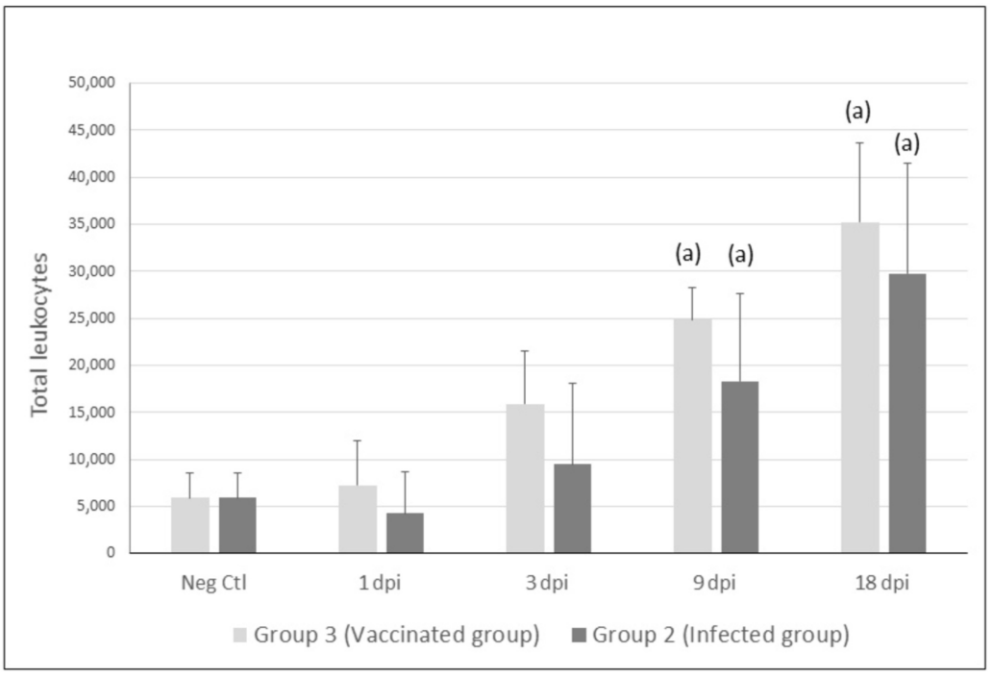


Fig. 2 Mean total number of leukocytes illustrating the effect of infection on peritoneal cell recruitment. Cell viability was assessed by trypan blue exclusion. "a" indicates significant variations between infected (Group 2, infected group), immunised (Group 3, vaccinated group) and control (Neg Ctl) sheep ($P < 0.05$) at 4 slaughtering time-points: 1, 3, 9 and 18 days post-infection (dpi).

Distribution of peritoneal leukocyte populations during infection

Leukocyte populations were characterised by a side scatter/forward scatter profile as shown in Fig. 3a, b. Dynamics of cell populations through the infection is shown in Fig.4a, b. The trend in all animals was an increase in the number of granulocytes and a decrease in both macrophages and lymphocytes throughout the infection.

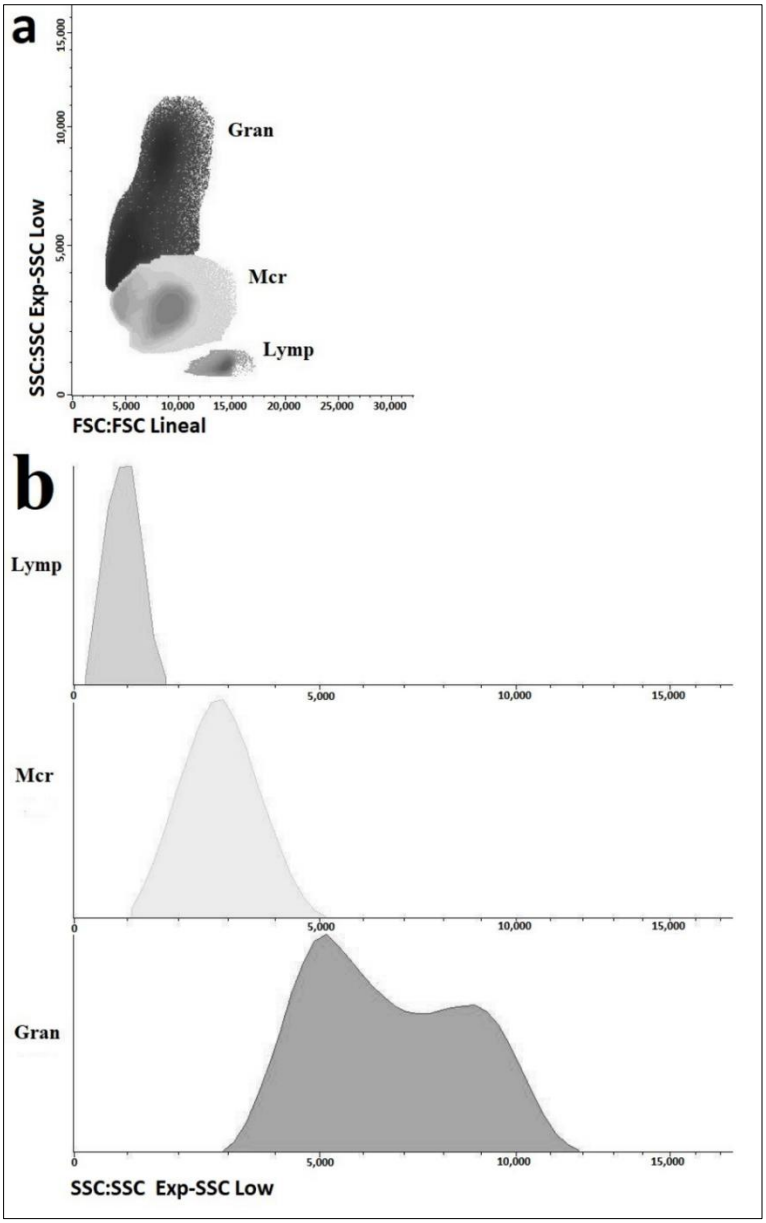
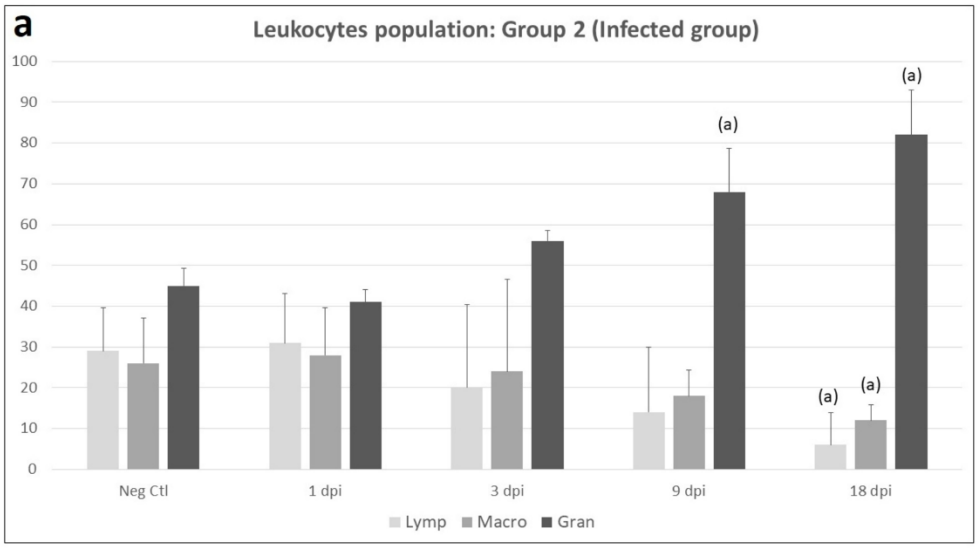


Fig. 3 Flow cytometry analysis of peritoneal cell population of sheep after peritoneal lavage characterised by a side scatter/forward scatter (SSC/FSC) profile. Distribution of major leukocyte populations on gated regions is represented by dot-plot (a) and histogram (b). Abbreviations: Lymp, lymphocytes; Mcr, macrophages; Gran, granulocytes.

Granulocytes were significantly increased in the infected group at 9 dpi ($U = 0$, $df = 8$, $P = 0.0129$) and 18 dpi ($U = 2$, $df = 8$, $P = 0.0032$). In the vaccinated group, a similar significant increment was detected at 9 dpi ($U = 3$, $df = 8$, $P = 0.0481$) and at 18 dpi ($U = 0$, $df = 8$, $P = 0.0071$). On the other hand, a significant decrease in the number of lymphocytes ($U = 3$, $df = 8$, $P = 0.0098$) and macrophages ($U = 6$, $df = 8$, $P = 0.0291$) was observed in the infected group at 18 dpi compared with the negative control group (Group 1).



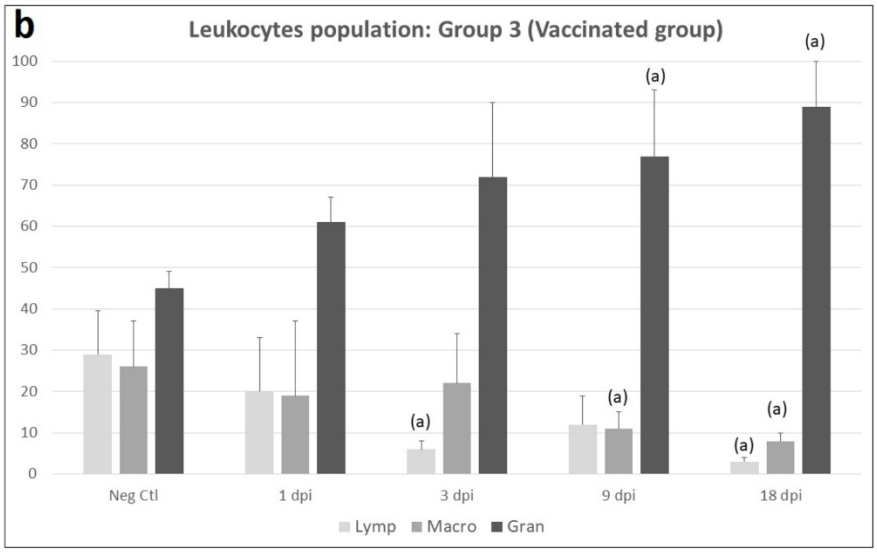


Fig. 4 Differential cell counts by flow cytometric analysis of peritoneal lavage leukocyte samples from uninfected (Neg Ctl, Group 1, a, b), infected (Group 2, a) and vaccinated sheep (Group 3, b). Each identified cell subset is expressed as a percentage of the total number of leukocytes. Values represent the mean ± SD. "a" indicates significant differences ($P< 0.05$) between groups. Abbreviations: Lymp, lymphocytes; Macro, macrophages; Gran, granulocytes.

Lymphocytes and macrophages were significantly decreased in vaccinated animals (U = 0, df = 8, P = 0.0011 and U = 1, df = 8, P = 0.0058, respectively) compared to negative control animals (Group 1). This decrease was also observed in the vaccinated animals at 3 dpi for lymphocytes (U = 6, df = 8, P = 0.0037) and at 9 dpi for macrophages (U = 2, df = 8, P = 0.0182), compared to the negative control group.

Hydrogen peroxide production by peritoneal leukocyte populations during infection

The results are shown in Fig. 5a, b. Production of H_2O_2 displayed a slightly different overall pattern in infected and vaccinated animals. During the initial stage of the infection, H_2O_2 production occurred mainly in the granulocyte cell type. In the infected group (Fig. 5a), the increase of H_2O_2 production by granulocytes was only observed at 18 dpi (U = 2, df = 8, P = 0.0002). Nevertheless, a significant decrease was observed at 3 dpi for monocytes/macrophages (U = 0, df = 8, P = 0.0296).

In vaccinated animals (Fig. 5b) granulocytes showed a significant increase (U = 0, df = 8, P<0.0001) in H_2O_2 production at 9 and 18 dpi, whereas no significant variations were observed in the response of monocytes/macrophages.

A statistically significant higher level of H_2O_2 production by granulocytes was observed in the vaccinated group than in the infected group at 9 dpi ($U = 0$, $df = 8$, $P<0.0001$).

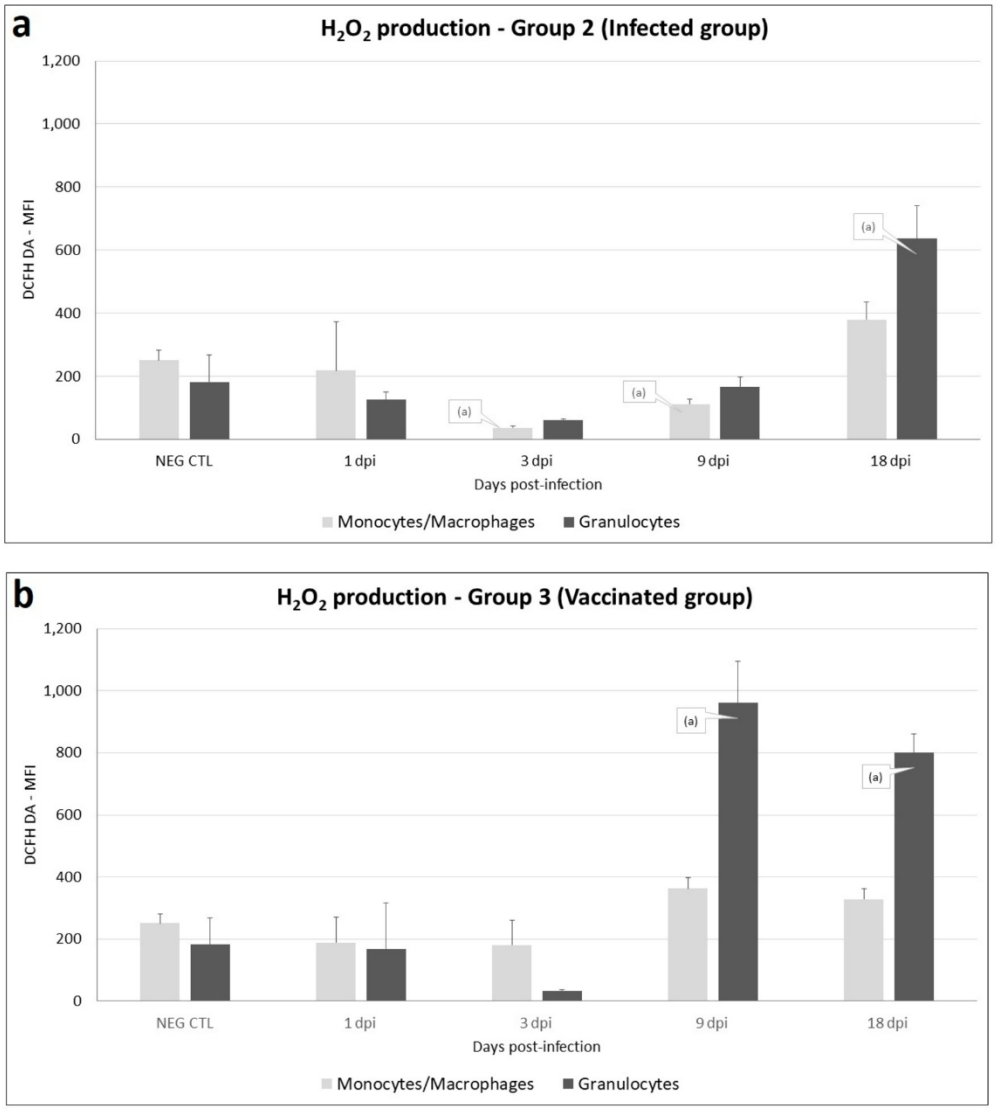


Fig. 5 Mean fluorescence intensity of H_2O_2 production (DCFH DA) in the peritoneal fluid of uninfected sheep (Neg. Ctl, Group 1, a, b), infected (Group 2, a) and vaccinated sheep (Group 3, b). Values represent the mean ± standard deviation, SD. "a" indicates significant differences ($P< 0.05$) between groups.

Nitric oxide production by peritoneal leukocyte populations during infection

The dynamic of NO production is shown in Fig. 6a, b. In the infected animals (Fig. 6a) a significant rise in NO production was detected by granulocytes at 9 dpi ($U = 0$, $df = 8$, $P = 0.0432$) and 18 dpi ($U = 0$, $df = 8$, $P = 0.0076$), and by monocytes/macrophages at 18 dpi ($U = 0$, $df = 8$, $P = 0.0022$). In vaccinated sheep (Fig. 6b) the NO production by granulocytes was statistically significant increased at 3 dpi ($U = 0$, $df = 8$, $P<0.0001$), 9 dpi ($U = 0$, $df = 8$, $P = 0.0026$) and 18 dpi ($U = 0$, $df = 8$, $P = 0.0133$), whereas production by monocytes/macrophages was significantly increased only at 3 dpi ($U = 0$, $df = 8$, $P<0.0001$).

Production of NO by granulocytes was statistically significant higher at 3 dpi ($U = 0$, $df = 8$, $P<0.0001$) and 9 dpi ($U = 3$, $df = 8$, $P = 0.0476$) in the vaccinated group than in the infected group.

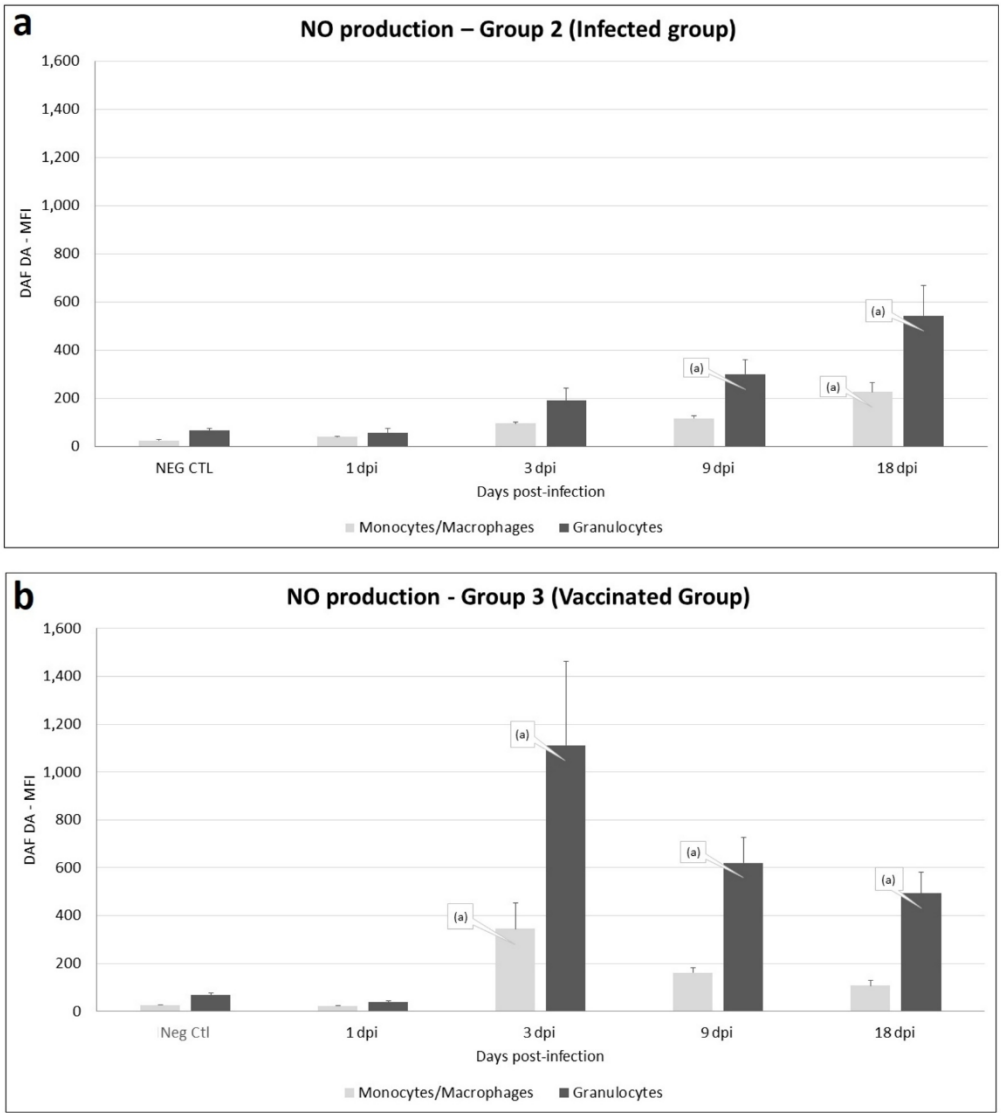


Fig. 6 Mean fluorescence intensity of NO production (DAF DA) in the peritoneal fluid of sheep uninfected (Neg Ctl, Group 1, a, b), infected (Group 2, a) and vaccinated sheep (Group 3, b). Values represent the mean ± standard deviation, SD. "a" indicates significant differences (P <0.05) between groups.

Discussion

This study focuses on the production of free radicals by leukocyte populations in peritoneal fluid during the early stages of fasciolosis, being the first in vivo study carried out in sheep infected and uninfected with *F. hepatica*. We have compared the response in sheep vaccinated with rFhCL1 and non-vaccinated animals and we have confirmed the existence of a noticeable NO response, mainly by granulocytes, in both infected and vaccinated animals.

The peritoneal cavity is a critical location in the development of *F. hepatica* infection as it is the route of migration of *F. hepatica* NEJ from the intestine to the liver and the site where an early immunomodulatory effect of the parasite likely plays a critical role in determining the ultimate outcome of the infection [9]. It has been suggested that effector mechanisms of NEJ killing and consequent immune protection are dependent on the activity of peritoneal leukocytes, as described for *F. hepatica* in rats [19] and F. gigantica in Indonesian thin tail sheep [15]. Both antibodies and free radicals (ROS and NO) produced by leukocytes have been considered effective elements in those protective peritoneal responses [14]. However, ROS and NO produced as a strategy to kill parasites during *F. hepatica*

infection have been also described as responsible for oxidative stress and hepatic damage in sheep [20], rats [21] and cattle [22].

In our experiment, peritoneal leukocyte populations in sheep increased immediately after infection, reaching significant levels at 9 and 18dpi in both infected and vaccinated groups, as previously described in rats experimentally challenged with *F. hepatica* [14]. From 9 dpi onwards, hepatic lesions could be detected, associated with the penetration of NEJ in the liver parenchyma. The cellular infiltration in hepatic lesions was mainly composed of eosinophils, macrophages and lymphocytes, whereas the peritoneal populations were mainly granulocytes, in increased proportions over the course of the infection. In our study, by using the flow cytometry assay, we could not discriminate the different cell population of granulocytes (neutrophils, eosinophils and basophils), but using immunocytochemistry staining, we have determined that eosinophils occurred in more than 95% of total peritoneal granulocyte populations (data not shown) [23]. The predominance of granulocytes and more specifically of eosinophils in the peritoneal cell population have been also observed in the early stage of infection in rats [11,24], in goats by immunohistochemical studies [17, 25] and in sheep by transcriptome analysis [26].

By means of the flow cytometry technique, we could assess the intracellular production of free radicals (H_2O_2 and NO) by peritoneal macrophages and granulocytes, although we could not identify the different oxidative response of neutrophils and eosinophils. The intracellular production of both H_2O_2 and NO by peritoneal leukocytes was stimulated during the early stages of infection, in the infected group and in the vaccinated group, as previously described in rats [14]. However, these authors found macrophages as the most significant cell type at the initial stage of the infection (7 dpi) in contrast to our findings in sheep, in which granulocytes were proved to be more relevant cells at all time-points of the study.

We have also found that macrophages and particularly granulocytes from vaccinated animals showed a significantly higher production of free radicals, mainly at 9 and 18 dpi. This is consistent with our previous work where a partial protective response was described in experimental trials with rFhCL1, that could be related to eosinophils and free radical (NO) production in the early stage of infection [17, 18].

The role of H_2O_2 in *F. hepatica* infection remains unclear. Transcriptome studies in mice revealed production of ROS as one the most significant pathways undergoing changes during immunoprotection [27] but it has also been related to oxidative stress and

pathology in chronic infection in sheep [20]. Moreover, different in vitro studies indicate that *F. hepatica* NEJ possess a unique ability to resist killing by reactive oxygen species released by sheep innate immune effector cells, which may involve the high expression of antioxidant enzymes such as superoxide dismutase, glutathione S-transferase (GST) or peroxiredoxin [28–31].

In our study, both macrophages and granulocytes were involved in the increase of NO production occurring between 3 and 18 dpi. Previous studies in rats have highlighted the involvement and complementary role of these two cell populations in the peritoneal stage of the infection [11,14,31]. In fact, Piedrafita et al. [15] described an antibody-dependent cell-mediated killing of NEJ involving both macrophages and eosinophils NO production in the resistance of ITT sheep to F. gigantica. Our previous vaccination study suggested that inducible nitric oxide synthase (iNOS) expression and subsequent NO production could be important for an effective response against the early migrating liver fluke [17]. Although the early production of NO we have detected in that study seemed to have little effect on the development of the infection and in the final fluke burden at the end of the experiment [18]. Consistent protective responses in sheep has not been achieved and the precise effective mechanisms of protection has not been yet

elucidated [9]. It has been hypothesised that NO and iNOS might play an important role in *F. hepatica* pathogenesis, possibly as an effective mechanism for killing migrating NEJ, as it has been previously shown to occur in resistant rats [31] or maybe as an expression of M1 macrophages activation which are known to be related to the development of Th1 responses required for protection [18,32, 33]. Recent transcriptomic studies have revealed that modifications in the NO signalling pathway may be a necessary condition for immunoprotection in mice [27] or, on the contrary, downregulation of iNOS might be a paramount factor during the non-protective response occurring in sheep [34]. In our study, we have not detected an inhibition in NO production in the early phase of infection. In another study, we found a low level of variation in iNOS expression in peritoneal macrophages by immunocytochemistry [35]. Those differences suggest that iNOS gene, protein expression and NO production in the initial stages of the infection may differ, with the protein probably remaining active for a longer time than the gene.

In conclusion, we have observed a clear leukocyte response in the peritoneal cavity of the sheep in the early stage of *F. hepatica* infection. The leukocyte populations, mainly granulocytes, exhibited a metabolic response with intracellular production of both H_2O_2 and NO. The effect of those free radicals on the NEJ and migrating juveniles it is still

unclear, since NEJ and migrating juveniles appear to be unaffected by those molecules as they reached the liver and evolved to mature stages. Further studies are needed to provide a broader insight on the biomolecular mechanisms involved in the evasion of the immune response of this parasite in the early stages of infection.

Conclusions

Experimental infection induced an increase in the total number of leukocytes within the abdominal cavity at 9 and 18 dpi which was characterised by an increase in the number of granulocytes and a decrease of both macrophages and lymphocytes. Production of both H_2O_2 and NO by peritoneal cells was increased in vaccinated and non-vaccinated animals. Granulocytes were mainly involved in H_2O_2 production, whereas granulocytes and macrophages were predominant in NO production. Vaccinated animals produced a significantly higher level of H_2O_2 and NO than infected animals.

Abbreviations

rFhCL1: recombinant cathepsin L1 of *Fasciola hepatica*; **H_2O_2**: hydrogen peroxide; **NO**: nitric oxide dpi: days post-infection **ROS**: reactive oxygen species; **TCBZ**: triclabendazole; **NEJ**: newly excysted

juveniles; **wpv**: weeks post-vaccination; **GST**: glutathione S-transferase **iNOS**: inducible nitric oxide synthase

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Ethics approval

This experiment was performed in accordance with the University of Cordoba Bioethics Committee (no. 7119) and European (86/609/CEE) and Spanish Directives (RD 223/1988).

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this study are included within the article. Raw data are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RPC, LB, FJMM, RZ, VHM, JP and AMM conceived and designed the study. RPC, LB and RZ collected and analysed the samples. RPC, LB and AMM performed the statistical analysis. All authors read and approved the final manuscript.

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References

1.Spithill TW, Smooker PT, Sexton JL, Bozas E, Morrison CA, Creany J, et al. Development of vaccines against *Fasciola hepatica* In: Dalton JP, editor. Fasciolosis.Wallingford, UK:CABI Publishing; 1999. p. 377–410.

2. Piedrafita D, Spithill TW, Smith RE, Raadsma HW. Improving animal and human health through understanding liver fluke immunology. Parasite Immunol. 2010;32:572–581.

3. Behm CA, Sangster NC. Pathology, pathophysiology and clinical aspects. In: Dalton JP, editor. Fasciolosis. Wallingford, UK:CABI Publishing; 1999. p. 185–224.

4.Olaechea F, Lovera V, Larroza M, Raffo F, Cabrera R. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Vet Parasitol. 2011;178:364–6.

5. Brockwell YM, Elliott TP, Anderson GR, Stanton R, Spithill TW, Sangster NC. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int J Parasitol Drugs Drug Resist. 2013;4:48–54.

6. Novobilský A, Höglund J. First report of closantel treatment failure against *Fasciola hepatica* in cattle. Int J Parasitol Drugs Drug Resist. 2015;5:172–7.

7. Venturina VM, Alejandro MA, Baltazar CP, Abes NS, Mingala CN. Evidence of *Fasciola* spp. resistance to albendazole, triclabendazole and bromofenofos in water buffaloes (*Bubalus bubalis*). Ann Parasitol. 2015;61:283–9.

8. Dalton JP, Robinson MW, Mulcahy G, O'Neill SM, Donelly S. Immunomodulatory molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic development. Vet Parasitol. 2013;195:272–5.

9. Molina-Hernández V, Mulcahy G, Pérez J, Martínez-Moreno Á, Donnelly S, O'Neill SM, et al. *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. Vet Parasitol. 2015;208:101–11.

10. Wandurska-Nowak E, Wisniewska J. Release of nitric oxide during experimental trichinellosis in mice. Parasitol Res. 2002;88:708–11.

11. Sibille P, Tliba O, Boulard CH. Early and transient cytotoxic response of peritoneal cells from *Fasciola hepatica*-infected rats. Vet Res. 2004;35:576–84.

12. Armour J, Dargie JD. Immunity to *Fasciola hepatica* in rat. Successful transfer of immunity by lymphoid-cells and by serum. Exp Parasitol. 1974;35:381–8.

13. Smith NC, Ovingston KS, Boray JC. *Fasciola hepatica* free radical generation by peritoneal leukocytes in challenged rodents. Int J Parasitol. 1992;22:281–6.

14. Jedlina L, Kozak-Ljunggren M, Wedrychowicz H. In vivo studies of the early, peritoneal, cellular and free radical response in rats infected

with *Fasciola hepatica* by flow cytometric analysis. Exp Parasitol. 2011;128:291–7.

15. Piedrafita D, Estuningsih E, Pleasance J, Prowse R, Raadsma HW, Meeusen ENT, et al. Peritoneal lavage cells of Indonesian thin-tail sheep mediate antibody-dependent superoxide radical cytotoxicity in vitro against newly excysted juvenile *Fasciola gigantica* but not juvenile *Fasciola hepatica*. Infect Immun. 2007;75:1954–63.

16. Collins PR, Stack CN, O'Neill SM, Doyle S, Ryan T. et al. Cathepsin L1, the major protease involved in liver fluke (*Fasciola hepatica*) virulence: propeptide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells. J Biol Chem. 2004;279:17038–46.

17. Zafra R, Perez-Ecija RA, Buffoni L, Pacheco IL, Martinez-Moreno A, LaCourse EJ, et al. Early hepatic and peritoneal changes and immune response in goats vaccinated with a recombinant glutathione transferase sigma class and challenged with *Fasciola hepatica*. Res Vet Sci. 2013;94:602–9.

18. Pacheco IL, Abril N, Morales-Prieto N, Bautista MJ, Zafra R, Escamilla A, et al. Th1/Th2 balance in the liver and hepatic lymph nodes of vaccinated and unvaccinated sheep during acute stages of infection with *Fasciola hepatica*. Vet Parasitol. 2017;238:61–5.

19. Kesik M, Jedlina-Panasiuk L, Kozak-Cieszczyk M, Płucienniczak A, Wedrychowicz H. Enteral vaccination of rats against *Fasciola hepatica* using recombinant cysteine proteinase (cathepsin L1). Vaccine. 2007;25:3619–28.

20. Saleh, M. A. Circulating oxidative stress status in desert sheep naturally infected with *Fasciola hepatica*. Vet Parasitol. 2008;154:262–9.

21. Bottari NB, Mendes RE, Lucca NJ, Schwertz CI, Henker LC, Olsson DC, et al. Oxidative stress associated with pathological lesions in the liver of rats experimentally infected by *Fasciola hepatica*. Exp Parasitol. 2015;159:24–8.

22. Mendes EA, Mendes TA de O, dos Santos SL, Menezes-Souza D, Bartholomeu DC, Martins IVF, et al. Expression of IL-4, IL-10 and IFN-

gamma in the liver tissue of cattle that are naturally infected with *Fasciola hepatica*. Vet Parasitol. 2013;195:177–82.

23. Ruiz-Campillo MT, Hernandez VM, Escamilla A, Perez J, Martinez-Moreno A, Donnelly S, et al. Immune signatures of pathogenesis in the peritoneal compartment during early infection of sheep with *Fasciola hepatica*. Sci Rep.2017;7:2782.

24. Van Millingen FJ, Cornelissen JBWJ, Hendriks IM, Gaasenbeek CPH, Bokhout BA. Protection to *Fasciola hepatica* in the gut mucosa of immune rats is associated with infiltrates of eosinophils, IgG1 and IgG2a antibodies around the parasites. Parasite Immunol. 1998;20:285–92.

25. Zafra R, Perez-Ecija RA, Buffoni L, Moreno P, Bautista MJ, Martinez-Moreno A, et al. Early and late peritoneal and hepatic changes in goats immunized with recombinant cathepsin L1 and infected with *Fasciola hepatica*. J Comp Pathol. 2013;148:373–84.

26. Alvarez Rojas CA, Ansell BR, Hall RS, Gasser RB, Young ND, Jex AR, et al. Transcriptional analysis identifies key genes involved in

metabolism, fibrosis/tissue repair and the immune response against *Fasciola hepatica* in sheep liver. Parasit Vectors.2015;8:124.

27. Rojas-Caraballo J, López-Abán J, Moreno-Pérez DA, Vicente B, Fernández-Soto P, del Olmo E, et al. Transcriptome profiling of gene expression during immunisation trial against *Fasciola hepatica*: identification of genes and pathways involved in conferring immunoprotection in a murine model. BMC Infect Dis. 2017;17:94.

28. Piedrafita D, Spithill TW, Dalton JP, Brindley PJ, Sandeman MR, Wood PR, et al. Juvenile *Fasciola hepatica* are resistant to killing *in vitro* by free radicals compared with larvae of *Schistosoma mansoni*. Parasite Immunol. 2000;22:287–95.

29. Donnelly S, O'NeillSM, Sekiya M, Mulcahy G, Dalton JP. Thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. Infect Immun. 2005;73:166–73.

30. Donnelly S, Stack CM, O'Neill SM, Sayed, AA, Williams DL, Dalton, JP. Helminth 2-Cys peroxiredoxin drives Th2 responses through

a mechanism involving alternatively activated macrophages. FASEB J. 2008;22:4022–32.

31. Piedrafita D, Parsons JC, Sandeman RM, Wood PR, Estuningsih SE, Partoutomo S, et al. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile *Fasciola hepatica* in vitro is mediated by reactive nitrogen intermediates. Parasite Immunol. 2001;23:473–82.

32. Flynn RJ, Irwin JA, Olivier M, Sekiya M, Dalton JP, Mulcahy G. Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet Immunol Immunopathol. 2007;120:31–40.

33. Garza-Cuartero L, O'Sullivan J, Blanco A, McNair J, Welsh M, Flynn RJ, et al. *Fasciola hepatica* infection reduces *Mycobacterium bovis* burden and mycobacterial uptake and suppresses the pro-inflammatory response. Parasite Immunol. 2016;38:387–402.

34. Fu Y, Chryssafidis AL, Browne JA, O'Sullivan J, McGettigan PA, Mulcahy G. Transcriptomic Study on Ovine Immune Responses to *Fasciola hepatica* Infection. PLoS Negl Trop Dis.2016;10:e0005015.

35. Ruiz-Campillo MT, Molina-Hernández V, Pérez J, Pacheco IL, Pérez R, Escamilla A. Study of peritoneal macrophage immunophenotype in sheep experimentally infected with *Fasciola hepatica*. Vet Parasitol. 2018;257:34–9.

DISCUSIÓN

Actualmente se plantea como una cuestión urgente y decisiva para el control de la fasciolosis el desarrollo de novedosos y alternativos métodos de control, entre ellos, de forma destacada, la obtención de vacunas comercialmente aceptables (Hillyer, 2005; McManus y Dalton, 2006; Spithill y cols., 2012). Entre los motivos que llevan a esta situación se halla, con una gran relevancia, el creciente número de casos descritos de resistencias al triclabendazol (Overend y Bowen, 1995; Fairweather, 2009; Brockwell y cols., 2014; Kelley y cols., 2016) como principio activo de elección contra la fasciolosis ovina. Igualmente, la constante preocupación por el tema de los residuos químicos y la sostenibilidad ambiental.

Con la finalidad de obtener vacunas que otorguen con gran garantía una protección frente a la fasciolosis la mayoría de los esfuerzos han sido encaminados al estudio de la respuesta inmunitaria y de antígenos con potencial inmunogénico durante las fases tardías de la infección (Ramajo y cols., 2001; Martínez-Fernández y cols., 2004; Guasconi y cols., 2012). Si bien se han obtenido algunos resultados prometedores (Golden y cols., 2010; Kęsik-Brodacka y cols., 2017; Wesołowska y cols., 2018a), es de vital importancia conocer los acontecimientos inmunológicos durante las primeras fases de la infección, donde las formas juveniles desenquistadas (NEJ) de *F. hepatica* llegan al intestino y atraviesan la pared intestinal,

migrando a través de la cavidad peritoneal en dirección al hígado. El motivo por el cual se dirige la atención a las formas juveniles es conocer mecanismos que interfieran con la migración, alimentación y la evasión del sistema inmunitario del hospedador en los estadios iniciales de la infección antes de que el daño sea considerable y cuando el parásito está relativamente expuesto a la respuesta inmunitaria del hospedador. Por este motivo, los estudios vacunales e inmunológicos han evolucionado hacia el uso de proteínas específicas de estadio (Jayaraj y cols., 2009). Recientemente se ha descrito una nueva familia de proteínas (14-3-3) que, estando presente en protozoos y helmintos (Siles-Lucas y cols., 2000; van Hemmert y cols., 2001; Koyama et al, 2001; Gadahi y cols., 2016), se han asociado a la superficie, estructuras externas, extractos de secreción, así como al control del ciclo celular en las NEJ (Robinson y cols., 2009; Hernández-González y cols., 2010; De la Torre-Escudero y cols., 2011). Esta familia de proteínas se ha utilizado en ensayos vacunales frente a diferentes helmintos con resultados prometedores (Schechtman y cols., 2001; Siles-Lucas y cols., 2003). Asimismo, estudios previos de proteómica han revelado reactividad de la molécula 14-3-3 tanto en los bordes externos de la ventosa oral como en la superficie de las formas juveniles desenquistadas de *Fasciola hepatica* junto con una función de señalización celular (Hernández-González y

cols., 2010). De manera que con todo lo descrito hasta el momento se sientan las bases que justifican nuestro primer estudio en el que describimos por primera vez la utilización de la proteína recombinante Fh14-3-3z en un ensayo vacunal en ovinos infectados experimentalmente con *F. hepatica*. La eficacia de esta nueva proteína se valoró atendiendo a parámetros parasitológicos de carga parasitaria y eliminación de huevos en heces, y patológicos, analizando el daño hepático originado por los adultos. Los resultados revelaron que las ovejas inmunizadas con rFh14-3-3z en adyuvante Montanide™ ISA 71 VG (Seppic®) no desarrollaron protección significativa frente a *F. hepatica*. A su vez, la tasa de implantación y la carga parasitaria fue similar en todos los grupos estudiados. En este ensayo vacunal no se han observado diferencias entre el grupo vacunado y los controles ni en la dinámica de eliminación de huevos ni en el tamaño de las *F. hepatica* adultas. Sin embargo, la carga parasitaria y la eliminación de huevos en heces mostró una débil correlación en todos los grupos, coincidiendo con lo descrito previamente en otros estudios con ovinos (Duménigo y cols., 1999) y bovinos (Radfar y cols., 2015).

En relación a la respuesta inmunitaria humoral se ha comprobado la producción de una fuerte respuesta consistente en elevados niveles de IgG1 y IgG2 específicos frente a rFh14-3-3z, donde el isotipo

predominante fue IgG1. Estos resultados se mantienen en la línea de lo descrito en ensayos anteriores (Buffoni y cols., 2012) donde la respuesta humoral predominante se caracteriza por la producción de IgG1, demostrando la inmunomodulación realizada por el parásito polarizando la respuesta inmunitaria hacia una vía Th2, no protectora en su caso (O'Neill y cols., 2001; Vukman y cols., 2013). La inducción de respuestas Th1/Th2 son esenciales para el desarrollo de inmunidad protectora frente a *F. hepatica*. Si bien una intensa respuesta de tipo 2 está asociada con la reducción de la carga parasitaria y la severidad de la enfermedad en muchas infecciones por nematodos (Hewitson y cols., 2009; Moreau y Chauvin, 2010), la infección por *F. hepatica* induce una respuesta no protectora de tipo 2 en ovejas y bovinos (Piedrafita y cols., 2004; Flynn y cols., 2010; Dalton y cols., 2013). Los resultados obtenidos en nuestro primer trabajo son similares a los descritos por otros autores en ratones (Schechtman y cols., 2001; Siles-Lucas y cols., 2003), donde se obtuvieron altos niveles de ambos isotipos después de la inmunización. No se ha descrito efecto de reinfección en el nivel de anticuerpos en animales inmunizados después de la infección, de la misma manera que la infección no provoca producción de anticuerpos en los animales no vacunados. La falta de producción de dichos isotipos frente a esta familia de proteínas en animales infectados observada en

nuestro estudio fue descrita previamente por Siles-Lucas y cols. (2003) y Uribe y cols. (2007) en ratones infectados con *E. multilocularis* y *Schistosoma bovis*, respectivamente. Sin embargo, otros estudios han descrito una producción significativa de anticuerpos anti-14-3-3 en animales únicamente infectados con *S. mansoni* y *S. japonicum* (Schechtman y cols., 2001; Qian y cols., 2012). Estos hallazgos sugieren que la producción de inmunoglobulinas puede variar en los diferentes tipos de infecciones, así como entre las diferentes especies de parásitos pertenecientes a un mismo género. Otro de los motivos que pueden explicar este bajo nivel de respuesta humoral se encuentra en la utilización de proteínas recombinantes en lugar de emplear su forma nativa o en la conformación de los epítopos, que pueden ser o no reconocidos por diferentes animales en un determinado estudio (Buffoni y cols., 2012; Dewilde y cols., 2008; Kumar y cols., 2012; González-Hernández y cols., 2018).

Entre los parámetros que se utilizan como indicadores de protección vacunal se encuentra la dimensión del daño hepático originado por el trematodo (Mendes y cols., 2010b; Pérez-Ecija y cols., 2010). Nuestros resultados demuestran que la mayoría de los animales presenta un daño hepático severo y extenso y no se encuentran diferencias significativas entre el grupo vacunado y el control. Este hallazgo, junto con la ausencia

de reducción de carga parasitaria en animales vacunados, sugiere que la inmunización con rFh14-3-3z, que desencadena altos niveles de protección frente a otras infecciones por helmintos en modelos murinos (Zhang y cols., 2001; Siles-Lucas y cols., 2003; Uribe y cols., 2007; Yang y cols., 2016), no es un candidato apropiado contra *F. hepatica* en ovinos.

El fallo vacunal que se presenta es consistente con los resultados encontrados en otros estudios de nuestro grupo donde se han inmunizado cabras con glutatión S-transferasa (Buffoni y cols., 2010; Zafra y cols., 2010) o con una combinación de catepsina L1, peroxirredoxina y Sm14 (Buffoni y cols., 2012). De la misma forma, Maggioli y cols. (2016) tampoco evidenciaron que se produjera una reducción de la carga parasitaria en bovinos inmunizados con tiorredoxina glutatión reductasa (TGR) e infectados con *F. hepatica*. Aunque estudios previos sí han conseguido obtener niveles de protección significativa en esta especie (Golden y cols., 2010), así como en ovinos (Piacenza y cols., 1999; Villa-Mancera y Méndez-Mendoza, 2012), la tendencia actual va dirigida a la utilización de vacunas multivalentes eficaces en otras parasitosis (Nisbet y cols., 2016) en ensayos de infección con *F. hepatica* (Piacenza y cols., 1999). Éste es uno de los objetivos de nuestros estudios, así como del Proyecto Europeo H2020 PARAGONE (H2020-SFS-2014-2-635408-

PARAGONE, Vaccines for animal parasites, Ref.: 635408), enmarcado en la consecución de vacunas frente a distintas parasitosis en animales de producción.

La situación actual en el desarrollo de vacunas abre una doble vertiente con la utilización de nuevos candidatos vacunales (Cwiklinski y Dalton, 2018) y la necesidad de una mejor compresión de la interacción entre parásito-hospedador y el estudio de la infección en fases iniciales (Tliba y cols., 2002; Hoyle y cols., 2003; Raadsma y cols., 2007). Es por ello que los objetivos de nuestros estudios se han centrado en la compresión de lo acontecido a nivel inmunológico en cavidad peritoneal atendiendo a distintos momentos temporales de la infección, tanto tempranos como tardíos, con los que poder hacer una completa comparativa y tener una visión más amplia de la actuación inmunológica frente a dicho parásito. La cavidad peritoneal es una localización crítica desde un punto de vista inmunológico en el desarrollo y transcurso de numerosas infecciones por helmintos. En el caso de *F. hepatica* esta localización orgánica es vital para el desarrollo de la infección e, igualmente, es la ruta de migración hasta el hígado para las formas juveniles una vez han atravesado la pared intestinal. Dicha cavidad es el lugar donde los efectos inmunomoduladores tempranos de *F. hepatica* juegan un papel crucial en los resultados de la infección (Molina-Hernández y cols., 2015). Se

hace necesario por ello estudiar y conocer en profundidad las respuestas innata y adquirida desencadenadas durante la infección y, especialmente, durante el proceso migratorio de las formas juveniles a través del peritoneo, así como los principales tipos celulares involucrados en ellas, como linfocitos y macrófagos. A su vez, el empleo de citometría de flujo, como una de las técnicas más idóneas para este abordaje, permite obtener con mayor precisión un conocimiento más exacto y veraz sobre los mecanismos inmunológicos: características físicas de las poblaciones celulares, dobles marcajes para la identificación fenotípica y estudio del ciclo celular (Serradell y cols., 2009; Jedlina y cols., 2011).

Una primera base para el conocimiento inmunológico a nivel peritoneal lo constituye el comportamiento de las poblaciones de leucocitos que actúen durante esta fase de la infección. En nuestro estudio pudimos corroborar como tras la infección la población de leucocitos peritoneales sufrió un incremento, alcanzando niveles significativos a los 9 y 18 dpi en animales infectados y vacunados en comparación con los animales no infectados, estando en concordancia con lo descrito por Jedlina y cols. (2011) en ratas infectadas experimentalmente con *F. hepatica*. Las lesiones hepáticas pueden evidenciarse desde el día 9 y se asocian a la invasión de las formas juveniles en el parénquima hepático. Si bien el infiltrado celular encontrado en las lesiones estuvo compuesto

principalmente por linfocitos, macrófagos y eosinófilos, la población dominante en la cavidad peritoneal fueron los granulocitos. No fue posible en nuestro estudio mediante citometría de flujo discernir las diferentes subpoblaciones de granulocitos (neutrófilos, eosinófilos y basófilos), aunque, por el contrario, mediante el uso de tinciones de inmunohistoquímica determinamos que los eosinófilos representan más del 95% del total de granulocitos (datos no mostrados) (Ruiz-Campillo y cols., 2017). El predominio de granulocitos, y especialmente de eosinófilos, en células peritoneales ha sido observado en estudios de infecciones tempranas en ratas (van Millingen y cols., 1998; Sibille y cols., 2004), cabras (Zafra y cols., 2013a,b) y ovejas (Alvarez Rojas y cols., 2015).

En la población leucocitaria descrita a nivel peritoneal, macrófagos y células dendríticas están consideradas células presentadoras de antígenos (APCs), las cuales juegan un papel crucial en la respuesta inmunológica innata y local, mientras que los linfocitos T son conocidos como los elementos clave por regular la respuesta inmunitaria del hospedador a través de varios mecanismos como la producción de citoquinas.

Actualmente se ha demostrado que muchas moléculas de *F. hepatica* producen severos efectos en estas células (Hamilton y cols., 2009; Dowling y cols., 2010; Jedlina y cols., 2011; Vukman y cols., 2013;

Rodríguez y cols., 2015; Aldridge y O'Neill, 2016; Rodríguez y cols., 2017), por ello muchos estudios se han centrado en estas poblaciones celulares en particular. Teniendo en cuenta que la respuesta inmunitaria contra *F. hepatica* se cree que es más efectiva durante los estadios migratorios peritoneales iniciales o hepáticos (Piedrafita y cols., 2001), nuestro objetivo ha sido determinar el fenotipo inmunológico y la dinámica de la población celular peritoneal, que son una de las primeras células inmunocompetentes involucradas en la respuesta inmunitaria temprana una vez las NEJ han entrado en la cavidad peritoneal, y llevar a cabo un estudio comparativo entre los estadios iniciales y tardíos de la infección.

En nuestro estudio hemos analizados subpoblaciones linfocitarias (TCD4, TCD8 y $WC^{+}\gamma\delta$), población de macrófagos (CD14), células presentadoras de antígenos (MHCII) y células dendríticas (CD83). Hemos observado que la dinámica de las subpoblaciones linfocitarias no muestra importantes variaciones en la cavidad peritoneal durante los estadios tempranos y tardíos de la infección, mientras que los macrófagos (CD14) tienden a desarrollar una inclinación decreciente a lo largo de la infección. Por su parte, las subpoblaciones de MHCII y CD83 presentan una tendencia creciente durante las fases iniciales de la infección, siendo ésta decreciente en la fase tardía de la infección.

Además, al inicio de la infección, cuando las formas larvarias de *F. hepatica* están migrando a través de la cavidad peritoneal (3-9 dpi), las células presentadoras de antígenos (MHCII y CD83) se redujeron significativamente; y más tarde, a los 9-18 dpi, cuando el estadio migratorio peritoneal del parásito se supone que ha finalizado, hubo un reducción significativa de CD14, y las tres poblaciones se redujeron de nuevo significativamente en los estadios tardíos de la infección en comparación con el grupo control no infectado.

En relación a la población de linfocitos, las células TCD4 fueron la subpoblación más predominante en los estadios tempranos y tardíos de la infección y fue la única que se modificó durante los estadios iniciales, con un significativo descenso a los 1 y 18 dpi. Las células TCD8, la segunda población, no varió significativamente a lo largo de la infección, pero las células $WC^{+}\gamma\delta$, la población minoritaria, se incrementaron ligeramente en los estadios crónicos. Según nuestro conocimiento, no hay estudios previos *in vivo* en rumiantes infectados con *F. hepatica* sobre la dinámica del fenotipo linfocitario peritoneal durante los estadios tempranos y tardíos de la infección. En cabras infectadas por *F. hepatica* nuestro grupo ha estudiado previamente la dinámica de TCD4, TCD8 y $WC^{+}\gamma\delta$ de sangre periférica durante la fase crónica de la infección, y se constató una reducción significativa en TCD4 a las 5 y 12 spi respecto

del grupo no infectado, mientras que no hubo diferencias significativas en TCD8 y $WC^{+}\gamma\delta$ (Zafra y cols., 2013c). La diferente dinámica de TCD4 y $WC^{+}\gamma\delta$ en estadios crónicos del presente estudio comparado con estudios previos en cabras sugiere un comportamiento diferente de estas subpoblaciones peritoneales y periféricas, o puede ser debido al reclutamiento de subpoblaciones diferentes en las infecciones por *F. hepatica* en ovejas y cabras. Recientemente se ha publicado un incremento de la población total de linfocitos peritoneales únicamente a los 9 dpi en ovejas infectadas por *F. hepatica* respecto del grupo control negativo, y se observó un descenso a los 18 dpi probablemente debido al aumento de un reclutamiento de eosinófilos (Ruiz-Campillo y cols., 2018). A este respecto, en algunos modelos animales experimentalmente infectados con helmintos entéricos, se ha descrito una población de TCD4 persistente, en una frecuencia altamente significativa, en la cavidad peritoneal una vez superada la infección (Steinfelder y cols., 2017), algo que nosotros particularmente vimos en nuestro estudio en los estadios crónicos, cuando se piensa que todos los trematodos adultos están establecidos en el hígado. La población de linfocitos TCD4 ha sido previamente descrita como la principal población para el desarrollo de resistencia a las infecciones de helmintos (Gill y cols., 1993; Balic y cols., 2000), debido a su influencia en la polarización de la respuesta

inmunitaria en reinfecciones subsiguientes y también al control de mecanismos inmunorreguladores durante las infecciones por helmintos por medio de la secreción de citoquinas (Jedlina y cols., 2011).

Un resultado inesperado en nuestro estudio fue la observación de la falta de aumento significativo en la población de células T peritoneales durante las fases iniciales, como ha sido descrito en ratas infectadas por *F. hepatica*, en las cuales TCD4 y TCD8 peritoneales se incrementaron significativamente en los estadios iniciales (Jedlina y cols., 2011). De hecho, la movilización de estas células T (TCD4, TCD8 y $WC^{+}\gamma\delta$) en el órgano en el cual la infección se estableció ha sido documentado en infecciones helmínticas en rumiantes (Mitra y cols., 2004). En cabras infectadas hemos detectado previamente que las lesiones hepáticas tempranas debido a la penetración de las formas juveniles en el hígado ocurren entre los 7-9 dpi, con un notable infiltrado celular rodeando las lesiones iniciales, compuesto principalmente por TCD4 y TCD8 (Zafra y cols., 2013b). Por lo tanto, se podría hipotetizar que como la migración de las NEJ de *F. hepatica* a través de la cavidad peritoneal solamente ocurre durante los primeros 4-6 dpi (Moazeni y Ahmadi, 2016), este periodo puede ser insuficiente para desarrollar un incremento significativo en el reclutamiento de células T peritoneales, que podría no

afectar la movilización de las subpoblaciones de linfocitos en el hígado durante los estadios tempranos.

Con respecto a las células $WC^{+}\gamma\delta$, se detectó un incremento en su porcentaje únicamente en el estadio tardío de la infección. La influencia de la variación de la dinámica de $WC^{+}\gamma\delta$ observada en la respuesta inmunitaria durante las fases iniciales y tardías de la infección aún permanece sin dilucidar. Hay evidencia de que las células T γδ podrían jugar un importante papel en la respuesta inmunitaria porque pueden mediar actividades efectoras como la producción de IFN-γ o TNF-α (Hsu y cols., 2015). Además, en bovino las $WC^{+}\gamma\delta$ se muestra que actúan como APC para células T αβ (Lahmers y cols., 2005). Esto podría condicionar algunos procesos inmunorregulatorios que pueden tener un efecto en el resultado de la infección en animales reinfectados, ya que esta población celular se conoce que desarrolla actividad de memoria contra diferentes patógenos (Blumerman y cols., 2007; Chien y Konigshofer, 2007).

Uno de los hallazgos más considerables de nuestro estudio fue la influencia de la infección en las células peritoneales CD14, CD83 y MHCII a lo largo del tiempo. Se podría observar un descenso significativo en las fases iniciales en las tres subpoblaciones estudiadas: durante el periodo de migración peritoneal de las formas juveniles (3-9

dpi) para las poblaciones CD83 y MHCII, y en el momento de la penetración en el parénquima hepático para la población CD14. De manera similar, una marcada y significativa reducción de estas tres subpoblaciones se demostró en el estadio crónico cuando se compara con el grupo control negativo. Aunque hay muy pocos estudios enfocados en las dinámicas de los macrófagos peritoneales (CD14), células dendríticas (CD83) y MHCII durante la infección en rumiantes y los resultados son algo disimilares (Escamilla y cols., 2017; Ruiz-Campillo y cols., 2018), hay una fuerte evidencia de la modulación de la función de las APC por los antígenos de *F. hepatica*. En estudios previos *in vitro* llevados a cabo en ratones, los antígenos tegumentarios suprimieron la maduración y función de células dendríticas (Hamilton y cols., 2009; Aldridge y O'Neill, 2016), los glicanos de *F. hepatica* regularon el descenso de la expresión de MHCII (Rodríguez y cols., 2015) y algunas proteínas derivadas de productos de excreción-secreción y otros péptidos modularon la actividad de células dendríticas a través de diferentes mecanismos (Noya y cols., 2016; Ruiz-Jimenez y cols., 2017). En un trabajo realizado por nuestro grupo también se ha demostrado que *F. hepatica* también modula la respuesta oxidativa de macrófagos peritoneales, induciendo un incremento de la producción de óxido nítrico y peróxido de hidrógeno durante las fases iniciales de la infección en

ovejas como se muestra en nuestro tercer trabajo. De la misma manera, en otra publicación de nuestro grupo se describió previamente una inducción específica de la apoptosis en las poblaciones de leucocitos peritoneales en estadios tempranos de la infección de *F. hepatica* en ovejas (Escamilla y cols., 2017), que probablemente afecta a las subpoblaciones de CD14, MHCII y CD83 analizadas en este estudio.

En la fase tardía de la infección, los porcentajes de CD14, CD83 y MHCII se redujeron también significativamente en comparación con el control negativo. En este momento los parásitos adultos se localizan en los conductos biliares durante varias semanas y la actividad de algunos macrófagos y células dendríticas peritonales es todavía desconocida. Se ha sugerido que el peritoneo actúa como un importante órgano linfoide donde la acción de presentación antigénica a las células inmunes tiene lugar y es seguida por su migración al lugar inflamatorio (Mitra y cols., 2004) y el papel de algunas APC peritoneales particulares podría estar relacionado con la movilización de macrófagos y linfocitos en el infiltrado inflamatorio que rodea de los conductos biliares principales (Escamilla y cols., 2017). Sin embargo, estudios recientes de transcriptómica en ovejas infectadas con *F. hepatica* han revelado un complejo y diferente patrón de respuesta en estadios iniciales (Rojas y cols., 2016) y finales (Fu y cols., 2017) de la infección y la movilización

y funcionalidad de diferentes poblaciones celulares pueden ser moduladas de maneras diferentes en distintos tejidos del hospedador (Sachdev y cols., 2017). Las diferencias encontradas en nuestros estudios pueden hallar respuesta en las limitaciones como la imposibilidad de realizar doble marcaje, impidiendo determinar con una mayor precisión las poblaciones de estudio.

Los datos obtenidos nos han permitido describir el inmunofenotipo de la población de células peritoneales en ovejas infectadas y no infectadas con *Fasciola hepatica* durante los estadios iniciales y finales de la infección y hemos proporcionado datos novedosos en relación al reclutamiento de células inmunes a lo largo del curso de la infección. Según nuestro conocimiento, ésta es la primera documentación del uso de citometría de flujo en el estudio de la dinámica de células inmunocompetentes locales en la cavidad abdominal en el hospedador natural de *F. hepatica*.

Continuando con la actuación de los leucocitos peritoneales, en la infección por *Fasciola hepatica* se sugiere que algunas de estas poblaciones son las responsables de los mecanismos de destrucción de las NEJ y la consiguiente protección inmunintaria (Kesik y cols., 2007). Entre los elementos que se consideran eficaces en las respuestas protectoras a nivel peritoneal se encuentran los radicales libres (ROS y

NO) y la producción de anticuerpos (Jedlina y cols., 2011), sin embargo, los primeros también son los responsables del estrés oxidativo y el daño hepático en distintas especies mamíferas (Saleh, 2008; Mendes y cols., 2013; Bottari y cols., 2015).

Las dos clases principales de radicales libres producidas por las células del sistema inmune son las especies reactivas de oxígeno (ROS) y nitrógeno (RNS). Se ha demostrado que las NEJ de *F. hepatica* estimulan la producción de ambas clases de radicales por los macrófagos (Piedrafita y cols., 2004) que median la muerte de dichas formas juveniles (Piedrafita y cols., 2000, 2001). Dada la especial atención que merecen las fases iniciales de la infección, en nuestro tercer trabajo analizamos la producción intracelular de dos especies reactivas (H_2O_2 y NO) por parte de macrófagos y granulocitos mediante citometría de flujo, si bien no pudimos hacer una distinción entre la respuesta oxidativa de neutrófilos y eosinófilos. La producción intracelular de estos dos radicales libres en los leucocitos peritoneales fue estimulada durante los estadios tempranos de la infección, tanto en el grupo infectado como en el vacunado, de la misma manera que se ha descrito en ratas (Jedlina y cols., 2011). Sin embargo, estos autores encontraron que los macrófagos fueron el tipo celular más importante en este período de la infección (7 dpi) en contraposición con nuestros hallazgos en ovejas, donde los

granulocitos se evidenciaron como las células relevantes en todos los puntos del estudio.

En relación a otros ensayos de inmunización, en este estudio se ha podido comprobar que los macrófagos y, sobre todo, los granulocitos de animales vacunados mostraron una producción significativamente mayor de radicales libres, especialmente a los 9 y 18 dpi. Estos datos apoyan lo hallado en nuestro trabajo previo donde se describe una respuesta protectora parcial en un ensayo experimental con rFhCL1, que puede ser relacionada con eosinófilos y la producción de radicales libres (NO) en estadios tempranos (Zafra y cols., 2013a; Pacheco y cols., 2017).

El papel del H_2O_2 en la infección por *F. hepatica* aún no está claro. Estudios de transcriptómica en ratones han revelado que la producción de ROS es una de las vías más eficientes para desencadenar cambios durante la inmunoprotección (Rojas-Caraballo y cols., 2017) pero también se ha relacionado con el estrés oxidativo y patología en infecciones crónicas en ovejas (Saleh, 2008). Además, diferentes estudios *in vitro* indican que las NEJ de *F. hepatica* poseen una especial habilidad para resistir su destrucción por las especies de oxígeno reactivo liberadas por las células de la respuesta inmunitaria innata, lo que puede implicar la alta expresión de enzimas antioxidantes como la superóxido

dismutasa, glutatión S-transferasa (GST) o peroxirredoxina (Piedrafita y cols., 2000, 2001; Donnelly y cols., 2005, 2008).

Nuestros análisis revelan que tanto los macrófagos como los granulocitos fueron partícipes del incremento en la producción de NO durante los 3 y 18 dpi. Este hecho ha sido remarcado con resultados de estudios previos en ratas sobre la implicación y el papel complementario de estas dos poblaciones celulares en la cavidad peritoneal durante la infección (Piedrafita y cols., 2001; Sibille y cols., 2004, Jedlina y cols., 2011). De hecho, Piedrafita y cols. (2007) describieron una destrucción de NEJ mediada por células dependiente de anticuerpos que involucraba la producción de NO por parte de macrófagos y eosinófilos en ovejas ITT resistentes a *F. gigantica*. En estudios de vacunación anteriores sugieren que la expresión de sintetasa inducible de óxido nítrico y la subsecuente producción de NO podría ser importante para una respuesta efectiva contra las formas migratorias (Zafra y cols., 2013a), si bien no parece producirse un gran efecto en el desarrollo de la infección y la carga parasitaria al final del experimento a pesar de la temprana producción de este radical (Pachecho y cols., 2017). Hasta el momento no se han conseguido respuestas protectoras consistentes en ovinos, así como tampoco se han esclarecido los mecanismos eficaces y precisos de protección (Molina-Hernández y cols., 2015). Se ha sugerido que el NO

y iNOS podrían jugar un importante papel en la patogénesis de *F. hepatica*, posiblemente como un eficaz mecanismo para la destrucción de las NEJ migratorias, como se ha mostrado previamente en ratas resistentes (Piedrafita y cols., 2001) o quizás como una expresión de la activación de los macrófagos M1 que se sabe están relacionado como el desarrollo de respuestas Th1 requeridas para protección (Flynn y cols., 2007; Garza-Cuartero y cols., 2016; Pachecho y cols., 2017). Estudios recientes de transcriptómica han revelado que modificaciones en la vía de señalización de NO puede ser una condición necesaria para la inmunoprotección de ratones (Rojas-Caraballo y cols., 2017) o, por el contrario, la regulación del descenso de iNOS podría ser un factor primordial durante las respuestas no protectoras ocurridas en ovejas (Fu y cols., 2016). En nuestro estudio no hemos detectado una inhibición en la producción de NO en las fases tempranas de la infección. En otro estudio encontramos un bajo nivel en la variación de la expresión de iNOS en macrófagos peritoneales por inmunocitoquímica (Ruiz-Campillo y cols., 2018). Esas diferencias sugieren que el gen iNOS, la expresión de proteínas y la producción de NO en las fases iniciales de la infección puede diferir, permaneciendo la proteína probablemente activa por un tiempo más largo que el gen.

En conclusión, hemos observado una clara respuesta leucocitaria en la cavidad peritoneal en ovejas en las fases iniciales de la infección por *F. hepatica*. Las poblaciones de leucocitos, principalmente los granulocitos, exhibieron una respuesta metabólica con producción intracelular de H_2O_2 y NO. El efecto de esos radicales libres en la migración de NEJ y las formas migratorias juveniles aún no está claro, ya que NEJ y estas formas migratorias parecen no verse afectadas por esas moléculas cuando alcanzan el hígado y evoluciona a estadios maduros.

CONCLUSIONES

1ª. El protocolo de vacunación empleado, con administración de una doble dosis de rFh14-3-3z en adyuvante Montanide, no ha inducido una respuesta protectora, ya que no se ha producido reducción del daño hepático, ni de la carga parasitaria ni de la eliminación de huevos en ovinos infectados con 150 metacercarias de *F. hepatica* (**Primer Estudio**).

2ª. La respuesta inmunitaria inducida por el antígeno recombinante rFh14-3-3z en la especie ovina se caracteriza por una significativa producción de anticuerpos específicos anti-rFh14-3-3z IgG1-IgG2, siendo la IgG1 el isotipo predominante (**Primer Estudio**).

3ª. Dicho antígeno Fh14-3-3z, sin embargo, no tiene un papel relevante en la interacción parásito-hospedador, debido a que no se producen anticuerpos específicos durante la infección por *F. hepatica* en la especie ovina (**Primer Estudio**).

4ª. La respuesta inmunitaria celular que se desarrolla en la cavidad peritoneal de los ovinos infectados con *F. hepatica* se caracteriza por un

incremento del número total de leucocitos durante las fases iniciales de la infección a los 9 y 18 dpi (**Tercer Estudio**).

5ª. La población leucocitaria más relevante en la respuesta celular peritoneal de ovinos infectados es la de granulocitos, cuyo porcentaje se ve significativamente aumentado y que condiciona una disminución relativa de macrófagos y linfocitos durante las fases tempranas de la infección a los 9 y 18 dpi (**Tercer Estudio**).

6ª. Se han identificado la intervención en la cavidad peritoneal durante la infección de los siguientes fenotipos inmunológicos celulares: TCD4, TCD8, $WC1^{+}\gamma\delta$, CD14, CD83 y MHCII, cuyos porcentajes mostraron variaciones durante las fases iniciales y tardías de la infección (**Segundo Estudio**).

7ª. En relación con las células responsables de la respuesta inmunitaria adquirida, los linfocitos, no se evidenció ninguna modificación significativa de las subpoblaciones linfocitarias TCD4, TCD8 y $WC1^{+}\gamma\delta$ en la cavidad peritoneal durante las fases iniciales de la infección. Sin embargo, durante la fase crónica, la subpoblación de linfocitos $WC1^{+}\gamma\delta$

aumenta significativamente en los animales infectados (**Segundo Estudio**).

8ª. El porcentaje de las poblaciones celulares peritoneales estudiadas en la respuesta innata se vio significativamente modificado durante la infección. La dinámica de CD14 en la cavidad peritoneal muestra una tendencia decreciente durante las fases iniciales y desciende significativamente durante las fases crónicas de la infección. Las células CD83 y MHCII desarrollan una tendencia creciente durante las fases iniciales de la infección para descender significativamente en la fase crónica de la infección (**Segundo Estudio**).

9ª. La respuesta oxidativa de los leucocitos peritoneales, considerada en base a la producción de peróxido de hidrógeno (H_2O_2) y óxido nítrico (NO), aumenta significativamente durante las fases iniciales de la infección. Este aumento en la producción de radicales libres fue mayor en animales inmunizados previamente con rFhCL1 (**Tercer Estudio**).

10ª. En el aumento de la respuesta oxidativa, los granulocitos constituyen la población celular que presenta una mayor producción de H_2O_2,

mientras que tanto macrófagos como granulocitos mostraron una alta capacidad de producción de NO (**Tercer Estudio**).

CONCLUSIONS

1. The protocol used in this study, which included vaccination on two occasions with rFh14-3-3z in Montanide adjuvant, does not induce a protective response, since there is no reduction of the liver damage and neither reduction of the fluke burden nor of eggs output in sheep infected with 150 metacercariae of *F. hepatica* (**First Study**).

2. The immne response induced by the recombinanat antigen Fh14-3-3z in sheep is characterised by a significant production of anti-rFh14-3-3z IgG1-IgG2, being IgG1 the predominant subclass (**First Study**).

3. The antigen Fh14-3-3z does not play a relevant role during the host-parasite interface, since no specific antibodies are produced during the infection with *F. hepatica* in sheep (**First Study**).

4. The cellular immune response developed in the peritoneal cavity of infected sheep is characterised by an increase in the total number of leukocytes during the early stages of infection at 9 and 18 dpi (**Third Study**).

5. The most relevant leukocyte population in the peritoneal cellular response of infected sheep are granulocytes, which percentage does significantly increase and imply a relative decrease of macrophages and lymphocytes during the early stages of infection at 9 and 18 dpi (**Third Study**).

6. The following cell subpopulations are present in the peritoneal cavity during *F. hepatica* infection: TCD4, TCD8, $WC1^{+}\gamma\delta$, CD14, CD83 and MHCII, which percentages from the total analysed cells show variations during the early and late stages of infection (**Second Study**).

7. In regard to the cells involved during the acquired immune response, the lymphocytes, no significant variation of the lymphocyte subpopulations TCD4, TCD8 and $WC1^{+}\gamma\delta$ in the peritoneal cavity occurs during the early stages of infection. However, $WC1^{+}\gamma\delta$ subpopulation is significantly increased in infected animals during the late stage of the infection (**Second Study**).

8. The recruitment of the peritoneal cell populations involved in the innate immune response are significantly modified during the infection.

The dynamic of the CD14 cells in the peritoneal cavity displays a decreasing trend during the early stages and was significantly decreased at the chronic stage of the infection. CD83 and MHCII cells develop an increasing trend during the early stages of infection, and are significantly decreased at the chronic stage of the infection (**Second Study**).

9. The oxidative response of peritoneal leukocytes, based on the hydrogen peroxide (H_2O_2) and nitric oxide (NO) production, is significantly increased during the early stages of infection in sheep. This increase in the free radical production is higher in immunised animals with rFhCL1 (**Third Study**)

10. During the increase of the oxidative response, granulocytes are the most relevant cell population involved in H_2O_2 production, whereas granulocytes and macrophages show a high capacity of NO production (**Third Study**).

REFERENCIAS BIBLIOGRÁFICAS

Acosta, D., Cancela, M., Piacenza, L., Roche, L., Carmona, C., Tort, J.F., 2008. *Fasciola hepatica* leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. Mol. Biochem. Parasitol. 158, 52-64.

Acosta, D., Goñi, F., Carmona, C., 1998. Characterization and partial purification of a leucine aminopeptidase from *Fasciola hepatica*. J. Parasitol. 84, 1–7.

Aldridge, A., O'Neill, S.M., 2016. *Fasciola hepatica* tegumental antigens induce anergic-like T cells via dendritic cells in a mannose receptor-dependent manner. Europ. J. Immunol. 46, 1180-1192.

Almeida, M.S., Torloni, H., Lee-Hoo, P., Vilar, M.M., Thaumaturgo, N., Simpson, A.J., Tendler, M., 2003. Vaccination against *Fasciola hepatica* infection using a *Schistosoma mansoni* defined recombinant antigen, Sm14. Parasite. Immunol. 25, 135–137.

Alvarado, R., To, J., Lund, M.E., Pinar, A., Mansell, A., Robinson, M.W., O'Brien, B.A., Dalton, J.P., Donnelly, S., 2016. The immune modulatory peptide FhHDM-1 secreted by the helminth *Fasciola*

hepatica prevents NLRP3 inflammasome activation by inhibiting endolysosomal acidification in macrophages. FASEB J. 31, 85-95.

Alvarez Rojas, C.A., Ansell, B.R., Hall, R.S., Gasser, R.B., Young, N.D., Jex, A.R., Scheerlink, J.P.Y., 2015. Transcriptional analysis identifies key genes involved in metabolism, fibrosis/tissue repair and the immune response against *Fasciola hepatica* in sheep liver. Parasit. Vectors. 8:124.

Alvarez-Sanchez, M.A., Mainar-Jaime, R.C., Perez-Garcia, J., Rojo-Vazquez, F.A., 2006. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet. Rec. 159, 424–425.

Andrews, S.J., 1999. The life cycle of *F. hepatica*. In: Dalton JP, editor. Fasciolosis. Wallingford, UK: CABI Publishing, pp. 1–29.

Armour, J., Dargie, J.D., 1974. Immunity to *Fasciola hepatica* in rat. Successful transfer of immunity by lymphoid-cells and by serum. Exp. Parasitol. 35, 381–388.

Balic, A., Bowles, V.M., Meeusen, E.N.T., 2000. Cellular profiles in the abomasal mucosa and lymph node during primary infection with *Haemonchus contortus* in sheep. Vet. Immunol. Immunopathol. 75, 109–120.

Barrera, B., Otero, J.A., Egido, E., Prieto, J.G., Seelig, A., Álvarez, A.I., Merino, G., 2012. The anthelmintic triclabendazole and its metabolites inhibit the membrane transporter ABCG2/BCRP. Antimicrob. Agents Chemother. 56, 3535–3543.

Baska, P., Zawistowska-Deniziak, A., Zdziarska, A.M., Wasyl, K., Wisniewski, M., Cywinska, A., Klockiewicz, M., Januszkiewicz, K., We˛drychowicz, H., 2013. *Fasciola hepatica* - the pilot study of in vitro assessing immune response against native and recombinant antigens of the fluke. Acta Parasitol. 58, 453-462.

Behm, C.A., Sangster, N.C., 1999. Pathology, pathophysiology and clinical aspects. In: Dalton JP, editor. Fasciolosis.Wallingford, UK: CABI Publishing, pp. 185–224.

Bennett, C.E., Threadgold, L.T., 1975. *Fasciola hepatica*: Development of caecal epithelium during migration in the mouse. Exp. Parasitol. 37, 426–441.

Blumerman, S.L., Herzig, C.T., Baldwin, C.L., 2007. WC1+ γδ T cell memory population is inducen by killed bacterial vaccine. Eur. J. Immunol. 37, 1204-1216.

Boray, J.C., 1963. The ecology of *Fasciola hepatica* with particular reference to its intermediate host in Australia. Proceedings of the 17th World Veterinary Congress, Hannover, pp.709–715.

Boray, J.C., 1969. Experimental fascioliasis in Australia. Adv. Parasitol. 7, 95-210.

Boray, J., Crowfoot, P., Strong, M., Allison, J., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. Vet. Rec. 113, 315–317.

Bottari, N.B., Mendes, R.E., Lucca, N.J., Schwertz, C.I., Henker. L.C., Olsson, D.C., Piva, M.M., Sangoi, M., Campos, L.P., Moresco R.N., Jaques, J.A., Da Silva A.S., 2015. Oxidative stress associated with pathological lesions in the liver of rats experimentally infected by *Fasciola hepatica*. Exp. Parasitol. 159, 24–8.

Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster N.C., 2013. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int. J. Parasitol. Drugs Drug Resist. 4, 48–54.

Buffoni, L., Zafra, R., Pérez-Écija, A., Martínez-Moreno, F.J., Martínez-Galisteo, E., Moreno, T., Pérez, J., Martínez-Moreno, A., 2010. Immune response in goats immunised with glutathione S-transferase and experimentally challenged with *Fasciola hepatica*. Parasitol. Int. 59, 147–153.

Buffoni, L., Martínez-Moreno, F.J., Zafra, R., Mendes, R.E., Pérez-Écija, A., Sekiya, M., Mulcahy, G., Pérez, J., Martínez-Moreno, A., 2012. Humoral immune response in goats immunised with cathepsin L1,

peroxiredoxin and Sm14 antigen and experimentally challenged with *Fasciola hepatica*. Vet. Parasitol. 185, 315–321.

Cadenas, E., Davies, K.J.A., 2000. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic. Biol. Med. 29, 222-230.

Cancela, M., Carmona, C., Rossi, S., Frangione, B., Goñi, F., Berasain, P., 2004. Purification, characterization, and immunolocalization of paramyosin from the adult stage of *Fasciola hepatica*. Parasitol. Res. 92, 441–448.

Casanueva, R., Hillyer, G.V., Ramajo, V., Oleaga, A., Espinoza, E.Y., Muro, A., 2001. Immunoprophylaxis against *Fasciola hepatica* in rabbits using a recombinant Fh15 fatty acid-binding protein, J. Parasitol. 87, 697–700.

Chaithirayanon, K., Grams, R., Vichasri-Grams, S., Hoffman, A., Korge, G., Viyanant, V., Upatham, E.S., Sobhon, P.O., 2006. Molecular and immunological characterization of encoding gene and 14-3-3 protein in *Fasciola gigantica*. Parasitol. 133, 763–775.

Chan, S.S.M., McConnell, I., Blacklaws, B.A., 2002. Generation and characterization of ovine dendritic cells derived from peripheral blood monocytes. Immunol. 107, 366–372.

Charlier, J., Vercruysse, J., Morgan, E., van Dijk, J., Williams, D.J., 2014. Recent advances in the diagnosis, impact on production and prediction of *Fasciola hepatica* in cattle. Parasitol. 141, 326–335.

Cheeseman, K.H, Slater, T.F., 1993. An introduction to free radicals chemistry. Br. Med. Bull. 49, 481-493.

Chen, M.G., Mott, K.E., 1990. Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. Trop. Dis. Bulletin 87, R1-R38.

Chien, Y. H., Konigshofer, Y., 2007. Antigen recognition by γδ T cells. Immunol. Rev. 215, 46–58.

Collins, P.R., Stack, C.N., O'Neill, S.M., Doyle, S., Ryan, T., Brennan, G.P., Mousley, A., Stewart, M., Maule, A.G., Dalton, J.P., Donnelly, S., 2004. Cathepsin L1, the major protease involved in liver fluke (*Fasciola*

hepatica) virulence: propeptide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells. J. Biol. Chem. 279, 17038–17046.

Cooper, K.M., Kennedy, D.G., Danaher, M., 2012. *ProSafeBeef* and anthelmintic drug residues - A case study in collaborative application of multi-analyte mass spectrometry to enhance consumer safety. Anal. Bioanal. Chem. 404, 1623–1630.

Creaney, J., Wilson, L., Dosen, M., Sandeman, R. M., Spithill, T.W., Parsons, J.C., 1996. *Fasciola hepatica*: irradiation-induced alterations in carbohydrate and cathepsin-B protease expression in newly excysted juvenile liver fluke. Exp. Parasitol. 83, 202-215.

Cwiklinski, K., Dalton, J.P., 2018. Advances in *Fasciola hepatica* research using 'omics' technologies. Int. J. Parasitol. 48, 321-331

Cwiklinski, K., de la Torre-Escudero, E., Trelis, M., Bernal, D., Dufresne, P.J., Brennan, G.P., O'Neill, S., Tort, J., Paterson, S., Marcilla, A., Dalton, J.P., Robinson, M.W., 2015. The extracellular vesicles of the helminth patogen, *Fasciola hepatica*: biogenesis pathways and cargo

mollecules involved in parasite pathogenesis. Moll. Cell. Proteomics. 14, 3258–3273.

Dalton, J.P., Brindley, P.J., Knox, D.P., Brady, C.P., Hotez, P.J., Donnelly, S., O'Neill, S.M., Mulcahy, G., Loukas, A., 2003a. Helminth vaccines: from mining genomic information for vaccine targets to systems used for protein expression. Int. J. Parasitol. 33, 621-640.

Dalton, J.P., McGonigle, S., Rolph, T.P., Andrews, S.J., 1996. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. Infect. Immun. 64, 5066–5074.

Dalton, J.P., Mulcahy, G., 2001. Parasite vaccines–a reality? Vet. Parasitol. 98, 149–167.

Dalton, J.P., Neill, S.O., Stack, C., Collins, P., Walshe, A., Sekiya, M., Doyle, S., Mulcahy, G., Hoyle, D., Khaznadji, E., Moire, N., Brennan, G., Mousley, A., Kreshchenko, N., Maule, A.G., Donnelly, S.M., 2003b. *Fasciola hepatica* cathepsin L-like proteases: biology, function, and

potential in the development of first generation liver fluke vaccines. Int. J. Parasitol. 30, 1173-1181.

Dalton, J.P., Robinson, M.W., Mulcahy, G., O'Neill, S.M., Donelly, S., 2013. Immunomodulatory molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic development. Vet. Parasitol. 195, 272–285.

Davies, C., Goose, J., 1981. Killing of newly excysted juveniles of *Fasciola hepatica* in sensitized rats. Parasite Immunol. 3, 81-96.

Davies, K.J.A., 1995. Oxidative stress: the paradox of aerobic life. Biochem. Soc. Symp. 61, 1-31.

Dawes, B., 1961. On the early stages of *Fasciola hepatica* penetrating into the liver of a experimental host, the mouse: a histological picture. J. Helminthol. R.T. Leiper supplement, 41-52.

De la Torre-Escudero, E., Manzano-Román, R., Valero, L., Oleaga, A., Pérez-Sánchez, R., Hernández-González, A., Siles-Lucas, M., 2011.

Comparative proteomic analysis of *Fasciola hepatica* juveniles and *Schistosoma bovis* schistosomula. J. Proteomics. 74, 1534–1544.

De Paulaa, R.C., Cassali, G.D., Negrão-Correaa, D., Guimarãesa, M.P., 2010. Development and pathology of *Fasciola hepatica* in CCL3-deficient mice. Vet. Parasitol. 173, 147-151.

Dewilde, S., Ioanitescu, A.I., Kiger, L., Gilany, K., Marden, M.C., Van Doorslaer, S., Vercruysse, J., Pesce, A., Nardini, M., Bolognesi, M., Moens, L., 2008. The hemoglobins of the trematodes *Fasciola hepatica* and *Paramphistomum epiclitum*: a molecular biological, physico-chemical, kinetic, and vaccination study. Protein. Sci. 17, 1653–1662.

Donnelly, S., O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P., 2005. Thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. Infect. Immun. 73, 166–173.

Donnelly, S., O'Neill, S. M., Stack, C. M., Robinson, M. W., Turnbull, L., Whitchurch, C., Dalton, J. P., 2010. Helminth cysteine proteases inhibit TRIF-dependent activation of macrophages via degradation of TLR3. J. Biol. Chem. 285, 3383–3392.

Donnelly, S., Stack, C.M., O'Neill, S.M., Sayed, A.A., Williams, D.L., Dalton, J.P., 2008. Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. FASEB J. 22, 4022–4032.

Dow, C., Ross, J.G., Tood, J.R., 1968. The histopathology of *Fasciola hepatica* infections in sheep. Parasitology. 58, 120-135.

Dowling, D.J., Hamilton, C.M., Donnelly, S., La Course, J., Brophy, P.M., Dalton, J., O'Neill, SM., 2010. Major secretory antigens of the Helminth *Fasciola hepatica* activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses. Infect. Immun. 78, 793–801.

Dröge, W., 2002. Free radicals in the physiological control of cell function. Physiol. Rev. 82, 47-95.

Duménigo, B.E., Espino, A.M., Finlay, C.M., Mezo, M., 1999. Kinetics of antibody-based antigen detection in serum and faeces of sheep experimentally infected with *Fasciola hepatica*. Vet. Parasitol. 86, 23–31.

Escamilla, A., Bautista, M.J., Zafra, R., Pacheco, I.L., Ruiz, M.T., Martínez-Cruz, S., Méndez, A., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2016. *Fasciola hepatica* induces eosinophil apoptosis in the migratory and biliary stages of infection in sheep. Vet. Parasitol. 216, 84–88.

Escamilla, A., Pérez-Caballero, R., Zafra, R., Bautista, M.J., Pacheco, I.L., Ruiz, M.T., Martínez-Cruz, S., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2017. Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infection in sheep. Vet. Parasitol. 238, 49–53.

Espino, A.M., Morales, A., Delgado, B., Rivera, F.M., Figueroa, O., Suárez, E., 2010. Partial immunity to *Fasciola hepatica* in mice after vaccination with FhSAP2 delivered as recombinant protein or DNA construct. Ethn. Dis., 17–23.

Euzéby, J., 1971. Les maladies vermineuses des animanux dometiques et leurs incidences sur la pathologie humaine. Tome II. Maladies dues aux plathelminthes. París, Francia: Vigot Frères Éditeurs.

Fairweather, I., 2009. Triclabendazole progress report, 2005-2009: an advancement of learning? J. Helminthol. 83, 139-150.

Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet. Parasitol. 180, 133–43.

Finlay, C.M., Stefanska, A.M., Coleman, M.M., Jahns, H., Cassidy, J.P., McLoughlin, R.M., Mills, K.H.G., 2017. Secreted products of *Fasciola hepatica* inhibit the induction of T cell responses that mediate allergy. Parasite Immunol. 39, 1–6.

Fiss, L., Adrien, M.A., Marcolongo-Pereira, C., Assis-Brasil, N.D., Sallis, E.S.V., Riet- Correa, F., Ruas, J.L., Schild, A.L., 2013. Subacute and acute fasciolosis in sheep in southern Brazil. Parasitol. Res. 112, 883-887.

Flynn, R.J., Irwin, J.A., Olivier, M., Sekiya, M., Dalton, J.P., Mulcahy, G., 2007. Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet. Immunol. Immunopathol. 120, 31–40.

Flynn, R.J., Mulcahy, G., 2008. Possible role for Toll-like receptors in interaction of *Fasciola hepatica* excretory/secretory products with bovine macrophages. Infect. Immun. 76, 678-684.

Flynn, R.J., Mulcahy, G., Elsheikha, H.M., 2010. Coordinating innate and adaptive immunity in *Fasciola hepatica* infection: implications for control. Vet. Parasitol. 169, 235–240.

Fu, Y., Browne, J.A., Killick, K., Mulcahy, G., 2017. Network Analysis of the Systemic Response to *Fasciola hepatica* Infection in Sheep Reveals Changes in Fibrosis, Apoptosis, Toll-Like Receptors 3/4, and B Cell Function. Front. Immunol. 8:485.

Fu, Y., Chryssafidis, A.L., Browne, J.A., O'Sullivan, J., McGettigan, P.A., Mulcahy, G., 2016. Transcriptomic Study on Ovine Immune Responses to *Fasciola hepatica* Infection. PLoS Negl. Trop. Dis. 10:e0005015.

Gadahi, J.A., Ehsan, M., Wang, S., Zhang, Z., Wang, Y., Yan, R., Song, X., Xu, L., Li, X., 2016. Recombinant protein of *Haemonchus contortus* 14-3-3 isoform 2 (rHcftt-2) decreased the production of IL-4 and

suppressed the proliferation of goat PBMCs in vitro. Exp. Parasitol. 171, 57–66.

Galtier, P., Eeckhoutte, C., Larrieu, G., 1987. *Fasciola hepatica*: liver enzymes in rats and interaction with chemical inducers. Exp. Parasitol. 63, 189-194.

Garcia-Campos, A., Ravidà, A., Linh, D., N., Cwiklinski, K., Dalton, J.P., Hokke, C.H., O 'Neill, S., Mulcahy, G., 2016. Tegument Glycoproteins and Cathepsins of Newly Excysted Juvenile *Fasciola hepatica* Carry Mannosidic and Paucimannosidic N-glycans. PLoS Negl. Trop. Dis. 10(5): e0004688.

Garza-Cuartero, L., Geurden, T., Mahan, S.M., Hardham, J.M., Dalton, J.P., Mulcahy, G., 2018. Antibody recognition of cathepsin L1-derived peptides in *Fasciola hepatica* infected and/or vaccinated cattle and identification of protective linear B-cell epitopes. Vaccine. 36, 958–968.

Garza-Cuartero, L., O'Sullivan, J., Blanco, A., McNair, J., Welsh, M., Flynn, R.J., Williams, D., Diggle, P., Cassidy, J., Mulcahy, G., 2016. *Fasciola hepatica* infection reduces *Mycobacterium bovis* burden and

mycobacterial uptake and suppresses the pro-inflammatory response. Parasite Immunol. 38, 387–402.

Genicot, B., Mouligneau, F., Lekeux, P., 1991. Economic and production consequences of liver fluke disease in double-muscled fattening cattle. J. Vet. Med. Series B. 38, 203-208.

Gill, H.S., Watson, D.L., Brandon, M.R., 1993. Monoclonal antibody to CD4+ T-cells abrogates genetic resistance to *Haemonchus contortus* in sheep. Immunol. 78, 43-49.

Gironès, N., Valero, M.A., García-Bodelón, M.A., Chico-Calero, I., Punzón, C., Fresno, M., Mas-Coma, S., 2007. Immune Suppression in Advanced Chronic Fascioliasis: An Experimental Study in a Rat Model. J. Infect. Dis. 195, 1504–1512.

Golden, O., Flynn, R.J., Read, C., Sekiya, M., Donnelly, S.M., Stack, C., Dalton, J.P., Mulcahy, G., 2010. Protection of cattle against a natural infection of *Fasciola hepática* by vaccination with recombinant cathepsin L1 (rFhCL1). Vaccine. 28, 5551–5557.

González-Lanza, C., Manga, Y., Del Pozo, E., Hidalgo, R., 1989. Dynamics of elimination of the eggs of *Fasciola hepatica* (Trematoda Digenea) in the faeces of cattle in the Porma basin, Spain. Vet. Parasitol. 34, 35-43.

González-Hernández, A., Borloo, J., Peelaers, I., Casaert, S., Leclercq, G., Claerebout, E., Geldhof, P., 2018. Comparative analysis of the immune responses induced by native versus recombinant versions of the ASP-based vaccine against the bovine intestinal parasite *Cooperia oncophora*. Int. J. Parasitol. 48, 41–49.

Graham-Brown, J., Hartley, C., Clough, H., Kadioglu, A., Baylis, M., Williams, D.J.L., 2018. Dairy heifers naturally exposed to *Fasciola hepatica* develop a type 2 immune response and concomitant suppression of leukocyte proliferation. Infect. Immun. 86, 1–13.

Guasconi, L., Serradell, M.C., Borgonovo, J., Garro, A.P., Varengo, H., Caffe, G., Masih, D.T., 2012. Immunization with crude antigens plus aluminium hydroxide protects cattle from *Fasciola hepatica* infection. J. Helminthol. 86, 64–69.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine, 3rd ed., Oxford University Press.

Hamilton, C.M., Dowling, D.J., Loscher, C.E., Morphew, R.M., Brophy, P.M., O'Neill, S.M., 2009. The *Fasciola hepatica* tegumental antigen suppresses dendritic cell maturation and function. Infect. Immun. 77, 2488–2498.

Haroun, E.M., Hillyer, G.V., 1986. Resistance to fasciolosis – a review. Vet. Parasitol. 20, 63–93.

Henker, L.C., Schwertz, C., Lucca, N.J., Piva, M.M., Prior, K.C., Baska, P., Norbury, L., Januszkiewicz, K., Dezen, D., Duarte, M.M.M.F., Moresco, R.N., Bertagnolli da Rosa, L., Mendes, R.E., 2017. Immune protection conferred by recombinant MRLC (myosin regulatory light chain) antigen in TiterMax Gold® adjuvant against experimental fasciolosis in rats. Vaccine. 35, 663-671.

Hernández-González, A., Valero, M.L., Sánchez del Pino, M., Oleaga, A., Siles-Lucas, M., 2010. Proteomic analysis of in vitro newly excysted

juveniles form *Fasciola hepatica*. Mol. Biochem. Parasitol. 172, 121–128.

Hewitson, J.P., Grainger, J.R., Maizels, R.M., 2009. Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity. Mol. Biochem. Parasitol. 167, 1–11.

Hillyer, G.V., 2005. *Fasciola* antigens as vaccines against fascioliasis and schistosomiasis. J. Helmintol. 79, 241-247.

Howell, A., Baylis, M., Smith, R., Pinchbeck, G., Williams, D., 2015. Epidemiology and impact of *Fasciola hepatica* exposure in high-yielding dairy herds. Prev. Vet. Med. 121, 41–48.

Hoyle, D. V, Taylor, D.W., 2003. The immune response of regional lymph nodes during the early stages of *Fasciola hepatica* infection in cattle. Parasite Immunol. 25, 221–229.

Hsu, H., Chen, C., Nenninger, A., Holz, L., Baldwin, C.L., Telfer, J.C., 2015. WC1 is a hybrid γδ TCR coreceptor and pattern recognition receptor for pathogenic bacteria. J. Immunol. 194, 2280-2288.

Jayaraj, R., Piedrafita, D., Dynon, K., Grams, R., Spithill, T.W., Smooker, P.M., 2009. Vaccination against fasciolosis by a multivalent vaccine of stage-specific antigens. Vet. Parasitol. 160, 230–236.

Jedlina, L., Kozak-Ljunggren, M., Wedrychowicz, H., 2011. *In vivo* studies of the early, peritoneal, cellular and free radical response in rats infected with *Fasciola hepatica* by flow cytometric analysis. Exp. Parasitol. 128, 291–297.

Keita, M., Vincendeau, P., Buguet, A., Cespuglio, R., Vallat, J.M., Dumas, M., Bouteille, B., 2000. Inducible nitric oxide synthase and nitrotyrosine in the central nervous system of mice chronically infected with *Trypanosoma brucei brucei*. Exp. Parasitol. 95, 19-27.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 32, 458–469.

Kelly, W.R., 1993. The liver and biliary system. In: Jubb K.V.F., Kennedy P.C. y Palmer N. (eds.). Pathology of domestic animals. pp. 319-407.

Kesik, M., Jedlina-Panasiuk, L., Kozak-Cieszczyk, M., Płucienniczak, A., Wedrychowicz, H., 2007. Enteral vaccination of rats against *Fasciola hepatica* using recombinant cysteine proteinase (cathepsin L1). Vaccine. 25, 3619–3628.

Kesik-Brodacka, M., Lipiec, A., Kozak Ljunggren, M., Jedlina, L., Miedzinska, K., Mikolajczak, M., Plucienniczak, A., Legocki, A.B., Wedrychowicz, H., 2017. Immune response of rats vaccinated orally with various plant-expressed recombinant cysteine proteinase constructs when challenged with *Fasciola hepatica* metacercariae. PLoS Negl. Trop. Dis. 11, 1–18.

Khallaayoune, K.H., Stromberg, B.E., Dakkak, A., Malone, J.B., 1991. Seasonal dynamics *of Fasciola hepatica* burdens in grazing Timahdit sheep in Morocco. Int. J. Parasitol. 21, 307-314.

Kolodziejczyk, L., Siemieniuk, E., Skrzydlewska, E., 2006. *Fasciola hepatica*: effects on the antioxidative properties and lipid peroxidation of rat serum. Exp. Parasitol. 113, 43-48.

Konopka, B., 1993. Occurrence of parasites in slaughter animals in Kielce región (central Poland) in 1987-1992. Med. Weter. 49, 373-374.

Koyama, T., Ohsawa, T., Shimada, S., Omata, Y., Xuan, X., Inoue, N., Maeda, R., Mikami, T., Saito, A., 2001. A 14-3-3 protein homologue is expressed in feline enteroepithelial-stages of *Toxoplasma gondii*. Vet. Parasitol. 96, 65–74.

Kumar, N., Anju, V., Gaurav, N., Chandra, D., Samanta, S., Gupta, S.C., Adeppa, J., Raina, O.K., 2012. Vaccination of buffaloes with *Fasciola gigantica* recombinant glutathione S-transferase and fatty acid binding protein. Parasitol. Res. 110, 419–426.

Lahmers, K.K., Norimine, J., Abrahamsen, M.S., Palmer, G.H., Brown, W.C., 2005. The CD4+ T cell immunodominant *Anaplasma marginale* major surface protein 2 stimulates γδ T cell clones that express unique T cell receptors. J. Leukoc. Biol. 77, 199-208.

Lally, N.C., Jenkins, M.C., Dubey, J.P., 1996. Development of a polymerase chain reaction assay for the diagnosis of neosporosis using

the *Neospora caninum* 14-3-3 gene. Mol. Biochem. Parasitol. 75, 169–178.

Lampe, K., Gottstein, B., Becker, T., Stahl-Hennig, C., Kaup, F.J., Mätz-Rensing, K., 2017. Immunization of rhesus macaques with *Echinococcus multilocularis* recombinant 14-3-3 antigen leads to specific antibody response. Parasitol. Res. 116, 435–439.

Maggioli, G., Acosta, D., Silveira, F., Rossi, S., Giacaman, S., Basika, T., Gayo, V., Rosadilla, D., Roche, L., Tort, J., Carmona, C., 2011a. The recombinant gut-associated M17 leucine aminopeptidase in combination with different adjuvants confers a high level of protection against *Fasciola hepatica* infection in sheep. Vaccine 29, 9057–9063.

Maggioli, G., Bottini, G., Basika, T., Alonzo, P., Salinas, G., Carmona, C., 2016. Immunization with *Fasciola hepatica* thioredoxin glutathione reductase failed to confer protection against fasciolosis in cattle. Vet. Parasitol. 224, 13–19.

Maggioli, G., Carambula, B., Carmona, C., 2004. Purification, Characterization, and Immunolocalization of a Thioredoxin Reductase From Adult *Fasciola hepatica*. 90, 205–211.

Maggioli, G., Silveira, F., Martin-Alonso, J.M., Salinas, G., Carmona, C., Parra, F., 2011b. A recombinant thioredoxin-glutathione reductase from *Fasciola hepatica* induces a protective response in rabbits. Exp. Parasitol. 129, 323–330.

Manga, Y., González-Lanza, C., Del Pozo, P.E., Hidalgo, R., 1990. Kinectis of *Fasciola hepatica* egg pasaje in the faeces of sheep in the Porna basin, Spain. Acta Parasitol. Pol. 35, 149-157

Manual of Veterinary Parasitological Laboratory Techniques. Ministry of Agriculture, Fisgeries and Food. Reference Book 418 (Third edition, 1986). Her Majesty's Stationery Office, London.

Maqbool, A., Hashmi, H.A., Shafique, M., Akhtar, T., Ahmad, M., Mahmood, F., 2000. Epidemiology and chemotherapy of fascioliasis in goats. Indian J. Anim. Res. 34, 33-36.

Martínez-Fernández, A.R., Nogal-Ruiz, J.J., López-Abán, J., Ramajo, V., Oleaga, A., Manga-González, Y., Hillyer, G.V., Muro, A., 2004. Vaccination of mice and sheep with Fh12 FABP from *Fasciola hepatica* using the new adjuvant/immunomodulator system ADAD. Vet. Parasitol. 126, 287-298.

Martínez-Moreno, A., Martínez-Cruz, M.S., Martínez, F.J., Gutiérrez, P.N., Hernández, S., 1996. Detection of antibodies to *Fasciola hepatica* excretory-secretory antigens in experimentally infected goats by enzime immunosorbent assay. Vet. Parasitol. 62, 247-252.

Mas-Coma, S., Bargues, M.D., Valero, M.A., 2005. Fascioliasis and other plant-borne trematode zoonoses. Int. J. Parasitol. 35, 1255-1278.

McGonigle, S.; Beall, M.J.; Pearce, E.J., 2002. Eukaryotic initiation factor 2α subunit associates with TGFβ receptors and 14-3-3ε and acts as a modulator of the TGFβ response. Biochemistry. 41, 579–587.

McManus, D.P., Dalton, J.P., 2006. Vaccines against the zoonotic trematodes *Schistosoma japonicum*, *Fasciola hepatica* and *Fasciola gigantica*. Parasitology. 133, pp. S43-S61

McNeilly, T.N., Nisbet, A.J., 2014. Immune modulation by helminth parasites of ruminants: implications for vaccine development and host immune competence. Parasite. 21, 51.

Meeusen, E., Lee, C.S., Rickard, M.D., Brandon, M.R., 1995. Cellular responses during liver fluke infection in sheep and its evasion by the parasite. Parasite Immunol. 17, 37-45.

Meeusen, E.N.T., Piedrafita, D., 2003. Exploiting natural immunity to helminth parasites for the development of veterinary vaccines. Int. J. Parasitol. 33, 1285-1290.

Mehmood, K., Zhang, H., Sabir, A.J., Abbas, R.Z., Ijaz, M., Durrani, A.Z., Saleem, M.H., Ur Rehman, M., Iqbal, M.K., Wang, Y., Ahmad, H.I., Abbas, T., Hussain, R., Ghori, M.T., Ali, S., Khan, A.U., Li, J., 2017. A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. Microb. Pathog. 109, 253–262.

Mello-Filho, A.C., Hoffmann, M.E., Meneghini, R., 1984. Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. Biochem. J. 218, 273-275.

Mendes, E.A., Mendes, T.A. de O, dos Santos, S.L., Menezes-Souza, D., Bartholomeu, D.C., Martins, I.V.F., Silvac, L.M., dos Santos Lima, W., 2013. Expression of IL-4, IL-10 and IFN-γ in the liver tissue of cattle that are naturally infected with *Fasciola hepatica*. Vet. Parasitol. 195, 177–182.

Mendes, R.E., Pérez-Ecija, R.A., Zafra, R., Buffoni, L., Martínez-Moreno, A., Dalton, J.P., Mulcahy, G., Pérez, J., 2010b. Evaluation of hepatic changes and local and systemic immune responses in goats immunized with recombinant Peroxiredoxin (Prx) and challenged with *Fasciola hepatica*. Vaccine 28 (16), 2832–2840.

Mendes, R.E., Pérez-Écija, A., Zafra, R., Buffoni, L., Martínez-Moreno, A., Pérez, J., 2010a. Evaluation of the Local Immune Response to *Fasciola hepatica* in Liver and Hepatic Lymph Nodes (HLN) of Goats Immunized with Cathepsin-L1 (CL1). J. Comp. Pathol. 141, 698–705.

Meng, M., He, S., Zhao, G., Bai, Y., Zhou, H., Cong, H., Lu, G., Zhao, Q., Zhu, X.Q., 2012. Evaluation of protective immune responses induced by DNA vaccines encoding *Toxoplasma gondii* surface antigen 1 (SAG1) and 14-3-3 protein in BALB/c mice. Parasit. Vectors. 5, 273.

Miller, C.M.D., Smith, N.C., Ikin, R.J., Boulter, N.B., Dalton, J.P., Donnelly, S., 2009. Immunological interactions between 2 common pathogens, Th1-inducing protozoan *Toxoplasma gondii* and the Th2-inducing helminth *Fasciola hepatica*. PLoS ONE. 4, e5692.

Mitchell, M., 1995. The distribution and epidemiology of liver fluke in New Zealand. Surveillance. 22, 22-26.

Mitra, R., Dharajiya, N., Kumari, L., Varalakshmi, C., Khar, A., 2004. Migration of antigen presenting cells from periphery to the peritoneum during an inflammatory response: role of chemokines and cytokines. FASEB J. 18, 1764-1766.

Moazeni, M., Ahmadi, A., 2016. Controversial aspects of the life cycle of *Fasciola hepatica*. Exp. Parasitol. 169, 81–89.

Mokhtarian, K., Akhlaghi, L., Meamar, A.R., Razmjou, E., Manouchehri Naeini, K., Gholami, S., Najafi Samei, M., Falak, R., 2016a. Serodiagnosis of fasciolosis by fast protein liquid chromatography fractionated excretory/secretory antigens. Parasitol. Res. 15, 1–9.

Mokhtarian, K., Akhlaghi, L., Mohamadi, M., Meamar, A.M., Razmjou, E., Khoshmirsafa, M., Falak, R., 2016b. Evaluation of anti-Cathepsin L1: a more reliable method for serodiagnosis of human fasciolosis. Trans. R. Soc. Trop. Med. Hyg. 110, 542–550.

Mokhtarian, K., Meamar, A.R., Khoshmirsafa, M., Razmjou, E., Masoori, L., Khanmohammadi, M., Akhlaghi, L., Falak, R., 2018. Comparative assessment of recombinant and native immunogenic forms of *Fasciola hepatica* proteins for serodiagnosis of sheep fasciolosis. Parasitol. Res. 117, 225–232.

Molina-Hernández, V., Mulcahy, G., Pérez, J., Martínez-Moreno, Á., Donnelly, S., O'Neill, S.M., Dalton, J.P., Cwiklinskia, K., 2015. *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. Vet. Parasitol. 208, 101–111.

Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in the Netherlands. Vet. Parasitol. 91, 153–158.

Morrison, C.A., Colin, T., Sexton, J.L., Bowen, F., Wicker, J., Friedel, T., Spithill, T.W., 1996. Protection of cattle against *Fasciola hepatica* infection by vaccination with glutathione S-transferase. Vaccine. 14, 1603–1612.

Moreau, E., Chauvin, A., 2010. Immunity against helminths: interactions with the host and the intercurrent infections. J. Biomed. Biotechnol. 2010:428593.

Motran, C.C., Silvane, L., Chiapello, L.S., Theumer, M.G., Ambrosio, L.F., Volpini, X., Celias, D.P., Cervi, L., 2018. Helminth infections: Recognition and modulation of the immune response by innate immune cells. Front. Immunol. 9, 1–12.

Mulcahy, G., Dalton, J.P., 1998. Vaccines in the control of liver fluke infection in ruminants: Current status and prospects. Ir. Vet. J. 51, 520-525.

Mulcahy, G., O'Connor, F., Clery, D., Hogan, S.F., Dowd, A.J., Andrews, S.J., Dalton, J.P., 1999. Immune responses of cattle to experimental anti-*Fasciola hepatica* vaccines. Res. Vet. Sci. 67, 27-33.

Mulcahy, G., O'Connor, F., McGonigle, S., Dowd, A., Clery, D., Andrews, S.J., Dalton, J.P., 1998. Correlation of specific antibody titre and avidity with protection in cattle immunized against *Fasciola hepatica*. Vaccine, 16, 932–939.

Nathan, C., Kie, Q., 1994. Nitric oxide synthases: roles, tolls and controls. Cell. 78, 915–918.

Navarro, J.A., Caro, M.R., Seva, J., Rosillo, M.C., Gomez, M.A., Gallego, M.C., 1996. Study of lymphocyte subpopulations in peripheral blood and secondary lymphoid organs in the goat using monoclonal antibodies to surface markers of bovine lymphocytes. Vet. Immunol. Immunopathol. 51, 147–156.

Nisbet, A.J., McNeilly, T.N., Greer, A.W., Bartley, Y., Oliver, E.M., Smith, S., Palarea-Albaladejo, J., Matthews, J.B., 2016. Protection of ewes against *Teladorsagia circumcincta* infection in the periparturient period by vaccination with recombinant antigens. Vet. Parasitol. 228, 130–136.

Novobilský, A., Höglund, J., 2015. First report of closantel treatment failure against *Fasciola hepatica* in cattle. Int. J. Parasitol. Drugs Drug Resist. 5, 172–177.

Noya, V., Brossard, N., Berasaín, P., Rodríguez, E., Chiale, C., Mazal, D., Carmona, C., Freire, T., 2016. A mucin-like peptide from *Fasciola hepatica* induces parasite-specific Th1-type cell immunity. Parasitol. Res. 115, 1053–1063.

Obenauer, J.C., Cantley, L.C., Yaffe, M.B., 2003. Scansite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res., 31, 3635–3641.

Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Vet. Parasitol. 178, 364–366.

O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H.G., Dalton, J.P., 2000. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite Immunol. 22, 147–155.

O'Neill, S.M., Mills, K.H., Dalton, J.P., 2001. *Fasciola hepatica* cathepsin L cysteine proteinase suppresses *Bordetella pertussis*-specific interferon-gamma production *in vivo*. Parasite Immunol. 23, 541–547.

Orbegozo-Medina, R.A., Martínez-Sernández, V., González-Warleta, M., Castro-Hermida, J.A., Mezo, M., Ubeira, F.M., 2018. Vaccination of sheep with Quil-A® adjuvant expands the antibody repertoire to the *Fasciola* MF6p/FhHDM-1 antigen and administered together impair the growth and antigen release of flukes. Vaccine 36, 1949–1957.

Ortiz, P., Scarcella, S., Cerna, C., Rosales, C., Cabrera, M., Guzmán, M., Lamenza, P., Solana, H., 2013. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Cajamarca (Peru): a clinical trial and in vivo efficacy test in sheep. Vet. Parasitol. 195, 118–121.

Overend, D.J., Bowen, F.L., 1995. Resistance of *Fasciola hepatica* to triclabendazole. Aust. Vet. J. 72, 275–276.

Pacheco, I.L., Abril, N., Morales-Prieto, N., Bautista, M.J., Zafra, R., Escamilla, A., Ruiz, M.T., Martínez-Moreno, A., Pérez, J., 2017. Th1/Th2 balance in the liver and hepatic lymph nodes of vaccinated and

unvaccinated sheep during acute stages of infection with *Fasciola hepatica*. Vet. Parasitol. 238, 61–65.

Pérez, J., Martín de las Mulas, J., Carrasco, L., Gutiérrez, P.N., Martínez-Cruz, M.S., Martínez-Moreno, A., 1999. Pathological and immunohistochemical study of the liver and hepatic lymph nodes in goats infected with one or more doses of *Fasciola hepatica*. J. Comp. Pathol. 120, 199-210.

Pérez-Caballero, R., Buffoni, L., Martínez-Moreno, F.J., Zafra, R., Molina-hernández, V., 2018. Expression of free radicals by peritoneal cells of sheep during the early stages of *Fasciola hepatica* infection. Parasit. Vectors. 11:500 (doi: 10.1186/s13071-018-3072-5).

Pérez-Ecija, R.A., Mendes, R.E., Zafra, R., Buffonni, L., Martínez-Moreno, A., Pérez, J., 2010. Pathological and parasitological protection in goats immunised with recombinant cathepsin L1 and challenged with *Fasciola hepatica*. Vet. J. 185, 351–353.

Piacenza, L., Acosta, D., Basmadjian, I., Dalton, J.P., Carmona, C., 1999. Vaccination with cathepsin L proteinases and with leucine

aminopeptidase induces high levels of protection against fascioliasis in sheep. Infect. Immun. 67, 1954–1961.

Piedrafita, D., Estuningsih, E., Pleasance, J., Prowse, R., Raadsma, H.W., Meeusen, E.N.T., Spithill, T.W., 2007. Peritoneal lavage cells of Indonesian thin-tail sheep mediate antibody-dependent superoxide radical cytotoxicity *in vitro* against newly excysted juvenile *Fasciola gigantica* but not juvenile *Fasciola hepatica*. Infect. Immun. 75, 1954–1963.

Piedrafita, D., Parsons, J.C., Sandeman, R.M., Wood, P.R., Estuningsih, S.E., Partoutomo, S., Spithill, T.W., 2001. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile *Fasciola hepatica in vitro* is mediated by reactive nitrogen intermediates. Parasite Immunol. 23, 473–482.

Piedrafita, D., Raadsma, H.W., Prowse, R., Spithill, T.W., 2004. Immunology of the host–parasite relationship in fasciolosis (*Fasciola hepatica* and *Fasciola gigantica*). Can. J. Zool. 82, 233–250.

Piedrafita, D., Spithill, T.W., Dalton, J.P., Brindley, P.J., Sandeman, M.R., Wood, P.R., Parsons, J.C., 2000. Juvenile *Fasciola hepatica* are resistant to killing *in vitro* by free radicals compared with larvae of *Schistosoma mansoni*. Parasite Immunol. 22, 287–295.

Piedrafita, D., Spithill, T.W., Smith, R.E., Raadsma, H.W., 2010. Improving animal and human health through understanding liver fluke immunology. Parasite Immunol. 32, 572–581.

Poglayen, G., Capelli, G., Martini, M., Zampiccoli, R., 1995. Epidemiologia delle parassitosi dell'aparato digerente del bovine nella provincia autonoma di Trento. Atti della Societa Italiana di Buiatria. 27, 483-489.

Prichard, R.K., Scholfield, P.J., 1971. *Fasciola hepatica*: cytochrome *c* oxidoreductases and effects of oxygen tension and inhibitors. Exp. Parasitol. 29, 215-222.

Qian, C.Y., Wang, J., Yu, C.X., Yin, X.R., Song, L.J., Zhang, W., Jin, Y., Ke, X.D., 2012. Characterization of IgG responses of rabbits to Sj14-

3-3 protein after experimental infection with *Schistosoma japonicum*. Parasitol. Res. 111, 2209–2211.

Raadsma, H.W., Kingsford, N.M., Suharyanta, Spithill, T.W., Piedrafita, D., 2007. Host responses during experimental infection with *Fasciola gigantica* or *Fasciola hepatica* in Merino sheep I. Comparative immunological and plasma biochemical changes during early infection. Vet. Parasitol. 143, 275-286

Radfar, M.H., Nourollahi-Fard, S.R., Mohammadyari, N., 2015. Bovine fasciolosis: prevalence, relationship between faecal egg count and worm burden and its economic impact due to liver condemnation at Rudstar abattoir, Northern Iran. J. Parasit. Dis. 39, 522–525.

Ramajo, V., Oleaga, A., Casanueva, P., Hillyer, G.V., Muro, A., 2001. Vaccination of sheep against *Fasciola hepatica* with homologous fatty acid binding proteins. Vet. Parasitol. 97, 35–46.

Rivera, F., Espino, A.M., 2016. Adjuvant-enhanced antibody and cellular responses to inclusion bodies expressing FhSAP2 correlates with protection of mice to *Fasciola hepatica*. Exp. Parasitol. 160, 31–38.

Roberts, J.A., Estuningsih, E., Wiedosari, E., Spithill, T.W., 1997a. Acquisition of resistance against *Fasciola gigantica* by Indonesian thin tail sheep. Vet. Parasitol. 73, 215-224.

Roberts, J.A., Widjavanti, S., Estuningsih, E., Hetzel, D.J., 1997b. Evidence for a major gene determing the resistance of Indonesian thin tail sheep against *Fasciola gigantica*. Vet. Parasitol. 68, 309-314.

Robinson, J.M., Badwey, J.A., 1994. Production of active oxygen species by phagocytic leukocytes. Immunol. Series. 60, 159-178.

Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P., Ranganathan, S., 2009. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host. Mol. Cell Proteomics. 8, 1891–1907.

Rodríguez, E., Carasi, P., Frigerio, S., da Costa, V., van Vliet, S., Noya, V., Brossard, N., van Kooyk, Y., García-Vallejo, J.J., Freire, T., 2017. *Fasciola hepatica* immunoregulates CD11c+ cells by interacting with the macrophage gal/GalNAc lectin. Front. Immunol. 8:264.

Rodríguez, E., Noya, V., Cervi, L., Chiribao, M.L., Brossard, N., Chiale, C., Carmona, C., Giacomini, C., Freire, T., 2015. Glycans from *Fasciola hepatica* Modulate the Host Immune Response and TLR-Induced Maturation of Dendritic Cells. PLoS Negl. Trop. Dis. 9, 1–26.

Rojas, C.A.A., Scheerlinck, J.P., Ansell, B.R.E., Hall, R.S., Gasser, R.B., Jex, A.R., 2016. Time-Course Study of the Transcriptome of Peripheral Blood Mononuclear Cells (PBMCs) from Sheep Infected with *Fasciola hepatica*. PLoS One. 11, 1-17.

Rojas-Caraballo, J., López-Abán, J., Moreno-Pérez, D.A., Vicente, B., Fernández-Soto, P., del Olmo, E., Patarroyo, M.A., Muro, A., 2017. Transcriptome profiling of gene expression during immunisation trial against *Fasciola hepatica*: identification of genes and pathways involved in conferring immunoprotection in a murine model. BMC Infect. Dis. 17:94.

Ruiz-Campillo, M.T., Hernandez, V.M., Escamilla, A., Perez, J., Martinez-Moreno, A., Donnelly, S., Dalton, J.P., 2017. Immune signatures of pathogenesis in the peritoneal compartment during early infection of sheep with *Fasciola hepatica*. Sci. Rep. 7:2782.

Ruiz-Campillo, M.T., Molina-Hernández, V., Pérez, J., Pacheco, I.L., Pérez, R., Escamilla, A., 2018. Study of peritoneal macrophage immunophenotype in sheep experimentally infected with *Fasciola hepatica*. Vet. Parasitol. 257, 34–39.

Ruiz-Jimenez, C., Celias, D., Silvane, L., Cervi, L., Espino, A.M., 2017. *Fasciola hepatica* Fatty Acid Binding Protein (Fh12) inhibits the activation of murine dendritic cells. J. Immunol. 198:1.

Rushton, B., 1977. Ovine fascioliasis following reinfection. Res. Vet. Sci. 22, 133-134.

Rushton, B., Murray, M., 1977. Hepatic pathology of a primary experimental infection of *Fasciola hepatica* in sheep. J. Comp. Pathol. 87, 459-470.

Sachdev, D., Gough, K.C., Flynn, R.J., 2017. The Chronic Stages of Bovine *Fasciola hepatica* Are Dominated by CD4 T-Cell Exhaustion. Front Immunol. 8:1002.

Saleh, M. A., 2008. Circulating oxidative stress status in desert sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. 154, 262–269.

Saleh, M.A., Osman, F.A., Abdel-Salam, M., 2005. Clinical follow up on bovine fascioliasis in the Egyptian oasis. Proc. Eighth Sci. Congr. Egypt. Soc. Cattle Dis. Assiut. 246-262.

Schechtman, D., Tarrab-Hazdai, R., Arnon, R., 2001. The 14-3-3 protein as a vaccine candidate against schistosomiasis. Parasite Immunol. 23, 213–217.

Schweizer, G., Braun, U., Deplazes, P., Torgerson, P.R., 2005. Estimating the financial losses due to bovine fasciolosis in Switzerland. Vet. Rec. 157, 188-193.

Sekiya, M., Mulcahy, G., Irwin, J.A., Stack, C.M., Donnelly, S.M., Xu, W., Collins, P., Dalton, J.P., 2006. Biochemical characterisation of the recombinant peroxiredoxin (FhePrx) of the liver fluke, *Fasciola hepatica*. FEBS Lett. 580, 5016–5022.

Serradell, M.C., Guasconi, L., Masih, D.T., 2009. Involvement of a mitochondrial pathway and key role of hydrogen peroxide during eosinophil apoptosis induced by excretory-secretory products from *Fasciola hepatica*. Mol. Biochem. Parasitol. 163, 95–106.

Sexton, J.L., Milner, A.R., Panaccio, M., Waddington, J., Wijffels, G., Chandler, D., Thompson, C., Wilson, L., Spithill, T.W., Mitchell, G., 1990. Glutathione S-transferase: novel vaccine against *Fasciola hepatica* infection in sheep. J. Immunol. 145, 3905–3910.

Sexton, J.L., Wilce, M.C., Colin, T., Wijffels, G.L., Salvatore, L., Feil, S., Parker, M.W., Spithill, T.W., Morrison, C.A., 1994. Vaccination of sheep against *Fasciola hepatica* with glutathione S-transferase. Identification and mapping of antibody epitopes on a three-dimesional model of the antigen. J. Immunol. 152, 1861–1872.

Sibille, P., Tliba, O., Boulard, C.H., 2004. Early and transient cytotoxic response of peritoneal cells from *Fasciola hepatica*-infected rats. Vet. Res. 35, 576–584.

Siles-Lucas, M.; Felleisten, R.S.J.; Hemphill, A.; Wilson, W.; Gottstein, B., 1998. Stage-specific expression of the 14-3-3 gene in *Echinococcus multilocularis*. Mol. Biochem. Parasitol. 91, 281–293.

Siles-Lucas, M., Nunes, C.P., Zaha, A., Breijo, M., 2000. The 14-3-3 protein is secreted by the adult worm of *Echinococcus granulosus*. Parasite Immunol. 22, 521–528.

Siles-Lucas, M., Merli, M., Mackenstedt, U., Gottstein, B., 2003. The *Echinococcus multilocularis* 14-3-3 protein protects mice against primary but not secondary alveolar echinococcosis. Vaccine. 21, 431–439.

Siles-Lucas, M., Uribe, N., López-Abán, J., Vicente, B., Orfao, A., Nogal-Ruiz, J.J., San Feliciano, A., Muro, A., 2007. The *Schistosoma bovis* Sb14-3-3ζ recombinant protein cross-protects against *Schistosoma mansoni* in BALB/c mice. Vaccine. 25, 7217–7223.

Sinclair, K.B., 1967. Pathogenesis of *Fasciola* and the other liver-flukes. Helminthol. Abst. 3, 115-134.

Sluchanko, N.N., Gusev, N.B., 2010. 14-3-3 proteins and regulation of cytoskeleton. Biochem. Mosc. 75, 1528–1546.

Smith, N.C., Ovingston, K.S., Boray, J.C., 1992. *Fasciola hepatica* free radical generation by peritoneal leukocytes in challenged rodents. Int. J. Parasitol. 22, 281–286.

Spithill, T.W., Carmona, C., Piedrafita, D., Smooker, P.M., 2012. Prospects for immunoprophylaxis against *Fasciola hepatica* (Liver Fluke). In: Caffrey, C.R. (Ed.), Parasitic Helminths: Targets, Screens, Drugs, and Vaccines, First ed. Wiley-VCH Verlag GmbH and Co., KGaA, Weinheim, pp. 467–486.

Spithill, T.W., Dalton J.P., 1998. Progress in development of liver fluke vaccines. Parasitol. Today. 14, 224-228.

Spithill, T.W., Smooker, P.T., Sexton, J.L., Bozas, E., Morrison, C.A., Creaney, J., Parsons, J.C., 1999. Development of vaccines against *Fasciola hepatica* In: Dalton JP, editor. Fasciolosis.Wallingford, UK: CABI Publishing, pp. 377–410.

Spithill, T.W., Piedrafita, D., Smooker, P.M., 1997. Immunological approaches for the control of fasciolosis. Int. J. Parasitol. 27, 1221–1235.

Steinfelder, S., Rausch, S., Michael, D., Kühl, A.A., Hartmann, S., 2017. Intestinal helminth infection induces highly functional resident memory CD4 + T cells in mice. Eur. J. Immunol. 47, 353–63.

Taylor, E.L., 1949. The epidemiology of fascioliasis in Britain. Report of the 14th International Veterinary Congress, London.

Teofanova, D., Hristov, P., Yoveva, A., Radoslavov, G., 2012. Native and Recombinant Fatty Acid Binding Protein 3 from *Fasciola hepatica* as a Potential Antigen. Biotechnol. Biotechnol. Equip. 26, 60–64.

Tian, A.L., Lu, M.M., Calderón-Mantilla, G., Petsalaki, E., Dottorini, T., Tian, X.W., Wang, Y., Huang, S.Y., Hou, J.L., Li, X., Elsheikha, H.M., Zhu, X.Q., 2018. A recombinant *Fasciola gigantica* 14-3-3 epsilon protein (rFg14-3-3e) modulates various functions of goat peripheral blood mononuclear cells. Parasit. Vectors. 11:152, 1–14.

Timanova-Atanasova, A., Jordanova, R., Radoslavov, G., Deevska, G., Bankov, I., Barrett, J., 2004. A native 13-kDa fatty acid binding protein from the liver fluke *Fasciola hepatica*. Biochim. Biophys. Acta Gen. Subj. 1674, 200–204.

Tliba, O., Chauvin, A., Le Vern, Y., Boulard, C., Sibille, P., 2002. Evaluation of the hepatic NK cell response during the early phase of *Fasciola hepatica* infection in rats. Vet. Res. 33, 327-332.

Uribe, N., Siles-Lucas, M., López-Abán, J., Esteban, A., Suarez, L., Martínez-Fernández, A., del Olmo, E., Muro, A., 2007. The Sb14-3-3ζ recombinant protein protects against *Schistosoma bovis* in BALB/c mice. Vaccine. 25, 4533–4539.

Urquhart, G.M., 1956. The pathology of experimental fascioliasis in the rabbit. J. Pathol. Bacteriol. 71, 301-310.

Ushio-Fukai, M., 2006. Localizing NADPH oxidase-derived ROS. Sci. STKE. 349, 1–6.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44-84.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M., Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact. 160, 1-40.

Van Hemmert, M.J., Steensma, H.Y., Van Heusden, G.P., 2001. 14-3-3 proteins: key regulators of cell division, signaling and apoptosis. Bioessays. 23, 936–946.

Van Millingen, F.J., Cornelissen, J.B.W.J., Hendriks, I.M., Gaasenbeek, C.P.H., Bokhout, B.A., 1998. Protection to *Fasciola hepatica* in the gut mucosa of immune rats is associated with infiltrates of eosinophils, IgG1 and IgG2a antibodies around the parasites. Parasite Immunol. 20, 285–292.

Venturina, V.M., Alejandro, M.A., Baltazar, C.P., Abes, N.S., Mingala, C.N., 2015. Evidence of *Fasciola* spp. resistance to albendazole,

triclabendazole and bromofenofos in water buffaloes (*Bubalus bubalis*). Ann. Parasitol. 61, 283–289.

Victor, B., Kanobana, K., Gabriël, S., Polman, K., Deckers, N., Dorny, P., Deelder, A.M., Palmblad, M., 2012. Proteomic analysis of *Taenia solium* metacestode excretion−secretion proteins. Proteomics 12, 1860–1869.

Villa-Mancera, A., Méndez-Mendoza, M., 2012. Protection and antibody isotype responses against *Fasciola hepatica* with specific antibody to pIII-displayed peptide mimotopes of cathepsin L1 in sheep. Vet. J. 194, 108–112.

Villa-Mancera, A., Quiroz-Romero, H., Correa, D., Ibarra, F., Reyes-Pérez, M., Reyes- Vivas, H., López-Velázquez, G., Gazarian, K., Gazarian, T., Alonso, R.A., 2008. Induction of immunity in sheep to *Fasciola hepatica* with mimotopes of cathepsin L selected from a phage display library. Parasitology. 135, 1437–1445.

Villa-Mancera, A., Reynoso-Palomar, A., Utrera-Quintana, F., Carreón-Luna, L., 2014. Cathepsin L1 mimotopes with adjuvant Quil A induces

a Th1/Th2 immune response and confers significant protection against *Fasciola hepatica* infection in goats. Parasitol. Res. 113, 243–250.

Vukman, K.V., Adams, P.A., Metz, M., Maurer, M., and O'Neill, S.M., 2013. *Fasciola hepatica* Tegumental Coat Impairs Mast Cells' Ability to Drive Th1 Immune Responses. J. Immunol. 43, 531-539

Wandurska-Nowak, E., Wisniewska, J., 2002. Release of nitric oxide during experimental trichinellosis in mice. Parasitol. Res. 88, 708–711.

Weidner, J.M., Kanatani, S., Uchtenhagen, H., Varas-Godoy, M., Schulte, T., Engelberg, K., Gubbels, M.J., Sun, H.S., Harrison, R.E., Archour, A., Barragan, A., 2016. Migratory activation of parasitized dendritic cells by the protozoan *Toxoplasma gondii* 14-3-3 protein. Cell. Microbiol. 18, 1537–1550.

Wesołowska, A., Basałaj, K., Norbury, L.J., Sielicka, A., Wędrychowicz, H., Zawistowska-Deniziak, A., 2018a. Vaccination against *Fasciola hepatica* using cathepsin L3 and B3 proteases delivered alone or in combination. Vet. Parasitol. 250, 15–21.

Wesołowska, A., Zawistowska-Deniziak, A., Norbury, L.J., Wilkowski, P., Januszkiewicz, K., Pyziel, A.M., Zygner, W., Wedrychowicz, H., 2016. Immune responses in rats and sheep induced by a DNA vaccine containing the phosphoglycerate kinase gene of *Fasciola hepatica* and liver fluke infection. Acta Parasitol. 61, 212–220.

Wesołowska, A., Zawistowska-Deniziak, A., Norbury, L.J., Wilkowski, P., Pyziel, A.M., Zygner, W., Wędrychowicz, H., 2018b. Lymphocyte responses of rats vaccinated with cDNA encoding a phosphoglycerate kinase of Fasciola hepatica (FhPGK) and *F. hepatica* infection. Parasitol. Int. 67, 85–92.

Wijffels, G.L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J., Bowen, F., Friedel, T., Spithill, T.W., 1994. Vaccination of sheep with purified cysteine proteinases of *Fasciola hepatica* decreases worm fecundity. Exp. Parasitol. 78, 132-148.

Yaffe, M.B., 2002. How do 14-3-3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett. 513, 53–57.

Yang, J., Zhu, W., Huang, J., Wang, X., Sun, X., Zhan, B., Zhu, X., 2016. Partially protective immunity induced by the 14-3-3 protein form *Trichinella spiralis*. Vet. Parasitol. 231, 63–68.

Young, I.S., Woodside, J.V., 2001. Antioxidants in health and disease. J. Clin. Pathol. 54, 176-86.

Zafra, R., Pérez-Écija, R.A., Buffoni, L., Mendes, R.E., Martínez-Moreno, A., Martínez- Moreno, F.J., Pérez, J., 2010. Evaluation of hepatic damage and local immune response in goats immunized with native glutathione S-transferase of *Fasciola hepatica*. J. Comp. Pathol. 143, 1101–1119.

Zafra, R., Perez-Ecija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martinez-Moreno, A., Mulcahy, G., Dalton, J.P., Pérez, J., 2013a. Early and late peritoneal and hepatic changes in goats immunized with recombinant cathepsin L1 and infected with *Fasciola hepatica*. J. Comp. Pathol. 148, 373–384.

Zafra, R., Perez-Ecija, R.A., Buffoni, L., Pacheco, I.L., Martinez-Moreno, A., LaCourse, E.J., Perally, S., Brophy, P.M., Pérez, J., 2013b.

Early hepatic and peritoneal changes and immune response in goats vaccinated with a recombinant glutathione transferase sigma class and challenged with *Fasciola hepatica*. Res. Vet. Sci. 94, 602–609.

Zafra, R., Pérez, J., Buffoni, L., Martínez-Moreno, F.J., Acosta, I., Mozos, E., Martínez-Moreno, A., 2013c. Peripheral blood lymphocyte subsets in *Fasciola hepatica* infected and immunised goats. Vet. Immunol. Immunopathol. 155, 135–138.

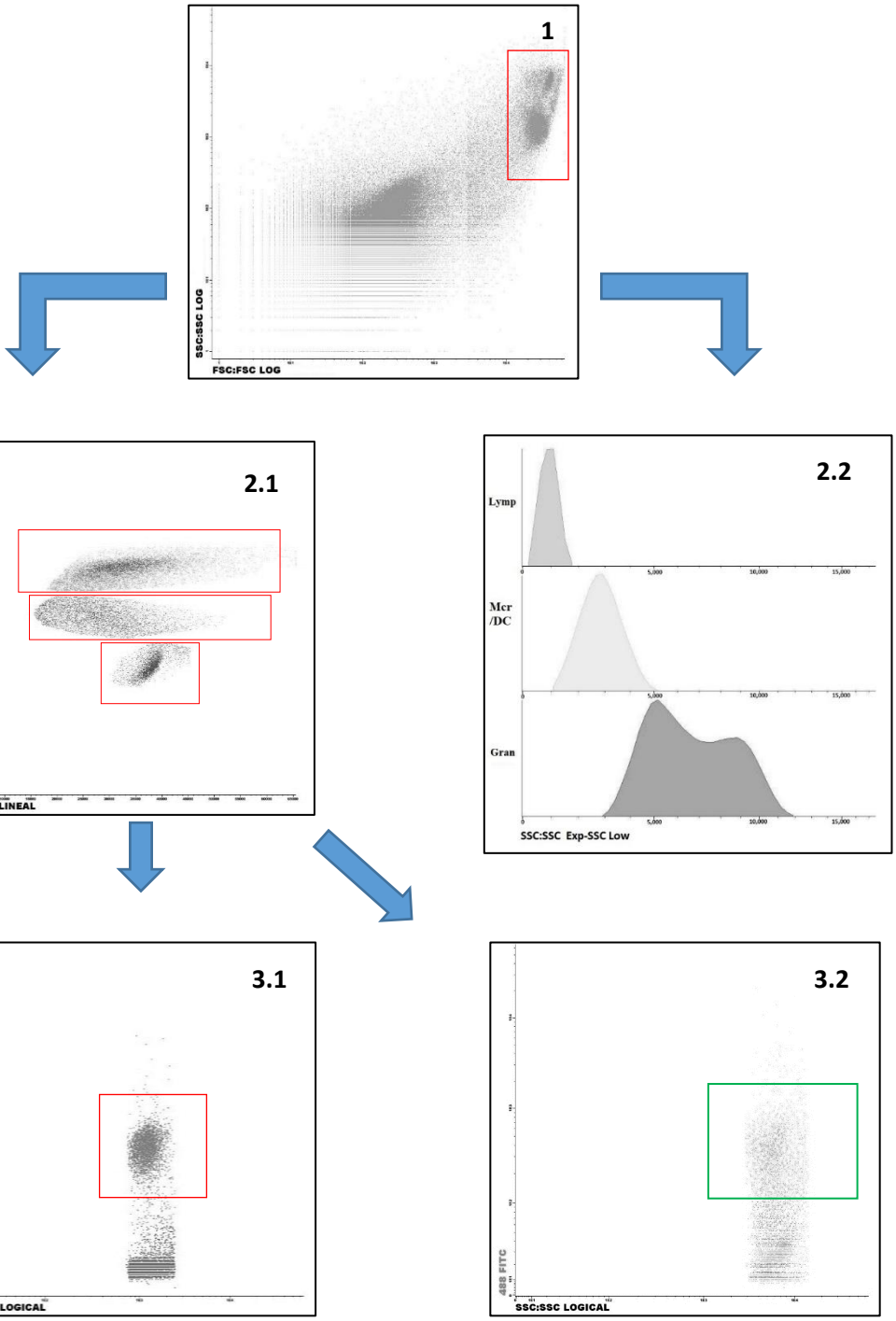
Zhang, Y.B., Taylor, M.G., Johansen, M.V., Bickle, Q.D., 2001. Vaccination of mice with a cocktail DNA vaccine induces a Th1-type immune response and partial protection against *Schistosoma japonicum* infection. Vaccine 20, 724–730.

ANEXOS

ANEXO I. MATERIAL SUPLEMENTARIO SEGUNDO ESTUDIO

Supplementary material.

Gating strategy.



1. **Dot-plot samples (1):** FSC and SSC in log scale are faced in order to exclude debris. Red square shows the leukocyte populations of interest.
2. **Dot-plot (2.1) and histogram (2.2):** once leukocyte populations are gated, lineal FSC is faced to log SSC so that white cells can be shown and identified properly. The histogram is an extra support for the correct gating in leukocyte subsets.
3. **Dot-plots for fluorochromes (3.1 and 3.2):** according to the fluorochrome in each antibody, RPE or FITC channels are faced to log SSC, and in each dot-plot it is only shown the subset of interest which was gated in dot-plot 2.

ANEXO II. PUBLICACIONES

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Pathological, immunological and parasitological study of sheep vaccinated with the recombinant protein 14-3-3z and experimentally infected with *Fasciola hepatica*

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ABSTRACT

In this study, the immunogenicity and protective capacity of a new recombinant vaccine candidate, the rFh14-3-3z protein was analysed in sheep experimentally challenged with *Fasciola hepatica*, in terms of fluke burden, faecal egg counts, hepatic damage and humoral immune response. Three groups of 8 animals each were used for study, group 1 was immunised with the rFh14-3-3z in Montanide adjuvant, whereas group 2 and 3 remained as adjuvant control and infection control groups, respectively. The parasitological analysis showed that no significant reduction in fluke burden, fluke size and faecal egg counts was detected. The extent of hepatic damage was very similar between groups. Nonetheless, animals immunised with the rFh14-3-3z protein induced the development of specific IgG1 and IgG2, being the IgG1 the predominant antibody; which confirms the immunogenicity of this protein in sheep. This is the first report of the 14-3-3z proteins as vaccine against the infection with *F. hepatica*.

1. Introduction

The trematode *Fasciola hepatica* is the causative agent of fasciolosis, a major parasitic disease of livestock and responsible for large economic losses to the agricultural industry. The parasite localises in the bile ducts and gallbladder of the animals and produces a severe traumatic hepatitis during the migratory and biliary stage that may lead to loss of the hepatic function. The life cycle of the parasite is complex and involves small pond freshwater snails of the genus *Galba* spp. as intermediate hosts. The disease mainly affects ruminants but other mammals such as pigs, horses, deer or wild animals may also get infected, as well as humans, particularly in endemic areas.

The use of anthelmintics is the most effective method for controlling the disease but resistance to different drugs has been recorded and is increasing worldwide (Moll et al., 2000; Alvarez-Sanchez et al., 2006; Olaechea et al., 2011; Novobilský and Höglund, 2015; Venturina et al., 2015), hence new control strategies such as immunological control may be a feasible option and the use of vaccines is still under development with many promising results (Dalton and Mulcahy, 2001; Almeida et al., 2003; Golden et al., 2010; Villa Mancera et al., 2014).

The 14-3-3 is a family of 24–33 kDa proteins expressed by unicellular and multicellular organisms which are known to play a key role in cell signaling pathways, activation of dendritic cells, cell division and apoptosis (Van Hemmert et al., 2001; Weidner et al., 2016). Within parasites, they have been identified and isolated from protozoans and helminths and recognised at the host-parasite interface (Victor et al., 2012). In a previous study, the 14-3-3 proteins were localized at the gut, tegument, reproductive organs and excretory-secretory products in the juvenile and adult stage of *F. gigantica*, and showed cross-reactivity with 14-3-3 proteins of other trematodes (Chaithirayanon et al., 2006). Later on, these proteins were seen to be associated with the surface and outer structures and oral sucker, secretion extracts and cell cycle control of newly excysted juveniles (NEJs) of *F. hepatica* (Robinson et al., 2009; Hernández-González et al., 2010; De la Torre-Escudero et al., 2011). These data suggest that the parasite 14-3-3 proteins might play an important role at the early stage of *F. hepatica* infection.

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The involvement of the 14-3-3z during the early and late stage of *F. hepatica* infection still needs to be fully elucidate. There are closely-related proteins which have shown immunomodulation capacity in ruminants infected with *Fasciola* sp. A recent study (Tian et al., 2018) reported that a recombinant 14-3-3e from *Fasciola gigantica* with 100% identity to 14-3-3 from *F. hepatica* stimulated IL-10 and TGF-β production by PBMCs and was also related to nitric oxide release and cell apoptosis. Moreover, it has been shown that immature flukes induce apoptosis of peritoneal leucocytes and eosinophils during the early stage of infection in sheep (Escamilla et al., 2016, 2017). In addition, there is further evidence that during the acute stage of infection with *F. hepatica* in sheep, when immature liver flukes burrow and migrate through the liver parenchyma, a polarised Th2 local immune response occurs in liver and hepatic lymph nodes (Pacheco et al., 2017).

The 14-3-3 protein family has been tested as prospective diagnostic or vaccine candidates (Lally et al., 1996; Schechtman et al., 2001; Siles-Lucas et al., 2007; Yang et al., 2016), though few reports are available about their use as vaccines against parasitic infections. Meng et al. (2012) used 14-3-3 proteins against the infection with *Toxoplasma gondii* and observed a protective immune response and the induction of a Th1 pathway profile in vaccinated mice, whereas Yang et al. (2016) reported high levels of Th1 cytokines and a significant protection in animals infected with *Trichinella spiralis*. In addition, Siles-Lucas et al. (2003) also obtained a significant protection against the infection with *Echinococcus multilocularis* in primarily infected mice, after vaccination with the protein 14-3-3 zeta (14-3-3z) isoform of the parasite. In trematode infections, similar results were recorded by Zhang et al. (2001); Siles-Lucas et al., (2007) and Uribe et al. (2007) in mice vaccinated with the 14-3-3z isoform of *S. japonicum* and of *Schistosoma bovis* and infected with *S. japonicum*, *S. mansoni* and *S. bovis*, respectively, in terms of reduction of parasite burden in vaccinated animals. Therefore, there is evidence to support that vaccination with 14-3-3z proteins may elicit significant protection against parasite plathyhelminths.

The aim of this study was to evaluate protection against *F. hepatica* infection in sheep immunised with the 14-3-3z of *F. hepatica*, by assessing the fluke burden, the hepatic damage and the humoral immune response. This is the first report of the use of 14-3-3z protein as vaccine candidate against the infection with *F. hepatica* in sheep.

2. Material and methods

2.1. Isolation and sequencing of the Fasciola hepatica 14-3-3z protein

Total RNA was extracted from a *F. hepatica* adult worm collected from a naturally infected cow at the Coreses slaughterhouse (Zamora, Spain) with the RNeasy Minikit (Quiagen, Spain), following the manufacturer's instructions. Total RNA was then subjected to reverse transcription with the First Strand cDNA Synthesis Kit (Roche, Spain). cDNA was then subjected to PCR with the following primers: Fh1433zFwd (5´- ggg*aattc*ccATGTCGCCGTGCTGGTTGAC) and Fh1433zRev (5´- gg*ctcgac*TTACTTGTCACCAGCATCAAC), containing the specific 14-3-3z sequence (GenBanck accession number MG518623) and the adaptors including restriction sites for *Eco*RI and *Xho*I. Primers were designed on the previously reported *F. gigantica* 14-3-3z sequence (GenBank accession number AY878648.1; Chaithirayanon et al., 2006), to cover the full length coding sequence, including the start and stop codons. PCR was performed in 35 cycles at 94 °C for 30 s, 48 °C for 30 s and 72 °C for 40 s, followed by a final extension step at 72 °C for 5 min. PCR product was electrophoresed in a 1% agarose gel in TBE together with the molecular weight markers (50 bp DNA Ladder; Thermo Fischer Scientific, Spain) and stained with ethidium bromide. The band at the expected molecular weight (777 base pairs) was excised and purified with the PureLink® Quick Gel Extraction Kit (Thermo Fischer Scientific, Spain). The purified band and the expression vector pGEX4T2 (GE Healthcare, Spain) were doubled digested with *Eco*RI and *Xho*I (Thermo Fischer Scientific, Spain), purified from an agarose gel as above-mentioned, and ligated with T4 DNA ligase (Thermo Fischer Scientific, Spain). The ligation reaction was used to transform competent *Escherichia coli* BL21 cells (GE Healthcare, Spain). Cells were grown overnight in agar plates containing ampicillin, Transformants were selected and used for protein expression, as described in Siles-Lucas et al. (2000). Protein purity and integrity after thrombin cleavage was checked in a 12% acrylamide gel stained with Coomassie blue, were a band of the expected molecular weight (25.3 kDa) was found (data not shown).

2.2. Vaccine preparation

The 14-3-3z recombinant protein of *F. hepatica* (rFh14-3-3z) was diluted in MontanideTM ISA 71 V G adjuvant (Seppic®). Each immunisation dose was prepared as follows: 100 µg of rFh14-3-3z was mixed with 100 µg of Montanide reaching a final volume of 1.5 ml per dose.

2.3. Animals

Twenty four female Merino-breed sheep 6 month old were used for the study. Prior to commencing the experimental trial, animals were individually identified using Avid Friendchip® microchips (Avid Identification Systems Inc, CA, USA), orally administered Diclazuril (Rumicox®, Esteve) and Ivermectin (Ivomec®, Merial) and confirmed to be free of liver fluke infection by faecal analyses and ELISA for *F. hepatica* specific antibodies. Sheep were housed in covered pens and fed daily with hay and commercial pelleted ration.

2.4. Experimental design

Sheep were randomly allocated into three groups of eight animals each. Animals from group 1 were immunised with the recombinant antigen rFh14-3-3z in Montanide adjuvant (Montanide™ISA 71 V G -Seppic®). Group 2 was immunised with Montanide adjuvant (adjuvant control group) and animals from group 3 were not immunised and remained as positive infection control. Immunisation was carried out subcutaneously on two occasions at intervals of four weeks.

Eight weeks after first immunisation all animals were orally challenged with a single dose of 150 metacercariae of *F. hepatica* (Ridgeway Research Ltd., St Briavels, UK) within a gelatine capsule using a bolus dosing gun. Fifteen weeks post-infection, sheep were euthanised by an intravenous injection of T61® (Intervet, Barcelona, Spain). This experiment was performed in accordance to the University of Cordoba Bioethics Committee (N. 7119) and European (86/609/CEE) and Spanish Directives (RD 223/1988).

2.5. Pathological methods

At necropsy, the liver was photographed on the visceral and diaphragmatic surface for gross evaluation, and liver pathology was assessed according to the following score (Table 1). Tissue samples from the left and right hepatic lobe were collected and fixed in 10% buffered formalin, embedded in paraffin wax and sections were stained with the haematoxylin eosin method for histopathological evaluation.

2.6. Parasitological methods

To assess egg output, faecal samples were individually collected weekly from week six after challenge until the end of the trial, and were analysed by a zinc sulphate-based flotation method using the McMaster chamber. During necropsy, the gallbladder was opened and liver was dissected, bile ducts were cut and opened and all flukes were collected, counted and measured. The liver was cut into small pieces and placed into warm water (40 C) for 30 min to collect remaining flukes.

Table 1
score for gross pathology assessment of hepatic damage in sheep.

Score	Pathology
0	Absolutely no pathology evident- liver normal colour, consistency and no visible signs of fluke lesions
1	Small areas of scar tissue and lesions, < 5% of the liver affected.
2	Moderate areas of scar tissue and lesions, occurring in 5-10% of the liver.
3	Moderate areas of scar tissue, thickening of bile ducts, small to moderate areas of necrosis, pus, 10-20% of liver affected
4	Moderate to large areas of scar tissue, thickened bile ducts evident. Moderate areas of necrosis, pus, haemorrhage. 20-30% of liver affected.
5	Large areas of scar tissue, thickened bile ducts. Multiple necrotic foci, pus, haemorrhage, severe degeneration and > 30% of the total liver affected.

2.7. Antibody detection

Blood samples were taken and plasma was collected to detect specific antibodies against rFh14-3-3z by ELISA. Briefly, 96 wells ELISA plates were coated (100 µl/well) with 5 µg/ml of rFh14-3-3z for IgG1 or 10 µg/ml for IgG2, diluted in 0.05 M carbonate–bicarbonate buffer pH 9.6 and incubated at 37 °C overnight. After 5 washes with phosphate buffer saline (PBS) 0.05% Tween 20, plates were blocked with 100 µl/well of blocking buffer containing 1% BSA diluted in PBS and incubated at 37 °C for 30 min. To detect IgG1, wells were washed and 100 µl/well of plasma diluted in blocking buffer was added and incubated at 37 °C for 30 min. Triple serial dilutions were performed to determine endpoint titre. Similarly, IgG2 was detected by adding 100 µl/well of plasma diluted at 1:10 and at 1:25. After washing, 100 µl/well of primary antibody diluted 1:5000 (mouse anti-bovine IgG1 and anti-bovine IgG2; 7500820–7500830 Cedi-Diagnostics), in blocking buffer was added and incubated at 37 °C for 30 min. After incubation, wells were washed and anti-mouse IgG-HRP was added at 37 °C for 30 min (AbD-Serotec, STAR13B). Plate was washed and 100 µl/well of Tetramethylbenzidine (TMB-Sigma) were added and incubated for 10 min at room temperature. The reaction was stopped by adding of 100 µl/well of 1 M sulfuric acid and optical density was measured at 450 nm using a microplate photometer (Multiskan™ FC, Thermo Scientific). Results are shown as antibody titre – $\log_{10}$ – for IgG1, and as optical density for IgG2.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). When significant differences were found, comparisons between groups were made using the Mann–Whitney *U* test for non-parametric distributions. Weekly data from each group was analysed by comparing each time-point values to pre-immunisation values. To assess the correlation between fluke burden and faecal egg counts, a Spearman correlation coefficient was performed. *P* values of 0.05 or lower were considered statistically significant.

3. Results

3.1. Parasitological results

The analysis of faecal egg output did not show significant differences between groups. (Fig. 1). Eggs were first detected at 8 wpi in group 1 and 9 wpi in group 2 and 3,

and all infected animals of the trial were positive with egg detection by the eleventh week post-infection.

The mean fluke burden and mean length of flukes are shown in Table 2. Fluke burden was 52.25 ± 16.71, 68.13 ± 23.79 and 51.13 ± 12.84 for group one, two and three, respectively, this means an implantation rate of 34.83 ± 11.14, 45.42 ± 15.86 and 34.08 ± 8.56. Although individual variation was high in all groups, statistical analyses showed no significant difference between groups. This result indicates that no reduction of fluke burden was observed in any of the vaccinated groups. Faecal egg count is shown in Fig. 1. The correlation coefficient between fluke burden and faecal egg count from each group in week 14 post-infection was of 0.09 for group 1 and of 0.3 for groups 2 and 3 and showed no significant differences.

3.2. Antibody response to rFh14-3-3z

Dynamics of plasma levels of specific anti-rFh14-3-3z IgG1 and IgG2 is presented in Fig. 2 and 3, respectively. All animals immunised with the recombinant antigen (group 1) developed an IgG1-IgG2 antibody response following immunisation. A sharp and significant increase in IgG1 was detected three weeks after immunisation, reaching the maximun value and showing a gradual decrease from that onwards ($P < 0.05$) (Fig. 2). For IgG2, a limited but significant production ($P < 0.05$) was observed only after vaccination in group 1 (Fig. 3). In contrast, no statistically significant rise in the specific antibody level of neither IgG1 nor IgG2 was detected in the immunised animals (group 1) after the experimental challenge. Comparison between groups showed that no production of specific anti-rFh14-3-3z antibodies was detected in animals from groups 2 and 3 during the trial.

3.3. Pathological results

Gross hepatic lesions were similar in all groups and consisted of fibrous scars, tortuous whitish tracts and patches over the liver surface, mainly located in the left hepatic lobe (Fig. 4). Bile ducts were whitish and enlarged. Individual analysis of the liver pathology within each group showed a mean score of 4 for groups 1 and 2, and a mean score of 4.5 for group 3. No statistical differences were detected between groups.

Histopathological changes were similar in the three infected groups and consisted of bile duct hyperplasia, fibrosis in inflammatory infiltration of eosinophils, lymphocytes and plasma cells in portal areas, granulomas with necrotic center surrounded by macrophages, lymphocytes and eosinophils, fibrotic areas in the hepatic parenchyma with variable amount of inflammatory infiltrate composed of eosinophils, lymphocytes, plasma cells and macrophages, some of them containing haemosiderin pigment.

4. Discussion

It is well known that sheep is the most susceptible animal species among ruminants to liver fluke infection, and many studies aimed at stimulating a protective immune response have been conducted in this host (Piacenza et al., 1999; Almeida et al., 2003; Maggioli et al., 2011; Wesolowska et al., 2016; Pacheco et al., 2017). We here report the results of the first attempt to use recombinant 14-3-3z protein as vaccine against the infection with *F. hepatica* in sheep. This protein family has previously been assessed in vaccination trials against helminth infections with promising results (Siles-Lucas et al., 2003, 2007; Uribe et al., 2007; Zhang et al., 2001), though no data is available about the use of the 14-3-3z against the liver fluke infection in ruminants. In this study, the analyses of the parasitological and pathological results, in terms of fluke burden and hepatic damage, indicate that sheep immunised with rFh14-3-4z protein in Montanide™ ISA 71 V G adjuvant (Seppic®) developed no significant protection against the experimental challenge. Vaccinated animals showed a similar implantation rate and fluke burden when compared with the control groups.

Consistent with these results, no statistical differences between groups were observed in neither faecal egg output nor fluke size. The analyses of the parasitological parameters showed a weak positive correlation between fluke burden and faecal egg counts in all groups, which lies along the same line of previous studies carried out in sheep

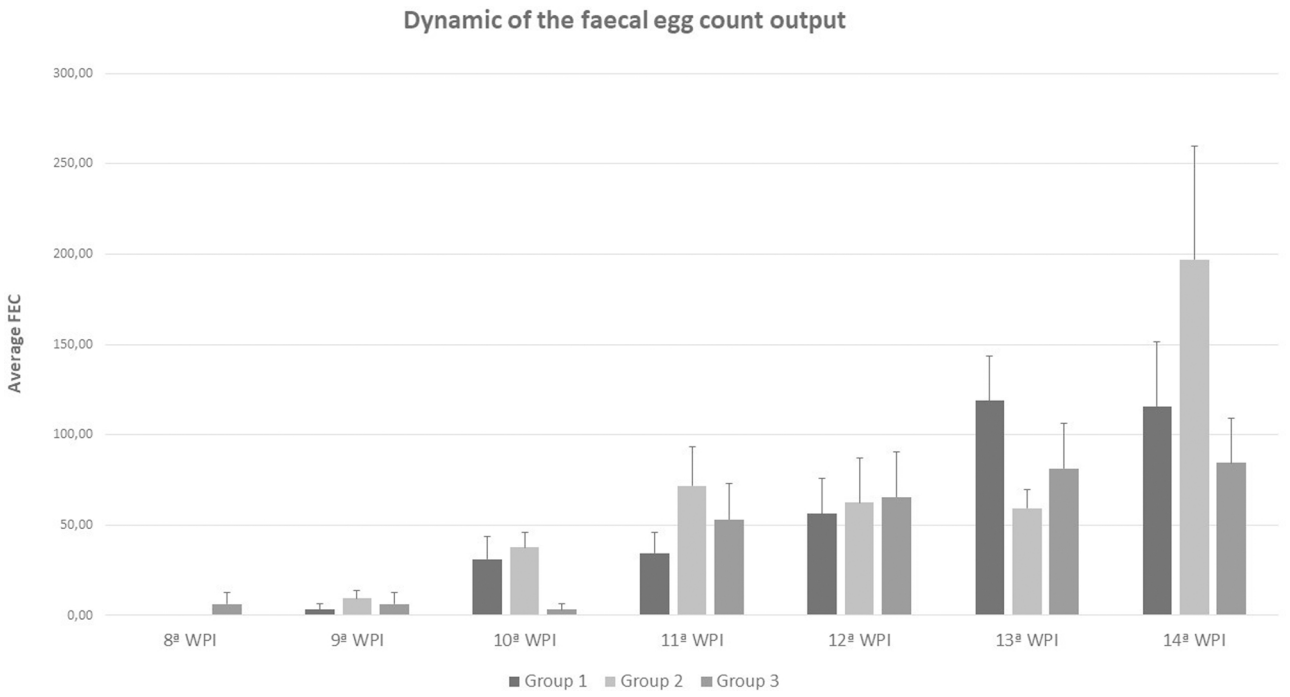

Fig. 1. Faecal egg output during the course of infection. Columns show the mean values of egg counts per group. Bars represent standard error. WPI, Weeks post-infection.

(Dumenigo et al., 1999) and cattle (Radfar et al., 2015) in which these two variables were seen to show a positive linear correlation.

On the other hand, vaccination produced a strong humoral immune response which consisted of high level of rFh14-3-3z-specific IgG1-IgG2, being IgG1 the predominant isotype. This observation confirms the immunogenicity of the 14-3-3 proteins in sheep and is in line with the results of previous studies. Schechtman et al. (2001) and Siles-Lucas et al. (2003) observed a high production of specific IgG1 and IgG2 after vaccination with 14-3-3 in mice, whereas recent studies showed that anti-14-3-3 antibodies were detectable as early as one week after vaccination (Yang et al., 2016) and for up to eight months post-vaccination (Lampe et al., 2017). Interestingly, no boosting effect on the antibody level was observed in the vaccinated animals after the experimental infection, likewise experimental challenge did not elicit antibody production in the unvaccinated animals. This lack on the production of a humoral immune response against 14-3-3 proteins in only-infected animals observed in our study, was previously described by Siles-Lucas et al. (2003) and Uribe et al. (2007) in primarily infected mice with *E. multilocularis* and *S. bovis*, respectively; in which the specific antibodies were detected at a very low level or not detected. On the contrary, other studies reported a significant production of specific anti-14-3-3 antibodies in unvaccinated and infected animals with *S. mansoni* and *S. japonicum* (Schechtman et al., 2001; Qian et al., 2012). These contrasting findings suggest that the production of specific 14-3-3 immunoglobulins may vary not only between the different helminths infections but also among parasite species belonging to the same genus, which is bewildering as 14-3-3 proteins are highly conserved among different species (Schechtman et al., 2001). Another reason for the absence of specific antibody production might be due to a difference in the nature of the native and the recombinant protein or in the conformational epitopes which are being or not being recognised by the different animals of the trial, since antibodies against the recombinant protein were detected in vaccinated sheep but not in infected-control animals. To date, no studies are available about the use of the native form of *F. hepatica* 14-3-3 protein hence, there is a lack of knowledge about the immunogenic capacity of the native Fh14-3-3; and few epitope mapping studies have been conducted in *F. hepatica* infections using immunodominant proteins. Sexton et al., (1994) observed a different level in the humoral immune response to peptides of the FhGST protein in vaccinated and infected sheep with *F. hepatica,* whereas a recent study showed that vaccinated cattle developing significant resistance against the infection produced antibodies against different peptides of the whole FhCL1 protein in protected and non-protected animals (Garza-Cuartero et al., 2018).

One of the key indicators of vaccine protection is the extent of hepatic damage produced by the liver flukes (Mendes et al., 2010; Pérez-Ecija et al., 2010). Our results indicate that the majority of animals showed a severely extensive damage and no significant differences were detected between vaccinated and control groups. This finding, together with the lack of fluke burden reduction observed in the vaccinated

Table 2
Fluke burden of immunised and control groups 15 weeks after challenge with *F. hepatica* metacercariae. Weight is expressed in grams and represents the total weight of the fluke burden per group. ± expresses standard deviation.

Group	Fluke burden	Implantation rate (%)	Length/Width (cm)	Weight (grams)
Group 1 (rFh14-3-3z + Adjuvant)	52.25 ± 16.71	34.83 ± 11.14	2.11 ± 0.20/0.87 ± 0.18	7.04 ± 2.96
Group 2 (Adjuvant control)	68.13 ± 23.79	45.42 ± 15.86	2.11 ± 0.28/0.79 ± 0.16	5.58 ± 4.33
Group 3 (Infection control)	51.13 ± 12.84	34.08 ± 8.56	1.89 ± 0.32/0.86 ± 0.15	6.00 ± 1.40

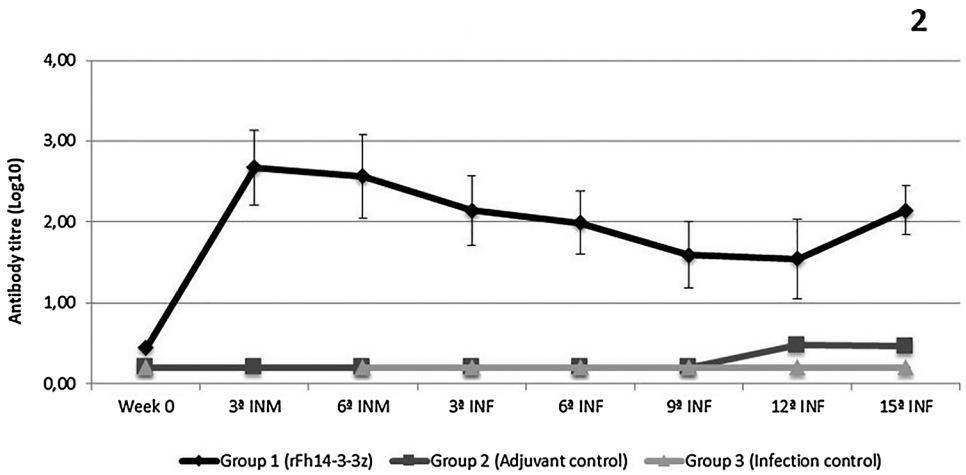

Fig. 2. Plasmatic levels of specific anti-rFh14-3-3z IgG1. Each point represents mean values of antibody titre log10. Bars at each point shows standard error.

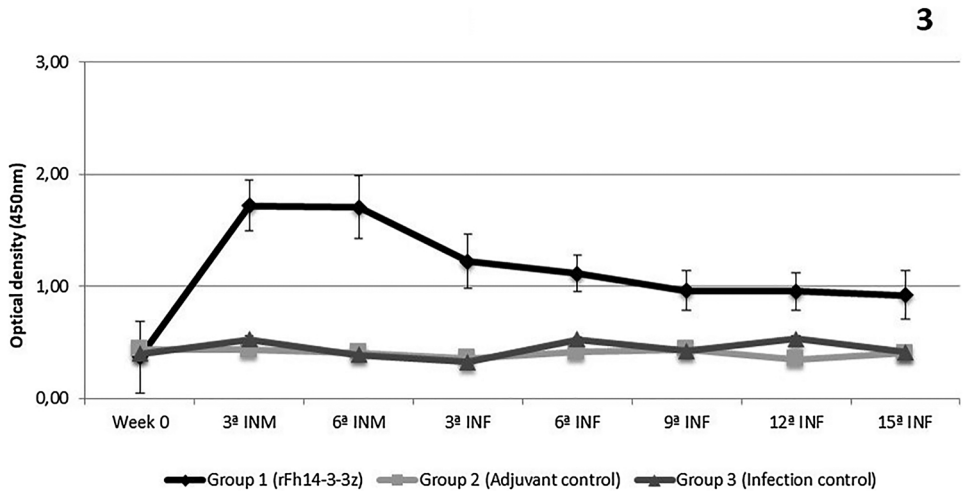

Fig. 3. Plasmatic levels of specific anti-rFh14-3-3z IgG2. Each point represents mean values of optical density measured at 450 nm. Bars at each point shows standard error.

animals suggest that vaccination with rFh14-3-3z, which elicited significant levels of protection against other helminth infections in murine models (Zhang et al., 2001; Siles-Lucas et al., 2003; Uribe et al., 2007; Yang et al., 2016) is not an appropriate vaccine candidate against *F. hepatica* infection in sheep.

The failure in producing a protective immune response observed in our study, is consistent with some previous vaccination trials reported by our group in which the immunisation with single antigens in goats did not induce reduction in the number of recovered flukes (Buffoni et al., 2010; Zafra et al., 2010; Buffoni et al., 2012). Similarly, Maggioli et al. (2016) also observed no significant reduction in fluke burden when using single antigens against the infection with *F. hepatica* in cattle. In contrast, some previous studies reported the development of significant protection when single peptides or proteins were used as vaccines in cattle (Golden et al., 2010) and sheep (Piacenza et al., 1999; Villa-Mancera and Méndez-Mendoza, 2012). It is possible that the protection failure in the vaccinated animals observed in this study might be attributed to several factors such as: the 14-3-3z proteins do not behave as immunodominant antigens or do not play a key role at the host-parasite interface in sheep fasciolosis, as seen by the absence of antibody production in the infected animals; the humoral immune response produced in the vaccinated animals was not strong enough or the antibodies lacked sufficient avidity and, hence were not able to neutralize the 14-3-3z-specific epitopes. It is worth to acknowledge that studies on the cellular immune response are also required to further explain this lack of protection.

5. Conclusions

Administration of a double dose of the rFh14-3-3z in Montanide adjuvant did not developed reduction in liver fluke burden nor hepatic damage in sheep challenged with 150 metacercariae of *F. hepatica*. The rFh14-3-3z was highly immunogenic as was demonstrated by the significant production of high level of anti-rFh14-3-3z IgG-IgG2, being IgG1 the predominant subclass. Non vaccinated and infected animals failed at producing specific antibodies, indicating a possible minor role of the Fh14-3-3z proteins during host-parasite interface. The vaccination protocol used in this study is not suitable for developing significant protection against the infection with *F. hepatica* in sheep. Since this is the first report of this protein as vaccine candidate against the infection with liver fluke in large animals, further studies using other animal models or following different immunisation protocols are still required to finally conclude the potencial capacity of the rFh14-3-3z proteins as vaccine.

Conflict of interest statement

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

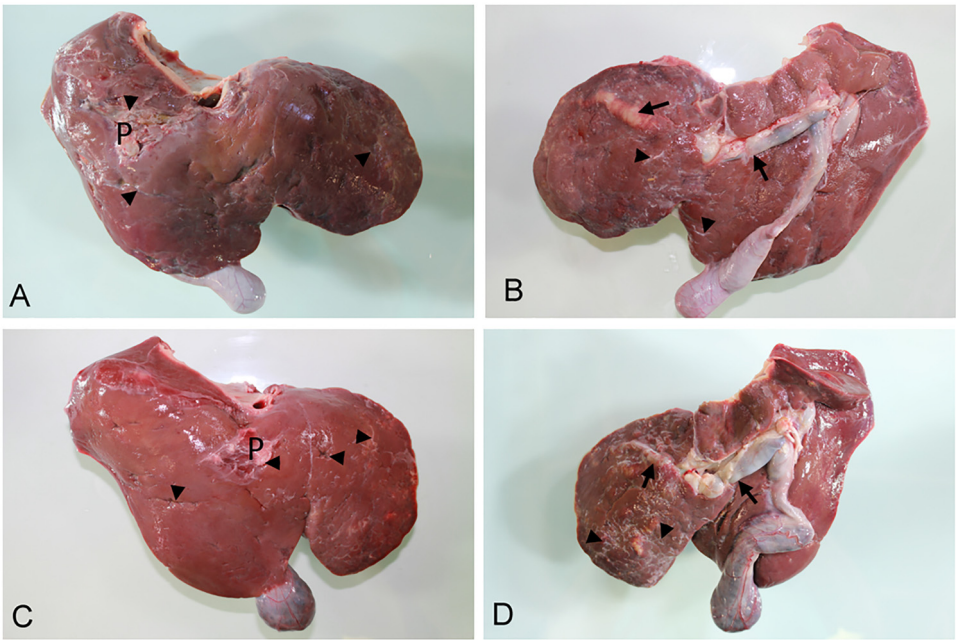

Fig. 4. Severe tortuous whitish tracts and scars (arrowheads) and patches (P) on the diaphragmatic and visceral surface of livers from group 1 (A–B) and group 3 (C–D). Bile ducts are enlarged and whitish (arrows).

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References

Almeida, M.S., Torloni, H., Lee-Hoo, P., Vilar, M.M., Thaumaturgo, N., Simpson, A.J., Tendler, M., 2003. Vaccination against *Fasciola hepatica* infection using a *Schistosoma mansoni* defined recombinant antigen, Sm14. Parasite Immunol. 25, 135–137.

Alvarez-Sanchez, M.A., Mainar-Jaime, R.C., Perez-Garcia, J., Rojo-Vazquez, F.A., 2006. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet. Rec. 159, 424–425.

Buffoni, L., Zafra, R., Pérez-Écija, A., Martínez-Moreno, F.J., Martínez-Galisteo, E., Moreno, T., Pérez, J., Martínez-Moreno, A., 2010. Immune response in goats immunised with glutathione S-transferase and experimentally challenged with *Fasciola hepatica*. Parasitol. Int. 59, 147–153.

Buffoni, L., Martínez-Moreno, F.J., Zafra, R., Mendes, R.E., Pérez-Écija, A., Sekiya, M., Mulcahy, G., Pérez, J., Martínez-Moreno, A., 2012. Humoral immune response in goats immunised with cathepsin L1, peroxiredoxin and Sm14 antigen and experimentally challenged with *Fasciola hepatica*. Vet. Parasitol. 185, 315–321.

Chaithirayanon, K., Grams, R., Vichasri-Grams, S., Hoffman, A., Korge, G., Viyanant, V., Upatham, E.S., Sobhon, P.O., 2006. Molecular and immunological characterization of encoding gene and 14-3-3 protein in *Fasciola gigantica*. Parasitol. 133, 763–775.

Dalton, J.P., Mulcahy, G., 2001. Parasite vaccines–a reality? Vet. Parasitol. 98, 149–167.

De la Torre-Escudero, E., Manzano-Román, R., Valero, L., Oleaga, A., Pérez-Sánchez, R., Hernández-González, A., Siles-Lucas, M., 2011. Comparative proteomic analysis of *Fasciola hepatica* juveniles and *Schistosoma bovis* schistosomula. J. Proteomics 74, 1534–1544.

Dumenigo, B.E., Espino, A.M., Finlay, C.M., Mezo, M., 1999. Kinetics of antibody-based antigen detection in serum and faeces of sheep experimentally infected with *Fasciola hepatica*. Vet. Parasitol. 86, 23–31.

Escamilla, A., Bautista, M.J., Zafra, R., Pacheco, I.L., Ruiz, M.T., Martínez-Cruz, S., Méndez, A., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2016. Fasciola hepatica induces eosinophil apoptosis in the migratory and biliary stages of infection in sheep. Vet. Parasitol. 216, 84–88.

Escamilla, A., Pérez-Caballero, R., Zafra, R., Bautista, M.J., Pacheco, I.L., Ruiz, M.T., Martínez-Cruz, S., Martínez-Moreno, A., Molina-Hernández, V., Pérez, J., 2017. Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infection in sheep. Vet. Parasitol. 238, 49–53.

Garza-Cuartero, L., Geurden, T., Mahan, S.M., Hardham, J.M., Dalton, J.P., Mulcahy, G., 2018. Antibody recognition of cathepsin L1-derived peptides in *Fasciola hepatica*-infected and/or vaccinated cattle and identification of protective linear B-cell epitopes. Vaccine 36, 958–968.

Golden, O., Flynn, R.J., Read, C., Sekiya, M., Donnelly, S.M., Stack, C., Dalton, J.P., Mulcahy, G., 2010. Protection of cattle against a natural infection of *Fasciola hepatica* by vaccination with recombinant cathepsin L1 (rFhCL1). Vaccine 28, 5551–5557.

Hernández-González, A., Valero, M.L., Sánchez del Pino, M., Oleaga, A., Siles-Lucas, M., 2010. Proteomic analysis of in vitro newly excysted juveniles form *Fasciola hepatica*. Mol. Biochem. Parasitol. 172, 121–128.

Lally, N.C., Jenkins, M.C., Dubey, J.P., 1996. Development of a polymerase chain reaction assay for the diagnosis of neosporosis using the *Neospora caninum* 14-3-3 gene. Mol. Biochem. Parasitol. 75, 169–178.

Lampe, K., Gottstein, B., Becker, T., Stahl-Hennig, C., Kaup, F.J., Mätz-Rensing, K., 2017. Immunization of rhesus macaques with *Echinococcus multilocularis* recombinant 14-3-3 antigen leads to specific antibody response. Parasitol. Res. 116, 435–439.

Maggioli, G., Acosta, D., Silveira, F., Rossi, S., Giacaman, S., Basika, T., Gayo, V., Rosadilla, D., Roche, L., Tort, J., Carmona, C., 2011. The recombinant gut-associated M17 leucine aminopeptidase in combination with different adjuvants confers a high level of protection against *Fasciola hepatica* infection in sheep. Vaccine 29, 9057–9063.

Maggioli, G., Bottini, G., Basika, T., Alonzo, P., Salinas, G., Carmona, C., 2016. Immunization with *Fasciola hepatica* thioredoxin glutathione reductase failed to confer protection against fasciolosis in cattle. Vet. Parasitol. 224, 13–19.

Mendes, R.E., Pérez-Ecija, R.A., Zafra, R., Buffoni, L., Martínez-Moreno, A., Dalton, J.P., Mulcahy, G., Pérez, J., 2010. Evaluation of hepatic changes and local and systemic immune responses in goats immunized with recombinant Peroxiredoxin (Prx) and challenged with *Fasciola hepatica*. Vaccine 28 (16), 2832–2840.

Meng, M., He, S., Zhao, G., Bai, Y., Zhou, H., Cong, H., Lu, G., Zhao, Q., Zhu, X.Q., 2012. Evaluation of protective immune responses induced by DNA vaccines encoding *Toxoplasma gondii* surface antigen 1 (SAG1) and 14-3-3 protein in BALB/c mice. Parasit. Vectors 5, 273.

Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in the Netherlands. Vet. Parasitol. 91, 153–158.

Novobilský, A., Höglund, J., 2015. First report of closantel treatment failure against *Fasciola hepatica* in cattle. Int. J. Parasitol. Drugs Drug Resist. 5, 172–177.

Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Vet. Parasitol. 178, 364–366.

Pacheco, I.L., Abril, N., Morales-Prieto, N., Bautista, M.J., Zafra, R., Escamilla, A., Ruiz, M.T., Martínez-Moreno, A., Pérez, J., 2017. Th1/Th2 balance in the liver and hepatic lymph nodes of vaccinated and unvaccinated sheep during acute stages of infection with *Fasciola hepatica*. Vet. Parasitol. 238, 61–65.

Pérez-Ecija, R.A., Mendes, R.E., Zafra, R., Buffonni, L., Martínez-Moreno, A., Pérez, J., 2010. Pathological and parasitological protection in goats immunised with recombinant cathepsin L1 and challenged with *Fasciola hepatica*. Vet. J. 185, 351–353.

Piacenza, L., Acosta, D., Basmadjian, I., Dalton, J.P., Carmona, C., 1999. Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. Infect. Immun. 67, 1954–1961.

Qian, C.Y., Wang, J., Yu, C.X., Yin, X.R., Song, L.J., Zhang, W., Jin, Y., Ke, X.D., 2012. Characterization of IgG responses of rabbits to Sj14-3-3 protein after experimental infection with *Schistosoma japonicum*. Parasitol. Res. 111, 2209–2211.

Radfar, M.H., Nourollahi-Fard, S.R., Mohammadyari, N., 2015. Bovine fasciolosis: prevalence, relationship between faecal egg count and worm burden and its economic impact due to liver condemnation at Rudstar abattoir, Northern Iran. J. Parasit. Dis. 39, 522–525.

Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P., Ranganathan, S., 2009. An

integrated transcriptomic and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*. Mol. Cell. Proteomics 8, 1891–1907.
Schechtman, D., Tarrab-Hazdai, R., Arnon, R., 2001. The 14-3-3 protein as a vaccine candidate against schistosomiasis. Parasite Immunol. 23, 213–217.
Sexton, J.L., Wilce, M.C., Colin, T., Wijffels, G.L., Salvatore, L., Feil, S., Parker, M.W., Spithill, T.W., Morrison, C.A., 1994. Vaccination of sheep against *Fasciola hepatica* with glutathione S-transferase. Identification and mapping of antibody epitopes on a three-dimesional model of the antigen. J. Immunol. 152, 1861–1872.
Siles-Lucas, M., Nunes, C.P., Zaha, A., Breijo, M., 2000. The 14-3-3 protein is secreted by the adult worm of *Echinococcus granulosus*. Parasite Immunol. 22, 521–528.
Siles-Lucas, M., Merli, M., Mackenstedt, U., Gottstein, B., 2003. The *Echinococcus multilocularis* 14-3-3 protein protects mice against primary but not secondary alveolar echinococcosis. Vaccine 21, 431–439.
Siles-Lucas, M., Uribe, N., López-Abán, J., Vicente, B., Orfao, A., Nogal-Ruiz, J.J., San Feliciano, A., Muro, A., 2007. The *Schistosoma bovis* Sb14-3-3ζ recombinant protein cross-protects against *Schistosoma mansoni* in BALB/c mice. Vaccine 25, 7217–7223.
Tian, A.L., Lu, M.M., Calderón-Mantilla, G., Petsalaki, E., Dottorini, T., Tian, X.W., Wang, Y., Huang, S.Y., Hou, J.L., Li, X., Elsheikha, H.M., Zhu, X.Q., 2018. Parasit. Vectors 11 (152). https://doi.org/10.1186/s13071-018-2745-4.
Uribe, N., Siles-Lucas, M., López-Abán, J., Esteban, A., Suarez, L., Martínez-Fernández, A., del Olmo, E., Muro, A., 2007. The Sb14-3-3ζ recombinant protein protects against *Schistosoma bovis* in BALB/c mice. Vaccine 25, 4533–4539.
Van Hemmert, M.J., Steensma, H.Y., Van Heusden, G.P., 2001. 14-3-3 proteins: key regulators of cell division, signaling and apoptosis. Bioessays 23, 936–946.
Venturina, V.M., Alejandro, M.A., Baltazar, C.P., Abes, N.S., Mingala, C.N., 2015. Evidence of *Fasciola spp*. resistance to albendazole, triclabendazole and bromofenofos in water buffaloes (*Bubalus bubalis*). Ann. Parasitol. 61, 283–289.
Victor, B., Kanobana, K., Gabriël, S., Polman, K., Deckers, N., Dorny, P., Deelder, A.M., Palmblad, M., 2012. Proteomic analysis of *Taenia solium* metacestode excretion−secretion proteins. Proteomics 12, 1860–1869.
Villa Mancera, A., Reynoso-Palomar, A., Utrera-Quintana, F., Carreón-Luna, L., 2014. Cathepsin L1 mimotopes with adjuvant Quil A induces a Th1/Th2 immune response and confers significant protection against *Fasciola hepatica* infection in goats. Parasitol. Res. 113, 243–250.
Villa-Mancera, A., Méndez-Mendoza, M., 2012. Protection and antibody isotype responses against *Fasciola hepatica* with specific antibody to pIII-displayed peptide mimotopes of cathepsin L1 in sheep. Vet. J. 194, 108–112.
Weidner, J.M., Kanatani, S., Uchtenhagen, H., Varas-Godoy, M., Schulte, T., Engelberg, K., Gubbels, M.J., Sun, H.S., Harrison, R.E., Archour, A., Barragan, A., 2016. Migratory activation of parasitized dendritic cells by the protozoan *Toxoplasma gondii* 14-3-3 protein. Cell. Microbiol. 18, 1537–1550.
Wesolowska, A., Zawistowska-Denisiak, A., Norbury, L.J., Wilkowski, P., Januszkiewicz, K., Pyziel, A.M., Zygner, W., Wedrychowicz, H., 2016. Immune responses in rats and sheep induced by a DNA vaccine containing the phosphoglycerate kinase gene of *Fasciola hepatica* and liver fluke infection. Acta Parasitol. 61, 212–220.
Yang, J., Zhu, W., Huang, J., Wang, X., Sun, X., Zhan, B., Zhu, X., 2016. Partially protective immunity induced by the 14-3-3 protein form *Trichinella spiralis*. Vet. Parasitol. 231, 63–68.
Zafra, R., Pérez-Écija, R.A., Buffoni, L., Mendes, R.E., Martínez-Moreno, A., Martínez-Moreno, F.J., Pérez, J., 2010. Evaluation of hepatic damage and local immune response in goats immunized with native glutathione S-transferase of *Fasciola hepatica*. J. Comp. Pathol. 143, 1101–1119.
Zhang, Y.B., Taylor, M.G., Johansen, M.V., Bickle, Q.D., 2001. Vaccination of mice with a cocktail DNA vaccine induces a Th1-type immune response and partial protection against *Schistosoma japonicum* infection. Vaccine 20, 724–730.

Parasites & Vectors

RESEARCH Open Access

Comparative dynamics of peritoneal cell immunophenotypes in sheep during the early and late stages of the infection with *Fasciola hepatica* by flow cytometric analysis

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Abstract

Background: The peritoneal cell populations (PCP) are thought to play a crucial role during the early immune response in *Fasciola hepatica* infection while newly excysted juveniles (NEJ) are migrating in the peritoneal cavity (PC) towards the liver. In this study, we aimed to determine the immunophenotypes of the PCP and to analyse the dynamics of the recruitment of the PCP during the early and late stage of the infection in sheep infected with *F. hepatica*.

Methods: Thirty-seven sheep were divided into three groups: Group 1 (n = 20) and 2 (n = 10) were challenged with *F. hepatica*, Group 3 (n = 7) was not infected and remained as uninfected control (UC). After the slaughtering, peritoneal lavages were carried out to isolate peritoneal cell populations at 1, 3, 9 and 18 days post-infection (dpi) for Group 1 and at 14 weeks post-infection (wpi) for Group 2 and 3. Flow cytometry was conducted to assess the dynamics of peritoneal cavity cell populations.

Results: TCD4 cells showed a significant decrease at 1 and 18 dpi when compared to UC; no statistical differences were detected for TCD8 and $WC1^{+}\gamma\delta$ during the early stage of the infection with respect to the UC. CD14 cells exhibited a decreasing trend, with a significant decrease at 9 and 18 dpi when compared to the UC. The dynamics of MHCII and CD83 cells showed a similar increasing pattern from 3 to 18 dpi. During the chronic stage, both TCD4 and TCD8 cells showed no significant differences when compared to the UC, although a slight but statistically significant higher level of $WC1^{+}\gamma\delta$ cells was observed. A lower percentage of antigen-presenting cells (APCs) was detected with respect to the UC.

Conclusions: The recruitment of the lymphocytes subsets did not show a significant increase during the course of the infection and only $WC1^{+}\gamma\delta$ cells displayed a significant increase at the chronic stage. For the CD14, a decreasing trend was observed during the early stage, which was statistically significant at the chronic stage of the infection. Peritoneal CD83 and MHCII cells developed an increasing trend during the early stage of infection, and showed a significant decrease at the late stage of the infection.

Keywords: Flow cytometry, NEJ, *Fasciola hepatica*, Peritoneal cells, Recruitment

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Background

Fasciola hepatica is a globally spread highly pathogenic trematode which mainly occurs in domestic ruminants as a chronic disease and produces major economic losses in terms of production loss and liver condemnation. Fasciolosis has been recognised by the WHO as a re-emerging neglected tropical disease and it is also of public health interest since it causes human infection as a food-borne parasitic disease; it is estimated that 2.4 million people are infected worldwide in over 70 countries [1].

It is well known that natural hosts do not develop an effective acquired resistance against the infection [2] and that anthelmintic treatment is the best means to control the infection. However, chemical residues in food and their impact on the environment [3, 4], as well as drug resistance reported in various countries [5–8], foster the study of new control methods such as vaccine development, although no vaccine formulation is commercially available to date.

The life-cycle of the parasite inside the animal host is complex: after the infection, the newly excysted juveniles (NEJ) penetrate the intestinal wall within the first two hours post-infection, enter the PC and migrate towards the liver, a process that usually takes about four to six days [9]. By the time *F. hepatica* reaches the mature stage inside its final location in the bile ducts, the disease has become a chronic infection and the immune system of the host has already been affected by the parasite: there is supporting evidence that *F. hepatica* has the capacity to immunomodulate the host's immune response [10–14].

At the early and late stage of the infection, NEJ and adults worms release a broad variety of antigenic molecules. Some of them include excretory-secretory products which mainly consist of proteins [15], exosome-like vesicles and tegument glycoproteins [16, 17] that may trigger local and systemic immune responses, hence the role of the peritoneal cell population is key for understanding the initial stage of the host-parasite interaction.

In *F. hepatica*-infected sheep at the initial stages of the infection, the peritoneal cavity fluid was recently reported to primarily consist of lymphocytes, macrophages and eosinophils, with lymphocytes and macrophages being the predominant cell populations and displaying a varying ratio along the course of infection [18]. Therefore, these two cell populations are considered as the main phenotypes of interest since they are thought to be one of the first immunocompetent cells involved in the early immune response once NEJ reach the PC. In this regard, it has been shown in a murine model that liver fluke NEJ are killed inside the peritoneal cavity [19], and in a previous study it has been observed that peritoneal macrophages (pMΦ) from rats developed cytotoxic mechanisms against *F. hepatica* NEJ [20]. Recently, it has been confirmed in an *in vivo* model that migrating *F. hepatica* NEJ causes alternative activation of pMΦ [18] and apoptosis in peritoneal leukocytes in sheep [21].

The aim of this study was to determine the immunophenotype of the PCP using flow cytometry to better comprehend its dynamics during the early and late stage of infection in sheep experimentally challenged with *F. hepatica*.

Methods

Animals

Thirty-seven female Merino-breed sheep, aged 6 months, were used for the study. Before commencing the study, all animals were confirmed to be free of *F. hepatica* infection by faecal analysis and by an in-house developed ELISA using microplates coated with recombinant *F. hepatica* cathepsin L1 (FhCL1) for detection of specific antibodies. Furthermore, all sheep were given Ivermectin (Noromectin®, Karizoo, Barcelona, Spain) and Diclazuril (Rumicox®, Esteve, Barcelona, Spain) in order to exclude potential presence of parasites. An individual clinical monitoring of the animals was conducted during the trial, which included a weekly clinical examination, and blood sampling for assessment of complete blood counts (data not shown). Simultaneously, faecal samples were taken weekly (from week 0 to week 14 of the trial) for detection of eggs belonging to gastrointestinal worms or *Eimeria* spp. oocysts. In addition, animals were housed in covered pens in clean and healthy conditions in order to avoid the appearance of other pathogens, and were fed daily with hay and commercial pelleted ration.

Experimental design

The study was designed to allow a comparative analysis of animals between the early and late stage of infection with *F. hepatica*, hence sheep were randomly allocated into three groups according to the slaughtering time. Group 1, 2 and 3 consisted of 20, 10 and 7 animals, respectively. At day 0 of the trial, animals from Group 1 (early stage) and 2 (late stage) were experimentally challenged with a single dose of 150 metacercariae of *F. hepatica* of bovine origin (Ridgeway Research Ltd., St Briavels, UK) administered in gelatine capsules, using a dosing gun. The twenty animals from Group 1 were sacrificed in batches of five sheep at each one of the following time points: 1, 3, 9 and 18 days post-infection (dpi). Animals from Group 2 and 3 were slaughtered 14 weeks post-infection (wpi). Animals from Group 3 were not infected and remained as uninfected control group (UC). All animals were humanly euthanised by an intravenous injection of T61® (Intervet, Barcelona, Spain).

Parasitological methods: egg output and liver fluke burden

Sheep of the late stage of infection study (Group 2) were sampled for detection of egg output and liver fluke burden. From week six post-infection (wpi) onwards, faecal samples were collected weekly from each animal and faecal examinations were performed using a flotation method for detection of *F. hepatica* eggs. In brief, 3 g of faeces were thoroughly mixed with 42 ml of saturated $ZnSO_4$ solution and eggs were counted using a modified McMaster method [22]. Each sample was analysed in duplicate and results were expressed as mean of eggs per gram of faeces.

During necropsy, the gall-bladder and the main bile ducts were opened and the liver was dissected and carefully examined for the presence of liver flukes. The liver was then cut into small pieces and placed into warm water (45 °C) for 30 min to collect remaining flukes which were not observed during bile duct opening. Finally, all liver flukes were counted.

Isolation of peritoneal cavity cells

A peritoneal lavage was conducted immediately after the slaughtering of each of the animals to obtain the peritoneal fluid. First, the ventral area of the abdomen was shaved and disinfected with polyvinylpyrrolidone iodine 10% (AGB, Madrid, Spain). A small incision (1–2 cm) was made in the skin over the midline and subcutaneous tissue was dissected. The *linea alba* and peritoneum were sectioned with blunt scissors to avoid haemorrhage. A 40-cm-long cannula connected to a syringe was inserted into the abdominal cavity and 60 ml sterile DPBS containing 9500 UI of heparin (warmed to 37 °C; Eurotubo®, Deltalab, Madrid, Spain) was injected into the abdominal cavity. After gently massaging the abdomen for 1 min, 40 ml of peritoneal fluid were withdrawn. Then, peritoneal fluid was centrifuged at 2300× *g* for 5 min and the supernatant was discarded. Cell pellets were resuspended again in DPBS and incubated for 15 min in an erythrolysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM disodium EDTA, dH_2O). A second centrifugation step (2300× *g* for 5 min) was performed to eliminate lysed erythrocyte membranes and the pellet was resuspended in 1 ml of medium. After that, the concentration of peritoneal cells was quantified using a Trypan Blue exclusion technique. The final concentration of the cells was then adjusted to 1×10^6 cells/ml in order to carry out a flow cytometry assay.

Cell population identification and isolation

Different monoclonal antibodies were used to determine the immunophenotype of the peritoneal cavity cell populations in uninfected and infected sheep at 1, 3, 9 and 18 dpi and at 14 wpi by flow cytometry analysis. Main lymphocyte subpopulations were identified separately as TCD4 and TCD8. WC1 was used to select the γδTCR cells. MHC class II was used for identification of APC, and CD14 was used as a marker for macrophages. CD83 was used to identify dendritic cells.

Flow cytometry acquisition was performed with a CyFlow Cube 6® cytometer (4 colours + FSC + SSC; Sysmex-Partec, Barcelona, Spain). Briefly, 200 µl of peritoneal fluid was diluted with 200 µl of phosphate buffered saline (PBS, pH = 7.2) and gently stirred. Afterwards, samples were incubated at 4 °C for 30 min in darkness with the different conjugated antibodies (Table 1) diluted at 1:400, according to the manufacturer's instructions. All antibodies, with the exception of the mouse anti-human CD83, have been previously used for flow cytometry assay in peripheral blood and lymphoid organs of ovines and goats [23, 24]. As negative control antibodies, mouse isotype control IgG1 and IgG2a [Bio-Rad (formerly AbD-Serotec) Kidlington, UK] were used for FITC and RPE labels, respectively.

Then, the samples were centrifuged at 2300× *g* for 5 min and the supernatant was discarded. After that, two

Table 1 Monoclonal antibodies used in flow cytometry analysis of peritoneal cavity cell populations

Peritoneal cell immunophenotypes	Antibodies	Fluorochromes	Clones	Reference
Isotype controls				
Control FITC	mouse control IgG1	FITC	–	MCA928F
Control RPE	mouse control IgG2a	RPE	–	MCA929PE
Cell populations				
TCD4	mouse anti-sheep CD4	RPE	clone 44.38	MCA2213PE
TCD8	mouse anti-bovine CD8	FITC	clone CC63	MCA837F
$WC1^+γδ$	mouse anti-bovine WC1	FITC	clone CC15	MCA838F
CD14	mouse anti-bovine CD14	FITC	clone CC-G33	MCA2678F
CD83	mouse anti-human CD83	FITC	clone HB15e	MCA1582F
MHCII	mouse anti-bovine MHC class II	FITC	clone IL-A21	MCA2445F

washes with PBS (centrifuged at 2300× *g* for 5 min) were carried out. Finally, cells were resuspended in PBS before acquisition.

Cells were identified by their morphological features gated as defined by their forward and side-scatter profiles (FSC *vs* SSC dot plot) in order to exclude cellular debris. Briefly, cellular debris were excluded by gating the leukocyte populations of interest based on the FSC-SSC (see panel 2.1 in Additional file 1: Figure S1). Once each of the leukocyte populations were defined as mentioned (i.e. lymphocytes, macrophages or dendritic cells), 10,000 events were counted and cell identification was then performed. The immunophenotype of interest was identified using the specific fluorescence channel *vs* SSC dot plots (see panels 3.1 and 3.2 in Additional file 1: Figure S1). For FITC-labelled antibodies, the FL1 channel (green detector, 488 nm laser, 536/40 nm) was used; for CD4-RPE antibody, the FL2 channel (orange detector, 488 nm laser, 590/50 nm) was used. The different peritoneal cell populations from all animals and from each of the slaughtering time points were determined separatedly, in different tubes. Having obtained the number of each immunophenotype, the percentage of the cell subset from the total leukocyte subpopulations was determined (on the basis of 10,000 events).

Results were analysed for changes in fluorescence and expressed as the mean of the percentage of each slaughtering time point. Data was analysed using Infinicyt™ Software v.1.8 (Cytognos, Salamanca, Spain).

Statistical analysis

Statistical analysis was performed with GraphPad Prism v.6.0 (GraphPad Software, Inc., San Diego, CA, USA). The Kolmogorov-Smirnov test was applied to evaluate if distributions were parametric. Comparison between pairs of groups were made using the two-tailed Mann-Whitney U-test for non-parametric distributions. Data from each stage of the infection (Groups 1 and 2) were statistically compared to data from control animals (Group 3). *P*-values of 0.05 or lower were considered statistically significant.

Results

Parasitological results

The number of parasites in animals from Group 2 is expressed as mean ± standard deviation (SD). The mean liver fluke burden was 67.78 ± 13.85 which represents an implantation rate of 45.19 ± 9.24%. The dynamics of egg output is shown in Fig. 1. Eggs were first detected at 8 wpi and all animals were positive to egg detection at 11 wpi. The egg output showed a progressive increasing trend from 10 wpi onwards, reaching a maximum level at 13 wpi.

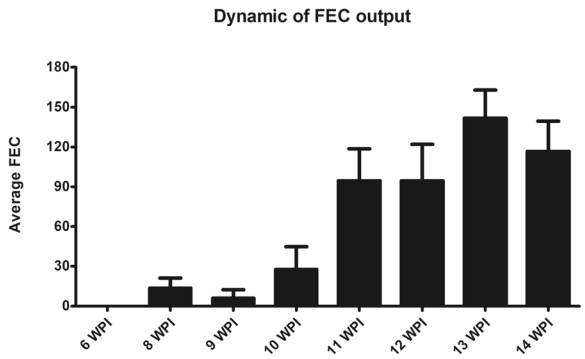

Fig. 1 Faecal egg output during the course of infection from animals of Group 2 (*n* = 10). Columns show the mean values of egg counts per week in infected animals from 6 until 14 wpi. Error bars represent standard error

In regard to the clinical monitoring of the animals during the trial, no clinical manifestations of disease, eggs or coccidian oocysts (apart from *F. hepatica* eggs in G2) were detected along the course of the trial.

Peritoneal leukocyte populations

Results of peritoneal leukocyte populations during the early and late stage of infection are expressed as the percentage (mean ± range) of each cell subpopulation (i.e. for TCD4, TCD8 and $WC1^{+}\gamma\delta$ results are expressed as the percentage of these three cell subsets of the total lymphocytes). The APC are represented as the percentage of MHCII. For macrophages and dendritic cells, CD14 and CD83, respectively, were used. Values are shown in Table 2.

Early stage of the infection

The total number of cells obtained from 40 ml of peritoneal fluid at 1, 3, 9 and 18 dpi was 598.16 × 10^6, 298.32 × 10^6, 3771.60 × 10^6 and 19,918.00 × 10^6, respectively (Table 2). The dynamics of the percentage of the peritoneal lymphocytes and APC in the negative and infected animals during the early stage of infection is shown in Fig. 2a-f. In the infected animals, flow cytometry analyses showed that lymphocyte cell subpopulation dynamics was different for TCD4, TCD8 and $WC1^{+}\gamma\delta$ cells. No significant differences with respect to the UC Group were detected for TCD8 and $WC1^{+}\gamma\delta$ cells, although slight non-significant variations were observed at different time points. The dynamics of TCD4 cells was irregular, with an initial decrease at 1 dpi ($U = 0$, $df = 8$, $P = 0.0357$), then an increase at 3 and 9 dpi and again a significant decrease at 18 dpi ($U = 1$, $df = 8$, $P = 0.0159$) when compared to animals of the UC.

Regarding the dynamics of APC, a different tendency was observed during the early stage of infection for CD14, MHCII and CD83 cells (Fig. 2d-f). CD14 cells

Table 2 Percentage of peritoneal cells

Cell populations	Percentage (%)					
	Group 1				Group 2	Group 3
	1 dpi (n = 5)	3 dpi (n = 5)	9 dpi (n = 5)	18 dpi (n = 5)	14 wpi (n = 10)	NegCtl (n = 7)
TCD4	12.42 (8.14–18.29)	27.24 (19.57–33.73)	37.00 (34.18–40.11)	18.68 (14.00–23.45)	33.68 (25.08–45.21)	30.98 (22.09–38.56)
TCD8	13.47 (10.13–15.65)	25.04 (18.98–28.92)	9.24 (7.52–12.42)	22.86 (17.73–34.22)	29.93 (13.25–41.55)	18.40 (2.59–22.91)
$WC1^+\gamma\delta$	5.28(4.30–6.40)	1.71 (1.13–2.44)	5.22 (3.40–7.69)	7.41 (4.85–10.93)	7.30 (4.57– 10.33)	2.28 (1.13–2.44)
CD14	60.15 (39.82–78.17)	63.48 (49.86–79.07)	41.42 (38.76–43.31)	31.84 (15.50–62.19)	19.18 (11.19–29.85)	69.44 (53.68–86.9)
CD83	22.78 (8.58–47.50)	2.17 (0.52–7.70)	11.89 (9.48–14.06)	23.21 (18.16–33.71)	6.17 (1.62– 8.00)	23.21 (20.75–27.43)
MHCII	48.68 (27.55–63.02)	9.04 (1.84–16.53)	22.54 (20.91–24.62)	40.23 (14.98–93.13)	6.66 (3.44–14.59)	62.00 (65.14–84.24)
Total cell count (40 ml)	598.16×10^6	298.32×10^6	3771.60×10^6	$19{,}918.00 \times 10^6$	1002.40×10^6	418.20×10^6
Concentration (cells/ml)	14.95×10^6	7.46×10^6	94.29×10^6	497.95×10^6	25.06×10^6	10.46×10^6

Cell immunonophenotypes are expressed as the mean of the percentage of each time point of challenged sheep from Group 1 (early stage) and from Group 2 (late stage) and UC animals (Neg. Ctl). The range of each immunophenotype are shown in parentheses. Total peritoneal cell counts and concentration of cells are expressed as the mean of each Group at the different time points

exhibited a decreasing trend, with a significant decrease at 9 (U = 0, df = 8, P = 0.0079) and at 18 dpi (U = 2, df = 8, P = 0.0317) when compared to the UC. The dynamics of MHCII and CD83 cells was quite similar with no significant modifications at 1 dpi and a significant decrease at 3 and 9 dpi with respect to the UC sheep. The decrease of MHCII cells was statistically significant at 3 dpi (U = 0, df = 8, P = 0.0079) and 9 dpi (U = 0, df = 8, P = 0.0079); CD83 cells were significantly reduced at 3 and 9 dpi (U = 0, df = 8, P = 0.0079) with respect to the uninfected sheep. When compared, both MHCII and CD83 cells showed the same increasing pattern from 3 to 18 dpi.

Late stage of the infection

The total number of cells obtained from animals of the Group 2 and 3 was 1002.40×10^6 and 418.20×10^6, respectively (Table 2).

At 14 wpi, the percentage of both TCD4 and TCD8 cells showed no significant differences compared to the uninfected group, although a slight but statistically significant higher level of $WC1^+\gamma\delta$ cells (U = 0, df = 15, P = 0.0079) was detected in the infected sheep (Fig. 3).

A noticeably lower percentage of CD14, MHCII and CD83 cells in infected animals was detected when compared to the uninfected sheep. Statistical analysis showed a significant decrease for the three cell populations (U = 0, df = 15, P = 0.0079).

Discussion

Peritoneal leukocyte population in sheep includes mainly lymphocytes and macrophages (and DC), with a minor proportion of eosinophils and neutrophils [21]. Within these cells, macrophages and DC are considered APC which play a crucial role in the innate and local immune response. Meanwhile, T lymphocytes are known to be of key importance as they immunoregulate the host immune response through various mechanisms such as cytokine production. Currently, many *F. hepatica* molecules have been shown to produce severe effects on these cell populations [13, 25–30], hence many studies are focusing on these particular cell populations. The host immune response against *F. hepatica* is believed to be more effective during the initial peritoneal or early hepatic migratory stages [20]. Therefore, we aimed to (i) determine the immunophenotype and the dynamics of the PCP, which are believed to be one of the main immunocompetent cells involved in the early immune response once NEJ have entered the PC, and to (ii) carry out a comparative study between the acute and chronic stage of the infection.

In our study we observed that the dynamics of the lymphocyte subsets did not show major variations in the peritoneal cavity during the early and late stage of the infection. Peritoneal macrophages showed a decreasing trend whereas MHCII and CD83 cells tended to develop an increasing trend along the infection. Indeed, at the beginning of the infection, when NEJ are migrating through the peritoneal cavity (3–9 dpi), the MHCII and CD83 cells were significantly reduced; later on, at 9–18 dpi, when the peritoneal migratory stage of the parasite is supposed to have finished, there was a significant reduction of CD14 cells. Subsequently, all three populations were again significantly reduced in the late stage of the infection in comparison to the UC.

TCD4 cells were the more prominent subpopulation both at the early and late stage of the infection and it was the only one that was modified during the early stage, with a significant decrease at 1 and 18 dpi. TCD8 cells, the second population, did not vary significantly along the infection, but $WC1^+\gamma\delta$ cells, the minority subpopulation, was slightly increased in the chronic stage. To our knowledge, there are no previous *in vivo*

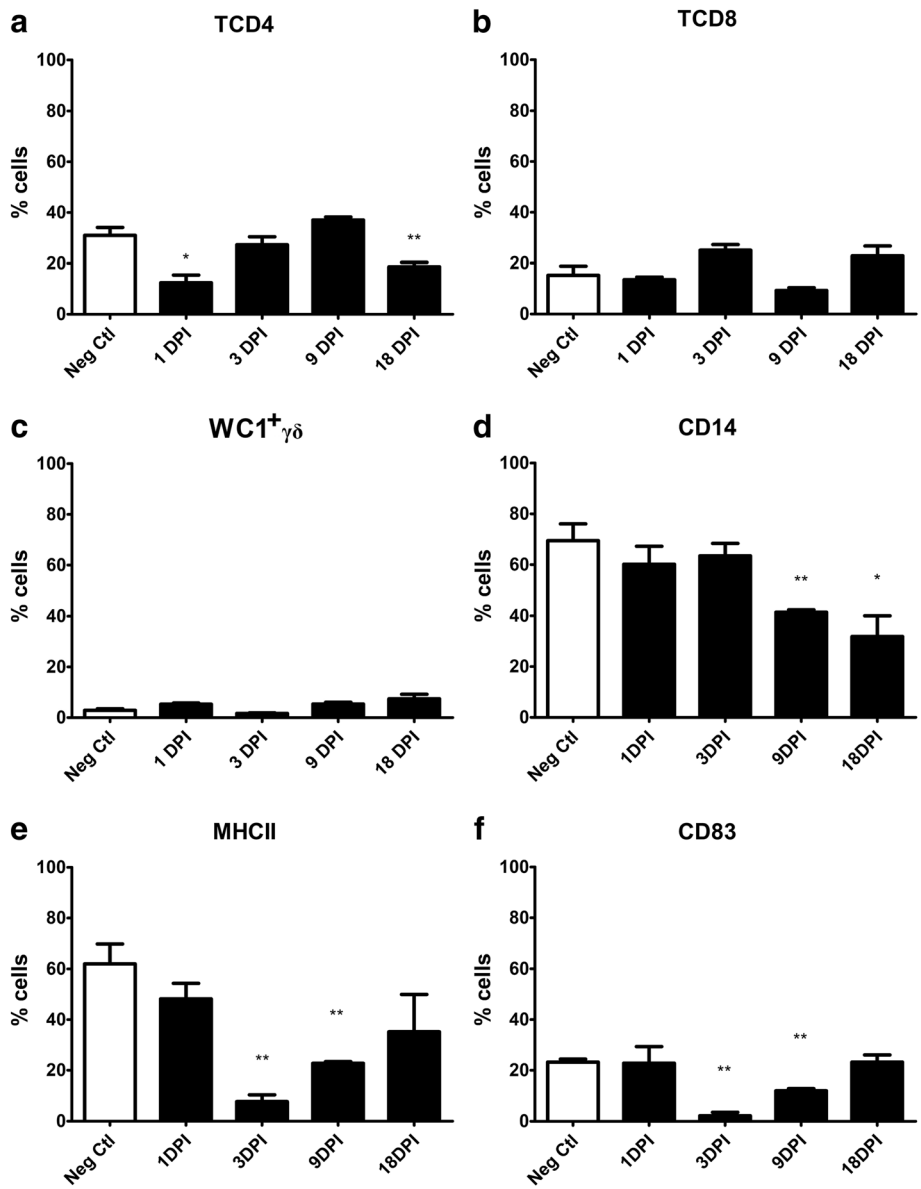

Fig. 2 Percentage of cell populations in the peritoneal fluid during the early stages of infection. The specific cell populations are shown as follows: (**a**) TCD4; (**b**) TCD8; (**c**) WC1^{+}γδ; (**d**) CD14; (**e**) MHCII; (**f**) CD83. Cell subsets of uninfected (Neg. Ctl) and infected sheep (Group 1) are shown in columns. Mann-Whitney U-test was used to compare data from Groups 1 and 3. Values represent the mean ± standard deviation (SD). Asterisks indicate different levels of significance between groups: *$P \leq 0.05$, **$P \leq 0.01$

studies in *F. hepatica*-infected ruminants regarding the dynamics of peritoneal lymphocyte phenotypes during the early and late stage of the infection. In *F. hepatica*-infected goats, our group previously studied the dynamics of circulating TCD4, TCD8 and WC1^{+}γδ cells from peripheral blood during chronic stages of infection, and a significant reduction of TCD4 cells was found at 5 and 12 wpi in respect to the UC, whereas no significant differences were noted for TCD8 and WC1^{+}γδ cells [31]. The different dynamics of TCD4 and WC1^{+}γδ cells in chronic stages of the present study compared to the previous study in goats suggests a different behaviour of peritoneal and peripheral TCD4 and WC1^{+}γδ cell subsets. Alternatively, this may be due to different cell subset recruitment in *F. hepatica*-infected sheep and goats. Recently it was reported that total peritoneal lymphocyte population was only increased at 9 dpi in *F. hepatica*-infected sheep in respect to UC, and a decrease was observed at 18 dpi, probably due to the increment in the recruitment of eosinophils [18]. In this regard, in some animal models experimentally infected with enteric helminths, there is

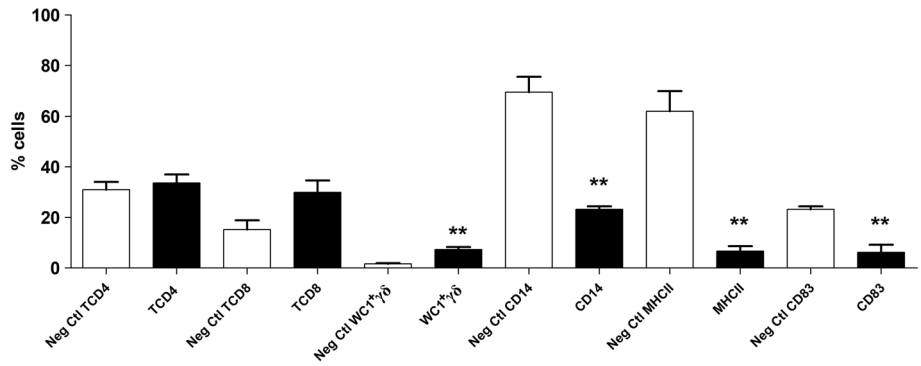

Fig. 3 Percentage of peritoneal cell populations during the late stages of infection. Each cell subset of uninfected (Neg. Ctl) and challenged animals (Group 2) are shown in columns. Mann-Whitney U-test was used to compare data from Groups 2 and 3. Values represent the mean ± standard deviation (SD). Asterisks indicate different levels of significance between groups: *$P \leq 0.05$, **$P \leq 0.01$

supporting evidence that once infection was overcome, a persistent TCD4 population, in a significantly high frequency, occurs at the peritoneal cavity [32], something we partly saw in our study at the chronic stage, when all liver flukes were thought to be already established in the liver. Due to their influence on the polarisation of the immune response in subsequent reinfections, TCD4 cells have been described as the major population for the development of resistance to helminth infection, as occurs with gastrointestinal nematodes [33, 34]. In addition, this cell population is also known to control immunoregulatory mechanisms during helminth infections by means of cytokine secretion [28].

An unexpected result of our study was the observation of a lack of significant increase in the peritoneal T cell population during the early stages, as it has been described in *F. hepatica*-infected rats, in which peritoneal TCD4 and TCD8 were significantly augmented at early stages [28]. In fact, the recruitment of these T cell subpopulations (TCD4, TCD8 and $WC1^{+}\gamma\delta$) in the organ in which the infection was established has been reported for ruminants in helminth infections [35]. In infected goats, we previously detected that the early hepatic lesions due to the penetration of the NEJ in the liver occurred in between 7 and 9 dpi, with a noticeable cellular infiltrate surrounding the initial hepatic lesions, mainly composed of TCD4 and TCD8 [31]. Therefore, it may be hypothesised that as *F. hepatica* NEJ migration through the PC only occurs during the first 4–6 dpi [9], this period might be insufficient to develop a significant increment in the recruitment of peritoneal T cells, which might not affect the recruitment of the lymphocyte subsets in the liver during the early stage.

With respect to the $WC1^{+}\gamma\delta$ T cells, an increase in the percentage was only detected at the late stage of the infection. The influence of the variation of the dynamics of $WC1^{+}\gamma\delta$ observed on the immune response during the early and late stage of the infection still remains to be elucidated. There is supporting evidence that γδ T cells may play an important role in the immune response as they can mediate effector activities such as production of INF-ɣ or TNF-α [36]. Moreover, in cattle the $WC1^{+}\gamma\delta$ T cells were shown to act as APC for αβ T cells [37]. This might condition some immunoregulatory processess which can have an effect on the outcome of the infection in reinfected animals, since this cell population is known to develop memory activity against different pathogens [38, 39].

One of the most noteworthy findings of our study was the influence of the infection on peritoneal CD14, CD83 and MHCII cells over time. A significant decrease could be observed in the early stage in the three studied subpopulations: during the period of peritoneal migration of juvenile flukes (3–9 dpi) for CD83 and MHCII cell populations, and at the time of penetration in the liver parenchyma for the CD14 cell population. Similarly, a marked and significant reduction of the three subpopulations was demonstrated at the chronic stage when compared to UC. Although there are very few studies focused on the dynamics of the peritoneal macrophages (CD14), dendritic cells (CD83) and MHCII cells during infection in ruminants, and the results of these studies are somehow dissimilar [18, 21], there is strong evidence of the modulation of APC functions by *F. hepatica* antigens. In previous *in vitro* studies conducted in mice, tegumental antigens were shown to suppress DC maturation and function [27, 30], *F. hepatica* glycans showed to downregulate the expression of MHCII [25] and some other ES-derived proteins and peptides modulated DC activity by different mechanisms [14, 40]. We have also demonstrated that *F. hepatica* also modulates the oxidative

response of pMΦ, inducing an increase in nitric oxide and hydrogen peroxide production during the early stage of infection in sheep [41]. Moreover, we previously described a specific induction of apoptosis in peritoneal leukocyte populations in the early stage of *F. hepatica* infection in sheep [21], which probably affects the subpopulations of CD14, MHCII and CD83 analysed in this study.

In the chronic stage of the infection, the percentages of the three APC subpopulations were also significantly reduced in comparison to UC. At this time, adult parasites have been located in the bile ducts for several weeks and the activity of some peritoneal macrophages and dendritic cells remains uncertain. It has been suggested that the peritoneum acts as an important lymphoid organ where presentation of antigen to the immune cells takes place and is followed by their migration to the inflammatory site [35] and the role of some particular peritoneal APC could be related to the recruitment of macrophages and lymphocytes in the inflammatory infiltrate surrounding the large bile ducts [21]. However, recent transcriptomic analysis in *F. hepatica*-infected sheep have revealed a complex and different pattern of response in early [42] and late stages of infection [43] and the recruitment and functionality of different cell populations can be modulated in different ways in the different tissues of the host [44].

In summary, we have analysed the cellular immune response elicited in the peritoneal cavity in sheep infected with *F.* hepatica and provided novel data regarding immune cell recruitment over the course of the infection. To our knowledge, this is the first report of the use of flow cytometry for the assessment of the dynamics of local immunocompetent cells at the abdominal cavity in the *F. hepatica* natural host.

Conclusions

We have identified the immunophenotype and the recruitment of the peritoneal cell population in sheep uninfected and infected with *F. hepatica* during the early and late stage of the infection. There is no statistically significant increment in the recruitment of the peritoneal TCD4, TCD8 and $WC1^{+}\gamma\delta$ cells along the course of infection, with the exception of $WC1^{+}\gamma\delta$ cells at the chronic stage. The dynamics of the CD14 cells recruitment at the peritoneal cavity displayed a decreasing trend during the early stage and was significantly decreased at the chronic stage of the infection. CD83 and MHCII cells developed an increasing trend during the early stage of infection, and were significantly decreased at the chronic stage of the infection.

Additional files

Additional file 1: Figure S1. Gating strategy for the identification of cell immunophenotypes by flow cytometric analysis. **1.** Dot-plot samples (1): FSC and SSC in log scale are faced in order to exclude debris. Red square shows the leukocyte populations of interest. **2.** Dot-plot (2.1) and histogram (2.2): once leukocyte populations are gatted, lineal FSC is faced to log SSC so that white cells can be shown and identified properly. The histogram is an extra support for the correct gating in leukocyte subsets. **3.** Dot-plots for fluorochromes (3.1 and 3.2): according to the fluorochrome in each antibody, RPE or FITC channels are faced to log SSC, and in each dot-plot it is only shown the subset of interest which was gated in dot-plot 2. (DOCX 378 kb)

Abbreviations

APC: Antigen presenting cells; DC: Dendritic cells; dpi: Days post-infection; NEJ: Newly excysted juveniles; PC: Peritoneal cavity; PCP: Peritoneal cell populations; pMΦ: Peritoneal macrophages; UC: Uninfected control; WHO: World Health Organization; wpi: Weeks post-infection

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Availability of data and materials

Data supporting the conclusions of this article are included within the article. Raw data are available from the corresponding author upon request.

Authors' contributions

RPC, JP, AMM and LB conceived and designed the study. RPC, FJMM, ILP, VMH, RZ, MTRC, AE and LB collected the samples and RPC analysed the samples. RPC and LB performed the statistical analysis. RPC, AMM and LB wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This experiment was performed in accordance with the University of Córdoba Bioethics Committee (code no. 1118) and European (2010/63/UE) and Spanish Directives (RD 1201/2005) on animal experimentation.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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References

1. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol. 2005;35:1255–78.
2. Molina-Hernández V, Mulcahy G, Pérez J, Martínez-Moreno Á, Donnelly S, O'Neill SM, et al. *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. Vet Parasitol. 2015;208:101–11.
3. Dalton JP, Mulcahy G. Parasite vaccines - a reality? Vet Parasitol. 2001;98: 149–67.
4. Cooper KM, Kennedy DG, Danaher M. *ProSafeBeef* and anthelmintic drug residues - a case study in collaborative application of multi-analyte mass spectrometry to enhance consumer safety. Anal Bioanal Chem. 2012;404: 1623–30.
5. Ortiz P, Scarcella S, Cerna C, Rosales C, Cabrera M, Guzmán M, et al. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Cajamarca (Peru): a clinical trial and *in vivo* efficacy test in sheep. Vet Parasitol. 2013; 195:118–21.
6. Brockwell YM, Elliott TP, Anderson GR, Stanton R, Spithill TW, Sangster NC. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int J Parasitol Drugs Drug Res. 2014;4:48–54.
7. Fairweather I. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet Parasitol. 2011;180:133–43.
8. Álvarez-Sánchez MA, Mainar-Jaime RC, Pérez-García J, Rojo-Vázquez FA. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet Rec. 2006;159:424–5.
9. Moazeni M, Ahmadi A. Controversial aspects of the life cycle of *Fasciola hepatica*. Exp Parasitol. 2016;169:81–9.
10. Dalton JP, Robinson MW, Mulcahy G, O'Neill SM, Donelly S. Immunomodulatory molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic development. Vet Parasitol. 2013;195:272–5.
11. Alvarado R, To J, Lund ME, Pinar A, Mansell A, Robinson MW, et al. The immune modulatory peptide FhHDM-1 secreted by the helminth *Fasciola hepatica* prevents NLRP3 inflammasome activation by inhibiting endolysosomal acidification in macrophages. FASEB J. 2016;31:85–95.
12. Finlay CM, Stefanska AM, Coleman MM, Jahns H, Cassidy JP, McLoughlin RM, et al. Secreted products of *Fasciola hepatica* inhibit the induction of T cell responses that mediate allergy. Parasite Immunol. 2017;39. https://doi.org/10.1111/pim.12460.
13. Rodríguez E, Carasi P, Frigerio S, da Costa V, van Vliet S, Noya V, et al. *Fasciola hepatica* immunoregulates CD11c+ cells by interacting with the macrophage gal/GalNAc lectin. Front Immunol. 2017;8:264.
14. Noya V, Brossard N, Berasaín P, Rodríguez E, Chiale C, Mazal D, et al. A mucin-like peptide from *Fasciola hepatica* induces parasite-specific Th1-type cell immunity. Parasitol Res. 2016;115:1053–63.
15. Hernández-González A, Valero ML, del PMS, Oleaga A, Siles-Lucas M. Proteomic analysis of *in vitro* newly excysted juveniles from *Fasciola hepatica*. Mol Biochem Parasitol. 2010;172:121–8.
16. Cwiklinski K, de la Torre-Escudero E, Trelis M, Bernal D, Dufresne PJ, Brennan GP, et al. The extracellular vesicles of the helminth patogen, *Fasciola hepatica*: biogenesis pathways and cargo mollecules involved in parasite pathogenesis. Moll Cell Proteomics. 2015;14:3258–73.
17. García-Campos A, Ravidà A, Nguyen DL, Cwiklinski K, Dalton JP, Hokke CH, et al. Tegument glycoproteins and cathepsins of newly excysted juvenile *Fasciola hepatica* carry mannosidic and paucimannosidic N-glycans. PLoS Negl Trop Dis. 2016;10:e0004688.
18. Ruiz-Campillo MT, Molina-Hernández V, Pérez J, Pacheco IL, Pérez R, Escamilla A, et al. Study of peritoneal macrophage immunophenotype in sheep experimentally infected with *Fasciola hepatica*. Vet Parasitol. 2018;257:34–9.
19. Davies C, Goose J. Killing of newly excysted juveniles of *Fasciola hepatica* in sensitized rats. Parasite Immunol. 1981;3:81–96.
20. Piedrafita D, Parsons JC, Sandeman RM, Wood PR, Estuningsih SE, Partoutomo S, et al. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile *Fasciola hepatica in vitro* is mediated by reactive nitrogen intermediates. Parasite Immunol. 2001;23:473–82.
21. Escamilla A, Pérez-Caballero R, Zafra R, Bautista MJ, Pacheco IL, Ruiz MT, et al. Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infections in sheep. Vet Parasitol. 2017;238:49–53.
22. Ministry of Agriculture, Fisheries and Food. Manual of Veterinary Parasitological Laboratory Techniques. Reference Book 418 (Third edition). London: Her Majesty's Stationery Office; 1986.
23. Navarro JA, Caro MR, Seva J, Rosillo MC, Gomez MA, Gallego MC. Study of lymphocyte subpopulations in peripheral blood and secondary lymphoid organs in the goat using monoclonal antibodies to surface markers of bovine lymphocytes. Vet Immunol Immunopathol. 1996;51:147–56.
24. Chan SSM, McConnell I, Blacklaws BA. Generation and characterization of ovine dendritic cells derived from peripheral blood monocytes. Immunology. 2002;107:366–72.
25. Rodríguez E, Noya V, Cervi L, Chiribao ML, Brossard N, Chiale C, et al. Glycans from *Fasciola hepatica* modulate the host immune response and TLR-induced maturation of dendritic cells. PLoS Negl Trop Dis. 2015;9:e0004234.
26. Dowling DJ, Hamilton CM, Donnelly S, La Course J, Brophy PM, Dalton J, et al. Major secretory antigens of the helminth *Fasciola hepatica* activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses. Infect Immun. 2010;78:793–801.
27. Hamilton CM, Dowling DJ, Loscher CE, Morphew RM, Brophy PM, O'Neill SM. The *Fasciola hepatica* tegumental antigen suppresses dendritic cell maturation and function. Infect Immun. 2009;77:2488–98.
28. Jedlina L, Kozak-Ljunggren M, Wedrychowicz H. *In vivo* studies of the early, peritoneal, cellular and free radical response in rats infected with *Fasciola hepatica* by flow cytometric analysis. Exp Parasitol. 2011;128:291–7.
29. Vukman KV, Adams PN, Metz M, Maurer M, O'Neill SM. *Fasciola hepatica* tegumental coat impairs mast cells' ability to drive Th1 immune responses. J Immunol. 2013;190:2873–9.
30. Aldridge A, O'Neill SM. *Fasciola hepatica* tegumental antigens induce anergic-like T cells *via* dendritic cells in a mannose receptor-dependent manner. Europ J Immunol. 2016;46:1180–92.
31. Zafra R, Pérez J, Buffoni L, Martínez-Moreno FJ, Acosta I, Mozos E, et al. Peripheral blood lymphocyte subsets in *Fasciola hepatica* infected and immunised goats. Vet Immunol Immunopathol. 2013;155:135–8.
32. Steinfelder S, Rausch S, Michael D, Kühl AA, Hartmann S. Intestinal helminth infection induces highly functional resident memory CD4 + T cells in mice. Eur J Immunol. 2017;47:353–63.
33. Gill HS, Watson DL, Brandon MR. Monoclonal antibody to CD4+ T-cells abrogates genetic resistance to *Haemonchus contortus* in sheep. Immunol. 1993;78:43–9.
34. Balic A, Bowles VM, Meeusen ENT. Cellular profiles in the abomasal mucosa and lymph node during primary infection with *Haemonchus contortus* in sheep. Vet Immunol Immunopathol. 2000;75:109–20.
35. Mitra R, Dharajiya N, Kumari L, Varalakshmi C, Khar A. Migration of antigen presenting cells from periphery to the peritoneum during an inflammatory response: role of chemokines and cytokines. FASEB J. 2004;18:1764–6.
36. Hsu H, Chen C, Nenninger A, Holz L, Baldwin CL, Telfer JC. WC1 is a hybrid γδ TCR coreceptor and pattern recognition receptor for pathogenic bacteria. J Immunol. 2015;194:2280–8.
37. Lahmers KK, Norimine J, Abrahamsen MS, Palmer GH, Brown WC. The CD4+ T cell immunodominant *Anaplasma marginale* major surface protein 2 stimulates γδ T cell clones that express unique T cell receptors. J Leukoc Biol. 2005;77:199–208.
38. Blumerman SL, Herzig CT, Baldwin CL. WC1+ γδ T cell memory population is inducen by killed bacterial vaccine. Eur J Immunol. 2007;37:1204–16.
39. Chien YH, Konigshofer Y. Antigen recognition by γδ T cells. Immunol Rev. 2007;215:46–58.
40. Ruiz-Jimenez C, Celias D, Silvane L, Cervi L, Espino AM. *Fasciola hepatica* fatty acid binding protein (Fh12) inhibits the activation of murine dendritic cells. J Immunol. 2017;198(Supplement 1):68 15.
41. Pérez-Caballero R, Buffoni L, Martínez-Moreno FJ, Zafra R, Molina-Hernández V. Expression of free radicals by peritoneal cells of sheep during the early stages of *Fasciola hepatica* infection. Parasit Vectors. 2018;11:500.
42. Rojas CAA, Scheerlinck JP, Ansell BRE, Hall RS, Gasser RB, Jex AR. Time-course study of the transcriptome of peripheral blood mononuclear cells (PBMCs) from sheep infected with *Fasciola hepatica*. PLoS One. 2016;11: e0159194.
43. Fu Y, Browne JA, Killick K, Mulcahy G. Network analysis of the systemic response to *Fasciola hepatica* infection in sheep reveals changes in fibrosis, apoptosis, toll-like receptors 3/4, and B cell function. Front Immunol. 2017;8:485.
44. Sachdev D, Gough KC, Flynn RJ. The chronic stages of bovine *Fasciola hepatica* are dominated by CD4 T-cell exhaustion. Front Immunol. 2017;8:1002.

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Parasites & Vectors

RESEARCH Open Access

Expression of free radicals by peritoneal cells of sheep during the early stages of *Fasciola hepatica* infection

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Abstract

Background: The majority of vaccination studies against infection with *F. hepatica* in a natural host have been conducted at the late stage of the infection when the host's immune response is already immunomodulated by the parasite towards a Th2 non-protective response. This study was aimed at analysing the dynamic of the cell populations present in peritoneal liquid and the production of free radicals by the peritoneal leukocytes in infected and vaccinated sheep with recombinant cathepsin L1 of *F. hepatica* (rFhCL1) in early stages of the infection.

Methods: Forty-five sheep were divided into three groups: Group 1 remained as negative control ($n = 5$), Group 2 ($n = 20$) was challenged with *F. hepatica* and Group 3 ($n = 20$) was vaccinated with rFhCL1 and challenged with *F. hepatica*. After the slaughtering, peritoneal lavages were carried out at 1, 3, 9 and 18 days post-infection (dpi) to isolate peritoneal cell populations. Flow cytometry was conducted to assess levels of hydrogen peroxide (H_2O_2) and nitric oxide (NO).

Results: There was a significant increase in the total number of leukocytes at 9 and 18 dpi in infected and vaccinated groups. Production of H_2O_2 was significantly increased in peritoneal granulocytes in both infected and vaccinated groups. Production of nitric oxide showed a significant rise in the granulocytes and monocytes/macrophages in infected and vaccinated sheep. The NO production by granulocytes at 3 and 9 dpi was significantly higher in the vaccinated than in the infected animals.

Conclusions: Experimental infection induced an increase in the total number of leukocytes within the abdominal cavity at 9 and 18 dpi, being more noticeable in vaccinated animals. Production of H_2O_2 occurred mainly in granulocytes of vaccinated and infected animals. Production of NO was incremented in vaccinated and non-vaccinated animals in all peritoneal cells. Vaccinated animals produced significant higher level of H_2O_2 and NO than infected animals.

Keywords: *Fasciola hepatica*, Nitric oxide, ROS, Peritoneal cells, Vaccines, Sheep

Background

Fasciolosis, caused by *Fasciola hepatica*, is a globally distributed parasitic disease that mainly affects ruminant livestock and causes great impact in terms of economic losses to the agricultural industry [1, 2]. The World Health Organization (WHO) recognises it as a food-borne trematode infection and as an important zoonotic disease. Immature and mature forms of the parasite inhabits the liver of the host and produces a hepatitis which may alter the liver function [3].

There are few effective strategies to control the disease though it is widely accepted that the use of anthelmintics is the best means to control the infection. Specific drugs differ in their efficacy as some of them may not affect early immature stages of the parasite, hence triclabendazole (TCBZ) has become the drug of choice in many countries. A key drawback lies on the anthelmintic resistance to various drugs including TCBZ which has been globally reported [4–7]. Consequently, in the past

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two decades, many attempts have been conducted to develop a viable vaccine showing diverse results in cattle and sheep. The majority of the vaccination studies have been focussed on the late stage of the infection when the immune response of the host is known to be already immunomodulated by the parasite towards a non-protective Th2 response and the inhibition of protective pro-inflammatory products [8, 9]. Therefore, studies in infected and vaccinated animals during the early stage of infection, when the parasite is migrating and establishing in the liver may shed light on the initial immunological pathways of the disease.

Previous studies have shown that some parasites can trigger free radical production by leukocytes including superoxide radical, nitric oxide, and hydrogen peroxide [10, 11]. In *F. hepatica* infections, resident peritoneal leukocytes such as macrophages might be involved in the killing of newly excysted juvenile liver flukes (NEJ). This may be accomplished by a parasite-specific antibody dependent mechanisms since most juvenile parasites are killed in the gut or the abdominal cavity, before reaching the liver [12]. In this way, some studies have shown an increase in the production of reactive oxygen species (ROS) produced by peritoneal leukocytes in rats infected with *Fasciola hepatica* [13, 14], which might be involved in the killing of migrating immature liver flukes during the early host-parasite interface. Moreover, free radical-induced cytotoxicity against NEJ of *Fasciola* sp. has been previously reported in infected sheep [15].

The aim of this work was to develop an *in vivo* study using flow cytometry in order to investigate the dynamic of the cell populations present in peritoneal liquid and the production of free radicals by leukocytes (macrophages and granulocytes) present in peritoneal liquid in infected animals (vaccinated and non-vaccinated) with recombinant cathepsin L1 of *F. hepatica* (rFhCL1) in early stages of the disease.

Methods

Animals and experimental design

Forty-five six-month-old male merino sheep obtained from a liver fluke-free farm were used for the experimental trial. Before beginning the study, animals were confirmed to be free of liver fluke infection by faecal analyses and ELISA for *F. hepatica* specific antibodies. Sheep were housed in covered pens and fed daily with hay and commercial pelleted ration.

Sheep were randomly divided into three groups: Group 1 consisted of 5 animals which were neither immunised nor experimentally challenged ($n = 5$), hence remained as negative control group, Group 2 consisted of 20 animals ($n = 20$) which were experimentally infected with *F. hepatica* (positive control group) and Group 3 consisted of 20 sheep which were immunised with rFhCL1 and experimentally challenged with *F. hepatica* ($n = 20$). In addition, animals from Groups 2 and 3 were subdivided into smaller groups of five animals each according to slaughtering day: 1, 3, 9 and 18 days post-infection (dpi).

Animals from Group 3 received the vaccine twice by subcutaneous inoculation on weeks 0 and 4 of the trial. At week 8 of the experiment, animals from Groups 2 and 3 were orally challenged with one single dose of 150 metacercariae of *F. hepatica* (Ridgeway Research Ltd., St Briavels, UK) administered in gelatine capsules using a dosing gun. As previously mentioned, five animals from Groups 2 and 3 were euthanised by an intravenous injection of T61® (Intervet, Barcelona, Spain) at each time-point; animals from the negative control group (Group 1) were euthanised on the 12th week of the trial.

Purification of recombinant *F. hepatica* cathepsin L1

Recombinant *F. hepatica* cathepsin L1 (rFhCL1) was expressed in the yeast *Pichia pastoris* and purified as described elsewhere [16]. Yeast transformants were cultured in 250 ml BMGY broth, buffered to pH 6.0, in 1 l baffled flasks at 30 °C until an OD_{600} of 2–6 was reached. Cells were harvested by centrifugation at 2000× *g* for 5 min and protein expression was induced by resuspending the cells in 50 ml BMMY broth, buffered at pH 6.0, 7.0 or 8.0, containing 1% methanol. The cultures were grown at 30 °C with shaking at 225× *rpm* for 3 days, and filter-sterilized methanol was added daily to maintain a final concentration of 1%. Recombinant proteins were purified from the yeast medium by affinity chromatography using Ni-NTA-agarose (Qiagen, Montreal, Canada). Briefly, a column prepared with 1 ml of resin was equilibrated by passing through 10 ml 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole. Ten millilitres of yeast media supernatant was mixed with 40 ml of the same buffer and applied to the column. The column was washed with 15 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole, and bound protein was eluted using 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 250 mM imidazole. Purified recombinant proteases were dialysed against phosphate buffered saline (PBS) and stored at -20 °C. Before immunisation, electrophoresis in polyacrylamide gel was carried out to check protein purity.

Vaccine preparation

The recombinant protein cathepsin L1 of *F. hepatica* (rFhCL1) was diluted in ISA 70 Montanide adjuvant. Each immunisation dose was prepared as follows: 100 μg of rFhCL1 was diluted in PBS containing 1 mg/ml of the adjuvant, reaching a final volume of 1 ml per dose.

Liver pathology

Necropsy was performed and the liver was removed and photographed on both visceral and diaphragmatic surface for gross evaluation. Liver tissue samples showing hepatic lesions were collected and fixed in 10% neutral buffered formalin for 24 h, then routinely processed and embedded in paraffin wax. Four-micron-thick tissue sections were stained with hematoxylin and eosin (H&E) for histopathology. Gross hepatic lesions during the early stages of infection in challenged animals (Groups 2 and 3) were counted using the Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

Antibody detection

Blood samples were taken at weeks 0 and 6 of the trial and at 1, 3, 9 and 18 days post-infection (dpi), and plasma was collected to detect specific IgG1 and IgG2 antibodies against rFhCL1 by ELISA. Briefly, 96-well ELISA plates were coated with 5 μg/ml of rFhCL1 (100 μl/well) diluted in 0.05 M carbonate-bicarbonate buffer pH 9.6 and incubated at 37 °C overnight. After 5 washes with phosphate buffer saline (PBS) 0.05% Tween 20, plates were blocked with 100 μl/well of blocking buffer containing 1% BSA diluted in PBS and incubated at 37 °C for 30 min. To detect IgG1, wells were washed and 100 μl/well of plasma diluted in blocking buffer was added and incubated at 37 °C for 30 min. Triple serial dilutions were performed to determine endpoint titre. Similarly, IgG2 was detected by adding 100 μl/well of plasma diluted at 1:25. After washing, 100 μl/well of primary antibody diluted 1:5000 (mouse anti-bovine IgG1 and anti-bovine IgG2; 7500820–7500830 Cedi-Diagnostics, Lelystad, The Netherlands), in blocking buffer was added and incubated at 37 °C for 30 min. After incubation, wells were washed and anti-mouse IgG-HRP (STAR13B, BIO-RAD - formerly AbD-Serotec-, Kidlington, UK) was added at 37 °C for 30 min. Plates were washed and 100 μl/well of tetramethylbenzidine (TMB; Sigma-Aldrich, Madrid, Spain) were added and incubated for 10 min at room temperature. The reaction was stopped by adding of 100 μl/well of 1 M sulphuric acid and optical density was measured at 450 nm using a microplate photometer (Multiskan^{TM} FC, Thermo Fisher Scientific, Madrid, Spain). Results are shown as antibody titre -$\log_{10}$-for IgG1, and as optical density for IgG2.

Isolation of peritoneal cell population

To collect peritoneal cell population, abdominal lavage of each sheep was immediately conducted after the slaughtering as previously described [17]. Briefly, the ventral region of the abdomen was sheared, shaved and disinfected using 10% polyvinylpyrrolidone iodine (AGB, Madrid, Spain). A 2 cm incision was made in the skin over the midline and subcutaneous tissue was dissected. The white line and peritoneum were sectioned with blunt scissors to avoid haemorrhage. A 40 cm long cannula connected to a syringe was inserted into the abdominal cavity and 40 ml sterile DPBS containing 9500 UI of heparin (Eurotubo®, Madrid, Spain) (warmed at 37 °C) was injected into the abdominal cavity. After softly massaging the abdominal cavity for 1 min, 40 ml of peritoneal fluid were withdrawn. Peritoneal fluid was centrifuged at 1500× *rpm* for 5 min and the supernatant was discarded. Cell pellets were resuspended again in DPBS and incubated for 15 min in an erythrolysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM disodium EDTA, dH_2O). A second centrifugation step (1500× *rpm* for 5 min) was performed to eliminate lysed erythrocyte membranes and the pellet was resuspended in 1 ml of medium. After that, the concentration of peritoneal cells was quantified using the Trypan Blue exclusion technique. The final concentration was adjusted to 1×10^6 cells/ml for analysis by flow cytometry assay.

Flow cytometry assay

Flow cytometry acquisition was performed with a CyFlow Cube 6® (Sysmex-Partec, Barcelona, Spain) cytometer. Leukocytes were identified by their characteristic appearance on a FSC-SSC dot plot and gated in order to exclude cellular debris. Ten thousand events were analysed for changes in fluorescence intensity (enzymatic activity) of macrophages and granulocytes. For oxidative metabolism, hydrogen peroxide (H_2O_2) and nitric oxide (NO) production, filtered light at green was used (488 nm FL1 channel). The results were analysed using FCS Express 4.0 (DeNovo Software, Los Angeles, USA) and Flowing Software 2.0 (Centre for Biotechnology, Turku, Finland).

In order to measure intracellular H_2O_2, the cell-permeable dye DCFH-DA (Dichloro-dihydro-fluorescein diacetate) was used. The non-fluorescence reduced form is converted into the fluorescent form when is oxidised and, in this way, the fluorescence can be detected by flow cytometry. A 200 μl sample of peritoneal cells were incubated in dark and 37 °C for 20 min with 1 ml of DCFH-DA 10 μM. After incubation, samples were centrifuged at 1500× *rpm* for 5 min and the supernatant was discarded. Two washes of PBS centrifuged at 1500× *rpm* for 5 min were carried out and the pellets are resuspended in DPBS.

Intracellular NO was measured using the cell-permeable dye DAF-2DA (4,5-diaminofluorescein diacetate), because the non-fluorescent reduced form is converted into the fluorescent form when oxidised, thus allowing the detection by flow cytometry. In the same way, a 200 μl sample of peritoneal cells was incubated in dark and 37 °C for 180 min. From this point onwards the methodology was similar to that described for DCFH-DA.

Statistical analysis

Statistical analysis was carried out with GraphPad Prism v.6.0 (GraphPad Software Inc., San Diego, CA, USA). The Kolmogorov-Smirnov test was applied to evaluate whether distributions were parametric. Comparison between pairs of groups was made using a two-tailed Mann-Whitney U-test for non-parametric distributions. $P < 0.05$ was considered statistically significant.

Results

Progression of infection: liver pathology and antibody production

No hepatic changes were observed in the negative control animals (Group 1). In infected and vaccinated animals (Groups 2 and 3), gross and histopathological hepatic changes were absent at 1 and 3 dpi, though tortuous whitish tracts and haemorrhagic spots, which mainly occurred along the left hepatic lobe, were detected at 9 and 18 dpi. Further description of the gross hepatic lesions as well as the histopathological changes have been previously described in our recent study [18].

The levels of plasmatic specific anti-rFhCL1 IgG1 and IgG2 are presented in Fig. 1a, b. All vaccinated animals with the recombinant antigen developed an IgG1-IgG2 antibody response following immunisation. In vaccinated animals, a statistically significant production of IgG1 was detected after immunisation at each slaughtering time-point ($U = 0$, $df = 8$, $P = 0.0006$ for 6 wpv; $U = 0$, $df = 8$, $P = 0.0097$ for 1, 9 and 18 dpi and $U = 3$, $df = 8$, $P = 0.0449$ for 3 dpi) and showing an increasing trend at the end of the trial (Fig. 1a). Production of IgG2 showed a similar pattern to that observed for IgG1 but was overall limited

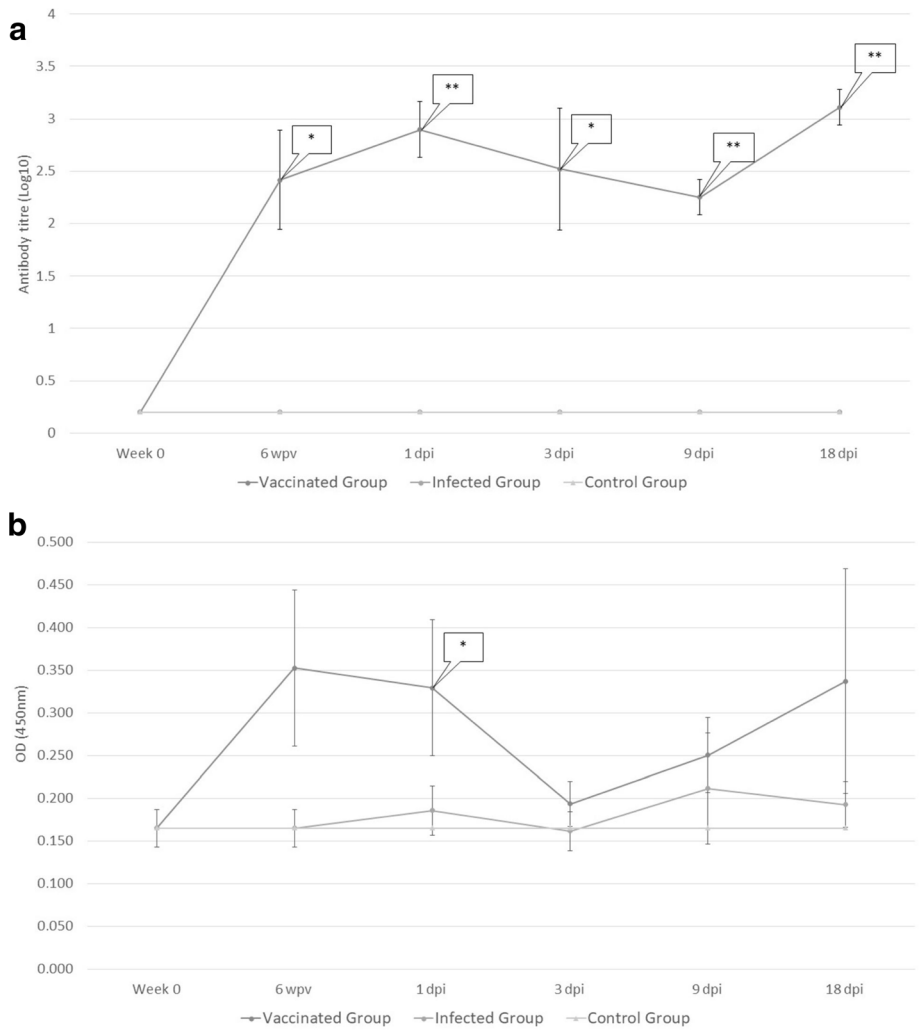

Fig. 1 Plasma levels of specific rFhCL1 IgG1 (**a**) and IgG2 (**b**). Each point represents mean values of antibody titre - log10 - (IgG1) and of optical density (IgG2) measured at 450 nm. Bars at each point represents tandard error. Immunisation with rFhCL1 developed a significant rise in the level of IgG1 isotype; dynamics of IgG1 in uninfected and infected sheep (Groups 1 and 2) shows a similar pattern, hence it is overlapped in the figure. Significant IgG2 production was detected only at 1 day post-infection (dpi) during the trial

and only statistically significant after immunisation (U = 2, df = 8, P = 0.0062; Fig. 1b). No production of specific anti-rFhCL1 IgG1 and IgG2 was detected in the negative and positive control groups (Groups 1 and 2).

Dynamics of peritoneal leukocytes during infection

The total number of leukocytes population from peritoneal liquid was assessed by flow cytometry and expressed as 1×10^6 cells/ml. There was a significant increase in the number of leukocytes at 9 and 18 dpi, in both infected (U = 3, df = 8, P = 0.0439 and U = 0, df = 8, $P < 0.0001$, respectively) and vaccinated (U = 0, df = 8, P = 0.0009 and U = 0, df = 8, $P < 0.0001$, respectively) groups in comparison with the negative control group (Fig. 2).

Distribution of peritoneal leukocyte populations during infection

Leukocyte populations were characterised by a side scatter/forward scatter profile as shown in Fig. 3a, b. Dynamics of cell populations through the infection is shown in Fig. 4a, b. The trend in all animals was an increase in the number of granulocytes and a decrease in both macrophages and lymphocytes throughout the infection.

Granulocytes were significantly increased in the infected group at 9 dpi (U = 0, df = 8, P = 0.0129) and 18 dpi (U = 2, df = 8, P = 0.0032). In the vaccinated group, a similar significant increment was detected at 9 dpi (U = 3, df = 8, P = 0.0481) and at 18 dpi (U = 0, df = 8, P = 0.0071). On the other hand, a significant decrease in the number of lymphocytes (U = 3, df = 8, P = 0.0098) and macrophages (U = 6, df = 8, P = 0.0291) was observed in the infected group at 18 dpi compared with the negative control group (Group 1).

Lymphocytes and macrophages were significantly decreased in vaccinated animals (U = 0, df = 8, P = 0.0011 and U = 1, df = 8, P = 0.0058, respectively) and in infected group (U = 3, df = 8, P = 0.0098 and U = 6, df = 8, P = 0.0291, respectively) at 18 dpi compared to negative control animals (Group 1). This decrease was also observed in the vaccinated animals at 3 dpi for lymphocytes (U = 6, df = 8, P = 0.0037) and at 9 dpi for macrophages (U = 2, df = 8, P = 0.0182), compared to the negative control group.

Hydrogen peroxide production by peritoneal leukocyte populations during infection

The results are shown in Fig. 5a, b. Production of H_2O_2 displayed a slightly different overall pattern in infected and vaccinated animals. During the initial stage of the infection, H_2O_2 production occurred mainly in the granulocyte cell type. In the infected group (Fig. 5a), the increase of H_2O_2 production by granulocytes was only observed at 18 dpi (U = 2, df = 8, P = 0.0002). Nevertheless, a significant decrease was observed at 3 dpi for monocytes/macrophages (U = 0, df = 8, P = 0.0296).

In vaccinated animals (Fig. 5b) granulocytes showed a significant increase (U = 0, df = 8, $P < 0.0001$) in H_2O_2 production at 9 and 18 dpi, whereas no significant variations were observed in the response of monocytes/macrophages.

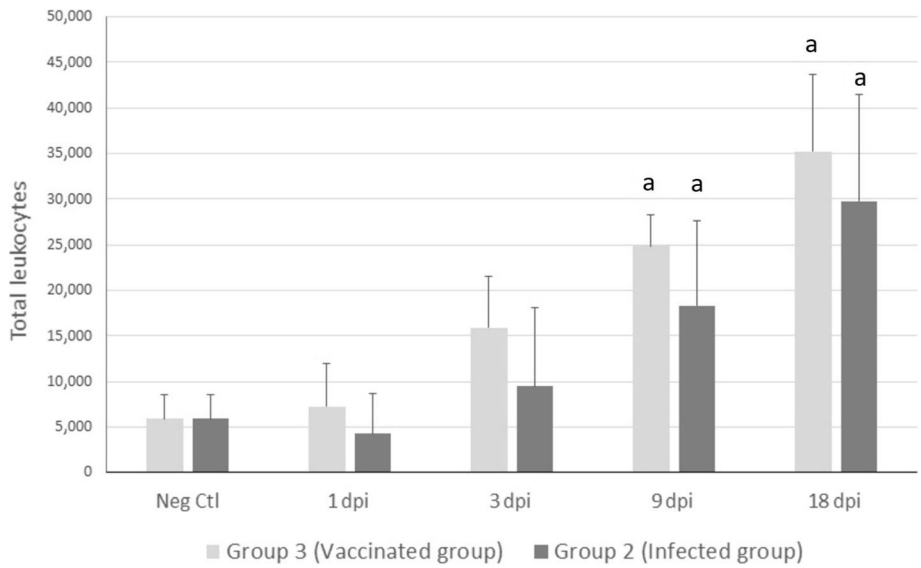

Fig. 2 Mean total number of leukocytes illustrating the effect of infection on peritoneal cell recruitment. Cell viability was assessed by trypan blue exclusion. "a" indicates significant variations between infected (Group 2, infected group), immunised (Group 3, vaccinated group) and control (Neg Ctl) sheep ($P < 0.05$) at 4 slaughtering time-points: 1, 3, 9 and 18 days post-infection (dpi)

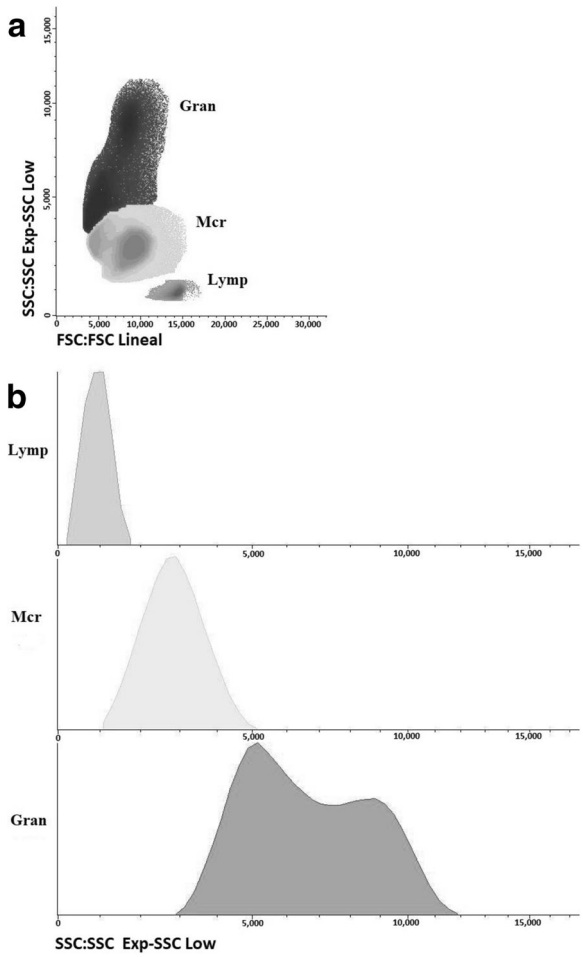

Fig. 3 Flow cytometry analysis of peritoneal cell population of sheep after peritoneal lavage characterised by a side scatter/forward scatter (SSC/FSC) profile. Distribution of major leukocyte populations on gated regions is represented by dot-plot (**a**) and histogram (**b**). *Abbreviations*: Lymp, lymphocytes; Mcr, macrophages; Gran, granulocytes

A statistically significant higher level of H_2O_2 production by granulocytes was observed in the vaccinated group than in the infected group at 9 dpi ($U = 0$, $df = 8$, $P < 0.0001$).

Nitric oxide production by peritoneal leukocyte populations during infection

The dynamic of NO production is shown in Fig. 6a, b. In the infected animals (Fig. 6a) a significant rise in NO production was detected by granulocytes at 9 dpi ($U = 0$, $df = 8$, $P = 0.0432$) and 18 dpi ($U = 0$, $df = 8$, $P = 0.0076$), and by monocytes/macrophages at 18 dpi ($U = 0$, $df = 8$, $P = 0.0022$). In vaccinated sheep (Fig. 6b) the NO production by granulocytes was statistically significant increased at 3 dpi ($U = 0$, $df = 8$, $P < 0.0001$), 9 dpi ($U = 0$, $df = 8$, $P = 0.0026$) and 18 dpi ($U = 0$, $df = 8$, $P = 0.0133$), whereas production by monocytes/macrophages was significantly increased only at 3 dpi ($U = 0$, $df = 8$, $P < 0.0001$).

Production of NO by granulocytes was statistically significant higher at 3 dpi ($U = 0$, $df = 8$, $P < 0.0001$) and 9 dpi ($U = 3$, $df = 8$, $P = 0.0476$) in the vaccinated group than in the infected group.

Discussion

This study focuses on the production of free radicals by leukocyte populations in peritoneal fluid during the early stages of fasciolosis, being the first *in vivo* study carried out in sheep infected and uninfected with *F. hepatica*. We have compared the response in sheep vaccinated with rFhCL1 and non-vaccinated animals and we have confirmed the existence of a noticeable NO response, mainly by granulocytes, in both infected and vaccinated animals.

The peritoneal cavity is a critical location in the development of *F. hepatica* infection as it is the route of migration of *F. hepatica* NEJ from the intestine to the liver and the site where an early immunomodulatory effect of the parasite likely plays a critical role in determining the ultimate outcome of the infection [9]. It has been suggested that effector mechanisms of NEJ killing and consequent immune protection are dependent on the activity of peritoneal leukocytes, as described for *F. hepatica* in rats [19] and *F. gigantica* in Indonesian thin-tail sheep [15]. Both antibodies and free radicals (ROS and NO) produced by leukocytes have been considered effective elements in those protective peritoneal responses [14]. However, ROS and NO produced as a strategy to kill parasites during *F. hepatica* infection have been also described as responsible for oxidative stress and hepatic damage in sheep [20], rats [21] and cattle [22].

In our experiment, peritoneal leukocyte populations in sheep increased immediately after infection, reaching significant levels at 9 and 18 dpi in both infected and vaccinated groups, as previously described in rats experimentally challenged with *F. hepatica* [14]. From 9 dpi onwards, hepatic lesions could be detected, associated with the penetration of NEJ in the liver parenchyma. The cellular infiltration in hepatic lesions was mainly composed of eosinophils, macrophages and lymphocytes, whereas the peritoneal populations were mainly granulocytes, in increased proportions over the course of the infection. In our study, by using the flow cytometry assay, we could not discriminate the different cell population of granulocytes (neutrophils, eosinophils and basophils), but using immuno cytochemistry staining, we have determined that eosinophils occurred in more than 95% of total peritoneal granulocyte populations (data not shown) [23]. The predominance of granulocytes and more specifically of eosinophils in the peritoneal cell populations have been also observed in the early stage of infection in rats [11, 24], in

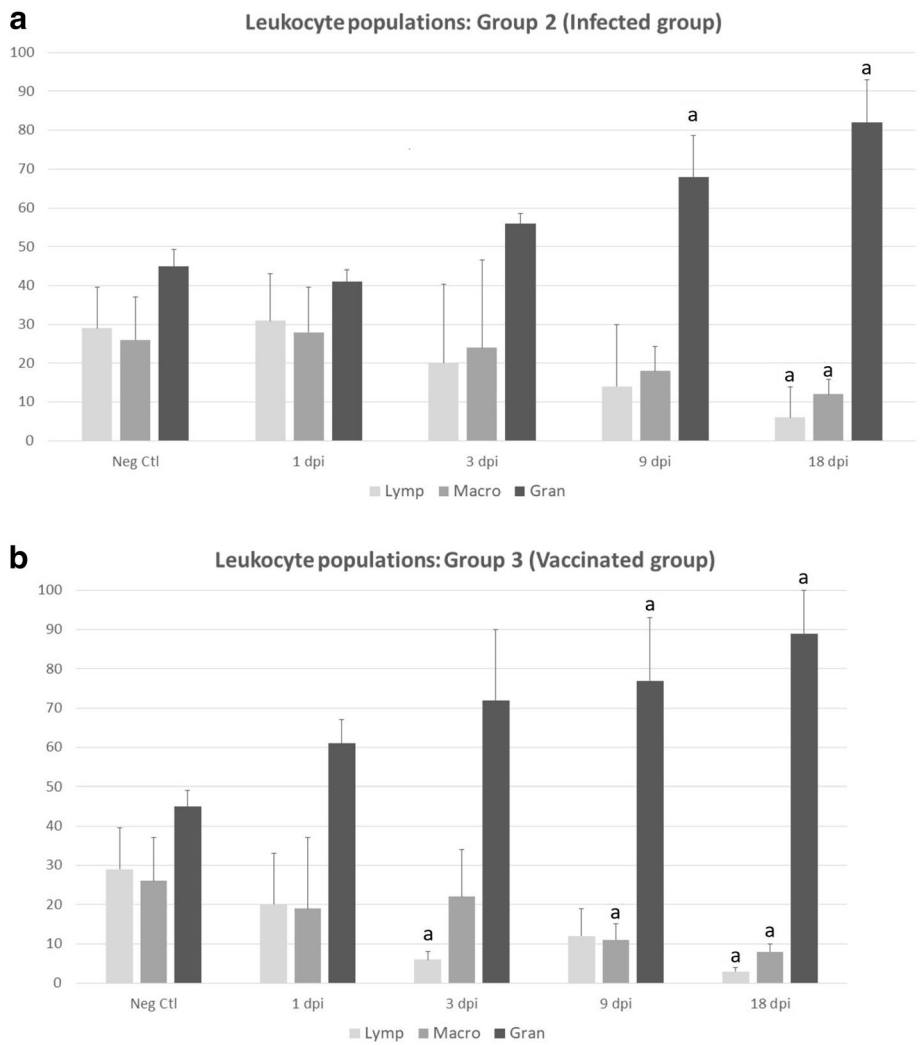

Fig. 4 Differential cell counts by flow cytometric analysis of peritoneal lavage leukocyte samples from uninfected (Neg Ctl, Group 1, **a**, **b**), infected (Group 2, **a**) and vaccinated sheep (Group 3, **b**). Each identified cell subset is expressed as a percentage of the total number of leukocytes. Values represent the mean ± SD. "a" indicates significant differences ($P < 0.05$) between groups. *Abbreviations*: Lymp, lymphocytes; Macro, macrophages; Gran, granulocytes

goats by immunohistochemical studies [17, 25] and in sheep by transcriptome analysis [26].

By means of the flow cytometry technique, we could assess the intracellular production of free radicals ($H_2O_2$and NO) by peritoneal macrophages and granulocytes, although we could not identify the different oxidative response of neutrophils and eosinophils. The intracellular production of both H_2O_2 and NO by peritoneal leukocytes was stimulated during the early stages of infection, in the infected group and in the vaccinated group, as previously described in rats [14]. However, these authors found macrophages as the most significant cell type at the initial stage of the infection (7 dpi) in contrast to our findings in sheep, in which granulocytes were proved to be more relevant cells at all time-points of the study.

We have also found that macrophages and particularly granulocytes from vaccinated animals showed a significantly higher production of free radicals, mainly at 9 and 18 dpi. This is consistent with our previous work where a partial protective response was described in experimental trials with rFhCL1, that could be related to eosinophils and free radical (NO) production in the early stage of infection [17, 18].

The role of H_2O_2 in *F. hepatica* infection remains unclear. Transcriptome studies in mice revealed production of ROS as one the most significant pathways undergoing changes during immunoprotection [27] but it has also been related to oxidative stress and pathology in chronic infection in sheep [20]. Moreover, different *in vitro* studies indicate that *F. hepatica* NEJ possess a unique ability to resist killing by reactive oxygen species released by

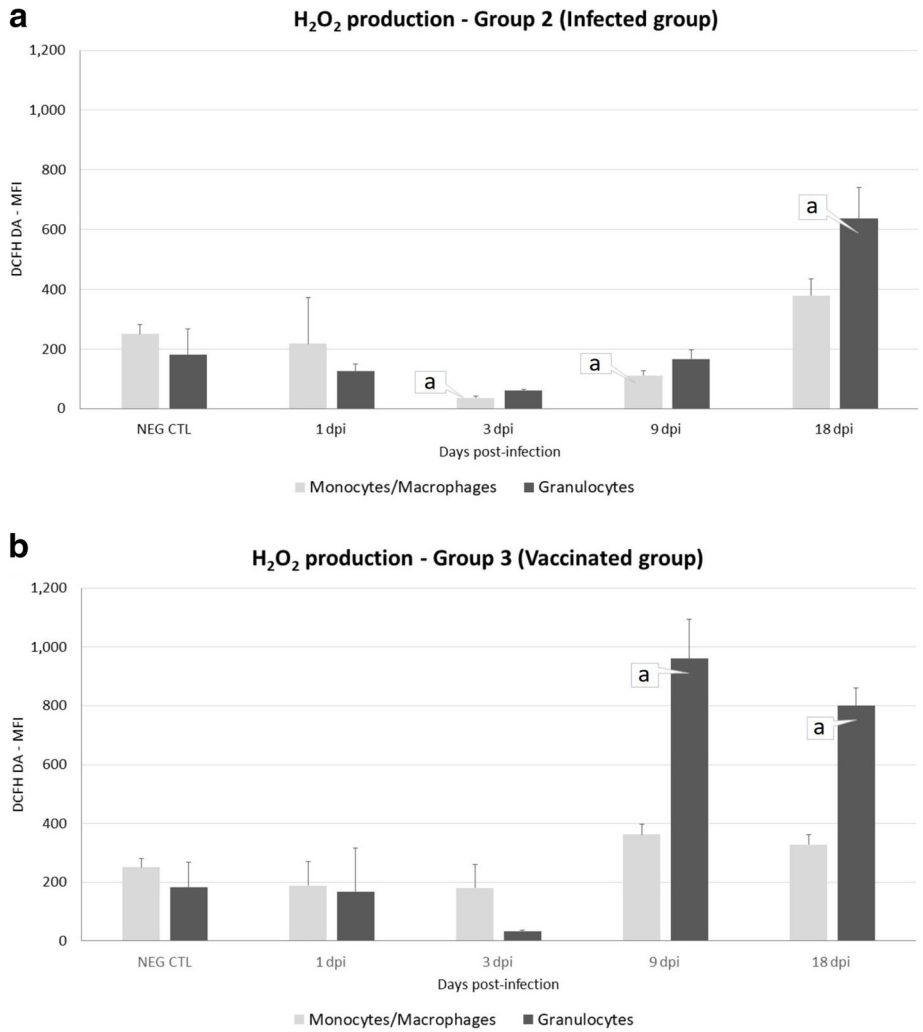

Fig. 5 Mean fluorescence intensity of H_2O_2 production (DCFH DA) in the peritoneal fluid of uninfected sheep (Neg. Ctl, Group 1, **a**, **b**), infected (Group 2, **a**) and vaccinated sheep (Group 3, **b**). Values represent the mean ± standard deviation, SD. "a" indicates significant differences ($P < 0.05$) between groups

sheep innate immune effector cells, which may involve the high expression of antioxidant enzymes such as superoxide dismutase, glutathione S-transferase (GST) or peroxiredoxin [28–31].

In our study, both macrophages and granulocytes were involved in the increase of NO production occurring between 3 and 18 dpi. Previous studies in rats have highlighted the involvement and complementary role of these two cell populations in the peritoneal stage of the infection [11, 14, 31]. In fact, Piedrafita et al. [15] described an antibody-dependent cell-mediated killing of NEJ involving both macrophages and eosinophils NO production in the resistance of ITT sheep to *F. gigantica*. Our previous vaccination study suggested that inducible nitric oxide synthase (iNOS) expression and subsequent NO production could be important for an effective response against the early migrating liver fluke [17]. Although the early production of NO we have detected in that study seemed to have little effect on the development of the infection and in the final fluke burden at the end of the experiment [18]. Consistent protective responses in sheep has not been achieved and the precise effective mechanisms of protection has not been yet elucidated [9]. It has been hypothesised that NO and iNOS might play an important role in *F. hepatica* pathogenesis, possibly as an effective mechanism for killing migrating NEJ, as it has been previously shown to occur in resistant rats [31] or maybe as an expression of M1 macrophages activation which are known to be related to the development of Th1 responses required for protection [18, 32, 33]. Recent transcriptomic studies have revealed that modifications in the NO signalling pathway may be a necessary condition for immunoprotection in mice [27] or, on the contrary, downregulation of iNOS might be a paramount factor during the non-protective response occurring in sheep [34]. In our study, we have

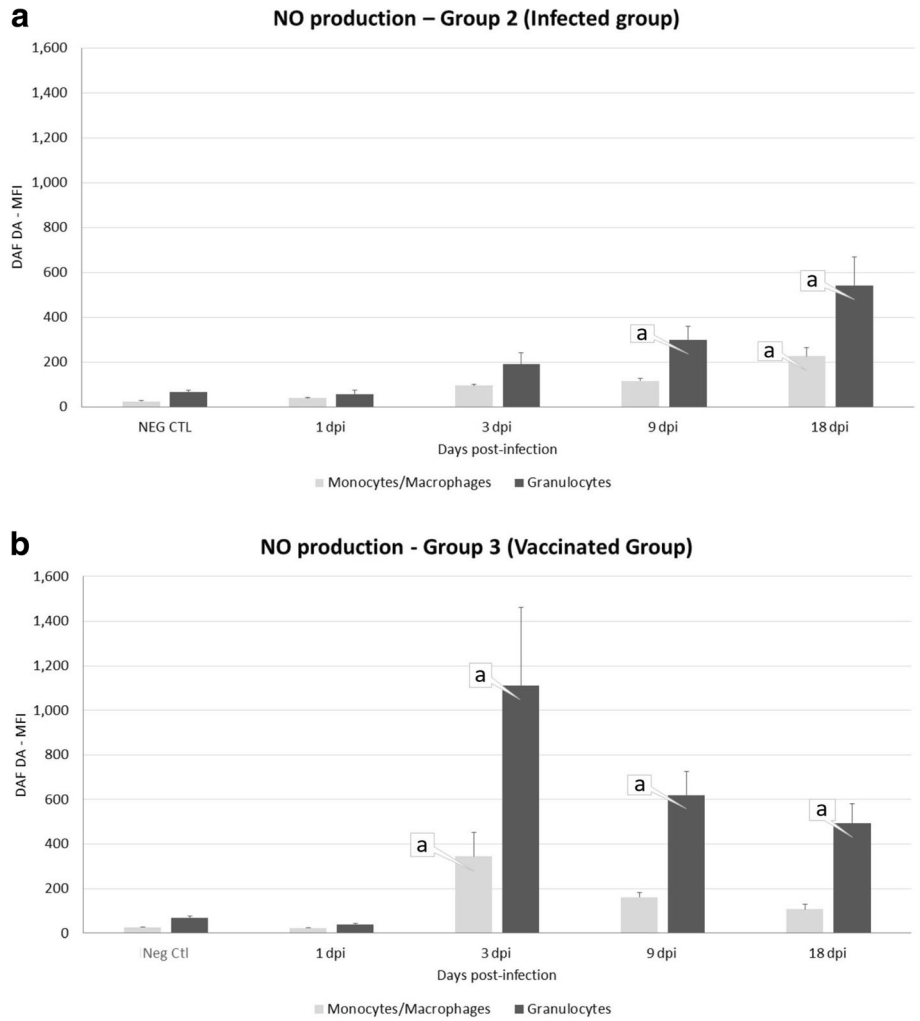

Fig. 6 Mean fluorescence intensity of NO production (DAF DA) in the peritoneal fluid of sheep uninfected (Neg Ctl, Group 1, **a**, **b**), infected (Group 2, **a**) and vaccinated sheep (Group 3, **b**). Values represent the mean ± standard deviation, SD. "a" indicates significant differences ($P < 0.05$) between groups

not detected an inhibition in NO production in the early phase of infection. In another study, we found a low level of variation in iNOS expression in peritoneal macrophages by immunocytochemistry [35]. Those differences suggest that iNOS gene, protein expression and NO production in the initial stages of the infection may differ, with the protein probably remaining active for a longer time than the gene.

In conclusion, we have observed a clear leukocyte response in the peritoneal cavity of the sheep in the early stage of *F. hepatica* infection. The leukocyte populations, mainly granulocytes, exhibited a metabolic response with intracellular production of both H_2O_2 and NO. The effect of those free radicals on the NEJ and migrating juveniles it is still unclear, since NEJ and migrating juveniles appear to be unaffected by those molecules as they reached the liver and evolved to mature stages. Further studies are needed to provide a broader insight on the biomolecular mechanisms involved in the evasion of the immune response of this parasite in the early stages of infection.

Conclusions

Experimental infection induced an increase in the total number of leukocytes within the abdominal cavity at 9 and 18 dpi which was characterised by an increase in the number of granulocytes and a decrease of both macrophages and lymphocytes. Production of both H_2O_2 and NO by peritoneal cells was increased in vaccinated and non-vaccinated animals. Granulocytes were mainly involved in H_2O_2 production, whereas granulocytes and macrophages were predominant in NO production. Vaccinated animals produced a significantly higher level of H_2O_2 and NO than infected animals.

Abbreviations
dpi: days post-infection; GST: glutathione S-transferase; H_2O_2: hydrogen peroxide; iNOS: inducible nitric oxide synthase; NEJ: newly excysted juveniles; NO: nitric oxide; rFhCL1: recombinant cathepsin L1 of *Fasciola hepatica*; ROS: reactive oxygen species; TCBZ: triclabendazole; wpv: weeks post-vaccination

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Availability of data and materials
Data supporting the conclusions of this study are included within the article. Raw data are available from the corresponding author upon request.

Authors' contributions
RPC, LB, FJMM, RZ, VHM, JP and AMM conceived and designed the study. RPC, LB and RZ collected and analysed the samples. RPC, LB and AMM performed the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This experiment was performed in accordance with the University of Cordoba Bioethics Committee (no. 7119) and European (86/609/CEE) and Spanish Directives (RD 223/1988).

Consent for publication
Not applicable.

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

Publisher's Note

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References

1. Spithill TW, Smooker PT, Sexton JL, Bozas E, Morrison CA, Creany J, et al. Development of vaccines against *Fasciola hepatica*. In: Dalton JP, editor. Fasciolosis. Wallingford, UK: CABI Publishing; 1999. p. 377–410.
2. Piedrafita D, Spithill TW, Smith RE, Raadsma HW. Improving animal and human health through understanding liver fluke immunology. Parasite Immunol. 2010;32:572–81.
3. Behm CA, Sangster NC. Pathology, pathophysiology and clinical aspects. In: Dalton JP, editor. Fasciolosis. Wallingford, UK: CABI Publishing; 1999. p. 185–224.
4. Olaechea F, Lovera V, Larroza M, Raffo F, Cabrera R. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Vet Parasitol. 2011;178:364–6.
5. Brockwell YM, Elliott TP, Anderson GR, Stanton R, Spithill TW, Sangster NC. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int J Parasitol Drugs Drug Resist. 2013;4:48–54.
6. Novobilský A, Höglund J. First report of closantel treatment failure against *Fasciola hepatica* in cattle. Int J Parasitol Drugs Drug Resist. 2015;5:172–7.
7. Venturina VM, Alejandro MA, Baltazar CP, Abes NS, Mingala CN. Evidence of *Fasciola* spp. resistance to albendazole, triclabendazole and bromofenofos in water buffaloes (*Bubalus bubalis*). Ann Parasitol. 2015;61:283–9.
8. Dalton JP, Robinson MW, Mulcahy G, O'Neill SM, Donelly S. Immunomodulatory molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic development. Vet Parasitol. 2013;195:272–5.
9. Molina-Hernández V, Mulcahy G, Pérez J, Martínez-Moreno Á, Donnelly S, O'Neill SM, et al. *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. Vet Parasitol. 2015;208:101–11.
10. Wandurska-Nowak E, Wisniewska J. Release of nitric oxide during experimental trichinellosis in mice. Parasitol Res. 2002;88:708–11.
11. Sibille P, Tliba O, Boulard CH. Early and transient cytotoxic response of peritoneal cells from *Fasciola hepatica*-infected rats. Vet Res. 2004;35:576–84.
12. Armour J, Dargie JD. Immunity to *Fasciola hepatica* in rat. Successful transfer of immunity by lymphoid-cells and by serum. Exp Parasitol. 1974;35:381–8.
13. Smith NC, Ovingston KS, Boray JC. *Fasciola hepatica* free radical generation by peritoneal leukocytes in challenged rodents. Int J Parasitol. 1992;22:281–6.
14. Jedlina L, Kozak-Ljunggren M, Wedrychowicz H. *In vivo* studies of the early, peritoneal, cellular and free radical response in rats infected with *Fasciola hepatica* by flow cytometric analysis. Exp Parasitol. 2011;128:291–7.
15. Piedrafita D, Estuningsih E, Pleasance J, Prowse R, Raadsma HW, Meeusen ENT, et al. Peritoneal lavage cells of Indonesian thin-tail sheep mediate antibody-dependent superoxide radical cytotoxicity *in vitro* against newly excysted juvenile *Fasciola gigantica* but not juvenile *Fasciola hepatica*. Infect Immun. 2007;75:1954–63.
16. Collins PR, Stack CN, O'Neill SM, Doyle S, Ryan T, et al. Cathepsin L1, the major protease involved in liver fluke (*Fasciola hepatica*) virulence: propeptide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells. J Biol Chem. 2004;279:17038–46.
17. Zafra R, Perez-Ecija RA, Buffoni L, Pacheco IL, Martinez-Moreno A, LaCourse EJ, et al. Early hepatic and peritoneal changes and immune response in goats vaccinated with a recombinant glutathione transferase sigma class and challenged with *Fasciola hepatica*. Res Vet Sci. 2013;94:602–9.
18. Pacheco IL, Abril N, Morales-Prieto N, Bautista MJ, Zafra R, Escamilla A, et al. Th1/Th2 balance in the liver and hepatic lymph nodes of vaccinated and unvaccinated sheep during acute stages of infection with *Fasciola hepatica*. Vet Parasitol. 2017;238:61–5.
19. Kesik M, Jedlina-Panasiuk L, Kozak-Cieszczyk M, Płucienniczak A, Wedrychowicz H. Enteral vaccination of rats against *Fasciola hepatica* using recombinant cysteine proteinase (cathepsin L1). Vaccine. 2007;25:3619–28.
20. Saleh MA. Circulating oxidative stress status in desert sheep naturally infected with *Fasciola hepatica*. Vet Parasitol. 2008;154:262–9.
21. Bottari NB, Mendes RE, Lucca NJ, Schwertz CI, Henker LC, Olsson DC, et al. Oxidative stress associated with pathological lesions in the liver of rats experimentally infected by *Fasciola hepatica*. Exp Parasitol. 2015;159:24–8.
22. Mendes EA, Mendes TA, dos Santos SL, Menezes-Souza D, Bartholomeu DC, Martins IVF, et al. Expression of IL-4, IL-10 and IFN-gamma in the liver tissue of cattle that are naturally infected with *Fasciola hepatica*. Vet Parasitol. 2013;195:177–82.
23. Ruiz-Campillo MT, Hernandez VM, Escamilla A, Perez J, Martinez-Moreno A, Donnelly S, et al. Immune signatures of pathogenesis in the peritoneal compartment during early infection of sheep with *Fasciola hepatica*. Sci Rep. 2017;7:2782.
24. Van Millingen FJ, Cornelissen JBWJ, Hendriks IM, Gaasenbeek CPH, Bokhout BA. Protection to *Fasciola hepatica* in the gut mucosa of immune rats is associated with infiltrates of eosinophils, IgG1 and IgG2a antibodies around the parasites. Parasite Immunol. 1998;20:285–92.
25. Zafra R, Perez-Ecija RA, Buffoni L, Moreno P, Bautista MJ, Martinez-Moreno A, et al. Early and late peritoneal and hepatic changes in goats immunized with recombinant cathepsin L1 and infected with *Fasciola hepatica*. J Comp Pathol. 2013;148:373–84.
26. Alvarez Rojas CA, Ansell BR, Hall RS, Gasser RB, Young ND, Jex AR, et al. Transcriptional analysis identifies key genes involved in metabolism, fibrosis/tissue repair and the immune response against *Fasciola hepatica* in sheep liver. Parasit Vectors. 2015;8:124.
27. Rojas-Caraballo J, López-Abán J, Moreno-Pérez DA, Vicente B, Fernández-Soto P, del Olmo E, et al. Transcriptome profiling of gene expression during

immunisation trial against *Fasciola hepatica*: identification of genes and pathways involved in conferring immunoprotection in a murine model. BMC Infect Dis. 2017;17:94.
28. Piedrafita D, Spithill TW, Dalton JP, Brindley PJ, Sandeman MR, Wood PR, et al. Juvenile *Fasciola hepatica* are resistant to killing *in vitro* by free radicals compared with larvae of *Schistosoma mansoni*. Parasite Immunol. 2000;22: 287–95.
29. Donnelly S, O'Neill SM, Sekiya M, Mulcahy G, Dalton JP. Thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. Infect Immun. 2005;73:166–73.
30. Donnelly S, Stack CM, O'Neill SM, Sayed AA, Williams DL, Dalton JP. Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. FASEB J. 2008;22:4022–32.
31. Piedrafita D, Parsons JC, Sandeman RM, Wood PR, Estuningsih SE, Partoutomo S, et al. Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile *Fasciola hepatica in vitro* is mediated by reactive nitrogen intermediates. Parasite Immunol. 2001;23:473–82.
32. Flynn RJ, Irwin JA, Olivier M, Sekiya M, Dalton JP, Mulcahy G. Alternative activation of ruminant macrophages by *Fasciola hepatica*. Vet Immunol Immunopathol. 2007;120:31–40.
33. Garza-Cuartero L, O'Sullivan J, Blanco A, McNair J, Welsh M, Flynn RJ, et al. *Fasciola hepatica* infection reduces *Mycobacterium bovis* burden and mycobacterial uptake and suppresses the pro-inflammatory response. Parasite Immunol. 2016;38:387–402.
34. Fu Y, Chryssafidis AL, Browne JA, O'Sullivan J, McGettigan PA, Mulcahy G. Transcriptomic Study on Ovine Immune Responses to *Fasciola hepatica* Infection. PLoS Negl Trop Dis. 2016;10:e0005015.
35. Ruiz-Campillo MT, Molina-Hernández V, Pérez J, Pacheco IL, Pérez R, Escamilla A. Study of peritoneal macrophage immunophenotype in sheep experimentally infected with *Fasciola hepatica*. Vet Parasitol. 2018;257:34–9.