UNIVERSIDAD DE LA REPÚBLICA
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Estudio de las respuestas inmunes asociadas a resistencia y susceptibilidad frente a la infección por *Echinococcus granulosus*

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"Estudio de las respuestas inmunes asociadas a resistencia y susceptibilidad frente a la infección por *Echinococcus granulosus*"

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A mis viejos, Beatriz y Juan, gracias por ser y gracias por estar…

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RESUMEN

Los helmintos parásitos son un grupo de metazoarios muy diverso y complejo, cuya principal característica en común es su habilidad de generar infecciones crónicas en sus hospederos naturales. Dentro de las helmintiasis, la equinococosis quística (EQ) es una parasitosis causada por el estadío larvario de *Echinococcus granulosus*, y es considerada una zoonosis de gran relevancia por afectar tanto al ser humano como a una amplia variedad de especies ganaderas productivas. Interesantemente, la infección por *E. granulosus* es capaz de durar varios años de manera asintomática en sus hospederos intermediarios, por lo que se ha postulado que el parásito tiene la habilidad de modular la respuesta del hospedero en favor de su sobrevida. En este sentido, los fenómenos inmunológicos responsables de la resistencia o susceptibilidad del hospedero a la infección han sido escasamente estudiados. Por lo tanto, el presente trabajo se realizó a efectos de profundizar en el conocimiento de las respuestas inmunes potencialmente protectoras desencadenadas por hospederos intermediarios. Para ello, se realizaron estudios en humanos y modelos murinos focalizados en demostrar que las respuestas inmunes montadas por ciertos hospederos tendrían un papel crucial en limitar el establecimiento y/o desarrollo de la EQ.

En primer lugar, se caracterizó el perfil serológico de una cohorte de individuos residentes en una zona endémica para EQ en Uruguay; que si bien fueron serológicamente positivos, no fueron diagnosticados imagenológicamente como pacientes con EQ durante un período de seguimiento de 2 años. En forma paralela, se caracterizaron también sendas cohortes de pacientes con EQ confirmada por cirugía y de donantes sanos. Los resultados mostraron que los individuos seropositivos pero normales por ultrasonido para EQ, presentaron un perfil serológico particularmente interesante, capaz de diferenciarlos de los dos grupos control. Asimismo, las características de dicho perfil sugieren que son capaces de mediar potenciales funciones efectoras antiparasitarias. En este sentido, aunque nuestro trabajo no describe una relación causal inequívoca anticuerpos-resistencia, el perfil serológico descripto podría asociarse con un cierto nivel de resistencia potencial a la infección.

En segundo lugar, se estableció un modelo de susceptibilidad diferencial a la infección por *E. granulosus* utilizando el modelo murino de EQ secundaria, mediante el uso de las cepas de ratones Balb/c y C57Bl/6. En este sentido, se demostró que la cepa Balb/c es más permisiva al desarrollo de la infección secundaria, mientras que la C57Bl/6 tiene comparativamente un nivel de resistencia mayor. Una vez establecido dicho modelo, se analizaron las diferencias entre la respuesta inmune generada por ratones Balb/c y C57Bl/6 infectados con *E. granulosus*. Los resultados indican que los anticuerpos naturales tendrían un papel relevante sobre el grado de susceptibilidad a la infección. Además, se mostró que la polarización diferencial de la respuesta de anticuerpos inducidos en ambas cepas de ratones, se correlacionaría con diferencias en la eficiencia de las mismas frente al desafío con *E. granulosus*.
Por otro lado, teniendo en cuenta que el establecimiento de la infección en el modelo experimental es un fenómeno anatómicamente confinado a la cavidad peritoneal, se evaluó el papel de las células peritoneales durante la fase temprana de la infección. Los resultados obtenidos apoyan firmemente la sugerencia existente para cestodos parásitos, en cuanto a que la susceptibilidad del hospedero experimental frente a la infección requiere de respuestas potentes de tipo Th2. Asimismo, se mostró que la producción temprana de óxido nítrico por células peritoneales podría ser un factor contribuyente a la resistencia en el modelo de infección secundaria, y se mostró por primera vez, que los anticuerpos naturales tendrían un papel importante como mediadores innatos de respuestas de citotoxicidad celular dependiente de anticuerpos. Finalmente, se demostró que únicamente los ratones con baja susceptibilidad a la infección (C57Bl/6), generan una respuesta de anticuerpos inducidos tempranamente, capaz de potenciar mecanismos efectores con actividad protoscolicida.

En suma, estos resultados en su conjunto, confirman nuestra hipótesis de partida, es decir, que las respuestas inmunes desencadenadas por los hospederos intermediarios tienen un papel crucial en limitar el establecimiento y/o desarrollo del metacestodo de *E. granulosus*. De esta forma, la exposición a estadíos infectivos del parásito no siempre derivaría en un establecimiento exitoso de la infección.

Capítulo 1

Introducción

1.- INTRODUCCIÓN

Los helmintos parásitos son un grupo diverso y complejo de metazoarios compuesto por numerosas especies que difieren en aspectos como tamaño, ciclo de vida e impacto clínico; sin embargo, todos ellos comparten una característica importante y es su capacidad de generar infecciones crónicas en sus hospederos específicos [Wiria *et al.*, 2012]. El grupo de patologías derivadas de la infección por helmintos parásitos forma parte de las denominadas Enfermedades Tropicales Desatendidas (del inglés: *Neglected Tropical Diseases*), que en su conjunto afectan a un tercio de la población mundial [Hotez *et al.*, 2011]. Asimismo, dentro de estas enfermedades, sólo las helmintiasis afectan a 1 de cada 4 individuos en el planeta [Hotez *et al.*, 2008].

En la actualidad se reconocen más de 20 especies de helmintos parásitos capaces de afectar directamente al ser humano, la mayoría de los cuales generan infecciones inicialmente leves, asintomáticas y raramente son motivo de consulta directa en los centros de salud. Sin embargo, por ejemplo en niños, la persistencia de helmintiasis gastrointestinales suele derivar en desnutrición, retardo en el crecimiento físico y en el desarrollo intelectual, así como pueden ser responsables de deficiencias cognitivas en la edad adulta [Wiria *et al.*, 2012].

Un fenómeno interesante y común a la mayoría de las infecciones por helmintos parásitos es la ausencia "clara" de sintomatología observable. Se supone que las helmintiasis han co-existido durante millones de años con los seres humanos (y lo siguen haciendo), habiéndose alcanzado cierto nivel de armonía entre los parásitos y sus hospederos, de forma tal que el parásito se asegure su sobrevida por largos períodos de tiempo. A su vez, esta misma adaptación sería beneficiosa para el hospedero ya que restringiría el daño en tejidos y órganos propios generado durante su respuesta inmune. De hecho, en la actualidad se postula que en la mayoría de los individuos infectados por helmintos parásitos, su sistema inmune se encuentra en "equilibrio" con el invasor, mientras que solo en una proporción pequeña de casos el mismo no se alcanza (o se desgasta por otros factores), y es a raíz de ello que se generan las manifestaciones clínicas asociadas a las helmintiasis [Wiria *et al.*, 2012].

1.1. *Echinococcus granulosus*

Las equinococosis (en sus formas quística, alveolar y poliquística) son un grupo de zoonosis causadas por parásitos cestodos del género *Echinococcus*. En particular, la equinococosis quística (EQ) tiene como agente etiológico a *Echinococcus granulosus* y es considerada actualmente por la Organización Mundial de la Salud (OMS) como una enfermedad desatendida [Cucher *et al.*, 2015]. Estimaciones sobre el impacto económico de esta enfermedad han sugerido pérdidas mundiales anuales de entre 1 y 3.6 millones de DALYs (del inglés *Disability-Adjusted Life Year*) a causa de EQ en humanos [Craig *et al.*, 2007], y de 2 billones de dólares americanos en la industria a causa de EQ en especies ganaderas [Budke *et al.*, 2006]. La magnitud de dichas pérdidas se debe, en parte, a que esta zoonosis es cosmopolita y de amplia distribución mundial, siendo especialmente endémica (e incluso hiper-endémica) en gran parte del territorio oeste de China y en el cono sur de América del Sur (principalmente en el sur de Brasil, las regiones montañosas de Perú, Argentina, Chile y Uruguay) [Cucher *et al.*, 2015].

Desde una perspectiva taxonómica, *E. granulosus* está compuesto de numerosas variantes inicialmente identificadas por J.D. Smyth y Z. Davies en 1974, denominándolas "cepas fisiológicas" [Smyth y Davies, 1974]. Desde ese entonces, más cepas se identificaron en base a diversos criterios bioquímicos, fisiológicos, inmunológicos, anatómicos y de distribución geográfica [Thompson, 2008; Cucher *et al.*, 2015]. Luego, mediante el uso de técnicas de biología molecular se identificaron polimorfismos en el ADN mitocondrial de *E. granulosus* que correlacionaron con las cepas descritas, designándose desde entonces un genotipo a cada una de las mismas. En la actualidad, se han descrito 10 genotipos (G1-G10) y la "cepa león" (solo hallada en hospederos salvajes) [McManus, 2013]. Finalmente, mediante análisis filogenéticos mitocondriales recientes, la mayoría de los genotipos han sido re-clasificados como nuevas especies. Esta nueva clasificación genera el complejo *E. granulosus sensu lato (s.l.)* formado por las especies *E. granulosus sensu stricto* (*s.s.*) (genotipos G1/G2/G3), *E. equinus* (genotipo G4), *E. ortleppi* (genotipo G5), *E. canadensis* (genotipos G6/G7/G8/G9/G10) y *E. felidis* ("cepa león") [Thompson, 2008; Nakao *et al.*, 2010]. A modo general, *E. granulosus s.s.* (particularmente el genotipo G1) es el que presenta

mayor distribución mundial y es el responsable de la amplia mayoría de casos humanos de EQ. Asimismo, tanto *E. ortleppi* como todos los genotipos de *E. canadensis* han sido reportados como causantes de un número menor de casos de EQ en humanos, mientras que hasta el momento no existen reportes de casos debidos a *E. equinus* y *E. felidis* [Cucher *et al.*, 2015]. En Uruguay, la evidencia epidemiológica molecular sobre el complejo *E. granulosus s.l.* es muy escasa, y solo se ha reportado las circulación en ganado bovino de *E. granulosus s.s.* (genotipo G1) y *E. ortleppi* [Cucher *et al.*, 2015].

1.2. Ciclo de vida de *Echinococcus granulosus*

El ciclo de vida de *E. granulosus* (al igual que para todos los cestodos parásitos) requiere de dos hospederos para completar su ciclo: un hospedero definitivo donde el adulto se desarrolla en el intestino delgado y un hospedero intermediario en el cual el metacestode (o quiste hidático) normalmente se desarrolla en hígado y/o pulmón (Figura 1). El hospedero definitivo siempre es un carnívoro (perros y otros cánidos) que se infecta al ingerir protoscoleces (PSC) que se producen por multiplicación asexuada del metacestode. Dentro de un quiste hidático fértil puede haber varios miles de PSC y cada uno de ellos tiene la potencialidad de desarrollarse en un gusano adulto. Los gusanos adultos producen huevos (que contienen el embrión: la oncósfera) que son expulsados juntos con las heces del hospedero definitivo. Los huevos son capaces de sobrevivir en el ambiente por períodos variables y son infectivos después de su ingesta por parte de numerosas especies de hospederos intermediarios (ungulados domésticos y salvajes) [Thompson, 1995]. Una vez ingeridos por el hospedero intermediario, las oncósferas se activan en el intestino delgado y producen secreciones líticas que facilitan su pasaje a través de la mucosa intestinal hacia el sistema circulatorio del hospedero (venoso y linfático). Desde allí se distribuyen hacia hígado y/o pulmones principalmente, donde las oncósferas se desarrollan en quistes [Siracusano *et al.*, 2009].

El quiste hidático es unilocular y contiene un líquido claro y transparente, denominado líquido hidático, que lo mantiene distendido. En la pared se distinguen dos capas: la externa o laminar, formada por mucopolisacáridos, es acelular y de estructura laminada; mientras que la capa interna o membrana germinativa está constituida por

epitelio nucleado, prolifera y origina agregados de células que se vacuolizan y forman las denominadas vesículas prolígeras. A su vez, en el interior de estas vesículas se forman mediante gemación los PSC. Con el tiempo, las vesículas se desprenden de la membrana germinativa y flotan libremente en el interior del quiste (vesículas hijas); algunas se rompen y se liberan los PSC que se acumulan formando la denominada "arenilla hidatídica". El quiste hidático está envuelto por una capa adventicia formada por tejido conjuntivo del hospedero procedente de la reacción inflamatoria local [Siracusano *et al.*, 2009].

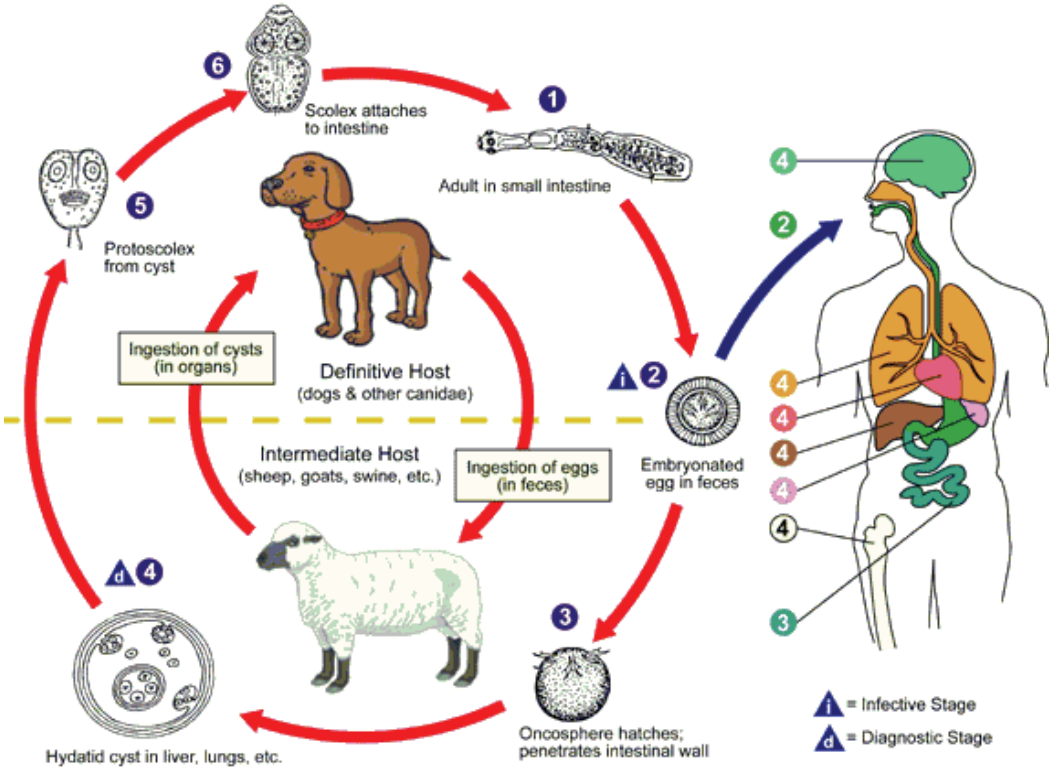

FIGURA 1. Ciclo de vida de *E. granulosus*.

(1) Gusano adulto, (2) Huevo embrionado, (3) Oncósfera, (4) Quiste hidático, (5) Protoscólex, (6) Escólex adherido a la mucosa intestinal.

[Tomado del sitio oficial del Centro para el Control y Prevención de Enfermedades del gobierno de los Estados Unidos: www.cdc.gov/dpdx/echinococcosis/].

1.3. Equinococosis quística

La EQ es una enfermedad crónica y compleja, considerándose a los humanos como hospederos intermediarios accidentales ya que no permiten que se complete el ciclo de vida del parásito al funcionar como un "callejón sin salida" biológico. El crecimiento de los metacestodos en humanos es lento (de 1 a 5 cm por año), pudiendo alcanzar en el hígado los 20 cm de diámetro. Con el tiempo se pueden formar septos internos y "quistes hijos" que perturban el patrón unilocular típico de los quistes jóvenes. En su desarrollo pueden comprimir estructuras adyacentes, fisurarse, romperse o infectarse. Cuando accidentalmente un quiste fértil se rompe, se produce una siembra interna de PSC que pueden originar nuevos quistes (infección secundaria) dada la plasticidad que presentan los PSC permitiéndoles evolucionar a quistes en el hospedero intermediario o a gusanos adultos en el hospedero definitivo [Thompson, 1995].

El diagnóstico y seguimiento de la EQ en humanos se basa en gran medida en el resultado de técnicas imagenológicas, principalmente ultrasonido (US), tomografía computarizada (TC) y resonancia magnética (RM). El US es el método de elección en la detección de quistes hidáticos hepáticos mostrando una sensibilidad del 93-98%, mientras que la TC (sensibilidad estimada en 90-97%) es útil para confirmar el diagnóstico inicial, y es capaz de detectar la presencia de "quistes hijos" y/o calcificados [Tsaroucha *et al.*, 2005; Mandal y Mandal, 2012]. Asimismo, la TC con contraste es la modalidad más recomendada para el estudio de posibles quistes hidáticos pulmonares, ya que el signo de "burbuja de aire" tiene una sensibilidad y especificidad del 85.7% y 96.6%, respectivamente, en el diagnóstico de quistes fisurados y/o infectados [Das y Das, 2011]. Por último, la RM es central en el diagnóstico de EQ cerebral [Tsaroucha *et al.*, 2005; Mandal y Mandal, 2012]. Finalmente, el uso de US ha permitido realizar una clasificación estandarizada de los quistes hidáticos según su "estado vital" (activos: CE1, CE2 y CE3a; transicionales: CE3b; inactivos: CE4 y CE5) por parte del "Grupo Informal de Trabajo sobre Equinococosis" (IWGE) de la OMS (Figura 2). Esta clasificación es útil para el médico clínico a la hora de seleccionar la mejor alternativa terapéutica para el paciente con EQ [Brunetti *et al.*, 2010; Brunetti *et al.*, 2016].

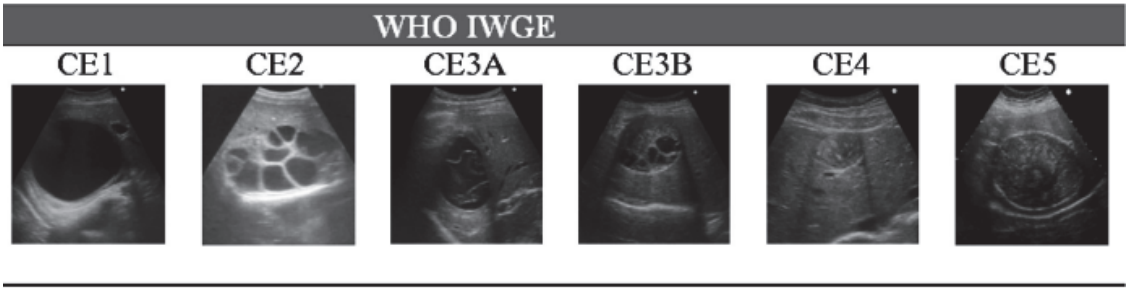

FIGURA 2. Clasificación estandarizada de quistes hidáticos según WHO-IWGE.
CE1, CE2 y CE3a corresponden a quistes activos, CE3b quistes transicionales, y CE4 y CE5 a quistes inactivos. [Tomada de Brunetti *et al.*, 2016].

Por otro lado, los métodos serológicos utilizados en el diagnósticos y seguimiento de pacientes con EQ se basan casi exclusivamente en la detección de anticuerpos IgG específicos contra antígenos (nativos o recombinantes) presentes en líquido hidático (mayoritariamente AgB). En este sentido, se han reportado varias desventajas en el uso de métodos serológicos debido a la moderada/baja sensibilidad-especificidad diagnóstica de los mismos, y al pobre valor pronóstico durante el seguimiento post-quirúrgico que presentan debido a la prolongada persistencia de los anticuerpos en circulación [Manzano-Román *et al.*, 2015]. Estas debilidades influyen negativamente en el uso de técnicas serológicas por parte de los médicos clínicos tratantes de pacientes con EQ.

El espectro clínico de la EQ va desde infecciones asintomáticas a severas, y raramente fatales. En la actualidad existen 4 aproximaciones para el manejo clínico de la enfermedad: para el caso de quistes activos las opciones son cirugía, técnicas percutáneas y/o quimioterapia, mientras que para quistes inactivos lo usual es el seguimiento continuo de su evolución. La decisión de cuál aproximación utilizar en cada paciente debería basarse en el estadío del quiste, su tamaño y localización anatómica, y las co-morbilidades potencialmente asociadas [Brunetti y Junghanss, 2009; Piccoli *et al.*, 2014; Rinaldi *et al.*, 2014].

1.4. Respuesta immune contra *E. granulosus* en hospederos intermediarios

En forma similar a lo que ocurre con otras infecciones por cestodos, la EQ desencadena en el hospedero intermediario respuestas inmunes que generalmente se asume no se asocian con inmunidad protectora. Por lo tanto, se ha sugerido ampliamente que el parásito podría ser capaz de evadir y/o modular activamente la respuesta del hospedero en favor de su sobrevida a través de mecanismos complejos poco conocidos aún. Por ello, diferenciaremos claramente la información existente referida a la respuesta inmune contra *E. granulosus* según el hospedero y tipo de infección, es decir, EQ "natural" en humanos y EQ "experimental" en ratones de laboratorio.

1.4.1. Respuesta immune contra *E. granulosus* en humanos

La respuesta inmune de humanos con EQ ha sido estudiada desde diversos ángulos a lo largo de los años; sin embargo, aún existen grandes deficiencias en el entendimiento del papel que desempeñan los distintos efectores inmunes en la defensa del hospedero.

1.4.1.1. Respuesta de anticuerpos en pacientes con EQ

La respuesta de anticuerpos en humanos con EQ ha sido ampliamente caracterizada. Sin embargo, a la misma no se le ha podido asignar aún una relevancia real en el contexto de la defensa del hospedero frente a *E. granulosus*. Durante la etapa crónica de la infección, se producen niveles elevados de anticuerpos específicos de los isotipos IgG, IgM e IgE, con predominio particular de las subclases IgG1 e IgG4 [Dessaint *et al.*, 1975; Craig, 1986; Pinon *et al.*, 1987; Wen y Craig, 1994; Sterla *et al.*, 1999; Daeki *et al.*, 2000; Li *et al.*, 2003; Khabiri *et al.*, 2006]. En este sentido, se ha demostrado que los niveles de subclases IgG específicas contra AgB serían buenos marcadores del status de la infección, ya que títulos elevados de IgG4 específica se asocian con las fases evolutivas de la EQ (quistes activos y transicionales según clasificación imagenológica), mientras que títulos específicos elevados de IgG1, IgG2 e IgG3 se asocian con las fases involutivas de la enfermedad (quistes inactivos según clasificación imagenológica)

[Daeki *et al.*, 2000]. Estos resultados sugerirían que la secuencia natural de fases evolutivas a fases involutivas en algunos individuos con EQ se relacionaría con cambios en el perfil de subclases IgG específicas contra determinados antígenos del parásito.

Además de las mencionadas particularidades serológicas de los pacientes con EQ dependientes de la historia natural del parásito en su hospedero, recientemente se ha reportado la influencia que tienen otros parámetros sobre la respuesta de anticuerpos en pacientes con EQ de localización hepática. Factores tales como la edad del paciente, el número de quistes y el tamaño de los mismos, también mostraron influencias sobre los resultados de las pruebas serológicas, tal como se había demostrado respecto al efecto de la actividad/viabilidad de los quistes [Lissandrin *et al.*, 2016].

Por otro lado, al comparar la respuesta humoral de pacientes con EQ primaria y pacientes con recidivas se observó que estos últimos presentan niveles mayores de IgE e IgG4 específicas contra líquido hidático [Riganò *et al.*, 1995a; Riganò *et al.*, 1995b; Riganò *et al.*, 1996; Hernández-Pomi *et al.*, 1997]. Así, la información disponible sobre la respuesta humoral en pacientes con EQ sugiere que la calidad de las mismas (en términos de isotipos y subclases de anticuerpos inducidas) podría condicionar el status de la infección.

1.4.1.2. Respuestas de citoquinas en pacientes con EQ

Los helmintos parásitos se caracterizan por inducir el desarrollo de potentes respuestas inmunes de tipo Th2 en sus hospederos [Díaz y Allen, 2007; Jenkins y Allen, 2010]. Sin embargo, a diferencia de la mayoría de los helmintos, la inmunidad contra cestodos parecería depender de efectores inmunes típicamente asociados a respuestas de tipo Th1, y *E. granulosus* no sería la excepción en su grupo [Hernández-Pomi *et al.*, 1997; Riganò *et al.*, 1999a; Riganò *et al.*, 1999b; Amri *et al.*, 2007; Amri *et al.*, 2009].

Los primeros estudios sobre la producción de citoquinas en pacientes con EQ comenzaron a mediados de 1990, e inicialmente compararon la respuesta de citoquinas de células mononucleares de sangre periférica (PBMC) de pacientes con

infección primaria y pacientes con recidivas, estimuladas *in vitro* con líquido hidático. En dichos estudios se reportó que los individuos con recidivas producen niveles significativamente mayores de IL-5, IL-4 e IL-10, con valores indetectables o muy bajos de IFN-γ respecto al grupo de pacientes con EQ primaria [Riganò *et al.*, 1995a; Riganò *et al.*, 1995b; Riganò *et al.*, 1996]. En consonancia con estos resultados, se reportó luego la presencia de un número mayor de eosinófilos circulantes en pacientes con recidivas respecto a los individuos con EQ primaria [Hernández-Pomi *et al.*, 1997].

Resultados recientes han confirmado la existencia de un perfil de citoquinas de tipo Th2 en pacientes con EQ, reforzando la hipótesis que dicho perfil no sería protector para el hospedero intermediario [Shan *et al.*, 2011]. A modo de ejemplo, en un reporte reciente donde se realizó un seguimiento por un período de 2 años a 50 pacientes con EQ operados y con quimioterapia antihelmíntica post-quirúrgica, se observó que solo en aquellos donde el tratamiento resultó exitoso los valores de concentración sérica de IL-4 e IL-10 descendieron significativamente. Además, aquellos pacientes con tratamiento no exitoso mostraron valores indetectables de IFN-γ en suero [Naik *et al.*, 2016].

Se ha reportado además, la existencia de una correlación entre los niveles de nitritos e IFN-γ producidos por PBMC de pacientes estimuladas *in vitro* con Ag5, sugiriéndose que la producción de óxido nítrico (NO) por parte del hospedero podría influir en el estatus de los quistes hidáticos [Ait-Aissa *et al.*, 2006]. En este sentido, se ha reportado además que las concentraciones séricas de nitritos en pacientes con recidivas es significativamente menor a la de individuos con EQ primaria, apoyando el posible papel anti-parasitario de la producción de NO [Zeghir-Bouteldja *et al.*, 2013].

Por otro lado, se ha sugerido también que las respuestas Th17 podrían actuar en forma sinérgica con las de tipo Th1 en la EQ humana [Mezioug y Touil-Boukoffa, 2012].

Estos y otros resultados [Touil-Boukoffa *et al.*, 1997; Amri *et al.*, 2007] sugerirían que la producción de NO inducida por IFN-γ podría constituir un mecanismo de defensa contra *E. granulosus* en humanos infectados, y por lo tanto, el perfil de la respuesta de citoquinas inducida en pacientes con EQ podría condicionar el pronóstico de la infección.

1.4.2. Respuesta immune contra *E. granulosus* en el modelo murino

Los ratones de laboratorio han sido extensamente utilizados como modelo biológico para el estudio de diversos aspectos relacionados a la inmunobiología de la infección por *E. granulosus*. Así, múltiples cepas de ratones y distintos estadíos parasitarios (además de diversas vías de inoculación) han sido utilizados para realizar dichas infecciones experimentales [Pennoit-De Cooman *et al.*, 1974; Hernández y Nieto, 1994; Baz *et al.*, 1995; Urrea-Paris *et al.*, 2001; Casado *et al.*, 2001; Siles-Lucas y Hemphill, 2002; Baz *et al.*, 2006; Cucher *et al.*, 2013]. En este sentido, el modelo murino de EQ secundaria ha sido ampliamente utilizado no solo para el estudio de aspectos básicos de la infección por *E. granulosus* [Baz *et al.*, 2006; Dematteis *et al.*, 1999; Dematteis *et al.*, 2003; Mourglia-Ettlin *et al.*, 2011; Cucher *et al.*, 2013], sino también para el ensayo preclínico de nuevos agentes quimioterapéuticos [Ceballos *et al.* 2010; Breijo *et al.*, 2011; Cumino *et al.*, 2012], estudios de potenciales candidatos vacunales [Hernández y Nieto, 1994; Hashemitabar *et al.*, 2005; Burgu *et al.*, 2007]; y de herramientas diagnósticas y/o de seguimiento [Denegri *et al.*, 1995; Ferragut *et al.*, 1998; Mamuti *et al.*, 2002]. En gran medida, el modelo de EQ secundaria ha ganado terreno sobre otros modos de infección (principalmente la primaria) ya que permite utilizar estadíos parasitarios no infectivos para el ser humanos y por ende, permite trabajar bajo condiciones de bioseguridad menos estrictas.

El modelo de EQ secundaria consiste en la inoculación de ratones por vía intraperitoneal (i.p.) con PSC viables y se basa en la capacidad que estos tienen para diferenciarse a quistes en hospederos intermediarios inmunocompetentes [Heath, 1970]. La cepa de ratones Balb/c ha sido las más utilizada como modelo y es a partir de la cual se ha obtenido la mayor parte de la información disponible.
La EQ secundaria experimental puede dividirse en dos etapas: una etapa temprana (hasta el día 20-30 p.i. aproximadamente) durante la cual se establece la infección (los PSC se diferencian a quistes) [Richards *et al.*, 1983], seguida de una etapa tardía o crónica en la cual los quistes ya establecidos crecen en tamaño y eventualmente se vuelven fértiles.

1.4.2.1. Respuesta de anticuerpos en ratones infectados con *E. granulosus*

Los aspectos inmunológicos de la respuesta del hospedero en el modelo murino de EQ secundaria han sido analizados en varios de sus aspectos. Al igual que en los estudios en pacientes con EQ, la relevancia que tienen las respuestas de anticuerpos contra el parásito no ha sido claramente establecida aún. Los estudios iniciales sobre la respuesta humoral en el modelo de infección secundaria mostraron que en la etapa crónica de la infección existen variaciones cuantitativas y cualitativas en la producción de anticuerpos en forma dependiente del estadío específico del parásito [Araj *et al.*, 1977; Liu *et al.*, 1992; Haralabidis *et al.*, 1995]. Asimismo, se ha mostrado que la respuesta de anticuerpos contra líquido hidático analizada durante la fase crónica de la infección, no se induce maduración de la avidez y que además dichos anticuerpos están dirigidos principalmente contra epítopes glucídicos (sensibles al tratamiento con meta-periodato de sodio) [Ferragut y Nieto, 1996]. Además, se observó que la relación entre los títulos de IgG1 e IgG3 específicas (IgG1/IgG3) contra antígenos somáticos de PSC (PSA), así como la avidez de los anticuerpos IgG3 específicos, decrece a lo largo del tiempo al igual que el reconocimiento de epítopes peptídicos [Severi *et al.*, 1997].

Por su parte, la respuesta de anticuerpos durante la etapa temprana de la infección experimental ha sido menos estudiada. Hasta el momento se ha reportado que ratones Balb/c con una semana de infección producen anticuerpos IgM e IgG específicos contra PSA a nivel sistémico [Dematteis *et al.*, 1999] y que dichas respuestas inducidas tempranamente serían en gran medida del tipo T-independientes dirigidas principalmente contra epítopes glucídicos [Baz *et al.*, 1999; Baz *et al.*, 2008]. Más recientemente, nuestro grupo reportó que en ratones Balb/c con 5 días de infección se producen niveles significativos de anticuerpos IgM e IgG2b específicos contra PSA secretados por plasmoblastos peritoneales [Mourglia-Ettlin *et al.*, 2011]. Esta respuesta específica inducida tempranamente podría ser relevante en el establecimiento de la infección, ya que (al menos *in vitro*) los PSC son altamente sensibles a la activación del sistema del complemento por vía clásica [Ferreira *et al.*, 1992].

1.4.2.2. Respuestas de citoquinas en ratones infectados con *E. granulosus*

En forma análoga a lo que sucede en humanos, los helmintos parásitos se caracterizan por potenciar el desarrollo de potentes respuestas inmunes de tipo Th2 también en modelos murinos [Díaz y Allen, 2007; Jenkins y Allen, 2010]. Sin embargo, análogamente a lo observado en humanos, la inmunidad contra *E. granulosus* en ratones infectados parecería depender de efectores inmunes típicamente asociados a respuestas de tipo Th1 [Rogan, 1998; Dematteis *et al.*, 1999; Dematteis *et al.*, 2003; Al-Qaoud y Abdel-Hafez, 2008; Mourglia-Ettlin *et al.*, 2011].

En este sentido, Rogan (1998) analizó el perfil de citoquinas producido por células de bazo de ratones Balb/c con infección crónica estimuladas *in vitro* con ConA, y observó que el mismo se caracteriza por una producción predominante de IL-10 e IL-4, con niveles reducidos de IFN-γ. Asimismo, las células periparasíticas en dichos animales producían predominantemente IL-10 al ser estimuladas *in vitro* [Rogan, 1998]. Por otro lado, el análisis del perfil de citoquinas en ratones implantados quirúrgicamente con quistes hidáticos viables de origen murino, mostró que 4 meses post-implantación las células de bazo y las células periparasíticas de aquellos animales con quistes inviables producían niveles significativamente elevados de IFN-γ, mientras que en los ratones con quistes aún viables la producción de IFN-γ era muy reducida [Rogan, 1998]. Estos resultados sugirieron que las respuestas de citoquinas de tipo Th2 serían inducidas por el parásito de forma tal de inhibir la producción de citoquinas de tipo Th1 potencialmente perjudiciales para el mismo.

Por otro lado, la respuesta de citoquinas en células de bazo de ratones con infección temprana estimuladas *in vitro* con PSA también se caracteriza por la producción predominante de citoquinas de tipo Th2 (IL-4, IL-5 e IL-10) sin detectarse niveles apreciables de IFN-γ [Dematteis *et al.*, 1999]. Asimismo, nuestro grupo ha reportado recientemente que en ratones Balb/c infectados existe una cinética bifásica en la expresión temprana de citoquinas a nivel local, es decir, en células peritoneales. Dicha cinética se caracteriza por una inducción inicial de citoquinas de tipo Th1 seguida de un cambio hacia un perfil de tipo Th2 aproximadamente al día 5 post-infección. Interesantemente, la expresión de IL-10 mostró un aumento igualmente

rápido al de las citoquinas Th1 pero sostenido a lo largo del tiempo [Mourglia-Ettlin *et al.*, 2011]. Claramente, esta respuesta no sería completamente eficiente contra *E. granulosus* ya que la infección igualmente logra establecerse.

En este sentido, evidencias indirectas sugieren que mecanismos asociados a los efectos del IFN-γ podrían ser relevantes en la eliminación temprana de un número importante de PSC, ya que células peritoneales adherentes de ratones Balb/c normales activadas con IFN-γ o IFN-γ/LPS muestran actividad protoscolicida *in vitro* [Dematteis *et al.*, 2003]. Estos y otros resultados [Al-Qaoud y Abdel-Hafez, 2008], apoyarían la hipótesis que el IFN-γ (citoquina clave en las respuestas de tipo Th1) tendría un papel central en la eliminación del parásito, y por lo tanto, el desarrollo temprano de una respuesta de citoquinas de tipo Th2 podría interpretarse como un mecanismo modulado y/o inducido activamente por el parásito en beneficio de su sobrevida.

1.5. Fenómenos de susceptibilidad/resistencia frente a la infección por *E. granulosus* en hospederos intermediarios

Los fenómenos de resistencia/susceptibilidad frente a la infección por *E. granulosus* en hospederos intermediarios (naturales y experimentales) han sido escasamente estudiados hasta el momento. En este sentido, se ha postulado que el perfil de la respuesta humoral y de citoquinas del hospedero determinaría en gran medida el éxito del establecimiento de la infección (susceptibilidad del hospedero) o la eliminación del parásito (resistencia del hospedero) [Yang *et al.*, 2012].

1.5.1. Evidencia y sugerencias provenientes de estudios en humanos

En humanos, existen muy pocos reportes donde básicamente se analicen asociaciones potenciales entre la probabilidad de ser más susceptible a la infección por *E. granulosus* como una función de la presencia en el individuo de determinados polimorfismos en genes relevantes para el sistema inmune [Azab *et al.*, 2004a; Al-Ghoury *et al.*, 2010; Kiper *et al.*, 2010; Yalcin *et al.*, 2010]. Por ejemplo, se ha reportado que en la población egipcia aquellos portadores de los alelos HLA-DR3 y –DR11 presentan mayor riesgo de padecer la enfermedad [Azab *et al.*, 2004a]. Por otro

lado, estudios realizados sobre la población yemení mostraron que los portadores del alelo HLA-DR16 serían más susceptibles al desarrollo de EQ, mientras que aquellos portadores de los alelos HLA-DR1, -DR8 y –DR52 mostrarían ciertos niveles de resistencia a la infección [Al-Ghoury *et al.*, 2010]. Es interesante destacar que el alelo HLA-DR1 también ha sido asociado con resistencia (junto al alelo HLA-B18) en niños turcos, mientras que en esta población el alelo HLA-DR15 se asociaría con susceptibilidad frente a la EQ [Yalcin *et al.*, 2010]. Por otro lado, Kiper *et al.* (2010) analizaron los polimorfismos existentes en los genes TAP1 y TAP2 en una cohorte de niños turcos con EQ, y reportaron que la heterocigosis u homocigosis en ciertos polimorfismos se asocian con distintos niveles de susceptibilidad a la infección.

Los resultados descriptos permitirían postular la existencia de cierto grado de predisposición inmunogenética responsable (al menos parcialmente) de la resistencia/susceptibilidad frente a la infección por *E. granulosus* en humanos. Esta predisposición influiría sobre el desarrollo de la respuesta inmune del hospedero contra el parásito condicionando la capacidad de este último de establecer una infección crónica [Yang *et al.*, 2012]. En este sentido, se ha reportado que aquellos pacientes con títulos muy altos de anticuerpos IgG específicos contra líquido hidático presentan una frecuencia significativamente elevada en cuanto al porte de los alelos HLA-DR3 y -DR11 [Azab *et al.*, 2004b], los cuales previamente se fueron reportados como alelos asociados a susceptibilidad [Azab *et al.*, 2004a]. Así, las respuestas de anticuerpos influenciadas por la carga inmunogenética del hospedero podrían estar relacionadas con fenómenos de resistencia/susceptibilidad frente a la infección por *E. granulosus* en humanos.

En este sentido, es interesante mencionar que a través de trabajos de campo realizados en diversas regiones endémicas para EQ a nivel mundial, se ha observado que el número de individuos seropositivos para antígenos del parásito siempre es superior al número de casos de EQ confirmados mediantes técnicas imagenológicas [Bonifacino *et al.*, 1991; Nahmias *et al.*, 1991; Zhang *et al.*, 1994; Cohen *et al.*, 1998; Shambesh *et al.*, 1999; Hernández *et al.*, 2005; Gavidia *et al.*, 2008]. Por ejemplo, en la región central de Perú se ha reportado que un 20% de la población resultó ser seropositiva contra antígenos de *E. granulosus*, mientras que solo un 3% fue

diagnosticada para EQ mediante técnicas imagenológicas [Gavidia *et al.*, 2008]. Resultados similares se han reportado en Yirka, un poblado ubicado en la región norte de Israel, donde si bien el 9% de los individuos examinados mostró resultados serológicos positivos para *E. granulosus*, solo el 1.6% fue finalmente diagnosticado para EQ [Nahmias *et al.*, 1991]. Estos resultados podrían sugerir que solo una proporción "pequeña" de desafíos humanos con oncósferas de *E. granulosus* resultarían en el establecimiento exitoso del parásito con el consiguiente desarrollo de EQ.

En Uruguay también se han reportado resultados similares [Bonifacino *et al.*, 1991; Cohen *et al.*, 1998]. Por ejemplo, en la región de Caraguatá (Tacuarembó) (Figura 3) se ha reportado que el 8.8% de los habitantes mostraron ser seropositivos para antígenos de *E. granulosus*, mientras que solo el 0.8% fue finalmente diagnosticado para EQ mediante estudios imagenológicos [Hernández *et al.*, 2005]. En este sentido, se ha sugerido que los anticuerpos específicos contra el parásito en los individuos seropositivos pero normales según diagnóstico imagenológico que residen en zonas endémicas para *E. granulosus*, podrían tener cierta función protectora frente al desarrollo de la EQ [Rogan *et al.*, 1992]. Sin embargo, las características de dichos anticuerpos, así como la frecuencia en la distribución de alelos del complejo HLA en dichos individuos, no han sido estudiadas hasta el momento.

FIGURA 3. Mapa político de Uruguay.
Se destacan la ubicación del departamento de Tacuarembó (en naranja) y la zona aproximada de Caraguatá (círculo negro), así como la localización del departamento de Montevideo (en verde).

1.5.2. Evidencias y sugerencias provenientes de estudios en modelos murinos

Los fenómenos de resistencia/susceptibilidad frente a la infección por *E. granulosus* en ratones no han sido estudiados en forma sistemática. Sin embargo, existe cierta evidencia indirecta que apoyaría la relevancia que las respuestas de anticuerpos y de citoquinas del hospedero experimental tendrían en dichos fenómenos. En este sentido, estudios realizados por nuestro grupo mostraron diferencias en la respuesta temprana de citoquinas en ratones Balb/c inoculados con una dosis menor de parásitos (500 PSC/ratón) respecto a la comúnmente utilizada en el modelo de EQ secundaria (2.000 PSC/ratón). La diferencia más notable entre ambos grupos fue la producción de IFN-γ por células de bazo estimuladas *in vitro* con PSA, ya que únicamente aquellos ratones infectados con la dosis menor de PSC produjeron niveles significativos de esta citoquina, lo cual se tradujo en un menor tamaño de los quistes hidáticos desarrollados [Dematteis *et al.*, 2003].

Por otra parte, tanto la respuesta de anticuerpos como la de citoquinas inducidas en un hospedero desafiado con un patógeno, dependen en gran medida de las características genéticas del hospedero. Dicha dependencia se ha asociado con fenómenos de susceptibilidad/resistencia frente a infecciones parasitarias. Un ejemplo clásico es la infección experimental por *Leishmania spp.*, en la cual los ratones de la cepa Balb/c son susceptibles a la infección, mientras que los ratones de la cepa C57Bl/6 son resistentes. Esta diferencia en susceptibilidad se asocia claramente con la respuesta de citoquinas inducida en cada cepa de ratones: los ratones de la cepa susceptible montan respuestas de citoquinas de tipo Th2 contra el parásito, mientras que los individuos de la cepa resistente desarrollan respuestas de citoquinas de tipo Th1 [Bogdan *et al.*, 1996; Etges y Muller, 1998; Launois *et al.*, 1998].

En el caso de susceptibilidad diferencial entre cepas de ratones frente a la infección por *E. granulosus* existen solo 2 reportes en la literatura donde se analiza este tópico, aunque con importantes diferencias en cuanto al estadío parasitario utilizado (y al origen del mismo), la vía de inoculación, las cepas de ratones en estudio y el punto temporal final de análisis parasitológico de la infección [Pennoit-De Cooman y De Rycke, 1970; Dempster *et al.*, 1991]. Por un lado, Pennoit-De Cooman y De Rycke

(1970) analizaron el resultado de infecciones con *E. granulosus* en las cepas de ratones albino AZ, albino Swiss y NMRI, mediante la inoculación por vía subcutánea de una alta dosis de PSC (10.000 PSC/ratón) obtenidos de quistes hidáticos de origen equino. En dicho estudio no hallaron diferencias significativas entre cepas de ratones en cuanto al número de quistes subcutáneos recuperados. Por otro lado, Dempster *et al*. (1991) han reportado que al administrar por vía oral oncósferas activadas a ratones endogámicos de distintas cepas (DBA, CBA, Balb/c y C57Bl/6) y exogámicos (CF-1), los ratones de la cepa Balb/c mostraron la mayor susceptibilidad a la infección, mientras que aquellos pertenecientes a la cepa C57Bl/6 resultaron ser los únicos totalmente refractarios. Interesantemente, la administración i.p. de oncósferas activadas tuvo un efecto diferente: mientras que los ratones de la cepa CBA fueron los más susceptibles a la infección, las cepas C57Bl/6 y Balb/c resultaron ser más resistentes aunque no totalmente refractarias [Dempster *et al*., 1991]. Si bien estos resultados apoyarían la existencia de fenómenos de resistencia/susceptibilidad frente a la infección por *E. granulosus* según la cepa de ratones utilizada, no existe hasta el momento ningún reporte sobre estos fenómenos en el modelo "clásico" de EQ secundaria.

2. HIPÓTESIS DE TRABAJO

La hipótesis central del presente trabajo se basa en que las respuestas inmunes desencadenadas por ciertos hospederos intermediarios (tanto humanos como ratones de laboratorio) tendrían un papel crucial en limitar el establecimiento y/o desarrollo del metacestodo de *E. granulosus*. De esta forma, la exposición (natural o experimental) a estadíos infectivos del parásito no siempre derivaría en un establecimiento exitoso de la infección.

3. OBJETIVOS

3.1. Objetivo general

Aportar al conocimiento básico de las respuestas inmunes potencialmente protectoras desencadenadas por hospederos intermediarios (humanos y ratones de laboratorio) frente a la infección por *E. granulosus*.

3.2. Objetivos específicos

1. Caracterizar serológicamente una cohorte de individuos residentes en Caraguatá (zona endémica para *E. granulosus*) que son seropositivos para el parásito pero no muestran resultados imagenológicos compatibles con EQ.

2. Establecer un modelo de susceptibilidad diferencial a la EQ secundaria utilizando las cepas de ratones Balb/c y C57Bl/6.

3. Analizar la relevancia de las respuestas de anticuerpos (naturales e inducidos) en la susceptibilidad a la infección en el modelo murino de EQ secundaria.

4. Evaluar el papel de la respuesta temprana de células peritoneales en la susceptibilidad a la infección en el modelo murino de EQ secundaria.

Capítulo 2

Antibody profiling in ultrasound normal individuals with positive serology for cystic echinococcosis

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Antibody profiling in ultrasound normal individuals with positive serology for cystic echinococcosis

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SUMMARY

Cystic echinococcosis is a zoonotic disease caused by the cestode parasite Echinococcus granulosus*. In endemic regions, seropositive individuals to* E. granulosus *usually and markedly outnumber image-confirmed cases of cystic echinococcosis, suggesting that some parasite challenges derive in unsuccessful infection establishments. However, it is still unknown whether such parasite-specific antibodies in healthy individuals might play a role in resistance/susceptibility to the infection. Therefore, we have here analysed the profile of antibodies recognizing* E. granulosus *antigens in seropositive but ultrasound normal individuals, as well as in surgery-confirmed patients and healthy donors. Our results showed that ultrasound normal individuals exhibited low avidity IgG antibodies, as well as low levels of parasite-specific IgG1 and IgG4 antibodies. In addition, they displayed significant levels of specific IgE, and thus, they revealed a uniquely high IgE: IgG4 ratio. Moreover, high levels of parasite-specific IgM were detected in such individuals, which showed characteristics of natural cross-reacting antibodies. Therefore, our results indicate that ultrasound normal individuals but seropositive for* E. granulosus *antigens exhibit a distinctive antibody profile. In this regard, possible associations between their antiparasite antibodies and potential resistance mechanisms to cystic echinococcosis are discussed.*

Keywords Echinococcus granulosus*, IgE, IgG4, natural IgM, resistance*

INTRODUCTION

Cystic echinococcosis (CE) is a parasite zoonotic disease with cosmopolitan distribution caused by the larval stage of the cestode *Echinococcus granulosus*, showing a worldwide prevalence of roughly six million infected people (1,2). Primary CE occurs in intermediate hosts (domestic and wild ungulates, being humans an accidental host) and derives from the ingestion of oncosphere-containing eggs, which later develop into metacestodes (hydatid cysts) mainly in the liver and lungs of the infected host. Secondary CE comes about after spillage of protoscoleces from a fertile cyst within an already infected intermediate host. This kind of infection results from protoscoleces developmental plasticity, which allows them to generate new cysts within intermediate hosts or adult worms if ingested by a definitive host. Dogs are the most common definitive host for *E. granulosus* and adult worms reside in their intestines, where they produce eggs containing oncospheres that are spread into the environment through dog faeces (2).

In helminth infections, antibodies are supposed to help in parasite clearance, to limit disease, and possibly to prevent parasite entry into the vasculature and adherence to mucosal surfaces (3). To those ends, the class and subclass of the antibody response is essential because each isotype/subclass has specific biological functions. Thus, it may be crucially important for a parasite-challenged host to mount the most appropriate antibody response, which is the one with the best chance of clearing the infection and/or controlling the disease. Although there is no direct evidence on the influence of intrinsic host factors on differential production of antibody isotypes, genetic background has been highlighted in susceptibility to several parasites (3). In human CE, the number, size, location and condition of the cysts influence parasite-specific IgG production, and only 60–80% of confirmed CE cases have been reported to be seropositive (4). However, parasite-specific IgG subclasses in patients with seropositive CE have been shown to differ when grouped according to infection stage (5,6), primary vs. relapsed disease (7) or chemotherapy responsiveness (8). In this regard, through analysis of the association between HLA polymorphisms and humoral response in patients with CE, it has been reported that

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patients with high antihydatid fluid IgG responses showed a significant increased frequency of HLA-DR3 and HLA-DR11 alleles (9), which in fact were previously reported to be susceptibility associated (10). Therefore, antibody responses could be related to infection susceptibility in human CE.

Field surveys in several endemic regions for *E. granulosus* around the world have shown that seropositive individuals usually and markedly outnumber image-confirmed CE cases (11–17), which might suggest that human challenge with *E. granulosus* not always derives in a successful infection establishment. For example, in central highlands of Peru, it has been reported that although roughly 20% of the population showed to be seropositive for *E. granulosus* antigens, only 3% were image-confirmed CE cases (17). Similar results were reported in the northern Israeli town of Yirka, where 9% of examined individuals showed to be seropositive while only 1·6% were diagnosed as patients with CE (12). Protective roles mediated by parasite-specific antibodies in healthy individuals residing in *E. granulosus* endemic areas have been suggested (18). In this regard, it was postulated that in highly endemic regions, there may be abundant low viability eggs able to induce immune responses without developing into cysts. Additionally, they suggested that the presence of eggs from other taeniid species might produce partial cross-protection to *E. granulosus* (18). Whatever the reason, parasite-specific antibodies in healthy individuals from endemic regions might be responsible for a certain degree of resistance to CE, although the individual nature of this response has not been determined yet.

In Uruguay, results on differences between prevalence of seropositive individuals and image-confirmed CE cases have also been previously reported (11,14). In fact, our group found that 8·8% of inhabitants from a highly endemic region for *E. granulosus* in Uruguay (Caraguatá) were seropositive, while only 0·8% were image diagnosed as patients with CE (16). The present work – which is a continuation of that report – aimed at analysing whether antibody profiles in ultrasound normal individuals with positive serology to *E. granulosus* antigens (termed UN individuals) could be associated with potential resistance mechanisms to CE. To that end, we analysed and compared the existing differences in specific antibodies between UN individuals, patients with CE and healthy donors.

MATERIALS AND METHODS

Serum samples

Samples belonged to the serum bank stored at −80°C reported in (16), according to Ethics Statements described therein. Sera were clustered into three groups: (i) UN individuals, (ii) patients with CE and (iii) healthy donors. For a detailed description of the populations (Table 1). UN individuals residing in an endemic region (Caraguatá) were defined as seropositive to *E. granulosus* antigens but negative for CE through image studies (sonography and X-rays) during a period of 2 years (16). Healthy donors' group was composed of residents from Caraguatá and Montevideo (nonendemic region) showing no significant differences between them in any studied parameter (data not shown).

Echinococcus granulosus crude antigens

Protoscoleces from *E. granulosus* were obtained by aseptic puncture of fertile bovine hydatid cysts and were washed several times with phosphate-buffered saline (PBS) pH 7·2 containing gentamicin (40 μg/mL). Protoscoleces somatic antigens (PSA) were obtained by ultrasound disruption according to (19), and protein content was assessed using BCA Protein Assay Reagent (Pierce) following manufacturer instructions. PSA was stored at −20°C until use.

Determination of total antibodies concentrations

Total IgG, IgM and IgA concentrations were determined by radial immunodiffusion using isotype-specific plates (Diffu-Plate, Biocientífica SA, Buenos Aires, Argentina). In each well, 5 μL of serum was added and then plates were

Table 1 Description of study groups. Serum samples were clustered into three groups: UN individuals, patients with CE and healthy donors

	UN individuals	CE patients	Healthy donors
n (F : M)	14 (12 : 2)	21 (11 : 10)	26 (13 : 13)
Median age (range)	30 (9–60)	43 (12–78)	31 (11–71)
E. granulosus serology	Positive	Positive	Negaive/Healthy[a]
Hydatid cyst	Non-suspected by sonography	Surgically confirmed	Non-suspected by sonograpy/Healthy[a]

[a]Within this group, 12 individuals resided in Caraguatá (seronegative without suspected hydatid cysts by sonography) (16), while the rest were healthy donors living in a nonendemic region in Uruguay (Montevideo) with no personal history of CE.

incubated at room temperature during 48/72 h following manufacturer instructions. Results were read manually.

Titration of parasite-specific IgG, IgM and IgA antibodies

PSA-specific IgG, IgM and IgA antibodies were determined by ELISA according to (20). Appropriate dilutions of each individual sample (in duplicate) were analysed, and the resulting mean value was reported. Specific IgM, IgG and IgA were determined using appropriate goat anti-human isotype antibodies labelled with peroxidase (Sigma or DAKO Sigma (St. Louis, MO, USA) or DAKO (Glostrup, Denmark)). Peroxidase activity was detected using *O*-phenylendiamine as chromophore (Sigma), and absorbance values were recorded at 492 nm. A pool of sera from CE patients with median-high titres of anti-PSA antibodies was used as a standard in every assay. Absorbance values for each sample were converted into antibody arbitrary units per mL (AU/mL) referred to this standard (21).

Titration of parasite-specific IgG and IgA subclasses

PSA-specific IgG1, IgG2, IgG3 and IgG4 antibodies were determined as described above using appropriate mouse anti-human IgG subclasses monoclonal antibodies labelled with biotin (Sigma) and streptavidin peroxidase (Sigma). Anti-PSA IgA1 and IgA2 antibodies were determined as described above using appropriate rabbit anti-human IgA subclasses antibodies (Nordick) followed by goat anti-rabbit IgG antibodies labelled with peroxidase (Thermo Scientific (Loughborough, UK)). In all cases, three appropriate dilutions of samples were analysed and the mean value was reported. A pool of sera from CE patients with median-high titre of anti-PSA antibodies was used as a standard in every assay. Absorbance values for each sample were converted into antibody arbitrary units per mL (AU/mL) referred to this standard (21).

Avidity index determination

Avidity index of anti-PSA IgG, IgM and IgA antibodies was determined by chaotropic elution (22), using potassium thiocyanate (KSCN) as a chaotropic agent. Briefly, appropriate dilutions of samples were dispensed in four wells of PSA-coated and blocked ELISA plates and incubated as usually. Then, 100 μL/well of PBS or 1·5 M KSCN in PBS was added in duplicates, and the plates were incubated at room temperature for 30 min. After washing the chaotropic solution, ELISA protocol was followed as described above. Mean value of absorbance in the absence of KSCN was assumed to represent 100% specific antibody binding. Avidity index was defined as the remaining absorbance percentage (mean value) after incubation with 1·5 M KSCN.

Titration of parasite-specific IgE antibodies

To determine anti-PSA IgE titres by ELISA, IgG antibodies were first depleted by incubating each serum sample with Protein G Agarose Fast Flow (Sigma) and following manufacturer instructions. Samples were analysed in duplicate, and the resulting mean value was reported. Anti-PSA IgE antibodies were determined as described above using appropriate rabbit anti-human IgE antibodies (DAKO) followed by goat anti-rabbit IgG antibodies labelled with peroxidase (Thermo Scientific). A pool of sera (IgG depleted) from CE patients with median-high titre of anti-PSA antibodies was used as a standard in every assay. Absorbance values for each sample were converted into antibody arbitrary units per mL (AU/mL) referred to this standard (21).

Statistics

Statistical differences between groups were assessed by nonparametric Mann–Whitney *U*-test and correlation analyses by Spearman's rank correlation test. In all cases, differences or correlations were regarded as significant with P-value $<0{\cdot}05$.

RESULTS

UN individuals and patients with CE differed in their antiparasite IgG profiles

Firstly, we analysed serum levels of parasite-specific IgG antibodies in the three groups. As expected, patients with CE showed significantly higher levels than healthy donors (Figure 1a), but UN individuals showed no differences with either groups (Figure 1a). Additionally, total IgG concentration was similar in every group (data not shown). Nevertheless, the avidity of those specific IgG antibodies showed similar values in UN individuals and in healthy donors, being in both cases significantly lower than in patients with CE (roughly threefold in terms of group median values) (Figure 1b).

On the other hand, results obtained from the analyses on parasite-specific IgG subclasses showed interesting differences (Figure 1c–f). In fact, UN individuals showed significantly lower levels of anti-PSA IgG1 and IgG4 antibodies than patients with CE did (approximately twofold and 300-fold in terms of group median values, respectively) (Figure 1c,f). No remarkable differences between

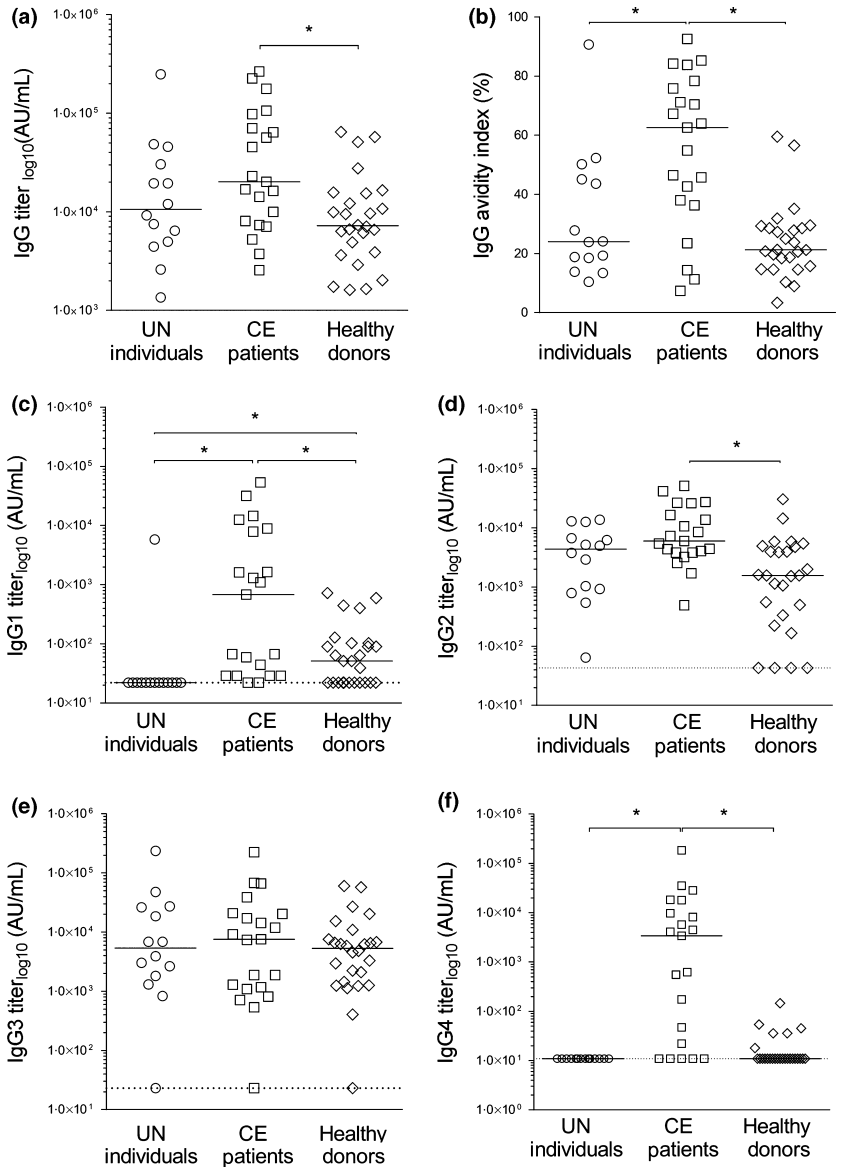


Figure 1 UN individuals and patients with CE differ in their antiparasite IgG profiles. Titration of anti-PSA IgG (a), IgG1 (c), IgG2 (d), IgG3 (e) and IgG4 (f) antibodies was performed by ELISA. Avidity index of anti-PSA IgG (b) was determined by ELISA with chaotropic elution. Values for each individual (symbols) and group median (horizontal lines) are shown. For IgG subclasses (c–f), detection threshold is shown as a dashed line. (*) Statistical significance ($P < 0{\cdot}05$).

groups were observed for parasite-specific IgG2 and IgG3 antibodies (Figure 1d,e).

Overall, our results showed important differences between UN individuals and patients with CE regarding specific IgG antibodies. Unlike patients with CE, UN individuals exhibited low avidity IgG antibodies, as well as low levels of PSA-specific IgG1 and IgG4 antibodies.

Antiparasite IgE levels were different in UN individuals, patients with CE and Healthy donors

Parasite-specific IgE production is a feature of helminth infections, and therefore, levels of anti-PSA IgE antibodies were analysed in the three groups. Results in Figure 2(a) show that patients with CE displayed higher levels of anti-PSA IgE than healthy donors did (80-fold in terms of group median values). Interestingly, UN individuals showed remarkable differences with CE patients and with healthy donors, being their anti-PSA IgE levels significantly lower and higher, respectively (11-fold and eightfold in terms of group median values, respectively) (Figure 2a).

On the other hand, the ratio between parasite-specific IgE and IgG4 levels has been suggested as an indicator of the efficiency of antibody responses in helminth infections due to IgG4 inhibitory effects on IgE biological functions (23). Thus, we calculated the IgE:IgG4 ratio in each individual from the three groups by dividing their levels of parasite-specific IgE by IgG4 levels. Results shown in

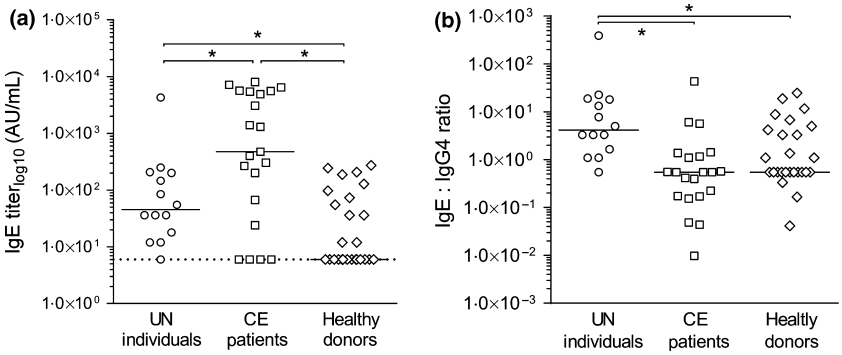


Figure 2 Antiparasite IgE levels are different in UN individuals, patients with CE and healthy donors. Titration of anti-PSA IgE (a) was performed by ELISA, and detection threshold is shown as a dashed line. Calculated ratios of anti-PSA IgE to IgG4 are shown (b). Values for each individual (symbols) and group median (horizontal lines) are shown. (*) Statistical significance ($P < 0{\cdot}05$).

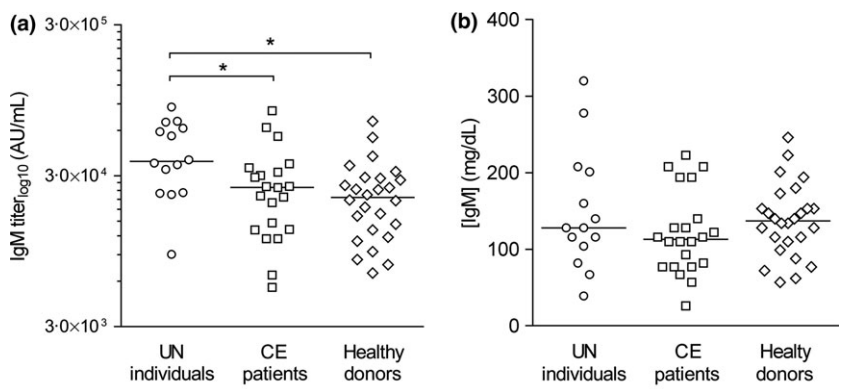
Figure 2(b) indicate that UN individuals exhibited significantly higher IgE:IgG4 ratios than patients with CE and healthy donors (eightfold in terms of group median values) (Figure 2b).

Summing up, our results showed that UN individuals displayed low levels – although significant – of antiparasite IgE antibodies in concomitance with significantly higher ratios of specific IgE:IgG4.

UN individuals showed a unique profile of parasite-specific IgM antibodies

Evaluation of antiparasite IgM levels showed outstanding results (Figure 3a). UN individuals displayed significantly higher values than any other group, while no differences in parasite-specific IgM levels were observed between patients with CE and healthy donors (Figure 3a). Interestingly, such differences were not associated with differences in total IgM concentration (Figure 3b).

Several reports have suggested that natural IgM – unlike induced IgM – represents a constant proportion of total serum IgM concentration, being therefore their levels linearly correlated (24–26). Therefore, we further analysed the interrelationships between parasite-specific IgM levels (either natural and/or induced) and total IgM concentrations in the three groups (Figure 3c). As expected, natural cross-reacting IgM levels in healthy donors showed an important positive and significant correlation with total



(c)

	r^2_s	*P*-value
UN individuals	0·6586	<0·05
CE patients	0·3845	0·09
Healthy donors	0·6639	<0·05

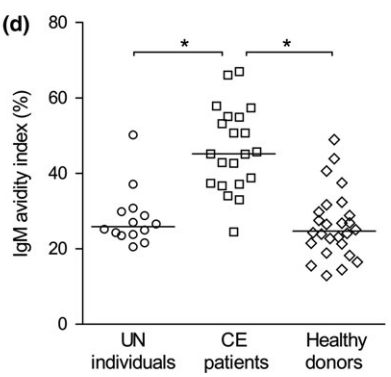


Figure 3 UN individuals show a unique profile of parasite-specific IgM antibodies. Titration of anti-PSA IgM (a) was performed by ELISA. Determination of total IgM concentration (b) was assessed by radial immunodiffusion. Avidity index of anti-PSA IgM (d) was determined by ELISA with chaotropic elution. Values for each individual (symbols) and group median (horizontal lines) are shown. (*) Statistical significance ($P < 0{\cdot}05$). Correlation analysis between levels of anti-PSA IgM and total IgM concentration was performed, and Spearman's correlation coefficients (r^2_s) and statistical significance (*P*-values) are shown for each group (c).

IgM concentrations (Figure 3c). On the contrary, in patients with CE, although both parameters displayed an appreciable interrelationship, it did not significantly correlate (Figure 3c). This result might be a consequence of the presence of both natural cross-reacting and parasite-induced IgM antibodies in patients with CE. Interestingly, correlation analysis in UN individuals showed a positive and significant correlation between parasite-specific IgM levels and total IgM concentrations, in a similar extent to the one observed in healthy donors (Figure 3c). In addition, avidity index determination in parasite-specific IgM antibodies showed similar values in UN individuals and healthy donors, while in patients with CE, values were significantly higher (Figure 3d). Thus, although it requires further confirmation, we hypothesize that parasite-specific IgM detected in the sera from UN individuals might belong to the pool of natural cross-reacting antibodies. Additionally, it is worth mentioning that the higher titres of anti-PSA IgM with low avidity indexes in UN individuals might probably reflect a higher concentration of parasite-specific IgM.

UN individuals and patients with CE exhibited no differences in antiparasite IgA antibodies

Total IgA concentration and levels of anti-PSA IgA and IgA subclasses were analysed in the three groups. However, no remarkable differences were observed between UN individuals and patients with CE in any IgA-related parameter (data not shown).

DISCUSSION

It has long been known that antigens from *E. granulosus* induce appreciable antibody responses in patients with CE. However, the immunological relevance of such responses has not been clearly determined yet. In this regard, most research on antibody responses in patients with CE has dealt with specific IgG and IgE responses, and the first studies on IgG subclasses in human CE indicated the existence of a switch from predominant IgG1 to IgG4 antibodies with disease progression (2). Thenceforth, the peculiar role of IgG4 in CE has been extensively studied and it is actually considered an immunological marker for the disease (5). IgG4 subclass is associated with prolonged, chronic infections, and it is a neither cytophilic nor complement-fixing antibody, which binds weakly to Fc receptors. Therefore, its induction has been associated with parasites' ability to evade the host immune response (3). In accordance with these studies, it has been reported that albendazole-treated patients exhibiting good therapeutic and clinical response to treatment had significantly lower IgG4 levels than poor or nonresponders, whereas IgG1 levels showed a reverse trend (27,28). In addition, higher IgG4 levels were seen in CE patients with progressive disease, while patients with stable disease showed higher IgG1 and IgG3 levels (8). Furthermore, specific IgG4 antibodies have been particularly associated with cysts development, growth and disease progression (CE evolutive phase), whereas IgG1, IgG2 and IgG3 responses occurred predominantly when cysts became infiltrated or were destroyed by the host (CE involutive phase) (6). Thus, *E. granulosus*-specific IgG subclasses seem to influence the natural history of human CE. In this regard, our results showed that UN individuals exhibited lower levels – in fact virtually undetectable – of parasite-specific IgG1 and IgG4 antibodies than patients with CE (Figure 1c,f). These results – at least for IgG4 antibodies – seem to be in accordance with the above-mentioned reports and suggest that low levels of antiparasite IgG4 in UN individuals might be related to the absence of ultrasound images compatible with hydatid cysts.

On the other hand, our results also showed that anti-PSA IgE levels in UN individuals were higher than in healthy donors and lower than in patients with CE (Figure 2a). Interestingly, IgE-mediated degranulation of effector cells was reported to be inhibited by parasite-specific IgG4 antibodies (23). Such inhibition results from the known competition between IgG4 and IgE for the antibody-fixation sites on mast cells and eosinophils, being bound-IgE – unlike bound-IgG4 – able to induce their degranulation (29). In fact, through histamine-release assays, it was found that among the four IgG subclasses, only IgG4 levels correlated with IgE-blocking activity and that such inhibition could be abolished by the selective depletion of serum IgG4 (30). In this regard, our results on the higher IgE:IgG4 ratio observed in UN individuals in comparison with CE patients (Figure 2b) might suggest the existence of a more efficient antibody response in those individuals. Thus, the fine-tuned balance between specific antibodies able to exert potent (e.g. IgE) or weak (e.g. IgG4) antiparasite activities could be suggested as a measurement of the effective/ineffective responses against human CE. Similar suggestions have been proposed for other helminth infections in humans (31).

Finally, our results also highlighted differences between groups regarding parasite-specific IgM, with UN individuals exhibiting the highest levels of anti-PSA IgM antibodies (Figure 3a). Moreover, their positive correlation with total IgM concentrations (Figure 3c) and their avidity values resembling natural cross-reacting antibodies in healthy donors (Figure 3d), suggested that antiparasite IgM in UN individuals probably belonged to the class of natural cross-reacting antibodies. Natural antibodies – being IgM

the predominant isotype – are produced at tightly regulated levels in the complete absence of external antigenic stimulation and provide early protection against several pathogens including viruses, bacteria, fungi and parasites (32–34). Polyreactivity is an outstanding feature of natural antibodies, providing hosts with pre-existing broad antibody repertoires which allow them to rapidly recognize and protect against pathogens that have not been encountered previously (32,34). In this sense, in CE, it was recognized that *E. granulosus* was more susceptible to immunological killing in early stages of infection, becoming more resistant at some later stage in its development. In such early stages – termed 'establishment phase' – the parasite is highly susceptible to antibody-mediated complement-dependent killing (35). In fact, *E. granulosus* oncosphere killing by complement has been shown to occur with sera from intermediate hosts (36–38), as well as from humans (18). In this regard – besides other important effector roles – natural IgM has been recognized as excellent complement activators (32,34). Therefore, our results might suggest that high levels of natural IgM recognizing *E. granulosus* antigens would be involved in some protection degrees observed in certain inhabitants residing in highly endemic regions for CE, an already described phenomenon in other infections (3,39).

In conclusion, we have here described the existence of remarkable different antibody profiles in patients with CE and UN individuals. It is important to note that the present work does not strictly describe an unequivocal causal effect of antibodies on human resistance to CE, because antibodies could merely express the result of a former host–parasite interaction that did not result in a successful infection due to several plausible causes (e.g. low challenging dose, exposure to senescent eggs). However, the higher IgE:IgG4 ratio as well as the higher levels of putative natural IgM antibodies in UN individuals could reflect an unsuccessful establishment of *E. granulosus*, and therefore, such individuals might represent a case of host resistance to CE.

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DISCLOSURE

All authors declare that there is no potential conflict of interest relevant to this article to declare.

COMPETING INTERESTS

The authors have no competing interests.

REFERENCES

1 Moro P & Schantz PM. Echinococcosis: a review. *Int J Infect Dis* 2009; **13**: 125–133.
2 Siracusano A, Delunardo F, Teggi A & Ortona E. Host-parasite relationship in cystic echinococcosis: an evolving story. *Clin Dev Immunol* 2012; **2012**: 639362.
3 Garraud O, Perraut R, Riveau G & Nutman TB. Class and subclass selection in parasite-specific antibody responses. *Trends Parasitol* 2003; **19**: 300–304.
4 Craig PS. *Echinococcus granulosus*: immunodiagnosis and vaccination, a perspective. *Parasitologia* 1997; **39**: 345–347.
5 Shambesh MK, Craig PS, Wen H, Rogan MT & Paolillo E. IgG1 and IgG4 serum antibody responses in asymptomatic and clinically expressed cystic echinococcosis patients. *Acta Trop* 1997; **64**: 53–63.
6 Daeki AO, Craig PS & Shambesh MK. IgG-subclass antibody responses and the natural history of hepatic cystic echinococcosis in asymptomatic patients. *Ann Trop Med Parasitol* 2000; **94**: 319–328.
7 Hernández-Pomi A, Borras-Salvador R & Mir-Gisbert A. Analysis of cytokine and specific antibody profiles in hydatid patients with primary infection and relapse of disease. *Parasite Immunol* 1997; **19**: 553–561.
8 Riganò R, Ioppolo S, Ortona E, *et al.* Long-term serological evaluation of patients with cystic echinococcosis treated with benzimidazole carbamates. *Clin Exp Immunol* 2002; **129**: 485–492.
9 Azab ME, Bishara SA, Helmy H, *et al.* Association of some HLA-DRB1 antigens with *Echinococcus granulosus* specific humoral immune response. *J Egypt Soc Parasitol* 2004; **34**: 183–196.
10 Azab ME, Bishara SA, Ramzy RM, Oteifa NM, El-Hoseiny LM & Ahmed MA. The evaluation of HLA-DRB1 antigens as susceptibility markers for unilocular cystic echinococcosis in Egyptian patients. *Parasitol Res* 2004; **92**: 473–477.
11 Bonifacino R, Malgor R, Barbeito R, *et al.* Seroprevalence of *Echinococcus granulosus* infection in a Uruguayan rural human population. *Trans R Soc Trop Med Hyg* 1991; **85**: 769–772.
12 Nahmias J, Goldsmith R, Schantz P, Siman M & el-On J. High prevalence of human hydatid disease (echinococcosis) in communities in northern Israel: epidemiologic studies in the town of Yirka. *Acta Trop* 1991; **50**: 1–10.
13 Zhang WB, Zhang ZZ, Alili H & Chi PS. An investigation of factors influencing the hyperendemic of echinococcosis in Xinjiang. *Chin J Zoonoses* 1994; **10**: 204–205.
14 Cohen H, Paolillo E, Bonifacino R, *et al.* Human cystic echinococcosis in a Uruguayan community: a sonographic, serologic, and epidemiologic study. *Am J Trop Med Hyg* 1998; **59**: 620–627.
15 Shambesh MA, Craig PS, Macpherson CN, Rogan MT, Gusbi AM & Echtuish EF. An extensive ultrasound and serologic study to investigate the prevalence of human cystic echinococcosis in northern Libya. *Am J Trop Med Hyg* 1999; **60**: 462–468.
16 Hernández A, Cardozo G, Dematteis S, *et al.* Cystic echinococcosis: analysis of the serological profile related to the risk factors in individuals without ultrasound liver changes living in an endemic area of Tacuarembó, Uruguay. *Parasitology* 2005; **130**: 455–460.
17 Gavidia CM, Gonzalez AE, Zhang W, *et al.* Diagnosis of cystic echinococcosis, central

Peruvian Highlands. *Emerg Infect Dis* 2008; **14**: 260–266.
18 Rogan MT, Craig PS, Zehyle E, Masinde G, Wen H & Zhou P. *In vitro* killing of taeniid oncospheres, mediated by human sera from hydatid endemic areas. *Acta Trop* 1992; **51**: 291–296.
19 Míguez M, Baz A & Nieto A. Carbohydrates on the surface of *Echinococcus granulosus* protoscoleces are immunodominant in mice. *Parasite Immunol* 1996; **18**: 559–569.
20 Mourglia-Ettlin G, Marqués JM, Chabalgoity JA & Dematteis S. Early peritoneal immune response during *Echinococcus granulosus* establishment displays a biphasic behavior. *PLoS Negl Trop Dis* 2011; **5**: e1293.
21 Baz A, Carol H, Fernández V, *et al.* *Echinococcus granulosus*: induction of T-independent antibody response against protoscolex glycoconjugates in early experimental infection. *Exp Parasitol* 2008; **119**: 460–466.
22 Pullen GR, Fitzgerald MG & Hosking CS. Antibody avidity determination by ELISA using thiocyanate elution. *J Immunol Methods* 1986; **86**: 83–87.
23 Yazdanbakhsh M, Kremsner PG & van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002; **296**: 490–494.
24 Platt JL, Lindman BJ, Geller RL, *et al.* The role of natural antibodies in the activation of xenogenic endothelial cells. *Transplantation* 1991; **52**: 1037–1043.
25 Vincent C, Serre G, Fournié B, Fournié A & Soleilhavoup JP. Natural IgG to epidermal cytokeratins vs. IgG to the stratum corneum of the rat oesophagus epithelium, so-called 'antikeratin antibodies', in rheumatoid arthritis and other rheumatic diseases. *J Autoimmun* 1991; **4**: 493–505.
26 Padilla ND, Ciurana C, van Oers J, Ogilvie AC & Hack CE. Levels of natural IgM antibodies against phosphorylcholine in healthy individuals and in patients undergoing isolated limb perfusion. *J Immunol Methods* 2004; **293**: 1–11.
27 Riganò R, Profumo E, Ioppolo S, *et al.* Immunological markers indicating the effectiveness of pharmacological treatment in human hydatid disease. *Clin Exp Immunol* 1995; **102**: 281–285.
28 Riganò R, Profumo E, Di Felice G, Ortona E, Teggi A & Siracusano A. *In vitro* production of cytokines by peripheral blood mononuclear cells from hydatid patients. *Clin Exp Immunol* 1995; **99**: 433–439.
29 Aalberse RC, Stapel SO, Schuurman J & Rispens T. Immunoglobulin G4: an odd antibody. *Clin Exp Allergy* 2009; **39**: 469–477.
30 Hussain R, Poindexter RW & Ottesen EA. Control of allergic reactivity in human filariasis. Predominant localization of blocking antibody to the IgG4 subclass. *J Immunol* 1992; **148**: 2731–2737.
31 Adjobimey T & Hoerauf A. Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation. *Ann Trop Med Parasitol* 2010; **104**: 455–464.
32 Panda S & Ding JL. Natural antibodies bridge innate and adaptive immunity. *J Immunol* 2015; **194**: 13–20.
33 Baumgarth N, Tung JW & Herzenberg LA. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Semin Immunopathol* 2005; **26**: 347–362.
34 Ehrenstein MR & Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* 2010; **10**: 778–786.
35 Rogan MT, Bodell AJ & Craig PS. Post-encystment/established immunity in cystic echinococcosis: is it really that simple? *Parasite Immunol* 2015; **37**: 1–9.
36 Heath DD, Holcman B & Shaw RJ. *Echinococcus granulosus*: the mechanism of oncosphere lysis by sheep complement and antibody. *Int J Parasitol* 1994; **24**: 929–935.
37 Heath DD & Lawrence SB. Antigenic polypeptides of *Echinococcus granulosus* oncospheres and definition of protective molecules. *Parasite Immunol* 1996; **18**: 347–357.
38 Lightowlers MW & Heath DD. Immunity and vaccine control of *Echinococcus granulosus* infection in animal intermediate hosts. *Parasitologia* 2004; **46**: 27–31.
39 Grönwall C, Vas J & Silverman GJ. Protective roles of natural IgM antibodies. *Front Immunol* 2012; **3**: 1–10.

Capítulo 3

Natural and induced antibodies contribute to differential susceptibility to secondary cystic echinococcosis of Balb/c and C57Bl/6 mice

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Natural and induced antibodies contribute to differential susceptibility to secondary cystic echinococcosis of Balb/c and C57Bl/6 mice

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C57Bl/6

ABSTRACT

Antibodies are key immune players in several helminth infections and animal models have been central for the identification of their mechanisms of protection. Murine secondary cystic echinococcosis is a useful model for studying *Echinococcus granulosus* immunobiology, being the immune profile mounted by the experimental host a determinant of parasite success or failure in infection establishment. In the present study, we analyzed infection outcome using Balb/c and C57Bl/6 mice strains, and compared their antibody responses in terms of quality and intensity. Our results showed that Balb/c is a highly susceptible strain to secondary cystic echinococcosis, while C57Bl/6 mice are quite resistant. Moreover, significant differences between strains were observed in natural and induced antibodies recognizing *E. granulosus* antigens, both at the systemic and peritoneal levels. Natural cross-reacting IgM, IgG2b and IgG3 antibodies were detected in sera from both strains but with different intensities, and – remarkably – natural IgG2b showed to be an intrinsic correlate of protection in both mice strains. Interestingly, naïve C57Bl/6 serum displayed a higher protoscolicidal activity, and heterologous – but not homologous – transference of C57Bl/6 naïve serum into Balb/c mice, significantly reduced their infection susceptibility. In the peritoneal cavity, different levels of natural cross-reacting IgM and IgG3 antibodies were detected in both mice strains, while cross-reacting IgG2b was detected only in C57Bl/6 mice. On the other hand, infected mice from both strains developed isotype-mixed antibody responses, with Balb/c mice biasing their response towards high avidity IgG1 and C57Bl/6 mice showing a predominance of mixed IgM/IgG2c/IgG2b/IgG3. In this regard, IgG1 levels showed to be a correlate of susceptibility in both mice strains. In conclusion, our results suggest that antibodies – either natural or induced – play a role in the susceptibility degree to murine secondary cystic echinococcosis.

1. Introduction

Chronic infections with helminth parasites have a significant impact on global public health, causing more than 2 billion human infections worldwide. Depending on the parasite species, they can cause varying degrees of mortality and morbidity rates (Wiria et al., 2012). Interestingly, although helminth parasites belong to a highly divergent animal group, they induce polarized and stereotyped Th2-type immune responses, with rare to no levels of Th1-type components (Díaz and Allen, 2007). For many – but not all – helminths, Th2-type responses mediate protection, but their effective immune components can differ between parasite species and different developmental stages of infection within a particular species. Such differences derive from the specific ecological niche occupied by the invading helminth at different stages of its life cycle, including the microenvironment where the parasite resides and the specific host–parasite interactions that subsequently occur there (Harris and Gause, 2011).

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As in most infections, antibody production occurs during helminth infections and the understanding of how natural cross-reacting and antigen-specific induced antibodies contribute to immunity against helminths would provide important insights into how protective immunity develops. Murine models of helminth infections are becoming increasingly important for identification of mechanisms of antibody-mediated protection and the specific immune effector cells that also contribute to protective immunity. Particularly, IgG and IgM have been shown to act as potent mediators of protective immunity following helminth infections (Harris and Gause, 2011). Evidence of protective immunity against helminth parasites has been obtained from observations generated by passive transference of immune serum, purified IgG or monoclonal antibodies into naïve experimental animals (McCoy et al., 2008; Gurish et al., 2004; Blackwell and Else, 2001; Rajan et al., 2005; Marcet et al., 2002; Attallah et al., 1999; Inaba et al., 2003; Harris et al., 2006; Herbert et al., 2002; Ligas et al., 2003). However, it is important to note that not all studies have reported such a protective effect (Liu et al., 2010; Wojciechowski et al., 2009; Harrison et al., 2008), suggesting that the ability of antibodies to mediate protective immunity depends on the parasite species investigated.

During helminth infections, antibodies are supposed to help in parasites clearance, to limit disease, and possibly to prevent parasite entry into the vasculature and adherence to mucosal surfaces (Garraud et al., 2003). To those ends, the class and subclass of the antibody response is essential because each isotype/subclass has specific biological functions. Thus, it is crucially important for an infected host to mount the most appropriate antibody response, which is the one with the best chance of clearing the infection and/or controlling the disease. Although there is no direct evidence that differential production of antibody isotypes is affected by intrinsic host factors, genetic background has been emphasized as a factor influencing susceptibility to experimental infection with parasites (Garraud et al., 2003).

Cystic echinococcosis (CE) is a zoonotic disease of cosmopolitan distribution caused by the larval stage of the cestode *Echinococcus granulosus*, showing a worldwide prevalence of roughly 1 million infected people (Moro and Schantz, 2009; Thompson, 2008; Siracusano et al., 2009). Primary CE occurs in intermediate hosts (domestic and wild ungulates, accidentally humans) and derives from the ingestion of eggs containing oncospheres, which later develop into metacestodes (also known as hydatid cysts) mainly in the liver and lungs of the infected host. Secondary CE comes about after spillage of protoscoleces from a fertile hydatid cyst within an already infected intermediate host. This kind of infection results from protoscoleces developmental plasticity, which allows them to develop into new cysts within intermediate hosts or into adult worms if ingested by a definitive host (usually dogs). The experimental model of secondary infection has been used to study host-parasite interactions, and is based on the intraperitoneal inoculation of viable protoscoleces into susceptible and immunocompetent mice (Heath, 1970). Experimental secondary CE in Balb/c mice can be divided into two stages: an early stage (until day 20–30 pi) with protoscoleces developing into hydatid cysts (Richards et al., 1983), and a late or chronic stage in which already differentiated cysts grow and eventually become fertile cysts (Heath, 1970).

Susceptibility and/or resistance phenomena in CE have been scarcely studied. To date only few reports have analyzed potential associations between immunological relevant genes (*e.g.*, polymorphisms in TAP-1/2 and HLA genes) and prognosis in human CE (Azab et al., 2004; Kiper et al., 2010; Yalcin et al., 2010). In murine CE it has been suggested that the antibody and cytokine profiles would determine the parasite success or failure to establish the infection and that such profiles would be tightly dependent on the host genetic background (Yang et al., 2012). However, infection outcome in different mice strains infected with *E. granulosus* has been poorly analyzed. In fact, only two reports have analyzed this topic but with important dissimilarities in terms of parasite stage and origin used, inoculation route, mice strains studied and infection end-point (Pennoit-De Cooman and De Rycke, 1970; Dempster et al., 1991). Indeed, for the current and globally accepted model of murine secondary CE, there are no reports to date comparing the influence of mice strain on the susceptibility to *E. granulosus* infection.

In the present study we have performed systematic parasitological analyses on the infection outcome using different murine genetic backgrounds (*e.g.*, Balb/c and C57Bl/6 strains) in order to determine differences in the infection outcome according to the mice strain used. Moreover, we have analyzed the specific humoral response in terms of quality and intensity in both mice strains, and therefrom we described potential immunological correlates of protection. In addition, we established the relevance of natural antibodies cross-reacting with *E. granulosus* antigens in the intrinsic mice strain susceptibility to secondary CE.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in compliance with Comisión Honoraria de Experimentación Animal (CHEA) from Universidad de la República, according to the Canadian Guidelines on Animal Care and the National Uruguayan Legislation N° 18.611. Experimental protocols were approved by the Ethics Committee of Facultad de Química (Universidad de la República) and were given the approval numbers 101900-001065-11 and 101900-001480-14.

2.2. Parasites and antigens

Protoscoleces from *E. granulosus* were obtained by aseptic puncture of fertile bovine hydatid cysts from Uruguayan abattoirs, and were washed several times with phosphate buffered saline (PBS) pH 7.2 containing gentamicin (40 μg/mL). Parasite viability was determined according to Dematteis et al. (1999). Only those batches with over 90% viability were used for experimental infections. Protoscoleces somatic antigens (PSA) were obtained by ultrasound disruption according to Míguez et al. (1996) and its protein content was assessed using BCA Protein Assay Reagent (Pierce) following manufacturer instructions. PSA was stored at −20 °C until use.

2.3. Mice and infections

Female Balb/c and C57Bl/6 mice were obtained from DILAVE (Uruguay) and housed at the animal facility of Instituto de Higiene (Montevideo, Uruguay). Experimental infections were performed with 6–8 weeks old mice, which were inoculated by the intraperitoneal (ip) route with 200 μL of a PBS suspension containing 2000 viable protoscoleces.

For long-term infections, mice from both strains ($n = 8$ per strain) were infected and bled at 0 (pre-infection), 1, 3, 6, 10, 15, 30 and 48 weeks post-infection (pi). Sera were obtained by regular means and stored at −80 °C until use. Immediately after the last bleeding, all mice were euthanized and their peritoneal cysts were counted and measured. Hydatid cyst volumes were calculated assuming cysts as sphere-shaped, and parasite load was defined as the arithmetic sum of every cyst volume within each mouse (Cucher et al., 2013).

For peritoneal antibody analyses and protoscolicidal activity assays, mice from both strains ($n = 15$ per strain) were infected. At 0 (pre-infection), 1 and 3 weeks pi, 5 mice per strain were bled (and sera stored at −80 °C), euthanized and their peritoneal cavities were washed with 1 mL of sterile PBS. After centrifugation during 7 min

at 1.200 rpm, cells-free peritoneal lavages were stored at −80 °C until use.

For serum transference experiments, naïve 6–8 weeks old Balb/c and C57Bl/6 mice ($n = 5$ per strain) were bled and their sera pooled by strain. After heat-inactivation (30 min at 56 °C), 300 μL of PBS-half-diluted pooled sera were ip inoculated into normal Balb/c mice ($n = 7$ for each pool). Balb/c control mice ($n = 10$) were ip inoculated with 300 μL of sterile PBS. Every mice from the 3 groups was infected as previously described 24 h post-transference. To assess infection outcome, mice were euthanized 41 weeks pi and their peritoneal cysts were counted.

2.4. Specific antibodies titration

Anti-PSA antibodies in sera and peritoneal exudates were measured by ELISA in individual samples according to Mourglia-Ettlin et al. (2011a). A pool of PSA-hyperimmunized sera from Balb/c and C57Bl/6 mice was used as standard and specific antibodies titers were expressed as arbitrary units referred to it. Specific IgM, IgG1, IgG2a/c, IgG2b and IgG3 were determined using appropriate goat or rabbit anti-mouse (isotype/subclass) antibodies labeled with peroxidase (Sigma). Peroxidase activity was detected using *O*-phenylendiamine as chromophore (Sigma), and absorbance values were recorded at 492 nm.

2.5. Avidity index determination

Avidity index of anti-PSA antibodies in sera and peritoneal exudates were determined according to Pullen et al. (1986). Briefly, appropriately diluted samples were dispensed in eight wells of PSA-coated and blocked ELISA plates and incubated as usually. Then, 100 μL/well of 0.1; 0.5; 1.5; 3.0; 4.0 and 6.0 M KSCN in PBS were added, and followed by incubation at room temperature for 30 min. After washing the chaotropic solutions, ELISA protocol was performed as described above. Absorbance values in the absence of KSCN were assumed to represent 100% specific antibody binding. Linear regression analyses of (% binding) *vs.* KSCN concentration were carried out, and the avidity index was calculated as the molar concentration of KSCN required to reduce antibody binding to 50%.

2.6. Determination of antibodies recognizing peptide epitopes

Percentage of antibodies in sera and peritoneal exudates recognizing *m*-periodate-resistant (peptide) epitopes in PSA was determined through treatment of PSA-coated ELISA plates with $NaIO_4$ according to Woodward et al. (1985). Briefly, PSA-coated and blocked ELISA plates were incubated during 1 h with 200 μL/well of 20 mM $NaIO_4$ in 50 mM acetate buffer pH 4.5 at room temperature. After washing, 250 μL of 50 mM $NaBH_4$ in PBS were added to each well and incubated for 30 min at room temperature. Then, appropriately diluted samples were dispensed in treated and non-treated wells and ELISA protocol was performed as described above. The percentage of absorbance values in treated wells respect to non-treated wells was defined as the percentage of antibodies recognizing *m*-periodate-resistant (peptide) epitopes in PSA.

2.7. Assessment of protoscolicidal activity

Protoscolicidal activity of sera and peritoneal exudates was assessed *in vitro* by incubating roughly 100 viable protoscoleces during 4 h at 37 °C with constant shaking in 100 μL of appropriately diluted individual samples. Sera and peritoneal exudates were tested at 2:3 and 4:5 final dilutions in PBS containing gentamicin (40 μg/mL), respectively. Heat-inactivated samples (30 min at 56 °C) and PBS alone were also tested. After incubation, parasites viability was determined according to Dematteis et al. (1999), and protoscolicidal activity was expressed as percentage of non-viable protoscoleces.

2.8. Statistics

Comparisons between mice strains were assessed by non-parametric Mann–Whitney *U* test. Follow-up studies within a strain were assessed by Wilcoxon matched-pairs signed rank test. Correlation analyses were assessed by Spearman's rank correlation test, and correlation coefficients (r^2_S) and *p*-values from Spearman's test are shown. In all cases differences were regarded as significant with $p < 0.05$.

3. Results

3.1. Balb/c mice are more susceptible to secondary CE than C57Bl/6 mice

Systematic analyses of *E. granulosus* infection outcome in different mice strains have been poorly performed. Thus, we studied the relative susceptibility to secondary CE in the most commonly used mice strains: Balb/c and C57Bl/6. To that end, we performed in parallel experimental infections with age-matched Balb/c and C57Bl/6 mice by inoculating 2000 viables protoscoleces in their peritoneal cavities. Forty-eight weeks pi, mice were sacrificed and two parasitological parameters useful to describe infection outcome were assessed: number of cysts and parasite load. Results in Fig. 1A show that Balb/c infected mice developed approximately 3 times more cysts (in terms of group median values) than their C57Bl/6 counterparts. On the other hand, comparison of parasite loads showed significantly higher values in Balb/c mice (approximately 6-fold higher in terms of group median values) (Fig. 1B). Interestingly, although Balb/c mice are larger than C57Bl/6 animals, no association between host body size and hydatid cyst volume was found. Indeed, when cysts from three independent experiments were arbitrarily grouped in three size-dependent categories (small, medium and large), it was shown that C57Bl/6 mice proportionally harboured larger cysts than Balb/c mice (mean values: 25% *vs.* 7%, respectively) (Fig. 1C). Therefore, although Balb/c mice showed to be a more permissive strain to secondary CE in terms of number of developed cysts and parasite loads, C57Bl/6 mice harboured a higher proportion of large cysts.

3.2. Intensity of systemic antibody response correlates with susceptibility to murine secondary CE

It is well known that specific antibody responses play a role in susceptibility/resistance to parasite infections. Particularly, in CE such a role has remained at least controversial, and as far as we know, no definitive data has been reported to date about the influence of the antibody response on the experimental infection outcome. Therefore, we analyzed the kinetics of systemic antibody responses specific for PSA in Balb/c and C57Bl/6 infected mice throughout a 48-week infection. Then, mice were sacrificed and infection outcome parameters were determined in order to perform correlation analyses. Considerable differences between mice strains regarding natural and induced antibodies recognizing parasite antigens were observed. Natural cross-reacting antibodies were detected in sera from both strains, being IgM titers higher in Balb/c mice (Fig. 2A) and IgG3 titers higher in C57Bl/6 animals (Fig. 2E). Once protoscoleces were inoculated, IgM titers became significantly higher in C57Bl/6 mice until 6 weeks pi (Fig. 2A). Interestingly, as soon as 1 week pi IgM significantly increased respect to baseline values only in C57Bl/6 mice, while IgM rise in Balb/c mice was delayed until 3 weeks pi (Fig. 2A). A similar kinetic profile was observed for induced IgG2b antibodies (Fig. 2D).

Table 1
IgG2b and IgG1 antibodies are immune correlates of protection and susceptibility to secondary CE, respectively. Spearman correlation analyses for Balb/c and C57Bl/6 mice were performed between parasitological data on infection outcome (number of cysts and parasite loads in each mouse at 48 weeks pi) and their respective antibody titers in every time-point studied. Significant correlations were observed only for induced IgG1 (positive) and natural IgG2b (negative) antibodies.

Induced IgG1								
	Balb/c				C57Bl/6			
	Cyst number		Parasite load		Cyst number		Parasite load	
Weeks pi	r^2_s	*p*-Value	r^2_s	*p*-Value	r^2_s	*p*-Value	r^2_s	*p*-Value
3	0.4962	0.0448	0.5238	0.0489	0.6347	0.0469	0.5476	0.0478
6	0.7619	0.0368	0.8333	0.0154	0.8743	0.0072	0.7381	0.0458
10	0.8095	0.0218	0.8195	0.0228	0.8144	0.0184	0.6191	0.0028
15	0.7857	0.0279	0.9048	0.0046	0.8623	0.0084	0.5238	0.0238
30	0.8333	0.0154	0.9048	0.0046	0.7545	0.0377	0.4762	0.0479
48	0.8095	0.0218	0.9248	0.0046	0.7904	0.0248	0.4286	0.0123
Natural IgG2b								
	Balb/c				C57Bl/6			
	Cyst number		Parasite load		Cyst number		Parasite load	
Weeks pi	r^2_s	*p*-Value	r^2_s	*p*-Value	r^2_s	*p*-Value	r^2_s	*p*-Value
0	−0.733	<0.0001	−0.746	<0.0001	−0.588	0.0476	−0.495	0.0489

Regarding IgG3, a sustained increase from 1 week pi onwards was observed in both mice strains, being IgG3 titers significantly higher in C57Bl/6 mice until 10 weeks pi (Fig. 2E). Induced IgG1 and IgG2a/c titers significantly increased in both strains from 1 week pi, predominating IgG1 titres in Balb/c mice and IgG2c titers in C57Bl/6 animals (Fig. 2B and C). Hence, although both mice strains developed isotype-mixed antibody responses, highly susceptible mice (Balb/c strain) biased their systemic response towards IgG1, while in less susceptible mice (C57Bl/6 strain) a predominance of mixed IgM–IgG2c–IgG2b–IgG3 was observed (Fig. 2). On the other hand, we performed Spearman's correlation analyses for each mice strain between parasitological data and antibody titers at every studied time point, and we observed significant correlations only for natural IgG2b and induced IgG1 antibodies (Table 1). Specific IgG1 titers showed a strong positive correlation with infection outcome parameters from 3 weeks pi onwards in both mice strains, suggesting that IgG1 polarization derives in a less efficient immune response (*i.e.*, more cysts and higher parasite loads within the infected host) (Table 1). On the other hand, natural IgG2b titers showed a negative correlation – in both mice strains – with infection outcome parameters (Table 1), suggesting that the higher the natural cross-reacting IgG2b titers, the more efficient the immune response (*i.e.*, less cysts and lower parasite loads within the infected host). Early-induced IgG2b responses also showed a negative correlation with infection outcome parameters, but without reaching statistical significance (data not shown). Summing up, results shown here point out that while IgG1 responses seem to be detrimental for the experimental host, IgG2b antibodies could be correlated with a protective response.

3.3. Quality of early systemic antibody response also differs between mice strains

Protective humoral responses have been shown to depend not only on antibody titers, but also on their quality characteristics. Therefore, we analyzed the avidity and recognition of peptide epitopes by serum specific antibodies in Balb/c and C57Bl/6 mice. Since the first month pi represent a crucial period for parasite establishment (Richards et al., 1983) and significant differences in most antibody isotypes were observed between strains (Fig. 2), we analyzed the quality of the humoral response at 0, 1 and 3 weeks pi.

First, when analyzing antibody avidity no significant differences between strains were observed, neither for natural cross-reacting IgM and IgG3 antibodies (Fig. 3A and E), nor for induced IgM, IgG1, IgG2a/c and IgG2b antibodies (Fig. 3A–D). It is worth noting that – independently of the mice strain analyzed – induced IgM one week pi showed a lower avidity index than natural IgM (Fig. 3A). Next, avidity maturation was assessed through paired-statistical analyses, and interestingly only Balb/c mice showed a significant IgG1 avidity maturation from 1 week to 3 weeks pi (Fig. 3B). On the other hand, although both strains significantly maturated IgG2a/c avidity at the same time, higher avidity increases were reached in C57Bl/6 mice (median of differences: 0.31 M *vs.* 0.26 M for C57Bl/6 and Balb/c, respectively) (Fig. 3C). No avidity maturation was observed for IgG3 in any strain, but Balb/c mice showed significantly higher values than their C57Bl/6 counterparts did at 3 weeks pi (Fig. 3E). Hence, our results showed that highly susceptible mice (Balb/c strain) developed an early unique IgG1 avidity maturation, while less susceptible mice (C57Bl/6 strain) maturated only the avidity of their IgG2c antibodies.

Second, we assessed the percentage of antibodies recognizing peptide epitopes in PSA. Although higher values for natural cross-reacting IgG3 were determined in Balb/c mice compared to their C57Bl/6 counterparts (Fig. 4E), no significant differences were observed for natural IgM (Fig. 4A). Similarly, no differences between strains were observed in the reactivity with peptide epitopes of induced IgG1, IgG2a/c and IgG2b antibodies (Fig. 4B–D). Regarding induced IgM and IgG3 antibodies, significant differences between strains were observed only at 3 weeks pi, being in both cases the percentages of antibodies reacting with peptide epitopes lower in C57Bl/6 mice (Fig. 4A and E). Interestingly – unlike Balb/c mice – the percentage of IgM recognizing peptide epitopes in C57Bl/6 mice decreased over time from 0 week (natural IgM) to 1 week pi, and then to 3 weeks pi (Fig. 4A). Additionally, only Balb/c mice showed a significant increase from 1 week to 3 weeks pi in the percentage of IgG3 reacting with peptide epitopes (Fig. 4E). Therefore, our results suggest that high percentages of natural and early-induced IgM and IgG3 recognizing peptide epitopes in PSA seem to be detrimental for the experimental host.

3.4. Early peritoneal antibody response is different in Balb/c and C57Bl/6 mice

Protoscoleces development into cysts in experimental secondary CE occurs within the murine peritoneal cavity. Thus, immune responses at the peritoneal level are of outstanding impor-

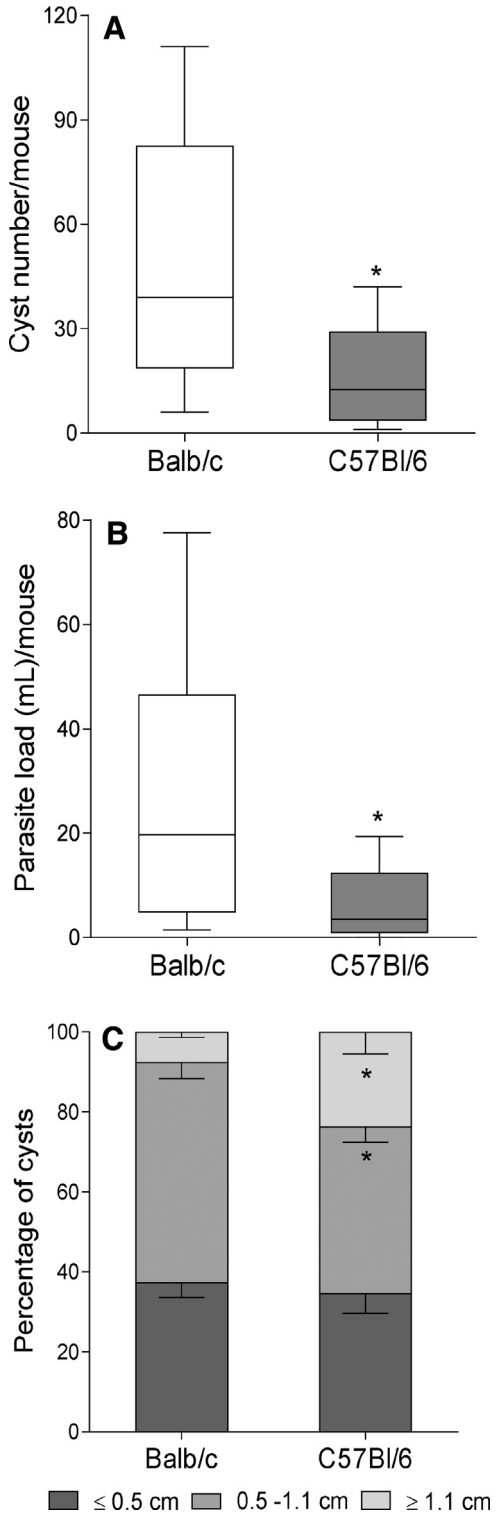

Fig. 1. Balb/c mice are more susceptible to secondary CE than C57Bl/6 mice. Balb/c and C57Bl/6 mice ($n = 8$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. All mice were euthanized 48 weeks pi, and their peritoneal cysts were recovered. Results are shown in box-and-whiskers plots for number of cysts (A) and parasite loads (B) in both mice strains. Cysts size distribution (grouped by diameters) in Balb/c and C57Bl/6 mice are shown as mean ± SEM of percentage of cysts within each size group (C). (*) Statistical significance ($p < 0.05$). Results are representative of three independent experiments.

tance for infection establishment, mainly during the early stages of the disease. Therefore, we inoculated ip 2000 viable protoscoleces into Balb/c and C57Bl/6 mice and analyzed the local specific antibody response in peritoneal exudates at 0, 1 and 3 weeks pi. Natural IgM and IgG3 peritoneal antibodies recognizing *E. granulosus* antigens were detected in both strains, with a reverse relationship respect to serum natural antibodies: IgM titers were higher in C57Bl/6 and IgG3 titers in Balb/c mice (Fig. 5A and E). Interestingly, natural peritoneal IgG2b was only detected in C57Bl/6 mice (Fig. 5D). Regarding induced antibodies, no differences between strains were shown for peritoneal IgM and IgG2a/c responses (Fig. 5A and C). Meanwhile, induced IgG2b reached significantly higher titers in C57Bl/6 mice at 3 weeks pi (Fig. 5D). Unlike C57Bl/6 mice, no titer increase over time was observed for induced IgG3 in Balb/c animals (Fig. 5E). Interestingly, peritoneal IgG1 was detected 1 week pi only in Balb/c mice with increasing values over time, while in C57Bl/6 mice IgG1 was only detected 3 weeks pi, reaching similar levels to 1 week pi values observed in Balb/c mice (Fig. 5B).

On the other hand, avidity and reactivity with peptide epitopes were also analyzed in peritoneal exudates when technically possible (Fig. 6). Unfortunately, no data could be obtained for IgG2b antibodies at any time point. Avidity index of natural peritoneal IgM was significantly higher in Balb/c mice, while no differences were observed for induced IgM between strains (Fig. 6A). No differences between Balb/c and C57Bl/6 mice were detected for IgG1 (Fig. 6B), as well as for natural and induced IgG3 (Fig. 6D). Interestingly – like induced IgG3 in serum – no variations in IgG3 avidity was observed at the peritoneal level in any mice strain (Fig. 6D). Meanwhile, peritoneal IgG2a/c avidity was significantly higher in C57Bl/6 mice 3 weeks pi (Fig. 6C). Finally, natural peritoneal IgM and IgG3 antibodies from C57Bl/6 mice showed higher recognition percentages of peptide epitopes than Balb/c mice (Fig. 6E and H). Interestingly, while values for induced IgM differed respect to natural IgM in both mice strains (Fig. 6E), no differences were observed between natural and induced IgG3 in any strain (Fig. 6H). Regarding IgG1 and IgG2a/c, no significant differences in recognition of peptide epitopes were observed between mice strains (Fig. 6F and G). Overall, our results showed important strain-specific differences in peritoneal exudates regarding natural and early-induced antibodies recognizing *E. granulosus* antigens, both in terms of intensity and quality.

3.5. In vitro *serum protoscolicidal activity depends on mice strain*

E. granulosus protoscoleces are known to be susceptible to complement killing, and it has been reported that in the presence of specific antibodies the tegumental membrane depolarization due to complement activation is faster and stronger than in their absence. Thus, in order to analyze the effects of strain-specific systemic and local antibodies on protoscoleces viability, we further studied the *in vitro* protoscolicidal activity of sera and peritoneal exudates from Balb/c and C57Bl/6 infected mice at 0, 1 and 3 weeks pi. Results in Fig. 7A show that serum protoscolicidal activity similarly increased over time in both mice strains. However, although normal sera from both strains displayed a significant protoscolicidal activity, C57Bl/6 sera showed an approximately 2-fold higher killing capacity (in terms of group median values) than Balb/c sera (Fig. 7A). Serum protoscolicidal activity seemed to be complement-mediated because heat-inactivated sera were unable to alter protoscoleces viability (Fig. 7B). Regarding peritoneal exudates, protoscolicidal activity could not be detected (data not shown), being not surprising because peritoneal content had to be diluted to be successfully recovered, and complement killing is known to be concentration-dependent. However, peritoneal exudates killing activity should not be completely discarded. Summing up, our results suggest that serum protoscolicidal activity increases

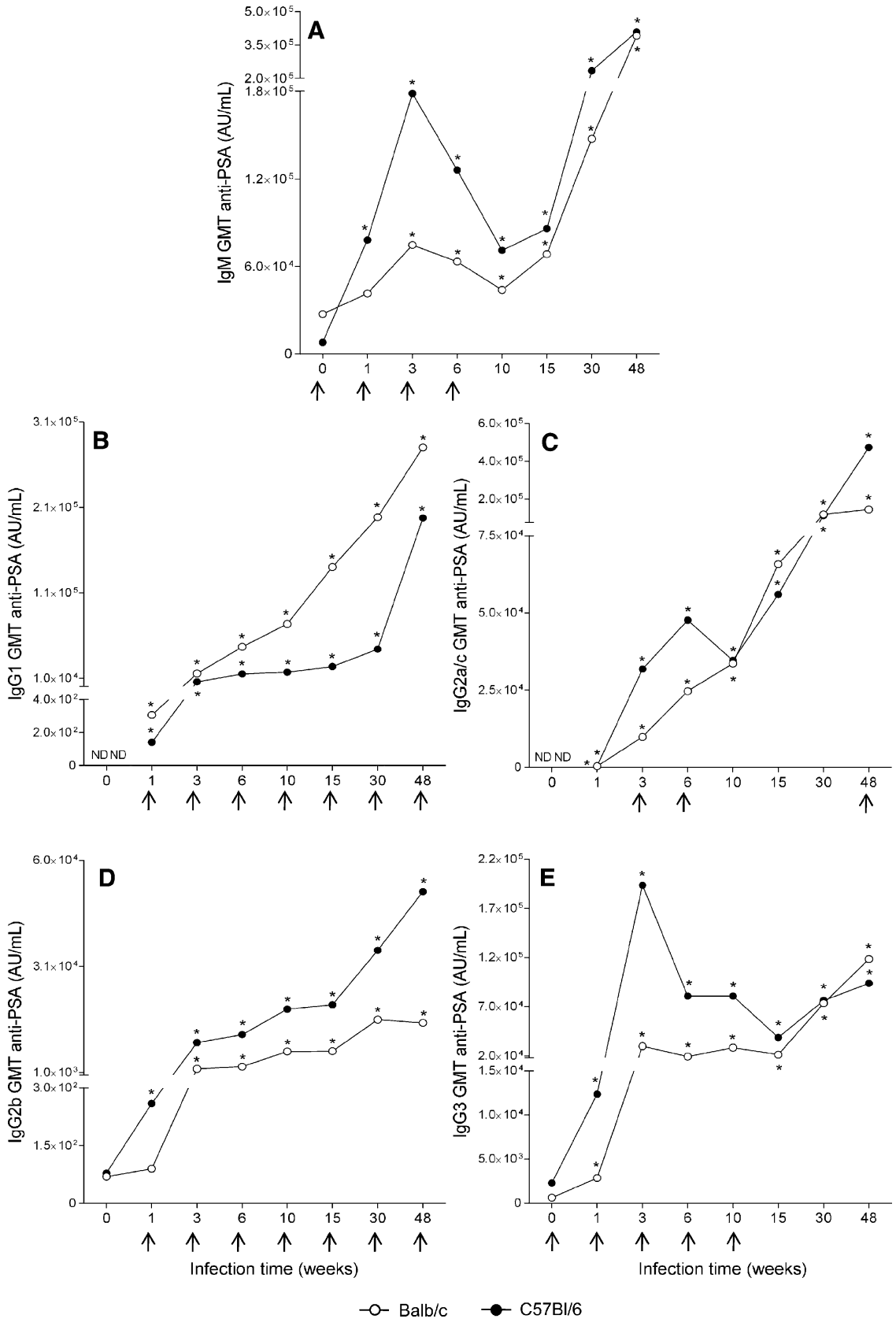

Fig. 2. Kinetics of serum antibody response to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 8$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice from both strains were bled at 0 (pre-infection), 1, 3, 6, 10, 15, 30 and 48 weeks pi, and serum anti-PSA IgM (A), IgG1 (B), IgG2a/c (C), IgG2b (D) and IgG3 (E) titers were determined by ELISA. Geometric mean titers (GMT) are shown for Balb/c (white circles) and C57Bl/6 (black circles) mice groups. Statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between time points and day 0 by asterisks (*). ND: not detected.

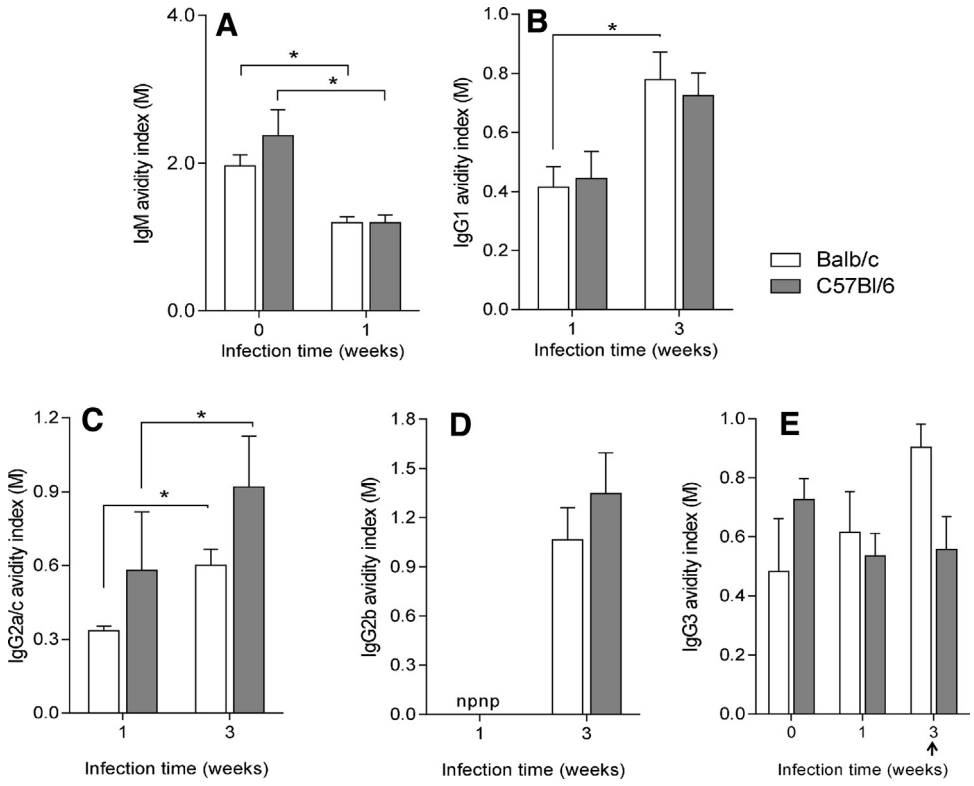

Fig. 3. Avidity analyses of early systemic antibodies to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 8$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice from both strains were bled prior to infection (0 week) and at different time-points. Avidity index for serum anti-PSA IgM (A), IgG1 (B), IgG2a/c (C), IgG2b (D) and IgG3 (E) antibodies in samples from 0, 1 and 3 weeks pi was determined by ELISA with chaotropic elution. Results are shown as mean ± SEM. Statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between time-points within a strain by asterisks (*). np: not performed.

with infection time in Balb/c and C57Bl/6 mice, and that normal C57Bl/6 serum shows an intrinsic higher protoscolicidal activity.

3.6. *Transference of normal C57Bl/6 serum to Balb/c mice increases their resistance to secondary CE*

Differences in intrinsic protoscolicidal activity between normal serum from Balb/c and C57Bl/6 mice may not necessarily be determined by differences in natural *E. granulosus* cross-reacting antibodies, but by intrinsic dissimilarities in complement activities. Thus, to assess if natural antibodies play a role in murine differential susceptibility to secondary CE, we performed passive transfer experiments of heat-inactivated serum from normal Balb/c or C57Bl/6 mice into Balb/c recipient mice 24 h before parasite challenge. Results in Fig. 7C show that transfer of naïve C57Bl/6 serum into Balb/c mice resulted in a significant reduction in the number of developed cysts 41 weeks pi. Interestingly, reduction in cyst number was approximately 3-fold (in terms of group median values), greatly resembling the usual difference observed in the number of developed cysts in Balb/c and C57Bl/6 infected mice (Fig. 1A). However, no protection was achieved with homologous serum transference (Fig. 7C). Therefore, our results suggest that natural antibodies recognizing *E. granulosus* antigens do play a role in infection resistance to secondary CE in C57Bl/6 mice.

4. Discussion

Balb/c and C57Bl/6 mice have been regarded as Th2- and Th1-prone strains, respectively, since they are widely known to differ in normal and pathological immune responses. For example, meanwhile C57Bl/6 mice have been shown to be highly susceptible to the experimental induction of organ-specific autoimmune diseases (Graus et al., 1993; Sun et al., 1997; Caspi et al., 1992; Avichezer et al., 2003), Balb/c mice usually display increased susceptibility to spontaneous and induced tumorigenesis (Ullrich et al., 1996; Medina, 1974; Kuraguchi et al., 2001). Furthermore, when infected by the intracellular parasite *Leishmania major*, C57Bl/6 mice develop protective Th1 immune responses while Balb/c mice show non-protective Th2 responses, being therefore resistant and susceptible strains to the infection, respectively (Reiner and Locksley, 1995; Belkaid et al., 2002). Such immune bias has been shown not only to rely on different MHC haplotypes, but also on profound differences in other key immune components. For example, splenic dendritic cells from Balb/c and C57Bl/6 mice differ in their expression level of several TLRs, as well as in the cytokine profile they produce in response to stimulation with microbial ligands (Liu et al., 2002). Moreover, in comparison with C57Bl/6 mice, Balb/c animals have been shown to exhibit higher frequencies of $CD4^+CD25^+$Treg cells in thymus and peripheral lymphoid organs, and their $CD4^+CD25^-$ responder T cells were shown to be more sensitive to $CD4^+CD25^+$Treg suppression (Chen et al., 2005). More recently, it has been shown that blood mast cell progenitors are less mature in C57Bl/6 mice than in Balb/c strain, probably affecting their migratory properties (Dahlin et al., 2013). All these differences would differentially influence the intrinsic susceptibility to diverse infections reported for C57Bl/6 and Balb/c mice.

Murine secondary CE model has been widely used to study not only basic aspects of *E. granulosus* biology and immunology (Baz et al., 2006; Dematteis et al., 1999, 2003; Mourglia-Ettlin et al., 2011a; Cucher et al., 2013), but also to test new chemotherapeutics or therapeutical protocols (Ceballos et al., 2010; Breijo et al., 2011; Cumino et al., 2012), vaccine candidates (Hernández and

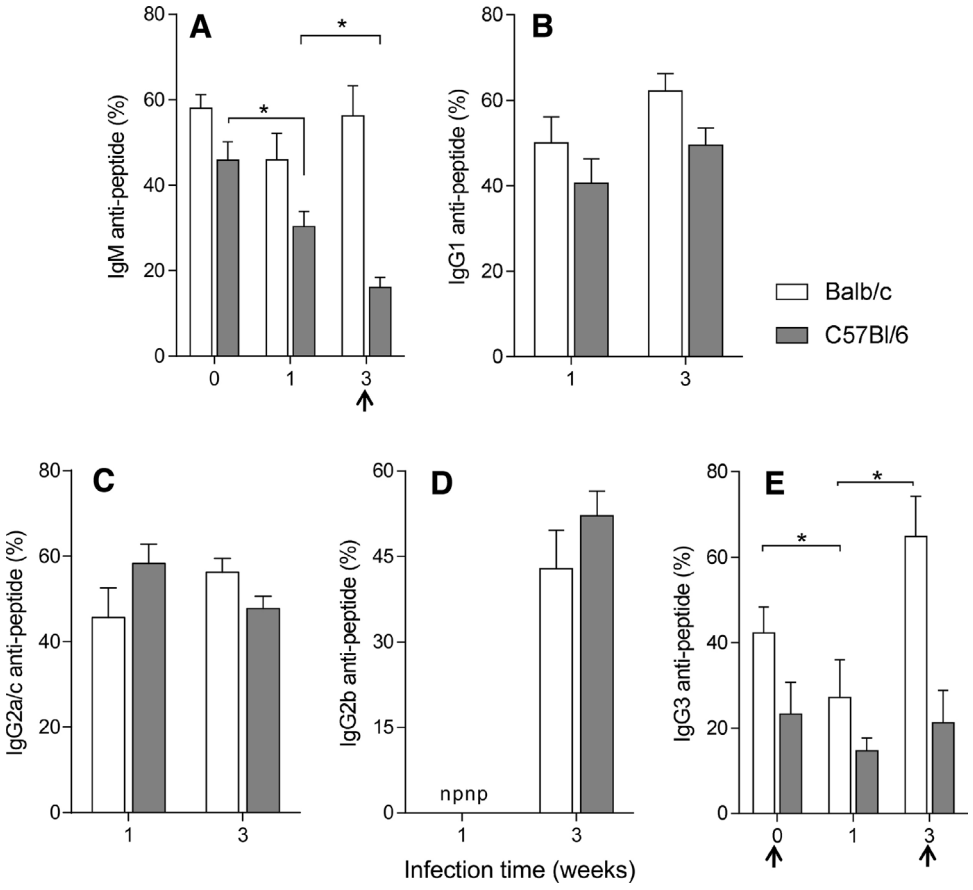

Fig. 4. Peptide epitopes recognition by early systemic antibodies to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 8$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice from both strains were bled prior to infection (0 week) and at different time-points. Recognition percentage of peptide epitopes by serum anti-PSA IgM (A), IgG1 (B), IgG2a/c (C), IgG2b (D) and IgG3 (E) antibodies in samples from 0, 1 and 3 weeks pi was determined by ELISA with periodate treatment. Results are shown as mean ± SEM. Statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between time-points within a strain by asterisks (*). np: not performed.

Nieto, 1994; Hashemitabar et al., 2005; Burgu et al., 2007), and diagnostic or follow-up tools (Denegri et al., 1995; Ferragut et al., 1998; Mamuti et al., 2002). Despite being Balb/c the most widely used mice strain in experimental secondary CE (Siles-Lucas and Hemphill, 2002; Baz et al., 2006), other strains – such as C57Bl/6 – have been utilized as well (Pennoit-De Cooman et al., 1974; Hernández and Nieto, 1994; Baz et al., 1995; Urrea-París et al., 2001; Casado et al., 2001; Cucher et al., 2013). Our results showed that Balb/c mice are more permissive to experimental secondary CE than C57Bl/6 mice, in terms of both number of developed hydatid cysts and parasite load (Fig. 1A and B). These results partially agree with an old report on strain-dependent outcome of murine experimental primary infections with *E. granulosus* oncospheres (Dempster et al., 1991). In that work, Dempster et al., (1991) reported that when *in vitro* activated oncospheres were orally administered, Balb/c mice showed the highest susceptibility to primary infection, while C57Bl/6 was an absolutely refractory strain to infection. Thus, Balb/c mice seem to be highly susceptible to *E. granulosus* independently of the administered parasite stage or inoculation route, while C57Bl/6 mice show less or no susceptibility.

Antibody responses have been shown to play a major role in susceptibility/resistance to parasite infections in several murine settings (McCoy et al., 2008; Gurish et al., 2004; Blackwell and Else, 2001; Rajan et al., 2005; Marcet et al., 2002; Attallah et al., 1999; Inaba et al., 2003; Harris et al., 2006; Herbert et al., 2002; Ligas et al., 2003). The profile of the antibody response mounted by an infected host is crucially important because of the effector functions able to be triggered by each antibody isotype/subclass. Although still not formally shown, host genetic background has been highlighted as an explanation for differential profiles of antibody responses (Garraud et al., 2003). In experimental CE there is no conclusive information about the role of antibody responses on the infection outcome. In the present work, we have evidenced the existence of considerable differences between Balb/c and C57Bl/6 mice strains regarding natural and induced antibodies recognizing *E. granulosus* antigens. Moreover, local (*e.g.*, peritoneal exudates) and systemic (*e.g.*, serum) antibody responses were also shown to differ significantly between mice strains.

Natural antibodies are produced at tightly regulated levels in the complete absence of external antigenic stimulation, and although IgM predominates among them, other isotypes are represented as well (Baumgarth et al., 2005). They provide early protection against pathogens, making them a crucial non-redundant component of the humoral immune system (Baumgarth et al., 2005). An outstanding feature of natural antibodies is their usual polyreactivity, which provides animals with pre-existing broad antibody reactivities that allows them to rapidly recognize and protect against pathogens that have not been encountered previously. Protective roles for natural antibodies have been described in numerous viral, bacterial, fungal, and parasitic infections (Ehrenstein and Notley, 2010; Panda and Ding, 2015). In the present work, we have shown the existence of natural antibodies cross-reacting with *E. granulosus* antigens at the systemic and local levels, but with differences in terms of titer and quality in Balb/c and C57Bl/6 mice. Serum natural IgM, IgG2b and IgG3 antibodies recognizing protoscoleces antigens were detected in both strains, with no significant differ-

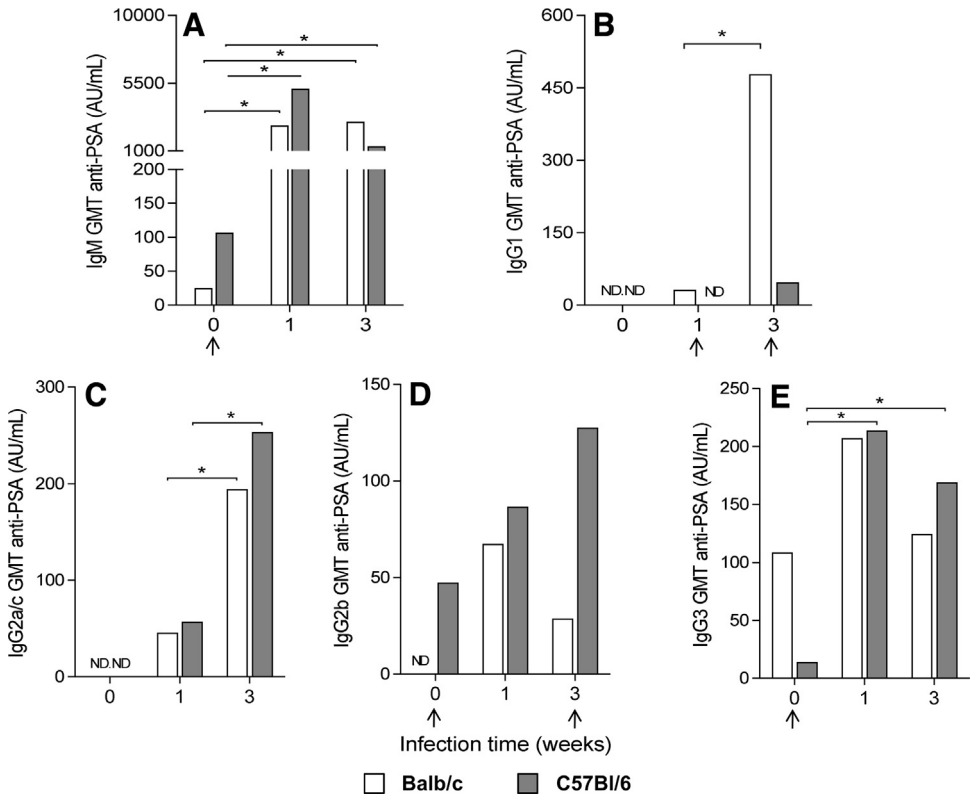

Fig. 5. Kinetics of the early peritoneal antibody response to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 15$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. At 0 (pre-infection), 1 and 3 weeks pi, 5 mice per strain were euthanized and their peritoneal cavities were washed with 1 mL of sterile PBS. Peritoneal anti-PSA IgM (A), IgG1 (B), IgG2a/c (C), IgG2b (D) and IgG3 (E) titers were determined by ELISA, and geometric mean titers (GMT) are shown for Balb/c and C57Bl/6 mice groups. Statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between time-points within a strain by asterisks (*). ND: not detected.

ences in IgG2b titers, but higher IgG3 and IgM titers in C57Bl/6 and Balb/c sera, respectively (Fig. 2A and E). Interestingly, we observed a significant negative correlation between natural IgG2b titers and infection outcome parameters in both mice strains, suggesting a protective role for natural IgG2b antibodies in murine secondary CE (Table 1). In this regard, functional studies on the anti-*E. granulosus* performance of normal sera from Balb/c and C57Bl/6 mice were carried out through different *in vitro* and *in vivo* approaches. Thus, we reported that normal C57Bl/6 serum displayed an intrinsic higher protoscolicidal activity *in vitro* (Fig. 7A), which seemed to be complement-mediated (Fig. 7B). Moreover, when normal heat-inactivated serum from C57Bl/6 mice was transferred into Balb/c animals before parasite challenge, we observed a significant reduction in infection outcome. Interestingly, no such a protection was achieved with transference of normal Balb/c serum (Figure 7C). Therefore, our results suggest that natural antibodies in C57Bl/6 mice cross-reacting with *E. granulosus* antigens could differentially contribute to their higher resistance to secondary CE.

The functions of natural antibodies not only depend on the ability to recognize pathogens, but also on the infection site. In experimental secondary CE, protoscoleces are intraperitoneally inoculated, and therefore, the very first natural antibodies able to interact with *E. granulosus* antigens are those pre-existing locally (*i.e.*, peritoneal natural antibodies). IgM and IgG3 peritoneal natural antibodies recognizing *E. granulosus* antigens were detected in both mice strains, showing the opposite titer relationship respect to serum natural antibodies—IgM was higher in C57Bl/6 and IgG3 in Balb/c mice (Fig. 5A and E). Notably, natural parasite cross-reacting IgG2b was only detected in peritoneal exudates from C57Bl/6 mice (Fig. 5D). Correlation analyses between antibodies in peritoneal exudates and infection outcome parameters could not be performed. However, as previously mentioned, serum natural IgG2b titers negatively correlated with infection outcome (Table 1), and interestingly such natural antibodies were only detected in peritoneal exudates from the most resistant mice strain (*e.g.*, C57Bl/6). This finding would strengthen the putative protective role of natural IgG2b in murine secondary CE. Regarding quality of antibodies in peritoneal exudates, we observed higher IgM avidity indexes in Balb/c mice (Fig. 6A). More interestingly, natural peritoneal IgM and IgG3 antibodies from C57Bl/6 mice were shown to recognize proportionally more peptide epitopes in protoscoleces antigens than those from Balb/c mice (Fig. 6E and H). Usually, m-periodate-resistant epitopes are assumed to represent protein antigens, while m-periodate-sensitive epitopes belong to carbohydrates. It is worth noting that in mice natural IgM and IgG3 preferentially recognize T-independent antigens (Baumgarth et al., 2005; Ehrenstein and Notley, 2010; Panda and Ding, 2015), and that carbohydrates in the surface of *E. granulosus* protoscoleces have been shown to be immunodominant in mice (Míguez et al., 1996). Moreover, we have previously reported that glycoconjugates from protoscoleces induce peritoneal B cells to produce Th2-type cytokines and – mainly – parasite non-specific polyclonal antibodies, suggesting that T-independent antigens could detrimentally polarize the early immune response (Mourglia-Ettlin et al., 2011b). Therefore, our results suggest that in C57Bl/6 mice the recognition bias of peritoneal natural IgM and IgG3 antibodies towards peptide epitopes in *E. granulosus* protoscoleces could render them more resistant to secondary CE.

The contrasts observed between *E. granulosus* cross-reacting natural antibodies from serum and peritoneal exudates within each mice strain might derive from differences in their cellular source. The precise producers of natural antibodies remain unclear;

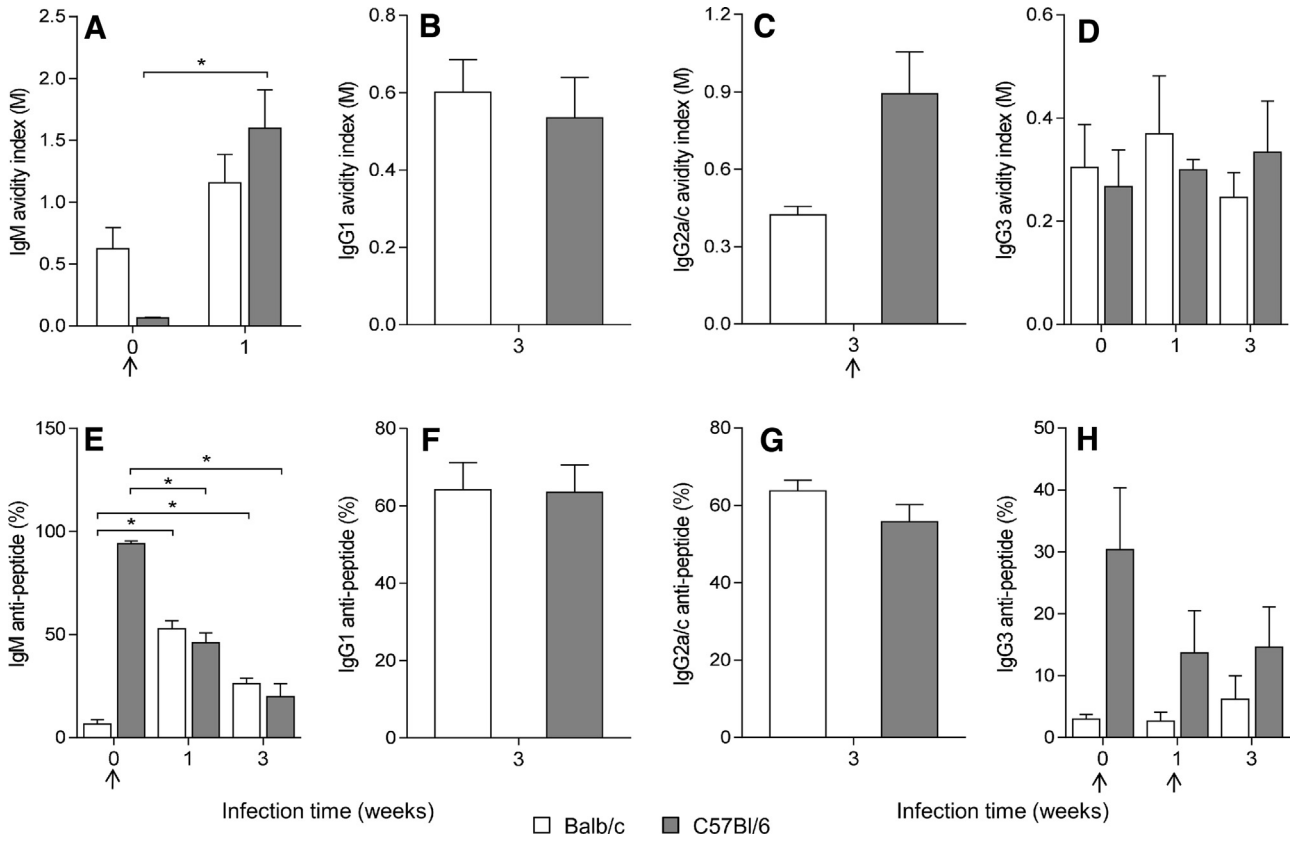

Fig. 6. Quality analyses of early peritoneal antibodies to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 15$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. At 0 (pre-infection), 1 and 3 weeks pi, 5 mice per strain were euthanized and their peritoneal cavities were washed with 1 mL of sterile PBS. Avidity index and recognition percentage of peptide epitopes by peritoneal anti-PSA IgM (A, E), IgG1 (B, F), IgG2a/c (C, G), and IgG3 (D, H) antibodies were determined by ELISA with chaotropic elution and periodate treatment, respectively. Results are shown as mean ± SEM. Statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between time-points within a strain by asterisks (*).

and although peritoneal B1 cells have been repeatedly implicated, Thurnheer et al. (2003) have shown that B1 cells contribute with approximately 50% of serum natural IgM, with B2 cells producing the remainder. Moreover, the compartment and environment where B cells reside have been suggested to govern natural antibodies repertoire and secretion rate (Ehrenstein and Notley, 2010). Therefore, given that B1 cells preferentially reside in the peritoneal cavity while B2 cells circulate systemically, it seems reasonably that the properties of serum and peritoneal natural antibodies differ.

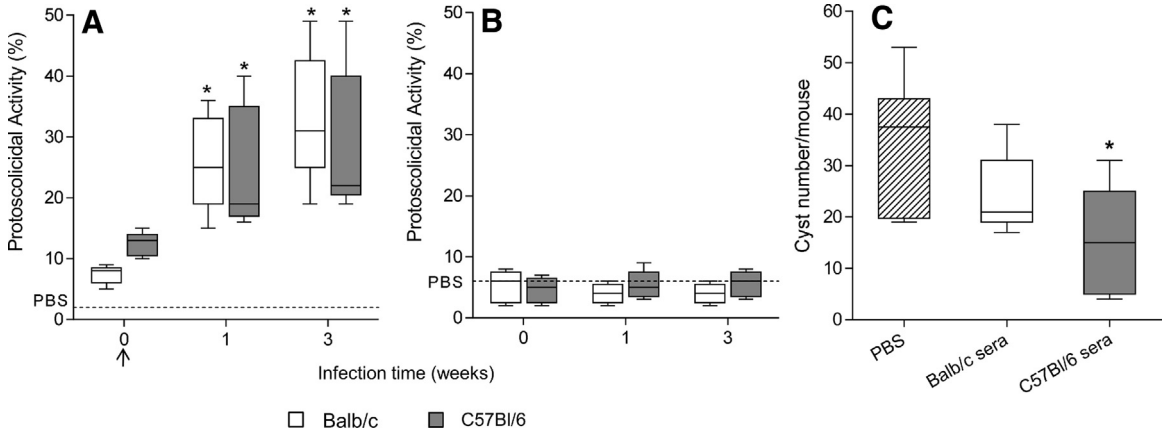

Fig. 7. *In vitro* and *in vivo* anti-parasite activity of Balb/c and C57Bl/6 mice sera.
For *in vitro* serum protoscolicidal activity assessment, Balb/c and C57Bl/6 mice ($n = 15$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. At 0 (pre-infection), 1 and 3 weeks pi, 5 mice per strain were bled and serum protoscolicidal activity was tested by incubating viable protoscoleces in appropriately diluted individual samples. Serum protoscolicidal activity was determined before (A) and after (B) heat-inactivation, and it was expressed as the percentage of non-viable protoscoleces post-incubation. Dashed lines represent mean values from in parallel incubations of protoscoleces with PBS (in sextuplicate). Results are shown in box-and-whiskers plots, and statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between time-points and day 0 by asterisks (*).
For *in vivo* assessment of serum anti-parasite activity, Balb/c mice were ip inoculated with heat-inactivated normal Balb/c or C57Bl/6 sera ($n = 7$ per strain serum), and 24 h later mice were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Balb/c control mice ($n = 10$) were ip inoculated with sterile PBS prior to protoscoleces inoculation. Infection outcome was assessed 41 weeks pi by counting peritoneal cysts (C). (*) Statistical significance ($p < 0.05$) respect to PBS group.

Besides their effector roles directly exerted on pathogens (*i.e.*, classical complement activation, neutralization, antibody-dependent cellular cytotoxicity, *etc.*), natural antibodies also assist in the priming of subsequent adaptive immune responses, linking the innate and adaptive immune systems (Ehrenstein and Notley, 2010; Panda and Ding, 2015). An interesting function assigned to natural antibodies – mainly to natural IgM – is that its presence is required for the development of normal antibody responses (Boes et al., 1998; Ehrenstein et al., 1998; Baumgarth et al., 2000). Such phenomenon can be explained through a broader process called antibody-mediated feedback regulation (Getahun and Heyman, 2006; Heyman, 2014). The outcome of antibody-mediated feedback regulation can either be an almost completely inhibited or a strongly enhanced antibody response, depending mainly on the nature of the antigen and the pre-existing antibody isotype involved (Getahun and Heyman, 2006; Heyman, 2014). Antibody responses to large particulate antigens – such as red blood cells or malaria parasites – are efficiently suppressed by any IgG subclass while enhanced by IgM. Contrarily, antibody responses to soluble protein antigens are enhanced by any IgG subclass while not affected by IgM (Sörman et al., 2014; Heyman, 2014). Our results show that C57Bl/6 mice mount an overall more robust antibody response against antigens from *E. granulosus* protoscoleces than Balb/c mice (Fig. 2). This finding could be at least partially explain by a differential antibody-mediated feedback regulation induced by peritoneal natural antibodies. Protoscoleces are very large particles and the predominance of natural IgM (enhancer) over IgG3 (suppressor) antibodies recognizing protoscoleces antigens in the peritoneal cavity of C57Bl/6 mice, would derive in an overall enhanced antibody response. Conversely, the predominance of peritoneal natural IgG3 over IgM in Balb/c mice would result in a less intense antibody response. These results are in line with the findings of Dai et al., (2009) who reported differences in natural glycan-specific IgM titers between Balb/c and C57Bl/6 mice, and suggested that such differences in pre-existing antibodies could influence the development of induced antibody responses against glycan-containing antigens.

During helminth infections, induced-antibodies are supposed to help in parasite clearance and to limit disease progression. Therefore, it is of outstanding importance for the host to polarize the class and subclass of its induced antibody response towards optimal effector functions against the parasite (Garraud et al., 2003). Our results showed that although both studied mice strains developed isotype-mixed antibody responses against protoscoleces antigens, Balb/c mice (highly susceptible) biased their systemic response towards IgG1, while in C57Bl/6 mice (more resistant) a predominance of mixed IgM/IgG2c/IgG2b/IgG3 was observed (Fig. 2). Moreover, correlation analyses suggested that IgG1 responses would be detrimental for the experimental host independently of its strain (Table 1), and statistical paired-analyses indicated that Balb/c mice – unlike C57Bl/6 counterparts – displayed an early IgG1 avidity maturation (Fig. 3B). IgG1 subclass does not activate the complement cascade and preferentially engages the inhibitory FcγRIIB (Nimmerjahn and Ravetch, 2005, 2006). On the other hand, IgM, IgG2a/c and IgG2b are excellent complement activators, and IgG2a/c and IgG2b have the highest cellular activation/inhibition ratio based on their preferential binding to activating FcγRs (Nimmerjahn and Ravetch, 2005, 2006). Protoscoleces from *E. granulosus* have been shown to be susceptible to the killing by activated macrophages (Jenkins et al., 1990; Dematteis et al., 2003) and the complement system (Ferreira et al., 1992, 2000; Breijo et al., 2008). Therefore, it is consistent that a bias in the induction of IgG1 (even high avidity IgG1) in Balb/c mice would result in a less efficient immune response against *E. granulosus*. Furthermore, the preferential induction of specific antibodies able to activate the complement cascade and to activate cellular responses observed in C57Bl/6 mice could at least partially explain their lower susceptibility to experimental CE.

5. Conclusions

In conclusion, we have here described the existence of differential susceptibility to secondary CE in Balb/c and C57Bl/6 mice, the two most widely used mice strains. While Balb/c mice seem to be highly susceptible to *E. granulosus* infection, C57Bl/6 animals are more resistant. In this regard, significant differences among strains in natural and induced antibodies recognizing *E. granulosus* antigens were described, both at the systemic and peritoneal level. Natural antibodies cross-reacting with *E. granulosus* antigens in C57Bl/6 mice would differentially contribute to their lower susceptibility to secondary CE. In addition, C57Bl/6 mice were shown to mount a more efficient antibody response against *E. granulosus* than their Balb/c counterparts. Altogether, our results suggest that antibody responses do play a role in resistance/susceptibility to murine secondary CE. Studies on other immune mediated mechanisms responsible for such differences in susceptibility are currently being carried out.

Disclosure

There are no potential conflicts of interest relevant to this article to report.

Acknowledgements

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References

Attallah, A.M., Attia, H., El-Nashar, E.M., Nawar, A., Abdel Kader, K., Ismail, H., El-Ebeidy, G., 1999. Induction of resistance against *Schistosoma mansoni* infection by passive transfer of an IgG2a monoclonal antibody. Vaccine 17 (18), 2306–2310.

Avichezer, D., Liou, G.I., Chan, C.C., Lewis, G.M., Wiggert, B., Donoso, L.A., Nickerson, J.M., Crawford, M.A., Caspi, R.R., 2003. Interphotoreceptor retinoid-binding protein (IRBP)-deficient C57BL/6 mice have enhanced immunological and immunopathogenic responses to IRBP and an altered recognition of IRBP epitopes. J. Autoimmun. 21, 185–194.

Azab, M.E., Bishara, S.A., Ramzy, R.M., Oteifa, N.M., El-Hoseiny, L.M., Ahmed, M.A., 2004. The evaluation of HLA-DRB1 antigens as susceptibility markers for unilocular cystic echinococcosis in Egyptian patients. Parasitol. Res. 92 (6), 473–477.

Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L.E., Herzenberg, L.A., Chen, J., 2000. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. J. Exp. Med. 192 (2), 271–280.

Baumgarth, N., Tung, J.W., Herzenberg, L.A., 2005. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Semin. Immunopathol. 26 (4), 347–362.

Baz, A., Hernández, A., Dematteis, S., Carol, H., Nieto, A., 1995. Idiotypic modulation of the antibody response of mice to *Echinococcus granulosus* antigens. Immunology 84 (3), 350–354.

Baz, A., Mourglia-Ettlin, G., Dematteis, S., 2006. Complexity and function of cytokine responses in experimental infection by *Echinococcus granulosus*. Immunobiology 211 (1–2), 3–9.

Belkaid, Y., Piccirillo, C.A., Mendez, S., Shevach, E.M., Sacks, D.L., 2002. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. Nature 420, 502–507.

Blackwell, N.M., Else, K.J., 2001. B cells and antibodies are required for resistance to the parasitic gastrointestinal nematode *Trichuris muris*. Infect. Immun. 69 (6), 3860–3868.

Boes, M., Esau, C., Fischer, M.B., Schmidt, T., Carroll, M., Chen, J., 1998. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. J. Immunol. 160 (10), 4776–4787.

Breijo, M., Anesetti, G., Martínez, L., Sim, R.B., Ferreira, A.M., 2008. *Echinococcus granulosus*: the establishment of the metacestode is associated with control of complement-mediated early inflammation. Exp. Parasitol. 118 (2), 188–196.

Breijo, M., Isnardi, F., Brauer, M., Schenker, R., Ferrari, M., Ferreira, A.M., 2011. An insect growth inhibitor – lufenuron – enhances albendazole activity against hydatid cyst. Vet. Parasitol. 181 (2–4), 341–344.
Burgu, A., Sarimehmetoğlu, O., Gonenc, B., Oge, H., Sahin, G., Aypak, S., 2007. Effects of immunization with purified specific proteins on the formation of secondary hydatid cysts in mice. Dtsch. Tierarztl. Wochenschr. 114 (9), 349–353.
Casado, N., Urrea-París, M.A., Moreno, M.J., Rodríguez-Caabeiro, F., 2001. Combined praziquantel and albendazole chemoprophylaxis in experimental hydatidosis. Parasitol. Res. 87 (9), 787–789.
Caspi, R.R., Grubbs, B.G., Chan, C.C., Chader, G.J., Wiggert, B., 1992. Genetic control of susceptibility to experimental autoimmune uveoretinitis in the mouse model. Concomitant regulation by MHC and non-MHC genes. J. Immunol. 148, 2384–2389.
Ceballos, L., Elissondo, C., Sánchez Bruni, S., Confalonieri, A., Denegri, G., Alvarez, L., Lanusse, C., 2010. Chemoprophylactic activity of flubendazole in cystic echinococcosis. Chemotherapy 56 (5), 386–392.
Chen, X., Oppenheim, J.J., Howard, O.M., 2005. Balb/c mice have more CD4+CD25+T regulatory cells and show greater susceptibility to suppression of their CD4+CD25-responder T cells than C57BL/6 mice. J. Leukocyte Biol. 78, 114–121.
Cucher, M., Mourglia-Ettlin, G., Prada, L., Costa, H., Kamenetzky, L., Poncini, C., Dematteis, S., Rosenzvit, M.C., 2013. *Echinococcus granulosus* pig strain (G7 genotype) protoscoleces did not develop secondary hydatid cysts in mice. Vet. Parasitol. 193 (1–3), 185–192.
Cumino, A.C., Nicolao, M.C., Loos, J.A., Denegri, G., Elissondo, M.C., 2012. *Echinococcus granulosus* tegumental enzymes as in vitro markers of pharmacological damage: a biochemical and molecular approach. Parasitol. Int. 61 (4), 579–585.
Dahlin, J.S., Heyman, B., Hallgren, J., 2013. Committed mast cell progenitors in mouse blood differ in maturity between Th1 and Th2 strains. Allergy 68, 1333–1337.
Dai, H., Zhang, Y., Lv, P., Gao, X.M., 2009. A study on the glycan specificity of natural antibody repertoires in rodents. Cell. Mol. Immunol. 6 (6), 453–459.
Dematteis, S., Baz, A., Rottenberg, M., Fernández, C., Orn, A., Nieto, A., 1999. Antibody and Th1/Th2-type responses in BALB/c mice inoculated with live or dead *Echinococcus granulosus* protoscoleces. Parasite Immunol. 21 (1), 19–26.
Dematteis, S., Rottenberg, M., Baz, A., 2003. Cytokine response and outcome of infection depends on the infective dose of parasites in experimental infection by *Echinococcus granulosus*. Parasite Immunol. 25 (4), 189–197.
Dempster, R.P., Berridge, M.V., Harrison, G.B., Heath, D.D., 1991. *Echinococcus granulosus*: development of an intermediate host mouse model for use in vaccination studies. Int. J. Parasitol. 21 (5), 549–554.
Denegri, G., Perez-Serrano, J., Casado, N., Rodriguez-Caabeiro, F., 1995. [13C]-nuclear magnetic resonance spectral profiles of serum from normal and *Echinococcus granulosus*-infected mice: a kinetic study. Parasitol. Res. 81 (2), 170–172.
Díaz, A., Allen, J.E., 2007. Mapping immune response profiles: the emerging scenario from helminth immunology. Eur. J. Immunol. 37 (12), 3319–3326.
Ehrenstein, M.R., Notley, C.A., 2010. The importance of natural IgM: scavenger, protector and regulator. Nat. Rev. Immunol. 10 (11), 778–786.
Ehrenstein, M.R., O'Keefe, T.L., Davies, S.L., Neuberger, M.S., 1998. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. Proc. Natl. Acad. Sci. U. S. A. 95 (17), 10089–10093.
Ferragut, G., Ljungström, I., Nieto, A., 1998. Relevance of circulating antigen detection to follow-up experimental and human cystic hydatid infections. Parasite Immunol. 20 (11), 541–549.
Ferreira, A.M., Breijo, M., Sim, R.B., Nieto, A., 2000. Contribution of C5-mediated mechanisms to host defence against *Echinococcus granulosus* hydatid infection. Parasite Immunol. 22 (9), 445–453.
Ferreira, A.M., Trecu, T., Reisin, I., 1992. Echinococcus granulosus: study of the in vitro complement activation by protoscoleces by measuring the electric potential difference across the tegumental membrane. Exp. Parasitol. 75 (3), 259–268.
Garraud, O., Perraut, R., Riveau, G., Nutman, T.B., 2003. Class and subclass selection in parasite-specific antibody responses. Trends Parasitol. 19 (7), 300–304.
Getahun, A., Heyman, B., 2006. How antibodies act as natural adjuvants. Immunol. Lett. 104 (1–2), 38–45.
Graus, Y.M., van Breda Vriesman, P.J., de Baets, M.H., 1993. Characterization of anti-acetylcholine receptor (AChR) antibodies from mice differing in susceptibility for experimental autoimmune myasthenia gravis (EAMG). Clin. Exp. Immunol. 92, 506–513.
Gurish, M.F., Bryce, P.J., Tao, H., Kisselgof, A.B., Thornton, E.M., Miller, H.R., Friend, D.S., Oettgen, H.C., 2004. IgE enhances parasite clearance and regulates mast cell responses in mice infected with *Trichinella spiralis*. J. Immunol. 172 (2), 1139–1145.
Harris, N., Gause, W.C., 2011. To B or not to B: B cells and the Th2-type immune response to helminths. Trends Immunol. 32 (2), 80–88.
Harris, N.L., Spoerri, I., Schopfer, J.F., Nembrini, C., Merky, P., Massacand, J., Urban Jr, J.F., Lamarre, A., Burki, K., Odermatt, B., Zinkernagel, R.M., Macpherson, A.J., 2006. Mechanisms of neonatal mucosal antibody protection. J. Immunol. 177 (9), 6256–6262.
Harrison, G.B., Pulford, H.D., Doolin, E.E., Pernthaner, A., Shoemaker, C.B., Hein, W.R., 2008. Antibodies to surface epitopes of the carbohydrate larval antigen CarLA are associated with passive protection in strongylid nematode challenge infections. Parasite Immunol. 30 (11–12), 577–584.
Hashemitabar, G.R., Razmi, G.R., Naghibi, A., 2005. Trials to induce protective immunity in mice and sheep by application of protoscolex and hydatid fluid antigen or whole body antigen of *Echinococcus granulosus*. J. Vet. Med. B Infect. Dis. Vet. Public Health 52 (5), 243–245.
Heath, D.D., 1970. The development of *Echinococcus granulosus* larvae in laboratory animals. Parasitology 60 (3), 449–456.
Herbert, D.R., Nolan, T.J., Schad, G.A., Lustigman, S., Abraham, D., 2002. Immunoaffinity-isolated antigens induce protective immunity against larval *Strongyloides stercoralis* in mice. Exp. Parasitol. 100 (2), 112–120.
Hernández, A., Nieto, A., 1994. Induction of protective immunity against murine secondary hydatidosis. Parasite Immunol. 16 (10), 537–544.
Heyman, B., 2014. Antibodies as natural adjuvants. Curr. Top. Microbiol. Immunol. 382, 201–219.
Inaba, T., Sato, H., Kamiya, H., 2003. Monoclonal IgA antibody-mediated expulsion of *Trichinella* from the intestine of mice. Parasitology 126 (Pt 6), 591–598.
Jenkins, P., Dixon, J.B., Rakha, N.K., Carter, S.D., 1990. Regulation of macrophage-mediated larvicidal activity in *Echinococcus granulosus* and *Mesocestoides corti* (Cestoda) infection in mice. Parasitology 100 (Pt 2), 309–315.
Kiper, N., Gerçeker, F., Utine, E., Yalçin, E., Pekcan, S., Cobanoğlu, N., Aslan, A., Köse, M., Doğru, D., Ozçelik, U., Ozgüç, M., 2010. TAP1 and TAP2 gene polymorphisms in childhood cystic echinococcosis. Parasitol. Int. 59 (2), 283–285.
Kuraguchi, M., Cook, H., Williams, E.D., Thomas, G.A., 2001. Differences in susceptibility to colonic stem cell somatic mutation in three strains of mice. J. Pathol. 193, 517–521.
Ligas, J.A., Kerepesi, L.A., Galioto, A.M., Lustigman, S., Nolan, T.J., Schad, G.A., Abraham, D., 2003. Specificity and mechanism of immunoglobulin M (IgM)- and IgG-dependent protective immunity to larval *Strongyloides stercoralis* in mice. Infect. Immun. 71 (12), 6835–6843.
Liu, Q., Kreider, T., Bowdridge, S., Liu, Z., Song, Y., Gaydo, A.G., Urban Jr, J.F., Gause, W.C., 2010. B cells have distinct roles in host protection against different nematode parasites. J. Immunol. 184 (9), 5213–5223.
Liu, T., Matsuguchi, T., Tsuboi, N., Yajima, T., Yoshikai, Y., 2002. Differences in expression of Toll-like receptors and their reactivities in dendritic cells in BALB/c and C57BL/6 mice. Infect. Immun. 70, 6638–6645.
Mamuti, W., Yamasaki, H., Sako, Y., Nakaya, K., Nakao, M., Lightowlers, M.W., Ito, A., 2002. Usefulness of hydatid cyst fluid of *Echinococcus granulosus* developed in mice with secondary infection for serodiagnosis of cystic echinococcosis in humans. Clin. Diagn. Lab. Immunol. 9 (3), 573–576.
Marcet, R., Díaz, A., Arteaga, E., Finlay, C.M., Sarracent, J., 2002. Passive protection against fasciolosis in mice by immunization with a monoclonal antibody (ES-7MoAb). Parasite Immunol. 24 (2), 103–108.
McCoy, K.D., Stoel, M., Stettler, R., Merky, P., Fink, K., Senn, B.M., Schaer, C., Massacand, J., Odermatt, B., Oettgen, H.C., Zinkernagel, R.M., Bos, N.A., Hengartner, H., Macpherson, A.J., Harris, N.L., 2008. Polyclonal and specific antibodies mediate protective immunity against enteric helminth infection. Cell Host Microbe 4 (4), 362–373.
Medina, D., 1974. Mammary tumorigenesis in chemical carcinogen-treated mice. I. Incidence in BALB-c and C57BL mice. J. Natl. Cancer Inst. 53, 213–221.
Míguez, M., Baz, A., Nieto, A., 1996. Carbohydrates on the surface of *Echinococcus granulosus* protoscoleces are immunodominant in mice. Parasite Immunol. 18 (11), 559–569.
Moro, P., Schantz, P.M., 2009. Echinococcosis: a review. Int. J. Infect. Dis. 13 (2), 125–133.
Mourglia-Ettlin, G., Amezcua-Vesely, M.C., Fraga, R., Baz, A., Merino, M.C., Gruppi, A., Dematteis, S., 2011b. *Echinococcus granulosus* glycoconjugates induce peritoneal B cell differentiation into antibody-secreting cells and cytokine production. Parasite Immunol. 33 (11), 621–631.
Mourglia-Ettlin, G., Marqués, J.M., Chabalgoity, J.A., Dematteis, S., 2011a. Early peritoneal immune response during *Echinococcus granulosus* establishment displays a biphasic behavior. PLoS Negl. Trop. Dis. 5 (8), e1293.
Nimmerjahn, F., Ravetch, J.V., 2005. Divergent immunoglobulin G subclass activity through selective Fc receptor binding. Science 310 (5753), 1510–1512.
Nimmerjahn, F., Ravetch, J.V., 2006. Fcgamma receptors: old friends and new family members. Immunity 24 (1), 19–28.
Panda, S., Ding, J.L., 2015. Natural antibodies bridge innate and adaptive immunity. J. Immunol. 194 (1), 13–20.
Pennoit-De Cooman, E., De Rycke, P.H., Van Outryve, E.J., 1974. Experimental secondary echinococcosis of *Echinococcus granulosus*. V. Quantitative aspects of the infection rate. Tropenmed. Parasitol. 25 (3), 338–344.
Pennoit-De Cooman, E., De Rycke, P.H., 1970. Experimental secondary echinococcosis of *Echinococcus granulosus*. I. Development in different strains of mice. Z. Parasitenkd. 34 (4), 362–370.
Pullen, G.R., Fitzgerald, M.G., Hosking, C.S., 1986. Antibody avidity determination by ELISA using thiocyanate elution. J. Immunol. Methods 86 (1), 83–87.
Rajan, B., Ramalingam, T., Rajan, T.V., 2005. Critical role for IgM in host protection in experimental filarial infection. J. Immunol. 175 (3), 1827–1833.
Reiner, S.L., Locksley, R.M., 1995. The regulation of immunity to *Leishmania major*. Annu. Rev. Immunol. 13, 151–177.
Richards, K.S., Arme, C., Bridges, J.F., 1983. *Echinococcus granulosus* equinus: an ultrastructural study of murine tissue response to hydatid cysts. Parasitology 86 (Pt 3), 407–417.
Siles-Lucas, M., Hemphill, A., 2002. Cestode parasites: application of in vivo and in vitro models for studies on the host–parasite relationship. Adv Parasitol. 51, 133–230.
Siracusano, A., Teggi, A., Ortona, E., 2009. Human cystic echinococcosis: old problems and new perspectives. Interdiscip. Perspect. Infect. Dis. 2009, ID 474368, http://dx.doi.org/10.1155/2009/474368

Sörman, A., Zhang, L., Ding, Z., Heyman, B., 2014. How antibodies use complement to regulate antibody responses. Mol. Immunol. 61 (2), 79–88.
Sun, B., Rizzo, L.V., Sun, S.H., Chan, C.C., Wiggert, B., Wilder, R.L., Caspi, R.R., 1997. Genetic susceptibility to experimental autoimmune uveitis involves more than a predisposition to generate a T helper-1-like or a T helper-2-like response. J. Immunol. 159, 1004–1011.
Thompson, R.C., 2008. The taxonomy, phylogeny and transmission of *Echinococcus*. Exp. Parasitol. 119 (4), 439–446.
Thurnheer, M.C., Zuercher, A.W., Cebra, J.J., Bos, N.A., 2003. B1 cells contribute to serum IgM, but not to intestinal IgA, production in gnotobiotic Ig allotype chimeric mice. J. Immunol. 170 (9), 4564–4571.
Ullrich, R.L., Bowles, N.D., Satterfield, L.C., Davis, C.M., 1996. Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation. Radiat. Res. 146, 353–355.
Urrea-París, M.A., Casado, N., Moreno, M.J., Rodriguez-Caabeiro, F., 2001. Chemoprophylactic praziquantel treatment in experimental hydatidosis. Parasitol. Res. 87 (6), 510–512.
Wiria, A.E., Djuardi, Y., Supali, T., Sartono, E., Yazdanbakhsh, M., 2012. Helminth infection in populations undergoing epidemiological transition: a friend or foe. Semin. Immunopathol. 34 (6), 889–901.
Wojciechowski, W., Harris, D.P., Sprague, F., Mousseau, B., Makris, M., Kusser, K., HonjoT, Mohrs, K., Mohrs, M., Randall, T., Lund, F.E., 2009. Cytokine-producing effector B cells regulate type 2 immunity to *H. polygyrus*. Immunity 30 (3), 421–433.
Woodward, M.P., Young Jr, W.W., Bloodgood, R.A., 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods 78 (1), 143–153.
Yalcin, E., Kiper, N., Tan, C., Ozcelik, U., Dogru, D., Cobanoglu, N., Kose, M., Pekcan, S., Aslan, A.T., Ersoy, F., 2010. The role of human leucocyte antigens in children with hydatid disease: their association with clinical condition and prognosis. Parasitol. Res. 106 (4), 795–800.
Yang, Y., Ellis, M.K., McManus, D.P., 2012. Immunogenetics of human echinococcosis. Trends Parasitol. 28 (10), 447–454.

Capítulo 4

Susceptibility and resistance to Echinococcus granulosus infection: Associations between mouse strains and early peritoneal immune responses

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Susceptibility and resistance to *Echinococcus granulosus* infection: Associations between mouse strains and early peritoneal immune responses

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ABSTRACT

In helminth infections, there are no easy associations between host susceptibility and immune responses. Interestingly, immunity to cestodes – unlike most helminths – seems to require Th1-type effectors. In this sense, we reported recently that Balb/c and C57Bl/6 mice are high and low susceptible strains, respectively, to experimental infection by *Echinococcus granulosus*. However, the role of the early cellular peritoneal response in such differential susceptibility is unknown. Here, we analyzed the kinetics of cytokines expression and cellular phenotypes in peritoneal cells from infected Balb/c and C57Bl/6 mice. Additionally, Principal Components Analysis (PCA) were conducted to highlight the most relevant differences between strains. Finally, the anti-parasite activities of peritoneal cells were assessed through *in vitro* systems. PCAs clustered C57Bl/6 mice by their early mixed IL-5/TNF-α responses and less intense expression of Th2-type cytokines. Moreover, they exhibited lower counts of eosinophils and higher numbers of macrophages and B cells. Functional studies showed that peritoneal cells from infected C57Bl/6 mice displayed greater anti-parasite activities, in accordance with higher rates of NO production and more efficient ADCC responses. In conclusion, mild Th2-responses and active cellular mechanisms are key determinants in murine resistance to *E. granulosus* infection, supporting the cestode immune exception among helminth parasites.

1. Introduction

Helminth parasites are metazoan pathogens characterized by establishing chronic infections and – depending on the species – by causing significant rates of mortality and morbidity. Currently, helminth infections are highly prevalent worldwide and more than 20 species are responsible for human infections (Wiria et al., 2012). Parasite infections and the corresponding host immune responses are products of a prolonged dynamic co-evolution between the host and the parasite. However, although helminth parasites belong to a highly divergent animal group, they usually induce polarized and stereotyped Th2-type immune responses, with rare to no levels of Th1-type components (Díaz and Allen, 2007; Anthony et al., 2007). In this regard, protection against many – but not all – helminths is Th2-type mediated, but their effective immune components can differ between parasite species and different developmental stages of infection within a particular species. Such differences derive from the specific ecological niche occupied by the invading helminth at different stages of its life cycle, including the microenvironment where the parasite resides and the specific host-parasite interactions that subsequently occur at that site (Harris and Gause, 2011).

The cestode parasite *Echinococcus granulosus* is the aetiological agent of cystic echinococcosis (CE), a zoonotic disease of cosmopolitan distribution caused by the infection with the parasite's larval stage (Moro and Schantz, 2009). Primary CE occurs in intermediate hosts (domestic and wild ungulates, accidentally humans) and derives from the ingestion of oncospheres-containing eggs, which later develop into metacestodes or hydatid cysts mainly in the liver and lungs of the infected host. Secondary CE comes about after spillage of protoscoleces (PSC) from a fertile cyst within an already infected intermediate host. This kind of infection results from PSC

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developmental plasticity, which lets them develop into new cysts within intermediate hosts or into adult worms if ingested by a definitive host (usually dogs). The experimental model of secondary infection has been used to study host-parasite interactions, and is based on the intraperitoneal inoculation of viable PSC into immunocompetent mice (Heath, 1970). In Balb/c animals, secondary CE can be divided into two stages: an early stage (until day 20–30 pi) with PSC developing into hydatid cysts (Richards et al., 1983), and a late or chronic stage in which already differentiated cysts grow and eventually become fertile cysts.

Murine models of helminth infections are becoming increasingly important for the elucidation of the immune mechanisms – and the specific effector cells associated – responsible for protection. In this sense, Balb/c and C57Bl/6 mice have been very useful in the study of susceptibility and/or resistance to infections because they have been regarded as Th2- and Th1-prone strains, respectively. However, for helminth infections no absolute rules can be stated regarding their susceptibility/resistance patterns, since they seem to depend on the parasite species studied. For example, while Balb/c and C57Bl/6 mice were shown to be susceptible and resistant strains to *Litomosoides sigmodontis* infection, respectively (Hoffmann et al., 2000); the inverse situation was observed in the infection model by *Heligmosomoides polygyrus* (Filbey et al., 2014). Moreover, in the murine model of cysticercosis by the cestode *Taenia crassiceps*, C57Bl/6 and Balb/c mice have been reported as resistant and susceptible strains, respectively (Terrazas, 2008).

Susceptibility and/or resistance phenomena in human CE have been scarcely studied; having only few reports so far analyzed potential associations between immunological relevant genes and disease prognosis. In this regard, profiles of antibody and cytokine responses have been suggested to determine the parasite success or failure in establishing the infection (Yang et al., 2012). On the other hand, Th1-type responses seem to mediate immunity against *E. granulosus* in murine infections (Rogan, 1998; Dematteis et al., 1999, 2003; Al-Qaoud and Abdel-Hafez, 2008; Mourglia-Ettlin et al., 2011), a fact also suggested for human CE (Hernández-Pomi et al., 1997; Riganò et al., 1999a,b; Amri et al., 2007, 2009). Regarding murine secondary CE, we have recently reported that Balb/c mice are significantly more permissive to infection than C57Bl/6 animals (Mourglia-Ettlin et al., 2016). Moreover, we described significant differences between strains in natural and induced antibodies recognizing *E. granulosus* antigens, suggesting that antibodies play a role in resistance/susceptibility phenomena (Mourglia-Ettlin et al., 2016).

In the present study, we performed a comparative analysis of the early peritoneal immune response in infected mice belonging to low (*e.g.*, C57Bl/6) and high (*e.g.*, Balb/c) susceptibility strains to secondary CE. Cytokine expression profiles as well as cellular phenotypes were determined, and by means of descriptive statistics (*e.g.*, Principal Components Analysis), we identified global immune patterns associated with differential degrees of susceptibility to murine *E. granulosus* infection. Finally, we established the relevance of anti-parasite effector mechanisms mediated by peritoneal cells in the intrinsic susceptibility of mouse strains to secondary CE.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in compliance with Comisión Honoraria de Experimentación Animal (CHEA) from Universidad de la República, according to the Canadian Guidelines on Animal Care and the National Uruguayan Legislation N°18.611. Experimental protocols were approved by the Ethics Committee of Facultad de Química (Universidad de la República) and were given the approval number 101900-001065-11.

2.2. Parasites, mice and infections

PSC from *E. granulosus* were obtained by aseptic puncture of fertile bovine hydatid cysts, and were washed several times with phosphate buffered saline (PBS) pH 7.2 containing gentamicin (40 μg/mL). Parasites viability was determined according to Dematteis et al. (1999). Only those batches with over 95% viability were used. Female Balb/c and C57Bl/6 mice were obtained from DILAVE (Uruguay) and housed at the animal facility of Instituto de Higiene (Montevideo, Uruguay). Experimental infections were performed with 6–8 weeks old mice, which were inoculated by the intraperitoneal route with 200 μL of a PBS suspension containing 2000 viable PSC. Globally, 52 mice were infected (26 from each strain) and 32 mice were used as controls (16 from each strain). The exact number of mice used in each experiment is stated in every experimental description and in each figure legend.

2.3. Peritoneal cells isolation

At different time-points post-inoculation (pi), infected Balb/c and C57Bl/6 mice ($n = 5$ per strain) were sacrificed by cervical dislocation prior inhalatory anesthesia. Uninfected mice ($n = 5$ per strain) were used as control groups (day 0 pi). Peritoneal cavities were extensively washed with cold sterile RPMI 1640 (for cell cultures) or PBS containing 40 μg/mL gentamicin (for mRNA extraction or flow cytometry) supplemented with 2% FCS. Red blood cells were lysed by treatment with RBC Lysing Buffer (Sigma) and remaining leukocytes were counted using Neubauer chamber and trypan blue staining.

2.4. Cytokine expression profiling

Cytokine profiles were assessed by qRT-PCR as previously described (Mourglia-Ettlin et al., 2011). Briefly, RNA extraction was performed using TRIzol® (Invitrogen) and DNA contamination was eliminated by DNase I treatment (Invitrogen) following manufacturer's recommendations. cDNA was then obtained by 1 μg RNA reverse transcription using MMLV-RT (Invitrogen) at 42 °C for 50 min. qPCR reactions were performed using mouse specific primers available under request for TNFα, IFN-γ, IL-2, IL-12_p35, IL-15, IL-4, IL-5, IL-6, IL-10, IL-13 and β-actin, using QuantiTect SYBR Green PCR Kit (QIAGEN) following manufacturer's instructions and 0.9 μM of each specific primer in a Rotor-Gene 6000 (Corbett Life Science). Conditions of cycling reactions were 95 °C for 15 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, followed by a melting curve rising from 72 °C to 90 °C. β-actin was used as a normalizing gene and relative mRNA amounts were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Cytokines expression changes (increase or decrease) were referred to control group values.

2.5. Peritoneal cells phenotyping

Peritoneal cells from 5-days-infected and control Balb/c and C57Bl/6 mice ($n = 5$ in each case) were identified by flow cytometry as previously described (Mourglia-Ettlin et al., 2011). Briefly, cell suspensions were pre-incubated with anti-mouse CD32/CD16 mAb (Mouse Fc Block, Becton Dickinson) for 30 min at 4 °C and then were incubated during 30 min at 4 °C with the following mAb anti-mouse: CD19-PE, CD3-FITC, CD4-PE, CD8-PE, CD49b-PE, F4/80-PE, Gr-1-FITC, Siglec-F-PE, and their specific isotype controls (Pharmingen, Becton Dickinson). Lymphocytes and non-lymphocytes were

identified through their typical FSC and SSC signals, and subpopulations were regarded as B cells ($CD19^+$), T $CD4^+$ cells ($CD3^+CD4^+$), T $CD8^+$ cells ($CD3^+CD8^+$), NK cells ($CD49b^+$), macrophages ($F4/80^{hi}$), neutrophils ($Gr\text{-}1^{hi}$) and eosinophils ($Siglec\text{-}F^+$). Acquisitions were performed using Cell Quest® software (Becton Dickinson) on a FACScalibur flow cytometer (Becton Dickinson). Data analyses were carried out with the free software *Flowing Software v.2.5.1* (www.flowingsoftware.com).

2.6. Principal Components Analysis

In order to identify immune patterns able to differentiate the early peritoneal responses between infected Balb/c and C57Bl/6 mice, we performed Principal Component Analysis (PCA) (Genser et al., 2007; Ringnér, 2008). Previous to conduct the PCA, data from cytokine expression profiles and from peritoneal cell phenotypes (5 days pi in both cases) were converted into matrices. Thus, a matrix of 10 objects (5 mice from each strain) and 10 variables (cytokines fold-changes); and, on the other hand, a matrix of 10 objects (5 mice from each strain) and 9 variables (number of cells in each population) were obtained. Data were scaled when necessary to work with values in the same order of magnitude, and analyses were based on correlations matrices. Factor weights (loadings) for the two Principal Components (PC) showing the largest eigenvalues are shown (Figs. 3 and 4). Loadings >|0.850| were considered the most relevant for the analyses. *STATISTICA® v.10* software was used for performing PCA and visualizing results.

2.7. Assessment of cell-dependent protoscolicidal activity

To determine the protoscolicidal activity of peritoneal cells from normal and infected Balb/c and C57Bl/6 mice, we performed an *in vitro* antibody-dependent cellular cytotoxicity (ADCC) assay according to Veerapathran et al. (2009). Sera used in the assay were pools from normal or 5-days-infected Balb/c or C57Bl/6 mice ($n = 5$ in each case). Briefly, roughly 50 viable PSC were incubated in 200 μL of complete RPMI 1640 (10% heat inactivated FCS, 100 μg/mL streptomycin, 100 U/mL penicillin, 10 mM L-glutamine and 50 μM 2-mercaptoethanol), with 1.5×106 peritoneal cells from individualized normal or infected Balb/c or C57Bl/6 mice ($n = 5$ per strain and condition). In parallel, ADCC activity was assessed in identical co-cultures performed in the presence of diluted (1:4) heat-inactivated homologous sera, matching the infection time of cells and sera. PSC cultured alone in complete RPMI 1640 (8 wells) were used as controls. Incubations were performed in 96-well plates during 48 h at 37 °C and 5% CO_2. Final viability of PSC in culture plates was determined under a light microscope, assuming still viable parasites those (either evaginated or not) without adhered cell clumps and showing normal signs of physical integrity. Results were expressed as the percentage of non-viable PSC in each well.

2.8. Determination of nitrites production

To analyze the spontaneous or parasite-induced production of nitrites by peritoneal cells from normal or infected Balb/c and C57Bl/6 mice, we quantified nitrites in culture supernatants by the Griess reaction (López-Collazo et al., 1998). On the one hand, 1.5×106 peritoneal cells from individualized normal Balb/c and C57Bl/6 mice ($n = 5$ per strain) were cultured in the presence or absence of roughly 50 viable PSC. On the other hand, 1.5×106 peritoneal cells from individualized 5-days-infected (or control) Balb/c and C57Bl/6 mice ($n = 5$ per strain) were cultured alone. Incubations in all cases were performed in 96-well plates with a final volume of 200 μL of complete RPMI 1640 during 48 h at 37 °C and 5% CO_2.

2.9. Statistics

Statistical analyses were assessed by non-parametric Mann–Whitney *U* test, being differences regarded as significant with $p < 0.05$.

3. Results

3.1. Infected Balb/c and C57Bl/6 mice displayed different early peritoneal cytokine profiles

Recently, we have shown that Balb/c and C57Bl/6 mice exhibit different susceptibility degrees to secondary CE, and such dissimilarities were associated with differences in natural and induced antibodies. In this regard, it is well known that the early cytokine profile influences antibody responses against pathogens. Therefore, we first analyzed the kinetics of cytokine expression in peritoneal cells from infected Balb/c and C57Bl/6 mice.

Results in Fig. 1A show the kinetics profile of Th1-type cytokines. In this regard, IL-12, IFN-γ and IL-15 displayed no gross differences between mouse strains (Fig. 1A). IL-12 expression showed a late significant repression in both strains, although it was earlier in Bab/c animals (Fig. 1A). Otherwise, although IFN-γ expression increased over time in both strains, C57Bl/6 mice showed an earlier induction (Fig. 1A). Regarding IL-15 expression, it showed no variation with infection time in C57Bl/6 mice, while only a punctual induction 3 days pi was observed in Balb/c animals (Fig. 1A). On the contrary, among Th1-type cytokines, TNF-α and IL-2 showed very different expression profiles in Balb/c and C57Bl/6 mice (Fig. 1A). While in Balb/c mice TNF-α expression was early and steadily repressed, in C57Bl/6 animals a sharp expression increase was observed 1 day pi (Fig. 1A). Regarding IL-2, Balb/c mice – unlike C57Bl/6 animals – showed a sustained induction since day 3 pi (Fig. 1A).

On the other hand, results in Fig. 1B show the kinetics profile of Th2-type cytokines. In this case, IL-6, IL-4 and IL-13 showed broadly similar behaviors between mouse strains. In fact, IL-6 expression displayed identical expression profiles in Balb/c and C57Bl/6 animals, both in terms of kinetics and intensity (Fig. 1B). Regarding IL-4 and IL-13, expression levels increased later in time, reaching Balb/c mice significantly higher values than C57Bl/6 animals (Fig. 1B). Interestingly, IL-5 and IL-10 expression displayed very different profiles between mouse strains (Fig. 1B). In C57Bl/6 mice, IL-5 expression showed an early and steady increase over time, whereas in Balb/c animals it significantly increased only at day 7 pi (Fig. 1B). On the contrary, unlike C57Bl/6 mice, IL-10 expression in Balb/c mice significantly increased since day 1 pi and showed steady values throughout the analyzed timeframe (Fig. 1B).

In summary, remarkable differences in cytokine expression profiles were observed in peritoneal cells from infected Balb/c and C57Bl/6 mice, both in terms of kinetics and intensity.

3.2. Peritoneal cell populations differ between Balb/c and C57Bl/6 mice in normal and infected individuals

In murine secondary CE, parasite establishment is a peritoneal phenomenon. Therefore, we characterized the cellular populations in the peritoneal cavity of infected Balb/c and C57Bl/6 mice. Since results on cytokine expression kinetics during early infection showed gross differences between mouse strains at day 5 pi (7 out of 10 studied cytokines), we focused on the analysis of the peritoneal cellular composition in 5-days infected mice (Fig. 2).

Regarding normal mice, interestingly differences were found for some cell populations between strains. C57Bl/6 animals showed roughly 2-fold higher counts of T $CD3^+CD4^+$ ($p = 0.0159$) and $CD3^+CD8^+$ cells ($p = 0.0079$) than Balb/c mice (Fig. 2A). On the con-

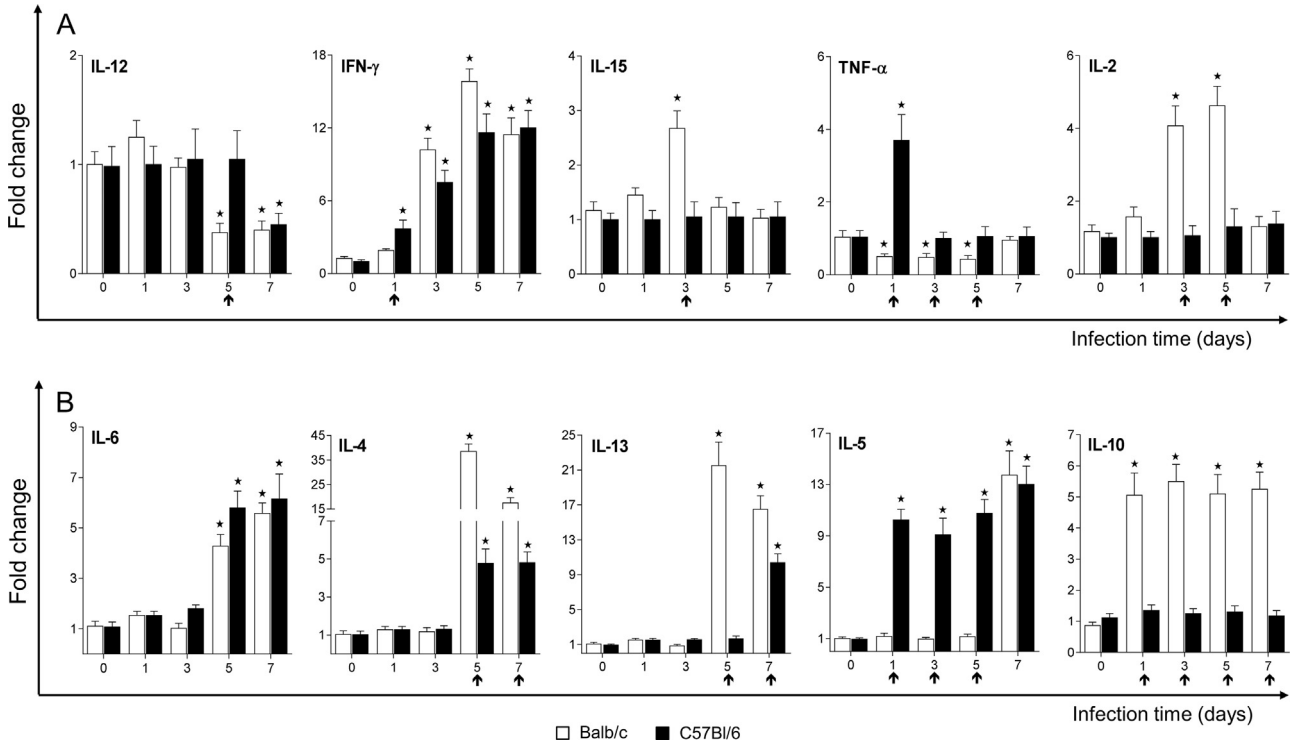


Fig. 1. Balb/c and C57Bl/6 mice exhibit different peritoneal cytokine profiles during early infection. Balb/c and C57Bl/6 mice ($n = 16$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice ($n = 4$ per strain) were sacrificed at 1, 3, 5 and 7 days pi and their total peritoneal cells were recovered. Uninfected mice ($n = 6$ per strain) were used for reference (day 0 pi, pre-infection). qRT-PCR was performed using specific primers for murine Th1-type (A) and Th2-type (B) cytokines. Relative mRNA levels were expressed respect to pre-infection group. Results are shown as mean ± SEM of fold-changes. Statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between time points and day 0 pi by asterisks (*).

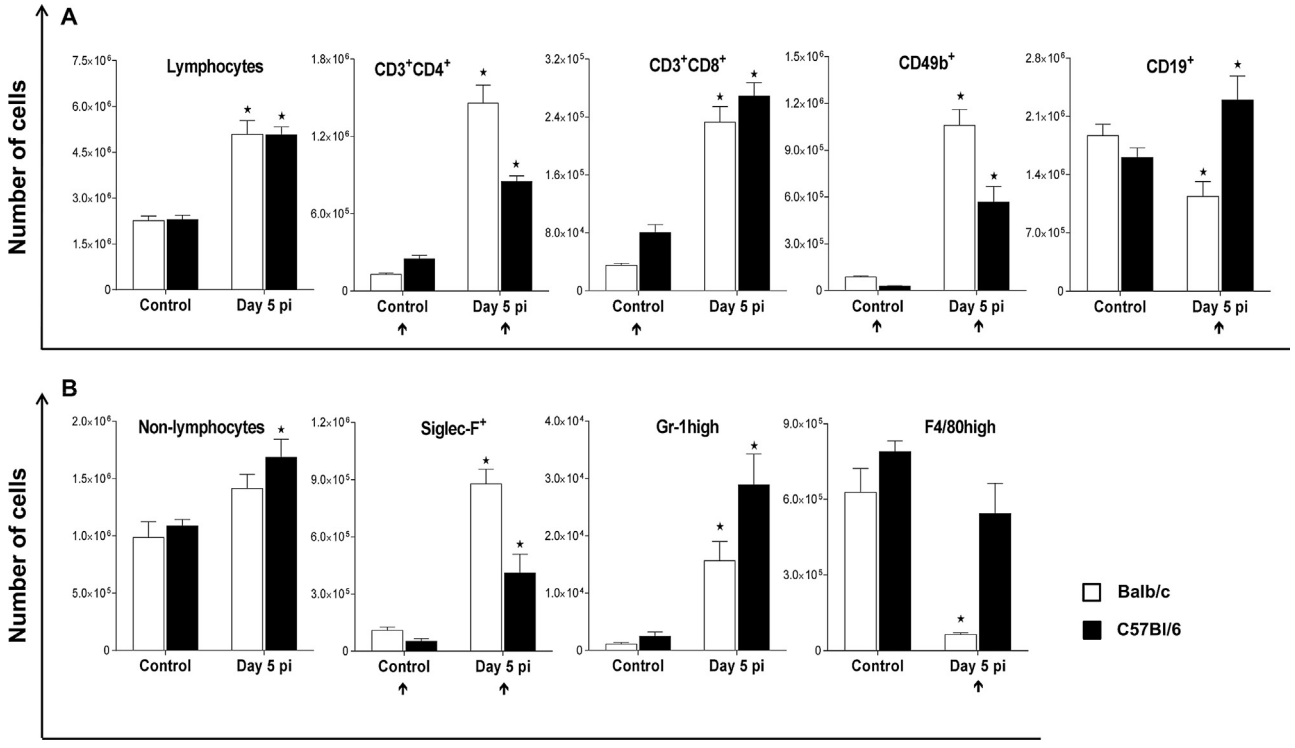
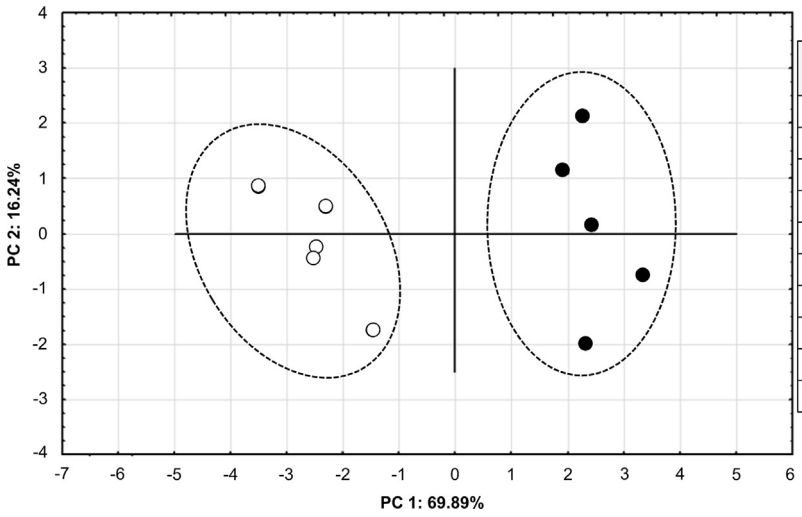
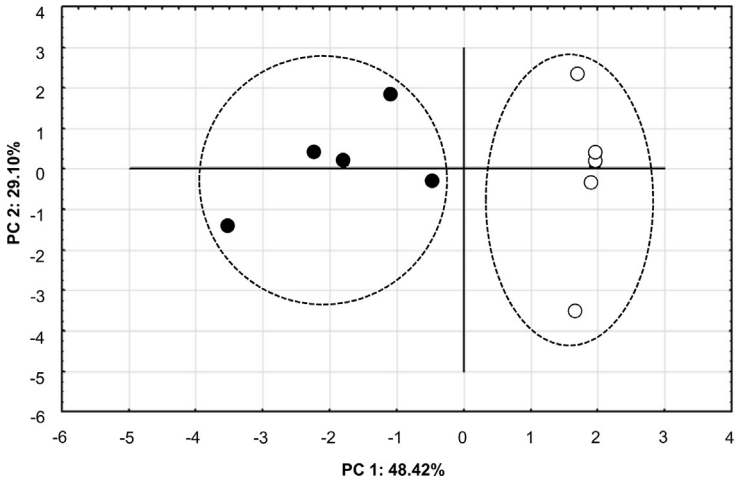


Fig. 2. Normal and 5-days-infected Balb/c and C57Bl/6 mice show differences in peritoneal cell populations. Balb/c and C57Bl/6 mice ($n = 5$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice were sacrificed 5 days pi and their peritoneal lymphocytes (A) and non-lymphocytes (B) were analyzed by flow cytometry. Uninfected mice ($n = 5$ per strain) were used for reference (day 0 pi, pre-infection). Results are shown as mean ± SEM of cell numbers. Statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between infected and uninfected mice within a strain by asterisks (*).



Variable	PC1 loadings	PC2 loadings
IL-12	0.769400	-0.507083
TNF-α	***0.864675***	0.033813
IL-15	-0.123266	***-0.986745***
IL-2	***-0.929406***	-0.228010
IFN-γ	-0.833098	0.402432
IL-4	***-0.983100***	-0.057822
IL-5	***0.946401***	0.177366
IL-6	0.725130	-0.192680
IL-10	***-0.853517***	-0.325840
IL-13	***-0.979790***	0.000474

Fig. 3. Clustering of infected Balb/c and C57Bl/6 mice through Principal Component Analysis of their peritoneal cytokine profiles 5 days pi. Studies of PCA were performed on a matrix of peritoneal cytokine expression: 10 objects (5 mice from each strain) and 10 variables (fold-changes of 10 different cytokines). Factor loadings for the two PCs with the largest eigenvalues are shown, and the most relevant for the study (those >|0.850|) are written in italics and bold. Balb/c and C57Bl/6 mice are depicted as white and black circles, respectively.



Variable	PC1 loadings	PC2 loadings
Lymphoid cells	-0.141955	***-0.975698***
$CD19^+$	***-0.903302***	-0.384612
$CD49b^+$	0.812464	-0.477872
$CD3^+CD4^+$	0.789594	-0.553239
$CD3^+CD8^+$	-0.528953	-0.458091
Non-lymphoid cells	-0.475907	-0.733310
$F4/80^+$	***-0.940619***	-0.173284
$Gr\text{-}1^+$	-0.330082	0.109321
$Siglec\text{-}F^+$	***0.859068***	-0.441226

Fig. 4. Clustering of infected Balb/c and C57Bl/6 mice through Principal Component Analysis of their peritoneal cell populations 5 days pi. Studies of PCA were performed on a matrix of peritoneal cell populations: 10 objects (5 mice from each strain) and 9 variables (cell number of 9 different populations). Factor loadings for the two PCs with the largest eigenvalues are shown, and the most relevant for the study (those >|0.850|) are written in italics and bold. Balb/c and C57Bl/6 mice are depicted as white and black circles, respectively.

trary, peritoneal cells from normal Balb/c mice displayed 3- and 2-fold higher values of NK cells ($p = 0.0079$, Fig. 2A) and eosinophils ($p = 0.0317$, Fig. 2B) than normal C57Bl/6 mice, respectively. Such contrasts in peritoneal cell composition between normal Balb/c and C57Bl/6 mice could differentially condition the development of the early local immune response against *E. granulosus*.

In fact, important differences between 5-days-infected Balb/c and C57Bl/6 mice were observed in several populations. Regarding the lymphoid compartment, although both mouse strains significantly increased counts of NK cells, T $CD3^+CD4^+$ and T $CD3^+CD8^+$ cells, Balb/c mice reached approximately 2-fold higher values of NK cells ($p = 0.0159$) and T $CD3^+CD4^+$ cells ($p = 0.0079$) than C57Bl/6 animals (Fig. 2A). Interestingly, remarkable differences were observed in the B cell compartment in infected mice, displaying significant increases and decreases in C57Bl/6 and Balb/c mice, respectively (Fig. 2A). Thereby, 5-days-infected C57Bl/6 mice showed roughly 2.5-fold higher counts in B cells than their Balb/c counterparts ($p = 0.0079$, Fig. 2A). On the other hand, only infected C57Bl/6 mice significantly increased counts of non-lymphoid cells roughly 1.5-fold respect to baseline values (Fig. 2B). In this regard, although eosinophils as well as neutrophils counts significantly increased in both strains, Balb/c mice reached roughly 2.5-fold higher values of eosinophils than C57Bl/6 animals ($p = 0.0159$, Fig. 2B). Interestingly, only Balb/c mice showed variations in the macrophage compartment, displaying a sharp counts decrease of approximately 10-fold respect to normal mice (Fig. 2B). Thus, 5-days-infected C57Bl/6 mice showed roughly 8.5-fold higher counts of macrophages than their Balb/c counterparts ($p = 0.0079$, Fig. 2B).

Overall, important differences were observed in the population composition of peritoneal cells from normal as well as from 5-days-infected Balb/c and C57Bl/6 mice.

3.3. *Clustering of Balb/c and C57Bl/6 infected mice through Principal Components Analysis of immunological parameters*

Since our main goal was to look broadly at immune profiles in infected Balb/c and C57Bl/6 mice in order to find differences in their early peritoneal immune responses, we performed Principal Components Analysis (PCA) to determine if any linear combination of cytokine expression profile or peritoneal cell populations might be

useful in differentiating both mouse strains. PCA is an algorithm of descriptive statistics commonly used to reduce the number of attributes used to represent a data set in order to identify patterns that stand out their similarities and differences. The procedure uses orthogonal transformations to convert the observations, which are presented as functions of possibly correlated variables, into a set of values of linearly uncorrelated variables called Principal Components (PC). We constructed two different matrices for PCA: one for cytokines fold-changes and another one for peritoneal cell populations, in both cases with data from 5-days-infected Balb/c and C57Bl/6 mice.

Firstly, PCA from the cytokine expression matrix yielded seven PCs, from which PC1 and PC2 accounted together for 86.13% of the total variability among individuals, and the biplot of PC1 *vs*. PC2 showed that Balb/c mice clustered away from C57Bl/6 individuals (Fig. 3). Accounting for 69.89% of the total variability, PC1 explains differences between mouse strains along the horizontal axis in the scoring plot. Analysis of the loading factors of PC1 showed that this component includes most of the cytokines studied, with IL-5 and TNF-α exhibiting the greatest positive loadings; and IL-4, IL-13, IL-2 and IL-10 the greatest negative loadings (Fig. 3). Accordingly, in moving from left to right in the biplot in Fig. 3, C57Bl/6 infected mice are those expressing the largest fold-changes in IL-5 and TNF-α, and the smallest fold-changes in IL-4, IL-13, IL-2 and IL-10. On the other hand, PC2 accounted for 16.24% of the total variability and mainly discriminates between individuals within a mouse strain, having IL-15 the greatest loading factor (Fig. 3).

Secondly, PCA from the matrix of peritoneal cell populations yielded nine PCs, from which PC1 and PC2 accounted together for 77.52% of the total variability between individuals, and the biplot of PC1 *vs*. PC2 showed that again Balb/c mice clustered away from C57Bl/6 individuals (Fig. 4). Accounting for 48.42% of the total variability, PC1 explains differences among mouse strains along the horizontal axis in the scoring plot. Analysis of the loading factors of PC1 showed that this component includes several of the cell populations studied, with eosinophils exhibiting the greatest positive loadings; and macrophages and B cells the greatest negative loadings (Fig. 4). Accordingly, in moving from right to left in Fig. 4, C57Bl/6 infected mice are those with the highest counts of macrophages and B cells, and the lowest counts of eosinophils. On the other hand, PC2 accounted for 29.10% of the total variability and mainly discriminates among individuals within a mouse strain, having total lymphocytes the greatest loading factors (Fig. 4).

In summary, our PCA studies suggest that the more resistant mouse strain to secondary CE (*e.g.*, C57Bl/6 mice) develops an early mixed IL-5/TNF-α response and an overall less intense Th2-type cytokine response, with higher counts of peritoneal macrophages and B cells than the more susceptible strain (*e.g.*, Balb/c mice).

3.4. *Peritoneal cells from infected C57Bl/6 mice – unlike Balb/c littermates – exhibit potent anti-parasite activities*

Activated peritoneal macrophages have been shown to exert protoscolicidal activity *in vitro*, which has been attributed to the production of reactive nitrogen species. Based on our results on significant differences in peritoneal cell populations and cytokine profiles between Balb/c and C57Bl/6 mice, we next analyzed the *in vitro* protoscolicidal activity of peritoneal cells from normal and 5-days-infected animals. Moreover, in order to assess the possible contribution of ADCC mechanisms to parasite killing, we performed such assays in the absence or presence of homologous heat-inactivated serum either from normal or 5-days-infected mice. Examples of live (Fig. 5A–C) and dead/damaged (Fig. 5D, E) PSC are shown.

Results in Fig. 5F show that peritoneal cells from normal Balb/c and C57Bl/6 mice display significant and similar protoscolicidal activity. Interestingly, when normal peritoneal cells from both strains were co-cultured with viable PSC, an also alike production of nitrites was observed (Fig. 5G), which could at least partially explain the similarity in protoscolicidal activity of normal peritoneal cells. Moreover, the addition of homologous normal serum

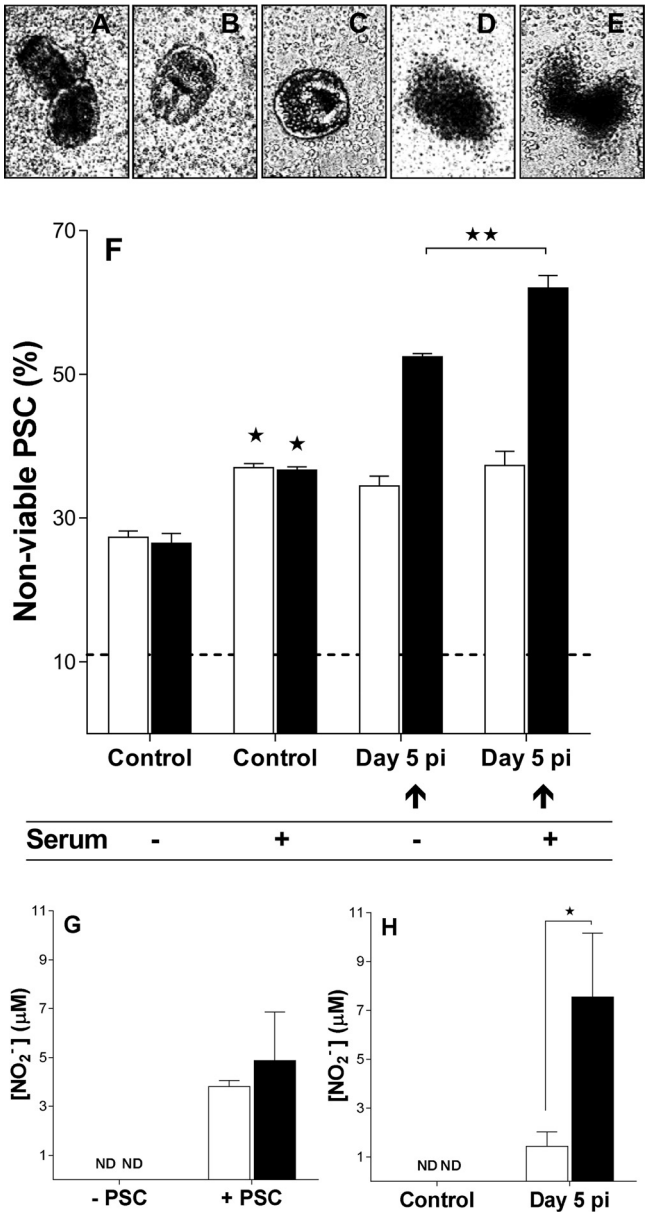


Fig. 5. *In vitro* protoscolicidal activity of peritoneal cells from normal and 5-days-infected Balb/c and C57Bl/6 mice. Balb/c and C57Bl/6 mice ($n = 5$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice were sacrificed 5 days pi and their peritoneal cells were recovered. Uninfected mice ($n = 5$ per strain) were used as controls. Cells were co-cultured with highly viable PSC during 48 h, and then PSC viability was determined under a light microscope. Examples of viable (A–C) and dead/damaged (D and E) PSC are shown. In parallel, co-cultures were performed in presence of homologous and infection-time-matched sera. Dashed line represents the mean value of PSC viability after incubation in complete medium alone. Results on parasite viability are expressed as mean ± SEM of the percentage of non-viable PSC (F). Secretion of nitrites by peritoneal cells was determined by the Griess reaction in 48 h-culture supernatants. Parasite-induced NO production was analyzed through co-cultures of peritoneal cells from uninfected mice with highly viable PSC (G). Infection-induced NO production was studied through incubation of peritoneal cells from 5-days-infected mice in complete medium alone (H). Results are shown as mean ± SEM of nitrites concentration. Statistical significance ($p < 0.05$) between strains is indicated by arrows (↑), and between infected and uninfected mice within a strain by asterisks (*).

to co-cultures significantly increased the protoscolicidal activity observed in both mouse strains (Fig. 5F), suggesting that natural antibodies cross-reacting with PSC antigens could play a role in the early anti-parasite response in murine secondary CE through ADCC mechanisms.

On the other hand, when co-cultures were performed with peritoneal cells from 5-days-infected mice, C57Bl/6 peritoneal cells showed a significantly higher anti-parasite activity than Balb/c animals ($p = 0.0079$, Fig. 5F). Such higher protoscolicidal activity could at least be partially explained by the higher levels of spontaneous nitrites secretion into the cultures by cells from 5-days-infected C57Bl/6 mice (Fig. 5H). In fact, peritoneal cells from infected C57Bl/6 mice secreted *in vitro* roughly 7-fold higher amounts of nitrites (in terms of group medians) than their Balb/c counterparts ($p = 0.0317$, Fig. 5H). Interestingly, addition of homologous and infection-time-matched serum to co-cultures of peritoneal cells from infected mice significantly increased anti-parasite activity only in C57Bl/6 co-cultures ($p = 0.0079$, Fig. 5F). This finding suggested that the very early antibody response induced in C57Bl/6 mice is unique in enhancing ADCC mechanisms against PSC of *E. granulosus*.

In summary, our results suggest that the differences in nitrites production by peritoneal cells and the ability of early-induced antibodies in triggering ADCC responses, could differentially contribute to susceptibility/resistance to secondary CE in Balb/c and C57Bl/6 mice.

4. Discussion

The host genetic background is known to influence the outcome of infectious diseases, and helminth infections are not an exception. Global patterns of murine susceptibility/resistance to helminth infections cannot be concluded, since they seem to depend on each parasite species. For example, while the intestinal nematodes *H. polygyrus* and *Trichuris muris* establish chronic infections in most mouse strains and induce Th2-type responses, Balb/c mice are more resistant to infection than C57Bl/6 animals (Maizels and Yazdanbakhsh, 2003; Patel et al., 2009). Interestingly, C57Bl/6 mice were shown to be resistant to *L. sigmodontis* – a nematode not strictly gastrointestinal – suggesting that the parasite can be killed by Th1-type responses (Saeftel et al., 2003). Thus, Th2-type responses are critical determinants in the outcome of nematode infections, but with different dynamics depending on the stage of the parasite and strain of the host (Reyes et al., 2009). Regarding infections by cestodes – the less studied class of helminths – available data is consistent with the general helminth literature in that they exhibit a strong propensity to drive Th2-type responses (Jenkins and Allen, 2010). However, protective immunity against cestodes seems to require Th1 cells (Emery et al., 1997; Rogan, 1998; Rodriguez-Sosa et al., 2002; Dematteis et al., 2003; Gottstein et al., 2006; Baz et al., 2006; Alonso-Trujillo et al., 2007; Terrazas, 2008; Jenkins and Allen, 2010).

Recently, our group reported that in the model of secondary CE, Balb/c and C57Bl/6 mice behave as high and low susceptible strains to *E. granulosus* infection, respectively (Mourglia-Ettlin et al., 2016). In the present work, we have shown that C57Bl/6 mice – unlike Balb/c animals – develop an early mixed IL-5/TNF-α response and an overall less intense Th2-type cytokine response at the peritoneal level. Indeed, infected C57Bl/6 mice displayed large fold-changes in IL-5 and TNF-α expression, with small (IL-4 and IL-13) or no (IL-10) variations in key Th2-type cytokines. These results are in accordance with previous reports suggesting that Th2-type responses are associated with susceptibility to *E. granulosus* infection in mice (Rogan, 1998; Dematteis et al., 1999, 2003) as well as in humans (Hernández-Pomi et al., 1997; Riganò et al., 1999a,b, 2001, 2004). In fact, we previously reported that the initial Th1-type response in the peritoneal cavity of infected Balb/c mice becomes Th2-biased by day 5 pi, suggesting that the blockade of possible early efficient immune mechanisms allows PSC to survive and to develop into hydatid cysts (a more immune-resistant parasite stage) (Mourglia-Ettlin et al., 2011). In addition, the forced production of the Th1-driving cytokine IL-12 in infected Balb/c mice was shown to induce protective immune mechanisms in the host (Al-Qaoud and Abdel-Hafez, 2008). Interestingly, unlike the case of most helminth parasites, immunity against cestodes seems to require Th1-type responses for inducing protection, since murine susceptibility to infection by *T. crassiceps* (Rodriguez-Sosa et al., 2002; Terrazas, 2008) or *E. multilocularis* (Emery et al., 1997; Vuitton, 2003) is also dependent on Th2-type responses.

Through PCA on peritoneal cytokine expression profiles induced by infection we have here shown that C57Bl/6 mice remarkably differed from Balb/c animals in their up-regulation of TNF-α and IL-5. In this regard, both cytokines have proved to be relevant in immunity against helminth parasites. In fact, Artis et al. (1999) revealed that TNF-α plays a critical role in the regulation of Th2-type cytokines. In that work, the authors showed that *in vivo* blockade of TNF-α in normally resistant mice to *T. muris* significantly delayed worm expulsion without altering IL-4, IL-5, or IL-13 production in the draining lymph node. Moreover, IL-13-mediated worm expulsion was also shown to be TNF-α dependent, and could be enhanced by administration of recombinant TNF-α (Artis et al., 1999). On the other hand, while no differences in *L. sigmodontis* survival were found between IL-5-deficient and C57BL/6 mice, IL-5 genetically deficient animals – unlike wild type littermates – were unable to generate a protective immune response when vaccinated with irradiated larvae (Le Goff et al., 2000). Interestingly, in the same infection model, IL-5 was shown to act synergistically with IFN-γ in reducing worm loads, and macrophage production of TNF-α was shown to be reduced in the absence of IL-5 and/or IFN-γ (Saeftel et al., 2003). In this regard, our results with the model of secondary CE showed a similar interdependency between IL-5, IFN-γ and TNF-α expression, since infected C57Bl/6 mice showed an early induction of IL-5 and IFN-γ with increased levels of TNF-α, while in infected Balb/c mice the early increase in IFN-γ expression without induction of IL-5 derived in repressed TNF-α expression. Therefore, peritoneal levels of TNF-α and IL-5 could be suggested as determining factors in susceptibility to murine secondary CE.

Preferential expression of immunoregulatory or immunosuppressive cytokines – like IL-10 – has been largely associated to host susceptibility in several helminth infections (Redpath et al., 2014), and particularly in echinococcosis—either alveolar or cystic (Vuitton, 2003; Baz et al., 2006). In human as well as in murine infections by *Echinococcus* spp., immunosuppressive effects of IL-10 have been related mainly to decreased production of NO (Godot et al., 2003; Amri et al., 2009) and to expansion of Treg cells (Nono et al., 2012; La et al., 2015). For example, Amri et al. (2009) showed that protoscolicidal activity of PBMC from healthy donors or CE patients is reduced by IL-10, and that such reduction correlated with a decrease in NO production. Accordingly, administration of IFNα-2a to mice infected with *E. multilocularis* decreased the high production of IL-10 by peritoneal macrophages and restored their oxidative metabolism (Godot et al., 2003). Interestingly, 75% of IFNα-2a-treated mice exhibited no hepatic lesions and 50% were fully protected, suggesting that IL-10 production is a susceptibility determinant in alveolar echinococcosis (Godot et al., 2003). On the other hand, E/S products from *E. multilocularis* metacestode vesicles induce high levels of IL-10 on naive murine DCs, which upon incubation with naive T cells led to a significant expansion of $Foxp3^+$ regulatory T cells *in vitro* (Nono et al., 2012). In this sense, results derived from the murine model of alveolar echinococcosis showed that engagement of PD-1 on Treg cells with PDL-1 on DCs promoted the secretion of IL-10 and TGF-β, which correlated with inhibition of T cell effector functions (La et al., 2015). Therefore, induction of

IL-10 is assumed to fulfill an important role for parasite persistence in echinococcosis. In this regard, our noteworthy results on IL-10 expression – Balb/c mice exhibited a significant and steady induction of IL-10 while C57Bl/6 mice did not change its baseline values – suggest that early IL-10 levels could be also a determining factor in susceptibility to murine secondary CE.

In addition to the cytokine patterns differentiating infected mice with low and high susceptibility to secondary CE, our PCA results on peritoneal populations highlighted that C57Bl/6 mice exhibited higher numbers of macrophages and B cells with lower counts of eosinophils than Balb/c animals. Regarding eosinophils, although shown to be potent effector cells for the killing of helminth parasites *in vitro*, their *in vivo* role has been more difficult to establish (Meeusen and Balic, 2000; Klion and Nutman, 2004). Indeed, Meeusen and Balic (2000) suggested that eosinophils do play a role in the killing of infective larval stages, but not in adult helminth parasites. In cestode infections, the metacestodes are particularly potent inducers of eosinophilia; however not only recruitment is important but also the activity of eosinophils may contribute to infection resistance (Makepeace et al., 2012). In this sense, a cysteine protease secreted by the metacestode of *E. multilocularis* was shown to degrade host eotaxin, preventing the infiltration of eosinophils into the peritoneal cavity of infected mice (Mejri and Gottstein, 2009). In the murine model of secondary CE using a mouse strain deficient in the complement component C5, higher levels of parasite establishment and larger cysts were observed than in wild type littermates, and those differences were associated with reduced levels of eosinophil infiltration in the C5-deficient strain (Ferreira et al., 2000). Thus, *Echinococcus* spp. may have evolved immune evasion strategies to limit eosinophil degranulation, as it has been demonstrated that recombinant human ECP can damage PSC of *E. granulosus in vitro* (Ramos et al., 2006). However, in spite of this data, eosinophilia is actually associated with an increased risk of recurrent infection with *E. granulosus* in humans (Hernández-Pomi et al., 1997). Therefore, our results on eosinophil recruitment in C5-sufficient mice – the more resistant strain exhibited lower eosinophil counts – better mimicked the odd human situation and suggest that an excessive local infiltrate of eosinophils may be associated with less efficient responses against *E. granulosus*.

In helminth infections, macrophages have been suggested to play crucial roles in the immune response, as they can initiate, modulate and be final effector cells. In this regard, although the number of macrophages have been shown to increase in the helminth-surrounding tissue – either through recruitment of blood precursors or through proliferation of resident macrophages – reports on their recruitment/proliferation in cestode infection models are virtually absent (Reyes and Terrazas, 2007; Rückerl and Allen, 2014). Indeed, few reports have dealt with macrophages in cestode infections, and as far as we know, none of them describes their relative or absolute number in the helminth-surrounding tissue (Reyes and Terrazas, 2007; Rückerl and Allen, 2014). Our results on murine secondary CE showed that infected C57Bl/6 mice exhibited higher numbers of peritoneal macrophages than Balb/c animals. In fact, such difference derived from a sharp decrease observed in Balb/c mice, while C57Bl/6 animals exhibited no significant variations in the number of peritoneal macrophages respect to uninfected controls. Interestingly, the spontaneous secretion of NO by peritoneal cells from infected C57Bl/6 mice – unlike Balb/c animals – was high, and NO has been shown to exert harmful effects on PSC (Dematteis et al., 2003). In this sense, the reduction in the number of macrophages observed in the most susceptible mouse strain could be associated with the lower production of NO by their peritoneal cells. In addition, B cells – particularly the peritoneal B1 subset – have been described as a source of NO (Bogdan, 2015), and interestingly their number showed an increase and decrease in infected C57Bl/6 and Balb/c mice, respectively. Therefore, differences in the number of peritoneal B cells may also contribute to the observed contrasts in NO production by peritoneal cells from both mouse strains. Thus, low counts of peritoneal macrophages and B cells – probably affecting the local production of NO – could be suggested as important contributing factor to murine susceptibility in secondary CE.

Finally, ADCC mechanisms exerted by different cell types have proved to be relevant in immunity against several helminth parasites (Moreau and Chauvin, 2010; Harris and Gause, 2011). However, a role for such mechanisms in immunity against cestodes has not been clearly stated. Our results on PSC killing by normal peritoneal cells showed that Balb/c and C57Bl/6 mice displayed significant but similar degrees of activities when homologous normal sera were used, suggesting an alike role for natural antibodies in "natural" ADCC regardless the mouse strain. However, ADCC responses by peritoneal cell from 5-days-infected mice were shown to be relevant only in C57Bl/6 animals, suggesting that their very early antibody response – unlike Balb/c mice – is efficient in enhancing ADCC mechanisms. These results are in accordance with our previous report showing that while Balb/c mice biased the early antibody response against PSC antigens towards IgG1, C57Bl/6 mice exhibited a predominance of mixed IgM/IgG2c/IgG2b/IgG3 specific antibodies (Mourglia-Ettlin et al., 2016). In this sense, IgG1 subclass preferentially engages the inhibitory FcγRIIB while IgG2a/c and IgG2b exhibit the highest cellular activation/inhibition ratio based on their preferential binding to activating FcγRs (Nimmerjahn and Ravetch, 2005, 2006). Therefore, it is consistent that an early IgG1 bias in Balb/c mice would result in less efficient ADCC responses against *E. granulosus* PSC in comparison with C57Bl/6 animals.

In conclusion, we have here described the existence of differences in the early peritoneal immune response in Balb/c and C57Bl/6 infected mice regarding secondary CE. Through PCA studies, we characterized the local immune response in the low susceptible mouse strain (*e.g.*, C57Bl/6) as an early mixed IL-5/TNF-α response with an overall less intense Th2-type cytokine pattern than in the high susceptible strain (*e.g.*, Balb/c). Moreover, also through PCA studies, we showed that infected C57Bl/6 mice exhibited higher numbers of peritoneal macrophages and B cells with lower counts of eosinophils than Balb/c animals. In this sense, differential production of NO by peritoneal cells from infected mice could be suggested as a contributing factor to infection susceptibility. Finally, we established the role of natural antibodies in mediating ADCC responses, and reported that only in C57Bl/6 mice the very early induced antibody response enhances parasite killing through ADCC. Altogether, our results suggest that the early peritoneal immune response is a key determinant factor in host susceptibility to murine secondary CE. Furthermore, the present work is not only relevant for the understanding of host-parasite interactions in *E. granulosus* infection, but also for improving our knowledge on cestodes immunobiology.

Conflict of interest

There are no potential conflicts of interest relevant to this article to report.

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References

Alonso-Trujillo, J., Rivera-Montoya, I., Rodríguez-Sosa, M., Terrazas, L.I., 2007. Nitric oxide contributes to host resistance against experimental *Taenia crassiceps* cysticercosis. Parasitol. Res. 100 (6), 1341–1350.

Al-Qaoud, K.M., Abdel-Hafez, S.K., 2008. The induction of T helper type 1 response by cytokine gene transfection protects mice against secondary hydatidosis. Parasitol. Res. 102 (6), 1151–1155.

Amri, M., Aissa, S.A., Belguendouz, H., Mezioug, D., Touil-Boukoffa, C., 2007. In vitro antihydatic action of IFN-gamma is dependent on the nitric oxide pathway. J. Interferon. Cytokine Res. 27 (9), 781–787.

Amri, M., Mezioug, D., Touil-Boukoffa, C., 2009. Involvement of IL-10 and IL-4 in evasion strategies of *Echinococcus granulosus* to host immune response. Eur. Cytokine Netw. 20 (2), 63–68.

Anthony, R.M., Rutitzky, L.I., Urban Jr., J.F., Stadecker, M.J., Gause, W.C., 2007. Protective immune mechanisms in helminth infection. Nat. Rev. Immunol. 7 (12), 975–987.

Artis, D., Humphreys, N.E., Bancroft, A.J., Rothwell, N.J., Potten, C.S., Grencis, R.K., 1999. Tumor necrosis factor alpha is a critical component of interleukin 13-mediated protective T helper cell type 2 responses during helminth infection. J. Exp. Med. 190 (7), 953–962.

Baz, A., Ettlin, G.M., Dematteis, S., 2006. Complexity and function of cytokine responses in experimental infection by *Echinococcus granulosus*. Immunobiology 211 (1-2), 3–9.

Bogdan, C., 2015. Nitric oxide synthase in innate and adaptive immunity: an update. Trends Immunol. 36 (3), 161–178.

Dematteis, S., Baz, A., Rottenberg, M., Fernández, C., Orn, A., Nieto, A., 1999. Antibody and Th1/Th2-type responses in BALB/c mice inoculated with live or dead *Echinococcus granulosus* protoscoleces. Parasite Immunol. 21 (1), 19–26.

Dematteis, S., Rottenberg, M., Baz, A., 2003. Cytokine response and outcome of infection depends on the infective dose of parasites in experimental infection by *Echinococcus granulosus*. Parasite Immunol. 25 (4), 189–197.

Díaz, A., Allen, J.E., 2007. Mapping immune response profiles: the emerging scenario from helminth immunology. Eur. J. Immunol. 37 (12), 3319–3326.

Emery, I., Liance, M., Leclerc, C., 1997. Secondary *Echinococcus multilocularis* infection in A/J mice: delayed metacestode development is associated with Th1 cytokine production. Parasite Immunol. 19 (11), 493–503.

Ferreira, A.M., Breijo, M., Sim, R.B., Nieto, A., 2000. Contribution of C5-mediated mechanisms to host defence against *Echinococcus granulosus* hydatid infection. Parasite Immunol. 22 (9), 445–453.

Filbey, K.J., Grainger, J.R., Smith, K.A., Boon, L., van, R., ooijen, N., Harcus, Y., Jenkins, S., Hewitson, J.P., Maizels, R.M., 2014. Innate and adaptive type 2 immune cell responses in genetically controlled resistance to intestinal helminth infection. Immunol. Cell Biol. 92 (5), 436–448.

Genser, B., Cooper, P.J., Yazdanbakhsh, M., Barreto, M.L., Rodrigues, L.C., 2007. A guide to modern statistical analysis of immunological data. BMC Immunol. 8, 27.

Godot, V., Harraga, S., Podoprigora, G., Liance, M., Bardonnet, K., Vuitton, D.A., 2003. IFN alpha-2a protects mice against a helminth infection of the liver and modulates immune responses. Gastroenterology 124 (5), 1441–1450.

Gottstein, B., Haag, K., Walker, M., Matsumoto, J., Mejri, N., Hemphill, A., 2006. Molecular survival strategies of *Echinococcus multilocularis* in the murine host. Parasitol. Int. (Suppl. 55), S45–9.

Harris, N., Gause, W.C., 2011. To B or not to B: B cells and the Th2-type immune response to helminths. Trends Immunol. 32 (2), 80–88.

Heath, D.D., 1970. The development of *Echinococcus granulosus* larvae in laboratory animals. Parasitology 60 (3), 449–456.

Hernández-Pomi, A., Borras-Salvador, R., Mir-Gisbert, A., 1997. Analysis of cytokine and specific antibody profiles in hydatid patients with primary infection and relapse of disease. Parasite Immunol. 19 (12), 553–561.

Hoffmann, W., Petit, G., Schulz-Key, H., Taylor, D., Bain, O., Le Goff, L., 2000. *Litomosoides sigmodontis* in mice: reappraisal of an old model for filarial research. Parasitol. Today 16 (9), 387–389.

Jenkins, S.J., Allen, J.E., 2010. Similarity and diversity in macrophage activation by nematodes, trematodes, and cestodes. J. Biomed. Biotechnol., 262609.

Klion, A.D., Nutman, T.B., 2004. The role of eosinophils in host defense against helminth parasites. J. Allergy Clin. Immunol. 113 (1), 30–37.

La, X., Zhang, F., Li, Y., Li, J., Guo, Y., Zhao, H., Pang, N., Ma, X., Wen, H., Fan, H., Ding, J., 2015. Upregulation of PD-1 on $CD4^+CD25^+$ T cells is associated with immunosuppression in liver of mice infected with Echinococcus multilocularis. Int. Immunopharmacol. 26 (2), 357–366.

Le Goff, L., Loke, P., Ali, H.F., Taylor, D.W., Allen, J.E., 2000. Interleukin-5 is essential for vaccine-mediated immunity but not innate resistance to a filarial parasite. Infect. Immun. 68 (5), 2513–2517.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25 (4), 402–408.

López-Collazo, E., Hortelano, S., Rojas, A., Boscá, L., 1998. Triggering of peritoneal macrophages with IFN-alpha/beta attenuates the expression of inducible nitric oxide synthase through a decrease in NF-kappaB activation. J. Immunol. 160 (6), 2889–2895.

Maizels, R.M., Yazdanbakhsh, M., 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat. Rev. Immunol. 3 (9), 733–744.

Makepeace, B.L., Martin, C., Turner, J.D., Specht, S., 2012. Granulocytes in helminth infection—who is calling the shots? Curr. Med. Chem. 19 (10), 1567–1586.

Meeusen, E.N., Balic, A., 2000. Do eosinophils have a role in the killing of helminth parasites? Parasitol. Today 16 (3), 95–101.

Mejri, N., Gottstein, B., 2009. *Echinococcus multilocularis* metacestode metabolites contain a cysteine protease that digests eotaxin, a CC pro-inflammatory chemokine. Parasitol. Res. 105 (5), 1253–1260.

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

Moro, P., Schantz, P.M., 2009. Echinococcosis: a review. Int. J. Infect. Dis. 13 (2), 125–133.

Mourglia-Ettlin, G., Marqués, J.M., Chabalgoity, J.A., Dematteis, S., 2011. Early peritoneal immune response during *Echinococcus granulosus* establishment displays a biphasic behavior. PLoS Negl. Trop. Dis. 5 (8), e1293.

Mourglia-Ettlin, G., Cucher, M., Arbildi, P., Rosenzvit, M., Dematteis, S., 2016. Natural and induced antibodies contribute to differential susceptibility to secondary cystic echinococcosis of Balb/c and C57Bl/6 mice. Immunobiology 221 (1), 103–115.

Nimmerjahn, F., Ravetch, J.V., 2005. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science 310 (5753), 1510–1512.

Nimmerjahn, F., Ravetch, J.V., 2006. Fcgamma receptors: old friends and new family members. Immunity 24 (1), 19–28.

Nono, J.K., Pletinckx, K., Lutz, M.B., Brehm, K., 2012. Excretory/secretory-products of *Echinococcus multilocularis* larvae induce apoptosis and tolerogenic properties in dendritic cells in vitro. PLoS Negl. Trop. Dis. 6 (2), e1516.

Patel, N., Kreider, T., Urban Jr., J.F., Gause, W.C., 2009. Characterisation of effector mechanisms at the host-parasite interface during the immune response to tissue-dwelling intestinal nematode parasites. Int. J. Parasitol. 39 (1), 13–21.

Ramos, A.L., Discipio, R.G., Ferreira, A.M., 2006. Eosinophil cationic protein damages protoscoleces in vitro and is present in the hydatid cyst. Parasite Immunol. 28 (8), 347–355.

Redpath, S.A., Fonseca, N.M., Perona-Wright, G., 2014. Protection and pathology during parasite infection: IL-10 strikes the balance. Parasite Immunol. 36 (6), 233–252.

Reyes, J.L., Terrazas, L.I., 2007. The divergent roles of alternatively activated macrophages in helminthic infections. Parasite Immunol. 29 (12), 609–619.

Reyes, J.L., Terrazas, C.A., Vera-Arias, L., Terrazas, L.I., 2009. Differential response of antigen presenting cells from susceptible and resistant strains of mice to *Taenia crassiceps* infection. Infect. Genet. Evol. 9 (6), 1115–1127.

Richards, K.S., Arme, C., Bridges, J.F., 1983. *Echinococcus granulosus* equinus: an ultrastructural study of murine tissue response to hydatid cysts. Parasitology 86 (Pt. 3), 407–417.

Riganò, R., Profumo, E., Ioppolo, S., Notargiacomo, S., Teggi, A., Siracusano, A., 1999a. Serum cytokine detection in the clinical follow up of patients with cystic echinococcosis. Clin. Exp. Immunol. 115 (3), 503–507.

Riganò, R., Profumo, E., Buttari, B., Teggi, A., Siracusano, A., 1999b. Cytokine gene expression in peripheral blood mononuclear cells (PBMC) from patients with pharmacologically treated cystic echinococcosis. Clin. Exp. Immunol. 118 (1), 95–101.

Riganò, R., Profumo, E., Bruschi, F., Carulli, G., Azzarà, A., Ioppolo, S., Buttari, B., Ortona, E., Margutti, P., Teggi, A., Siracusano, A., 2001. Modulation of human immune response by *Echinococcus granulosus* antigen B and its possible role in evading host defenses. Infect. Immun. 69 (1), 288–296.

Riganò, R., Buttari, B., De Falco, E., Profumo, E., Ortona, E., Margutti, P., Scottà, C., Teggi, A., Siracusano, A., 2004. *Echinococcus granulosus* -specific T-cell lines derived from patients at various clinical stages of cystic echinococcosis. Parasite Immunol. 26 (1), 45–52.

Ringnér, M., 2008. What is principal component analysis? Nat. Biotechnol. 26 (3), 303–304.

Rodriguez-Sosa, M., David, J.R., Bojalil, R., Satoskar, A.R., Terrazas, L.I., 2002. Cutting edge: susceptibility to the larval stage of the helminth parasite *Taenia crassiceps* is mediated by Th2 response induced via STAT6 signaling. J. Immunol. 168 (7), 3135–3139.

Rogan, M.T., 1998. T-cell activity associated with secondary infections and implanted cysts of *Echinococcus granulosus* in BALB/c mice. Parasite Immunol. 20 (11), 527–533.

Rückerl, D., Allen, J.E., 2014. Macrophage proliferation, provenance, and plasticity in macroparasite infection. Immunol. Rev. 262 (1), 113–133.

Saeftel, M., Arndt, M., Specht, S., Volkmann, L., Hoerauf, A., 2003. Synergism of gamma interferon and interleukin-5 in the control of murine filariasis. Infect. Immun. 71 (12), 6978–6985.

Terrazas, L.I., 2008. The complex role of pro- and anti-inflammatory cytokines in cysticercosis: immunological lessons from experimental and natural hosts. Curr. Top Med. Chem. 8 (5), 383–392.

Veerapathran, A., Dakshinamoorthy, G., Gnanasekar, M., Reddy, M.V., Kalyanasundaram, R., 2009. Evaluation of *Wuchereria bancrofti* GST as a vaccine candidate for lymphatic filariasis. PLoS Negl. Trop Dis. 3 (6), e457.

Vuitton, D.A., 2003. The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? Acta Trop. 85 (2), 119–132.

Wiria, A.E., Djuardi, Y., Supali, T., Sartono, E., Yazdanbakhsh, M., 2012. Helminth infection in populations undergoing epidemiological transition: a friend or foe? Semin Immunopathol. 34 (6), 889–901.

Yang, Y., Ellis, M.K., McManus, D.P., 2012. Immunogenetics of human echinococcosis. Trends Parasitol. 28 (10), 447–454.

Capítulo 5

Discusión Global y Conclusiones

El objetivo general del presente trabajo consistió en realizar aportes de relevancia al conocimiento básico de las respuestas inmunes potencialmente protectoras frente a la infección por *E. granulosus* en hospederos intermediarios. Para ello, se realizaron una serie de estudios tanto en humanos (hospederos naturales accidentales), como en modelos murinos de infección (hospederos experimentales). En ambos casos, los fenómenos de resistencia/susceptibilidad frente a *E. granulosus* han sido escasamente estudiados hasta el momento. Sin embargo, varios autores han propuesto que los perfiles de la respuesta de anticuerpos y de citoquinas desarrollados por el hospedero, determinarían en gran medida el éxito del establecimiento de la infección o la eliminación del parásito. Así, la inducción de distintos perfiles de respuesta podrían ser potencialmente asociados con casos de susceptibilidad y resistencia del hospedero, respectivamente [Yang *et al.*, 2012].

En primer lugar, la abundancia de reportes sobre: (a) el hecho que el número de individuos seropositivos para antígenos del parásito en regiones endémicas para la EQ siempre es superior al número de casos que luego son confirmados mediante técnicas imagenológicas [Bonifacino *et al.*, 1991; Nahmias *et al.*, 1991; Zhang *et al.*, 1994; Cohen *et al.*, 1998; Shambesh *et al.*, 1999; Hernández *et al.*, 2005; Gavidia *et al.*, 2008]; y (b) la sugerencia realizada por algunos autores en relación a que dichos anticuerpos podrían tener cierta función protectora frente al desarrollo de la enfermedad [Rogan *et al.*, 1992], confluyeron para la postulación del primer objetivo específico. Es así que se realizó una caracterización detallada del perfil de anticuerpos (totales y específicos contra antígenos del parásito) en una cohorte de individuos residentes en una zona endémica para EQ en Uruguay, que si bien fueron serológicamente positivos, no mostraron durante un período de seguimiento de 2 años imágenes compatibles con EQ [Hernández *et al.*, 2005]. Dichos individuos se denominaron grupo UN (Tabla 1, Cap.2, pág. 22). Paralelamente, se realizó la misma caracterización de la respuesta de anticuerpos en un grupo de pacientes con EQ confirmada quirúrgicamente (grupo CE) y en un grupo de donantes sanos (grupo HD) (Tabla 1, Cap.2, pág. 22).

En este sentido, los resultados obtenidos mostraron que los individuos del grupo UN presentan un perfil de anticuerpos específicos contra *E. granulosus* particularmente

interesante y capaz de diferenciarlos tanto de los individuos del grupo CE como de los del grupo HD. Concretamente, los individuos del grupo UN mostraron bajos niveles de IgG4 (Figura 1f, Cap.2, pág.24) pero niveles importantes de IgE (Figura 2a, Cap.2, pág.25). Esta peculiaridad hizo que los individuos del grupo UN mostraran una relación IgE/IgG4 significativamente mayor a la observada en los demás grupos en estudio (Figura 2b, Cap.2, pág.25). Esto sugiere que el balance fino entre las respuestas de anticuerpos con potentes acciones antiparasitarias (IgE) y aquellas con pocos o nulos efectos contra el parásito (IgG4), podría estar relacionado con una respuesta potencialmente más eficiente contra *E. granulosus*. Apoyando esta hipótesis, se han sugerido interpretaciones similares para otras helmintiasis humanas [Adjobimey y Hoerauf, 2010].

Además, es interesante destacar que los individuos del grupo UN mostraron niveles significativamente superiores de IgM contra antígenos del parásito (con características de IgM naturales polireactivas) respecto a los individuos de los grupos CE y HD (Figura 3, Cap.2, pág.25). En consonancia con estos resultados, se ha reportado que *E. granulosus* es más susceptible a los mecanismos efectores del sistema inmune de hospederos intermediaros durante la etapa de establecimiento de la infección, es decir en el estadío de oncósfera [Rogan *et al.*, 2015]. Las oncósferas son sensibles a la acción del sistema del complemento de los hospederos intermediarios [Heath *et al.*, 1994; Heath y Lawrence, 1996; Lightowlers y Heath, 2004], incluido el ser humano [Rogan *et al.*, 1992]. Por lo tanto, y teniendo en cuenta que las IgM naturales son excelentes activadoras del sistema complemento [Panda y Ding, 2015; Ehrenstein y Notley, 2010], nuestros resultados sugieren que los individuos del grupo UN podrían tener ciertos niveles potenciales de resistencia "innata" frente a la infección por *E. granulosus*. En la misma línea de razonamiento, se han realizado interpretaciones similares en el contexto de otras patologías humanas de etiología infecciosa [Grönwall *et al.*, 2012].

En suma, los resultados reportados en el Capítulo 2 mostraron que los individuos del grupo UN presentan un perfil de anticuerpos contra *E. granulosus* que les es propio y característico, y que los diferencia tanto de pacientes con EQ así como de donantes sanos. En este sentido, aunque nuestro trabajo no describe una relación causal

inequívoca sobre el efecto de los anticuerpos en una situación de resistencia frente a *E. granulosus*, resulta claro que las características del perfil de anticuerpos en los individuos del grupo UN sí podrían asociarse con una potencial situación de desafío parasitario sin establecimiento exitoso de la infección.

Es importante destacar que el establecimiento de la EQ en humanos es asintomático, y por lo tanto, resulta prácticamente imposible obtener datos sobre la respuesta inmune temprana contra *E. granulosus* en contextos de infecciones naturales. En este sentido, si bien el modelo de EQ secundaria tiene sus limitaciones, ya que no refleja los posibles efectos que tendría la infección primaria natural (dado que se utilizan ratones normales), igualmente ha resultado de gran ayuda para la interpretación de los sucesos inmunológicos que ocurren en la etapa crónica de la infección y para la generación de posibles hipótesis referidas a los eventos que ocurrirían en las etapas tempranas de la misma [Tamarozzi *et al.*, 2015].

En base a lo antes mencionado, nos planteamos como segundo objetivo establecer un modelo de susceptibilidad diferencial a la infección por *E. granulosus* utilizando el modelo murino de EQ secundaria, para luego analizar las diferencias en respuesta inmune contra el parásito en ratones infectados con distintos niveles de resistencia a la infección. Para ello, se realizaron infecciones secundarias experimentales en ratones de las cepas Balb/c y C57Bl/6, y posteriormente analizar parámetros parasitológicos sobre el resultado de la infección en ambas cepas. Estas cepas de ratones fueron seleccionadas en base a los resultados reportados previamente en el modelo murino de EQ primaria [Dempster *et al.*, 1991]. Asimismo, resulta interesante destacar que ambas cepas exhiben diferencias intrínsecas en el "sesgo inmunológico". En este sentido, se ha reportado que mientras los ratones Balb/c son propensos a un sesgo Th2, los C57Bl/6 son propensos hacia un sesgo Th1. Estas diferencias parecen no solo estar asociadas con el diferente haplotipo de MHC que cada una presenta, sino además con una variedad de contrastes adicionales en componentes claves del sistema inmune. Por ejemplo, perfil de expresión y actividad de receptores TLR en células dendríticas [Liu *et al.*, 2002]; frecuencia y capacidad supresora de células T reguladoras $CD4^{+}CD25^{+}$ naturales [Chen *et al.*, 2005]; tasa de maduración de

progenitores de mastocitos [Dahlin *et al.*, 2013]; y niveles de IgM naturales capaces de reconocer glicanos [Dai *et al.*, 2009], entre otras diferencias.

Las diferencias antes mencionadas, claramente podrían influir en la susceptibilidad intrínseca de los ratones Balb/c y C57Bl/6 frente a patógenos, en especial frente a la infección por *E. granulosus*, en la cual se ha sugerido que las respuestas polarizadas de tipo Th1 y Th2 en el hospedero intermediario podrían ser protectoras o ineficientes, respectivamente. Así, nuestros resultados muestran que la cepa Balb/c es más permisiva al desarrollo de la EQ secundaria, mientras que la C57Bl/6 tiene comparativamente un nivel de resistencia mayor (Figura 1, Cap.3, pág.34). Estos resultados, estarían en línea con reportes previos para el modelo murino de EQ primaria [Dempster *et al.*, 1991]. Por lo tanto, los ratones Balb/c podrían ser considerados como altamente susceptibles a la infección por *E. granulosus* independientemente del estadío parasitario infectante, mientras que los C57Bl/6 mostrarían nula o baja susceptibilidad según se trate de EQ primaria o secundaria, respectivamente.

Teniendo en cuenta la diferencia en susceptibilidad al establecimiento de la infección por *E. granulosus* entre ambas cepas, como tercer objetivo se analizaron las diferencias de la respuesta de anticuerpos (naturales e inducidos) contra antígenos de *E. granulosus*. Para ello, se realizaron infecciones secundarias en ratones Balb/c y C57Bl/6, y se evaluó simultáneamente la cinética de anticuerpos específicos así como las características de los anticuerpos naturales capaces de reconocer antígenos del parásito. Es importante considerar que el perfil de la respuesta de anticuerpos montada por un hospedero infectado es de crucial relevancia, ya que cada isotipo/subclase de inmunoglobulina es capaz de desencadenar distintas funciones efectoras, algunas de las cuales podrían tener potenciales acciones antiparasitarias [Garraud *et al.*, 2003]. De manera interesante, nuestros resultados mostraron diferencias importantes entre los ratones Balb/c y C57Bl/6, en cuanto a los anticuerpos naturales e inducidos capaces de reconocer antígenos de *E. granulosus*. Además, las respuestas sistémicas (en suero) y locales (en exudados peritoneales) también mostraron diferencias significativas entre ambas cepas.

Con respecto a los anticuerpos naturales con reactividad hacia antígenos de *E. granulosus*, se observaron diferencias importantes entre cepas, en términos de niveles y características funcionales, tanto a nivel sistémico (Figuras 2, 3 y 4, Cap.3, pág.35,36,37) como local (Figuras 5 y 6 Cap.3, pág.38,39). De hecho, se logró establecer que los niveles séricos de IgG2b naturales funcionarían como un potencial correlato de protección en ambas cepas de ratones (Tabla 1, Cap.3, pág.33). Además, la presencia de IgG2b natural solo en exudados peritoneales de ratones C57Bl/6 reafirmaría su posible participación en mecanismos protectores (Figura 5, Cap.3, pág.38). Asimismo, mediante estudios funcionales *in vitro* e *in vivo*, se demostró que los anticuerpos naturales séricos de ratones C57Bl/6 contribuirían positivamente a la mayor resistencia frente a la EQ secundaria observada en dichos animales (Figura 7, Cap.3, pág.39). Por último, el hecho que los ratones infectados de la cepa C57Bl/6 desencadenen respuestas globales de anticuerpos más robustas contra antígenos de *E. granulosus* que los ratones Balb/c (Figura 2, Cap.3, pág.35), podría explicarse al menos parcialmente, por un posible efecto diferencial de los anticuerpos naturales en cavidad peritoneal sobre la regulación por retroalimentación mediada por anticuerpos (del inglés *Antibody-Mediated Feedback Regulation*, AMFR) [Getahun y Heyman, 2006; Heyman, 2014]. Como se discute en el Capítulo 3, los PSC son partículas de gran tamaño y dada la predominancia de IgM natural (potenciadora del AMFR) sobre IgG3 natural (supresora del AMFR) detectada en la cavidad peritoneal de ratones C57Bl/6 (a diferencia de lo que se observa en ratones Balb/c), ello podría conducir a una potenciación global su respuesta de anticuerpos.

Por su parte, el análisis de las subclases de IgG inducidas contra antígenos de *E. granulosus*, mostró que ambas cepas de ratones polarizan las mismas en forma opuesta. De hecho, aunque en ambas cepas se observaron respuestas mezcladas (con presencia de todas las subclases de IgG), los ratones de alta susceptibilidad a la infección (Balb/c) mostraron una rápida y sostenida polarización hacia IgG1, mientras que en los individuos de baja susceptibilidad (C57Bl/6) se observó una predominancia de las subclases IgG2c, IgG2b e IgG3 (Figura 2, Cap.3, pág.35). Interesantemente, se pudo establecer además que los niveles séricos de IgG1 inducidas funcionarían como un potencial correlato de susceptibilidad a la infección en ambas cepas de ratones (Tabla 1, Cap.3, pág,33).

En este sentido, cabe destacar que IgG1 es una subclase no activadora del sistema complemento y es además el principal ligando para el receptor FcγRIIB, inhibidor de la activación celular (por ejemplo en macrófagos) [Nimmerjahn y Ravetch, 2005; Nimmerjahn y Ravetch, 2006]. Así, la inducción preferencial de anticuerpos capaces de activar el sistema complemento y de estimular respuestas celulares en los ratones C57Bl/6, a diferencia de lo observado en animales Balb/c, explicaría (al menos parcialmente) la diferencia en susceptibilidad a la EQ secundaria existente entre ambas cepas.

En suma, los resultados reportados en el Capítulo 3 demostraron la existencia de fenómenos de susceptibilidad diferencial a la infección secundaria por *E. granulosus* entre ratones de las cepas C57Bl/6 y Balb/c. Asimismo, se demostró el papel protector que jugarían los anticuerpos naturales en esta susceptibilidad diferencial. Finalmente, se reportó que la polarización diferencial de la respuesta de anticuerpos en ambas cepas se correlacionaría con diferencias en la eficiencia de las mismas frente al desafío con PSC de *E. granulosus*.

Finalmente, de acuerdo con el último objetivo propuesto, evaluamos el papel de la respuesta temprana de células peritoneales en el modelo murino de susceptibilidad diferencial a la EQ secundaria (Capítulo 4). El establecimiento de la infección en dicho modelo es un fenómeno anatómicamente confinado a la cavidad peritoneal y ocurre durante los primeros 20-30 días de infección [Richards *et al.*, 1983]. Por esta razón, las características y funcionalidad de las respuestas de células peritoneales en la etapa temprana de la infección, tendrían un papel relevante en los fenómenos de resistencia/susceptibilidad frente a *E. granulosus*. Es así que, se realizaron infecciones secundarias en ratones Balb/c y C57Bl/6, para luego y en forma simultánea, caracterizar la cinética de expresión de citoquinas en células de cavidad peritoneal. Además se analizó, en un punto temporal seleccionado, la presencia de diferentes fenotipos celulares presentes en cavidad peritoneal. Por otra parte, mediante ensayos *in vitro*, se valoró la posible actividad antiparasitaria de las células peritoneales.

En cuanto a la cinética de expresión de citoquinas, nuestros resultados mostraron diferencias importantes entre ratones infectados de ambas cepas (Figura 1, Cap.4,

pág.47). Con el objetivo de destacar las diferencias de mayor peso en cuanto a la expresión de citoquinas, se utilizó la herramienta de estadística descriptiva denominada "Análisis de Componentes Principales" (del inglés *Principal Components Analysis*, PCA). Así, reportamos en el Capítulo 4 que los ratones C57Bl/6, a diferencia de los Balb/c, desarrollaron una respuesta temprana mezclada de IL-5 y TNF-α en concomitancia con una menor intensidad de expresión de citoquinas asociadas al perfil de tipo Th2 (Figura 3, Cap.4, pág.48). En este sentido, estos resultados abalarían las sugerencias previamente reportadas sobre la asociación entre respuestas de tipo Th2 y la susceptibilidad a la infección por *E. granulosus* en el modelo murino [Rogan, 1998; Dematteis *et al.*, 1999; Dematteis *et al.*, 2003; Al-Qaoud y Abdel-Hafez, 2008; Mourglia-Ettlin *et al.*, 2011]. Además, apoyarían el concepto general sobre la excepcionalidad de los cestodos en el concierto de los helmintos parásitos, dado que la inmunidad contra estos (a diferencia de nematodos y trematodos) requeriría de mecanismos efectores de tipo Th1, mientras que la susceptibilidad a la infección dependería del desarrollo de respuestas potentes de tipo Th2. Resultados similares se han reportado para los cestodos parásitos *Taenia crassiceps* [Rodriguez-Sosa *et al.*, 2002; Terrazas, 2008] y *E. multilocularis* [Emery *et al.*, 1997; Vuitton, 2003].

En cuanto a las poblaciones celulares presentes en la cavidad peritoneal de ratones Balb/c y C57Bl/6 durante la infección temprana, nuestros resultados mostraron diferencias relevantes entre ambas cepas (Figura 2, Cap.4, pág.47). Nuevamente, para destacar las diferencias de mayor peso, realizamos PCA y así reportamos en el Capítulo 4 que los ratones C57Bl/6, a diferencia de los Balb/c, presentan un mayor número de macrófagos y células B en concomitancia con un menor recuento de eosinófilos a nivel peritoneal (Figura 4, Cap.4, pág.48). En este sentido, aunque los eosinófilos han sido descriptos generalmente como células con funciones antihelmínticas potentes, su funcionalidad no solo depende de su número [Makepeace *et al.*, 2012]. De hecho, una elevada eosinofilia en humanos se ha asociado con un riesgo mayor de padecer recidivas [Hernández-Pomi *et al.*, 1997]. Por lo tanto, nuestros resultados sobre el reclutamiento de eosinófilos estaría en la misma línea que la situación peculiar reportada en humanos, sugiriendo que un excesivo infiltrado local de eosinófilos podría asociarse con respuestas inmunes poco eficientes contra *E. granulosus*. Por otro lado, el número menor de macrófagos y células B peritoneales observado en los ratones

Balb/c parecería ser un posible factor de susceptibilidad en el modelo de EQ secundaria, probablemente por afectar negativamente la producción local de óxido nítrico (NO) capaz de dañar a los PSC inoculados (Figura 5, Cap.4, pág.49).

Finalmente, los resultados sobre la funcionalidad de las células peritoneales en cuanto a su actividad protoscolicida, mostraron que si bien los anticuerpos naturales presentes en ambas cepas son capaces de disparar mecanismos de citotoxicidad celular dependiente de anticuerpos (del inglés *Antibody-Dependent Cellular Cytotoxicity*, ADCC) por células peritoneales normales, solo los anticuerpos inducidos tempranamente en la cepa C57Bl/6 son capaces de potenciarlos significativamente (Figura 5, Cap.4, pág.49). Interesantemente, estos resultados se podrían relacionar con la cinética de producción de anticuerpos específicos reportada en el Capítulo 3 (Figura 2, pág.35), ya que como se mencionó previamente, los ratones Balb/c polarizan su respuesta tempranamente hacia IgG1, la cual interactúa preferencialmente con el receptor celular inhibidor FcγRIIB [Nimmerjahn y Ravetch, 2005; Nimmerjahn y Ravetch, 2006].

En suma, en el Capítulo 4 describimos la existencia de importantes diferencias entre la respuesta temprana de células peritoneales de ratones Balb/c y C57Bl/6 infectados. Los resultados obtenidos apoyarían firmemente la sugerencia existente para cestodos parásitos, en cuanto a que la susceptibilidad del hospedero frente a la infección requiere de respuestas potentes de tipo Th2. Asimismo, se mostró que la producción temprana de NO por células peritoneales podría ser un factor contribuyente a la resistencia en el modelo de EQ secundaria. Por último, se logró establecer un papel importante de los anticuerpos naturales como mediadores de respuestas ADCC "innatas", y se demostró que, a diferencia de lo observado en ratones Balb/c, el perfil de la respuesta temprana de anticuerpos inducidos en ratones C57Bl/6 sería capaz de potenciar mecanismos efectores que estarían asociados a su mayor resistencia a la EQ secundaria.

Finalmente, y a modo de comentario global, podemos decir que los resultados derivados del presente trabajo y reportados en los tres artículos presentados, sugieren fuertemente un papel relevante de los anticuerpos en cuanto a su función protectora

en la EQ, tanto en humanos como en el modelo murino de infección secundaria. En este sentido, los anticuerpos naturales capaces de reconocer antígenos de *E. granulosus* se destacarían como un factor clave en posibles fenómenos de resistencia/susceptibilidad frente a la EQ. Además, nuestros resultados apoyarían la hipótesis sobre la asociación entre respuestas de tipo Th1 y Th2 con resistencia y susceptibilidad, respectivamente, a la infección por *E. granulosus*. Por último, y considerando nuestra hipótesis de partida, mostramos que las respuestas inmunes desencadenadas por los hospederos intermediarios tienen un papel crucial en limitar el establecimiento y/o desarrollo del metacestodo de *E. granulosus*. En consecuencia, la exposición a estadíos infectivos del parásito no siempre deriva en un establecimiento exitoso de la infección.

Capítulo 6

Referencias Bibliográficas

Adjobimey T, Hoerauf A. **Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation.** *Ann Trop Med Parasitol*. 2010 ;104(6):455-64.

Ait-Aissa S, Amri M, Bouteldja R, Wietzerbin J, Touil-Boukoffa C. **Alterations in interferon-gamma and nitric oxide levels in human echinococcosis.** *Cell Mol Biol (Noisy-le-grand)*. 2006; 52(1):65-70.

Al-Ghoury AB, El-Hamshary EM, Azazy AA, Hussein EM, Rayan HZ. **HLA class II alleles: susceptibility or resistance to cystic echinococcosis in Yemeni patients.** *Parasitol Res*. 2010; 107(2):355-61.

Al-Qaoud KM, Abdel-Hafez SK. **The induction of T helper type 1 response by cytokine gene transfection protects mice against secondary hydatidosis.** *Parasitol Res*. 2008; 102(6):1151-5.

Amri M, Aissa SA, Belguendouz H, Mezioug D, Touil-Boukoffa C. **In vitro antihydatic action of IFN-gamma is dependent on the nitric oxide pathway.** *J Interferon Cytokine Res*. 2007; 27(9):781-7.

Amri M, Mezioug D, Touil-Boukoffa C. **Involvement of IL-10 and IL-4 in evasion strategies of *Echinococcus granulosus* to host immune response.** *Eur Cytokine Netw*. 2009; 20(2):63-8.

Araj GF, Matossian RM, Frayha GJ. **The host response in secondary hydatidosis of mice. I. Circulating antibodies.** *Z Parasitenkd*. 1977; 52(1):23-30.

Azab ME, Bishara SA, Ramzy RM, Oteifa NM, El-Hoseiny LM, Ahmed MA. **The evaluation of HLA-DRB1 antigens as susceptibility markers for unilocular cystic echinococcosis in Egyptian patients.** *Parasitol Res*. 2004a; 92(6):473-7.

Azab ME, Bishara SA, Helmy H, Oteifa NM, El-Hoseiny LM, Ramzy RM, Ahmed MA. **Association of some HLA-DRB1 antigens with *Echinococcus granulosus* specific humoral immune response.** *J Egypt Soc Parasitol*. 2004b; 34(1):183-96.

Baz A, Carol H, Fernández V, Mourglia-Ettlin G, Nieto A, Orn A, Dematteis S. ***Echinococcus granulosus*: induction of T-independent antibody response against protoscolex glycoconjugates in early experimental infection.** *Exp Parasitol*. 2008; 119(4):460-6.

Baz A, Ettlin GM, Dematteis S. **Complexity and function of cytokine responses in experimental infection by *Echinococcus granulosus*.** *Immunobiology*. 2006; 211(1-2):3-9.

Baz A, Hernández A, Dematteis S, Carol H, Nieto A. **Idiotypic modulation of the antibody response of mice to *Echinococcus granulosus* antigens.** *Immunology*. 1995; 84(3):350-4.

Baz A, Richieri A, Puglia A, Nieto A, Dematteis S. **Antibody response in CD4-depleted mice after immunization or during early infection with *Echinococcus granulosus*.** *Parasite Immunol*. 1999; 21(3):141-50.

Bogdan C, Gessner A, Solbach W, Röllinghoff M. **Invasion, control and persistence of *Leishmania* parasites.** *Curr Opin Immunol*. 1996; 8(4):517-25.

Bonifacino R, Malgor R, Barbeito R, Balleste R, Rodríguez MJ, Botto C, Klug F. **Seroprevalence of *Echinococcus granulosus* infection in a Uruguayan rural human population.** *Trans R Soc Trop Med Hyg*. 1991; 85(6):769-72.

Breijo M, Isnardi F, Brauer M, Schenker R, Ferrari M, Ferreira AM. **An insect growth inhibitor--lufenuron--enhances albendazole activity against hydatid cyst.** *Vet Parasitol*. 2011; 181(2-4):341-4.

Brunetti E, Heller T, Richter J, Kaminstein D, Youkee D, Giordani MT, Goblirsch S, Tamarozzi F. **Application of Ultrasonography in the Diagnosis of Infectious Diseases in Resource-Limited Settings.** *Curr Infect Dis Rep*. 2016; 18(2):6.

Brunetti E, Junghanss T. **Update on cystic hydatid disease.** *Curr Opin Infect Dis*. 2009; 22(5):497-502.

Brunetti E, Kern P, Vuitton DA. **Writing Panel for the WHO-IWGE. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans.** *Acta Trop*. 2010; 114(1):1-16.

Budke CM, Deplazes P, Torgerson PR. **Global socioeconomic impact of cystic echinococcosis.** *Emerg Infect Dis*. 2006; 12(2):296-303.

Burgu A, Sarimehmetoğlu O, Gonenc B, Oge H, Sahin G, Aypak S. **Effects of immunization with purified specific proteins on the formation of secondary hydatid cysts in mice.** *Dtsch Tierarztl Wochenschr*. 2007; 114(9):349-53.

Casado N, Urrea-París MA, Moreno MJ, Rodríguez-Caabeiro F. **Combined praziquantel and albendazole chemoprophylaxis in experimental hydatidosis.** *Parasitol Res*. 2001; 87(9):787-9.

Ceballos L, Elissondo C, Sánchez Bruni S, Confalonieri A, Denegri G, Alvarez L, Lanusse C. **Chemoprophylactic activity of flubendazole in cystic echinococcosis.** *Chemotherapy*. 2010; 56(5):386-92.

Chen X, Oppenheim JJ, Howard OM. **BALB/c mice have more $CD4^+CD25^+$ T regulatory cells and show greater susceptibility to suppression of their $CD4^+CD25^-$ responder T cells than C57BL/6 mice.** *J Leukoc Biol*. 2005; 78(1):114-21.

Cohen H, Paolillo E, Bonifacino R, Botta B, Parada L, Cabrera P, Snowden K, Gasser R, Tessier R, Dibarboure L, Wen H, Allan JC, Soto de Alfaro H, Rogan MT, Craig PS. **Human cystic echinococcosis in a Uruguayan community: a sonographic, serologic, and epidemiologic study.** *Am J Trop Med Hyg*. 1998; 59(4):620-7.

Craig PS, McManus DP, Lightowlers MW, Chabalgoity JA, Garcia HH, Gavidia CM, Gilman RH, Gonzalez AE, Lorca M, Naquira C, Nieto A, Schantz PM. **Prevention and control of cystic echinococcosis.** *Lancet Infect Dis*. 2007; 7(6):385-94.

Craig PS. **Detection of specific circulating antigen, immune complexes and antibodies in human hydatidosis from Turkana (Kenya) and Great Britain, by enzyme-immunoassay.** *Parasite Immunol*. 1986; 8(2):171-88.

Cucher M, Mourglia-Ettlin G, Prada L, Costa H, Kamenetzky L, Poncini C, Dematteis S, Rosenzvit MC. ***Echinococcus granulosus*** **pig strain (G7 genotype) protoscoleces did not develop secondary hydatid cysts in mice.** *Vet Parasitol*. 2013; 193(1-3):185-92.

Cucher MA, Macchiaroli N, Baldi G, Camicia F, Prada L, Maldonado L, Avila HG, Fox A, Gutiérrez A, Negro P, López R, Jensen O, Rosenzvit M, Kamenetzky L. **Cystic echinococcosis in South America: systematic review of species and genotypes of** ***Echinococcus granulosus sensu lato*** **in humans and natural domestic hosts.** *Trop Med Int Health*. 2015; doi: 10.1111/tmi.12647.

Cumino AC, Nicolao MC, Loos JA, Denegri G, Elissondo MC. ***Echinococcus granulosus*** **tegumental enzymes as in vitro markers of pharmacological damage: a biochemical and molecular approach.** *Parasitol Int*. 2012; 61(4):579-85.

Daeki AO, Craig PS, Shambesh MK. **IgG-subclass antibody responses and the natural history of hepatic cystic echinococcosis in asymptomatic patients.** *Ann Trop Med Parasitol*. 2000; 94(4):319-28.

Dahlin JS, Heyman B, Hallgren J. **Committed mast cell progenitors in mouse blood differ in maturity between Th1 and Th2 strains.** *Allergy*. 2013; 68(10):1333-7.

Dai H, Zhang Y, Lv P, Gao XM. **A study on the glycan specificity of natural antibody repertoires in rodents.** *Cell Mol Immunol*. 2009; 6(6):453-9.

Das A, Das SK. **"Air - bubble" sign - an uncommon presentation of a common disease.** *J Glob Infect Dis*. 2011; 3(3):310-2.

Dematteis S, Baz A, Rottenberg M, Fernández C, Orn A, Nieto A. **Antibody and Th1/Th2-type responses in BALB/c mice inoculated with live or dead** ***Echinococcus granulosus*** **protoscoleces.** *Parasite Immunol*. 1999; 21(1):19-26.

Dematteis S, Rottenberg M, Baz A. **Cytokine response and outcome of infection depends on the infective dose of parasites in experimental infection by** ***Echinococcus granulosus*****.** *Parasite Immunol*. 2003; 25(4):189-97.

Dempster RP, Berridge MV, Harrison GB, Heath DD. ***Echinococcus granulosus*****: development of an intermediate host mouse model for use in vaccination studies.** *Int J Parasitol*. 1991; 21(5):549-54.

Denegri G, Perez-Serrano J, Casado N, Rodriguez-Caabeiro F. **[13C]-nuclear magnetic resonance spectral profiles of serum from normal and *Echinococcus granulosus*-infected mice: a kinetic study.** *Parasitol Res*. 1995; 81(2):170-2.

Dessaint JP, Bout D, Wattre P, Capron A. **Quantitative determination of specific IgE antibodies to *Echinococcus granulosus* and IgE levels in sera from patients with hydatid disease.** *Immunology*. 1975; 29(5):813-23.

Díaz A, Allen JE. **Mapping immune response profiles: the emerging scenario from helminth immunology.** *Eur J Immunol*. 2007; 37(12):3319-26.

Ehrenstein MR, Notley CA. **The importance of natural IgM: scavenger, protector and regulator.** *Nat Rev Immunol*. 2010; 10(11):778-86.

Emery I, Liance M, Leclerc C. **Secondary *Echinococcus multilocularis* infection in A/J mice: delayed metacestode development is associated with Th1 cytokine production.** *Parasite Immunol*. 1997; 19(11):493-503.

Etges R, Müller I. **Progressive disease or protective immunity to *Leishmania major* infection: the result of a network of stimulatory and inhibitory interactions.** *J Mol Med (Berl)*. 1998; 76(6):372-90.

Ferragut G, Ljungström I, Nieto A. **Relevance of circulating antigen detection to follow-up experimental and human cystic hydatid infections.** *Parasite Immunol*. 1998; 20(11):541-9.

Ferragut G, Nieto A. **Antibody response of *Echinococcus granulosus* infected mice: recognition of glucidic and peptidic epitopes and lack of avidity maturation.** *Parasite Immunol*. 1996; 18(8):393-402.

Ferreira AM, Trecu T, Reisin I. ***Echinococcus granulosus*: study of the in vitro complement activation by protoscoleces by measuring the electric potential difference across the tegumental membrane.** *Exp Parasitol*. 1992; 75(3):259-68.

Garraud O, Perraut R, Riveau G, Nutman TB. **Class and subclass selection in parasite-specific antibody responses.** *Trends Parasitol*. 2003; 19(7):300-4.

Gavidia CM, Gonzalez AE, Zhang W, McManus DP, Lopera L, Ninaquispe B, Garcia HH, Rodríguez S, Verastegui M, Calderon C, Pan WK, Gilman RH. **Diagnosis of cystic echinococcosis, central Peruvian Highlands.** *Emerg Infect Dis*. 2008; 14(2):260-6.

Getahun A, Heyman B. **How antibodies act as natural adjuvants.** *Immunol Lett*. 2006; 104(1-2):38-45.

Grönwall *et al*., 2012 Grönwall C, Vas J, Silverman GJ. **Protective Roles of Natural IgM Antibodies.** *Front Immunol*. 2012; 3:66.

Haralabidis S, Karagouni E, Frydas S, Dotsika E. **Immunoglobulin and cytokine profile in murine secondary hydatidosis.** *Parasite Immunol*. 1995; 17(12):625-30.

Hashemitabar GR, Razmi GR, Naghibi A. **Trials to induce protective immunity in mice and sheep by application of protoscolex and hydatid fluid antigen or whole body antigen of *Echinococcus granulosus*.** *J Vet Med B Infect Dis Vet Public Health*. 2005; 52(5):243-5.

Heath DD, Holcman B, Shaw RJ. ***Echinococcus granulosus*: the mechanism of oncosphere lysis by sheep complement and antibody.** *Int J Parasitol*. 1994; 24(7):929-35.

Heath DD, Lawrence SB. **Antigenic polypeptides of *Echinococcus granulosus* oncospheres and definition of protective molecules.** *Parasite Immunol*. 1996; 18(7):347-57.

Heath DD. **The development of *Echinococcus granulosus* larvae in laboratory animals.** *Parasitology*. 1970; 60(3):449-56.

Hernández A, Cardozo G, Dematteis S, Baz A, Trias N, Nuñez H, Barragué A, López L, Fuentes J, López O, Ferreira C. **Cystic echinococcosis: analysis of the serological profile related to the risk factors in individuals without ultrasound liver changes living in an endemic area of Tacuarembó, Uruguay.** *Parasitology*. 2005; 130(Pt 4):455-60.

Hernández A, Nieto A. **Induction of protective immunity against murine secondary hydatidosis.** *Parasite Immunol*. 1994; 16(10):537-44.

Hernández-Pomi A, Borras-Salvador R, Mir-Gisbert A. **Analysis of cytokine and specific antibody profiles in hydatid patients with primary infection and relapse of disease.** *Parasite Immunol*. 1997; 19(12):553-61.

Heyman B. **Antibodies as natural adjuvants.** *Curr Top Microbiol Immunol*. 2014; 382:201-19.

Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. **Helminth infections: the great neglected tropical diseases.** *J Clin Invest*. 2008; 118(4):1311-21.

Hotez PJ, Mistry N, Rubinstein J, Sachs JD. **Integrating neglected tropical diseases into AIDS, tuberculosis, and malaria control.** *N Engl J Med*. 2011; 364(22):2086-9.

Jenkins SJ, Allen JE. **Similarity and diversity in macrophage activation by nematodes, trematodes, and cestodes.** *J Biomed Biotechnol*. 2010; 2010:262609.

Khabiri AR, Bagheri F, Assmar M, Siavashi MR. **Analysis of specific IgE and IgG subclass antibodies for diagnosis of *Echinococcus granulosus*.** *Parasite Immunol*. 2006; 28(8):357-62.

Kiper N, Gerçeker F, Utine E, Yalçin E, Pekcan S, Cobanoğlu N, Aslan A, Köse M, Doğru D, Ozçelik U, Ozgüç M. **TAP1 and TAP2 gene polymorphisms in childhood cystic echinococcosis.** *Parasitol Int*. 2010; 59(2):283-5.

Launois P, Tacchini-Cottier F, Parra-Lopez C, Louis JA. **Cytokines in parasitic diseases: the example of cutaneous leishmaniasis.** *Int Rev Immunol*.1998; 17(1-4):157-80.

Li J, Zhang WB, Wilson M, Ito A, McManus DP. **A novel recombinant antigen for immunodiagnosis of human cystic echinococcosis.** *J Infect Dis*. 2003; 188(12):1951-60.

Lightowlers MW, Heath DD. **Immunity and vaccine control of *Echinococcus granulosus* infection in animal intermediate hosts.** *Parassitologia*. 2004; 46(1-2):27-31.

Lissandrin R, Tamarozzi F, Piccoli L, Tinelli C, De Silvestri A, Mariconti M, Meroni V, Genco F, Brunetti E. **Factors Influencing the Serological Response in Hepatic *Echinococcus granulosus* Infection.** *Am J Trop Med Hyg*. 2016; 94(1):166-71.

Liu D, Lightowlers MW, Rickard MD. **Examination of murine antibody response to secondary hydatidosis using ELISA and immunoelectrophoresis.** *Parasite Immunol*. 1992; 14(3):239-48.

Makepeace BL, Martin C, Turner JD, Specht S. **Granulocytes in helminth infection -- who is calling the shots?** *Curr Med Chem*. 2012; 19(10):1567-86.

Mamuti W, Yamasaki H, Sako Y, Nakaya K, Nakao M, Lightowlers MW, Ito A. **Usefulness of hydatid cyst fluid of *Echinococcus granulosus* developed in mice with secondary infection for serodiagnosis of cystic echinococcosis in humans.** *Clin Diagn Lab Immunol*. 2002; 9(3):573-6.

Mandal S, Mandal MD. **Human cystic echinococcosis: epidemiologic, zoonotic, clinical, diagnostic and therapeutic aspects.** *Asian Pac J Trop Med*. 2012; 5(4):253-60.

Manzano-Román R, Sánchez-Ovejero C, Hernández-González A, Casulli A, Siles-Lucas M. **Serological Diagnosis and Follow-Up of Human Cystic Echinococcosis: A New Hope for the Future?** *Biomed Res Int*. 2015; 2015:428205.

McManus DP. **Current status of the genetics and molecular taxonomy of *Echinococcus* species.** *Parasitology*. 2013; 140(13):1617-23.

Mezioug D, Touil-Boukoffa C. **Interleukin-17A correlates with interleukin-6 production in human cystic echinococcosis: a possible involvement of IL-17A in immunoprotection against *Echinococcus granulosus* infection.** *Eur Cytokine Netw*. 2012; 23(3):112-9.

Mourglia-Ettlin G, Marqués JM, Chabalgoity JA, Dematteis S. **Early peritoneal immune response during *Echinococcus granulosus* establishment displays a biphasic behavior.** *PLoS Negl Trop Dis*. 2011; 5(8):e1293.

Nahmias J, Goldsmith R, Schantz P, Siman M, el-On J. **High prevalence of human hydatid disease (echinococcosis) in communities in northern Israel: epidemiologic studies in the town of Yirka.** *Acta Trop*. 1991; 50(1):1-10.

Naik MI, Tenguria RK, Haq E. **Detection of serum cytokines before and after pharmacological and surgical treatment in patients with cystic echinococcosis.** *J Helminthol*. 2016; 90(1):91-5.

Nakao M, Yanagida T, Okamoto M, Knapp J, Nkouawa A, Sako Y, Ito A. **State-of-the-art *Echinococcus* and *Taenia*: phylogenetic taxonomy of human-pathogenic tapeworms and its application to molecular diagnosis.** *Infect Genet Evol*. 2010; 10(4):444-52.

Nimmerjahn F, Ravetch JV. **Divergent immunoglobulin g subclass activity through selective Fc receptor binding.** *Science*. 2005; 310(5753):1510-2.

Nimmerjahn F, Ravetch JV. **Fcgamma receptors: old friends and new family members.** *Immunity*. 2006; 24(1):19-28.

Panda S, Ding JL. **Natural antibodies bridge innate and adaptive immunity.** *J Immunol*. 2015; 194(1):13-20.

Pennoit-De Cooman E, De Rycke PH, Van Outryve EJ. **Experimental secondary echinococcosis of *Echinococcus granulosus*. V. Quantitative aspects of the infection rate.** *Tropenmed Parasitol*. 1974; 25(3):338-44.

Pennoit-De Cooman E, De Rycke PH. **Experimental secondary echinococcosis of *Echinococcus granulosus*. I. Development in different strains of mice.** *Z Parasitenkd*. 1970; 34(4):362-70.

Piccoli L, Tamarozzi F, Cattaneo F, Mariconti M, Filice C, Bruno A, Brunetti E. **Long-term sonographic and serological follow-up of inactive echinococcal cysts of the liver: hints for a "watch-and-wait" approach.** *PLoS Negl Trop Dis*. 2014; 8(8):e3057.

Pinon JM, Poirriez J, Lepan H, Geers R, Penna R, Fernández D. **Value of isotypic characterization of antibodies to *Echinococcus granulosus* by enzyme-linked immuno-filtration assay.** *Eur J Clin Microbiol*. 1987; 6(3):291-5.

Richards KS, Arme C, Bridges JF. ***Echinococcus granulosus equinus*: an ultrastructural study of murine tissue response to hydatid cysts.** *Parasitology*. 1983; 86 (Pt 3):407-17.

Riganò R, Profumo E, Di Felice G, Ortona E, Teggi A, Siracusano A. **In vitro production of cytokines by peripheral blood mononuclear cells from hydatid patients.** *Clin Exp Immunol*. 1995a; 99(3):433-9.

Riganò R, Profumo E, Ioppolo S, Notargiacomo S, Ortona E, Teggi A, Siracusano A. **Immunological markers indicating the effectiveness of pharmacological treatment in human hydatid disease.** *Clin Exp Immunol*. 1995b; 102(2):281-5.

Riganò R, Profumo E, Teggi A, Siracusano A. **Production of IL-5 and IL-6 by peripheral blood mononuclear cells (PBMC) from patients with *Echinococcus granulosus* infection.** *Clin Exp Immunol*. 1996; 105(3):456-9.

Riganò R, Profumo E, Buttari B, Teggi A, Siracusano A. **Cytokine gene expression in peripheral blood mononuclear cells (PBMC) from patients with pharmacologically treated cystic echinococcosis.** *Clin Exp Immunol*. 1999a; 118(1):95-101.

Riganò R, Profumo E, Ioppolo S, Notargiacomo S, Teggi A, Siracusano A. **Serum cytokine detection in the clinical follow up of patients with cystic echinococcosis.** *Clin Exp Immunol*. 1999b; 115(3):503-7.

Rinaldi F, De Silvestri A, Tamarozzi F, Cattaneo F, Lissandrin R, Brunetti E. **Medical treatment versus "Watch and Wait" in the clinical management of CE3b echinococcal cysts of the liver.** *BMC Infect Dis*. 2014; 14:492.

Rodriguez-Sosa M, David JR, Bojalil R, Satoskar AR, Terrazas LI. **Cutting edge: susceptibility to the larval stage of the helminth parasite *Taenia crassiceps* is mediated by Th2 response induced via STAT6 signaling.** *J Immunol*. 2002; 168(7):3135-9.

Rogan MT, Bodell AJ, Craig PS. **Post-encystment/established immunity in cystic echinococcosis: is it really that simple?** *Parasite Immunol*. 2015; 37(1):1-9.

Rogan MT, Craig PS, Zehyle E, Masinde G, Wen H, Zhou P. **In vitro killing of taeniid oncospheres, mediated by human sera from hydatid endemic areas.** *Acta Trop*. 1992; 51(3-4):291-6.

Rogan MT. **T-cell activity associated with secondary infections and implanted cysts of *Echinococcus granulosus* in BALB/c mice.** *Parasite Immunol*. 1998; 20(11):527-33.

Severi MA, Ferragut G, Nieto A. **Antibody response of *Echinococcus granulosus* infected mice: protoscolex specific response during infection is associated with decreasing specific IgG1/IgG3 ratio as well as decreasing avidity.** *Parasite Immunol*. 1997; 19(12):545-52.

Shambesh MA, Craig PS, Macpherson CN, Rogan MT, Gusbi AM, Echtuish EF. **An extensive ultrasound and serologic study to investigate the prevalence of human cystic echinococcosis in northern Libya.** *Am J Trop Med Hyg*. 1999; 60(3):462-8.

Shan JY, Ji WZ, Li HT, Tuxun T, Lin RY, Wen H. **TLR2 and TLR4 expression in peripheral blood mononuclear cells of patients with chronic cystic echinococcosis and its relationship with IL-10.** *Parasite Immunol*. 2011; 33(12):692-6.

Siles-Lucas M, Hemphill A. **Cestode parasites: application of in vivo and in vitro models for studies on the host-parasite relationship.** *Adv Parasitol*. 2002; 51:133-230.

Siracusano A, Teggi A, Ortona E. **Human cystic echinococcosis: old problems and new perspectives.** *Interdiscip Perspect Infect Dis.* 2009; 2009:474368.

Smyth JD, Davies Z. **Occurrence of physiological strains of *Echinococcus granulosus* demonstrated by in vitro culture of protoscoleces from sheep and horse hydatid cysts.** *Int J Parasitol.* 1974; 4(4):443-5.

Sterla S, Sato H, Nieto A. ***Echinococcus granulosus* human infection stimulates low avidity anticarbohydrate IgG2 and high avidity antipeptide IgG4 antibodies.** *Parasite Immunol.* 1999; 21(1):27-34.

Tamarozzi F, Mariconti M, Neumayr A, Brunetti E. **The intermediate host immune response in cystic echinococcosis.** *Parasite Immunol.* 2015; doi: 10.1111/pim.12301.

Terrazas LI. **The complex role of pro- and anti-inflammatory cytokines in cysticercosis: immunological lessons from experimental and natural hosts.** *Curr Top Med Chem.* 2008; 8(5):383-92.

Thompson RC. **The taxonomy, phylogeny and transmission of *Echinococcus*.** *Exp Parasitol.* 2008; 119(4):439-46.

Thompson RCA. In ***Echinococcus and* Hydatid Disease**, Eds. Thompson RCA and Lymbery AJ. Oxford: CAB International, UK; 1995: 1–50.

Touil-Boukoffa C, Sancéau J, Tayebi B, Wietzerbin J. **Relationship among circulating interferon, tumor necrosis factor-alpha, and interleukin-6 and serologic reaction against parasitic antigen in human hydatidosis.** *J Interferon Cytokine Res.* 1997; 17(4):211-7.

Tsaroucha AK, Polychronidis AC, Lyrantzopoulos N, Pitiakoudis MS, J Karayiannakis A, Manolas KJ, Simopoulos CE. **Hydatid disease of the abdomen and other locations.** *World J Surg.* 2005; 29(9):1161-5.

Urrea-París MA, Casado N, Moreno MJ, Rodriguez-Caabeiro F. **Chemoprophylactic praziquantel treatment in experimental hydatidosis.** *Parasitol Res.* 2001; 87(6):510-2.

Vuitton DA. **The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite?** *Acta Trop.* 2003; 85(2):119-32.
Wen H, Craig PS. **Immunoglobulin G subclass responses in human cystic and alveolar echinococcosis.** *Am J Trop Med Hyg.* 1994; 51(6):741-8.

Wiria AE, Djuardi Y, Supali T, Sartono E, Yazdanbakhsh M. **Helminth infection in populations undergoing epidemiological transition: a friend or foe?** *Semin Immunopathol.* 2012; 34(6):889-901.

Yalcin E, Kiper N, Tan C, Ozcelik U, Dogru D, Cobanoglu N, Kose M, Pekcan S, Aslan AT, Ersoy F. **The role of human leucocyte antigens in children with hydatid disease:**

their association with clinical condition and prognosis. *Parasitol Res*. 2010; 106(4):795-800.

Yang Y, Ellis MK, McManus DP. **Immunogenetics of human echinococcosis.** *Trends Parasitol*. 2012; 28(10):447-54.

Zeghir-Bouteldja R, Amri M, Bouaziz S, Mezioug D, Touil-Boukoffa C. **Comparative study of nitric oxide (NO) production during human hydatidosis: relationship with cystic fluid fertility.** *Parasitol Res*. 2013; 112(2):649-54.

Zhang WB, Zhang ZZ, Alili H, Chi PS. **An investigation of factors influencing the hyperendemic of echinococcosis in Xinjiang.** *Chin J Zoonoses*. 1994; 10:204–205.