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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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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-

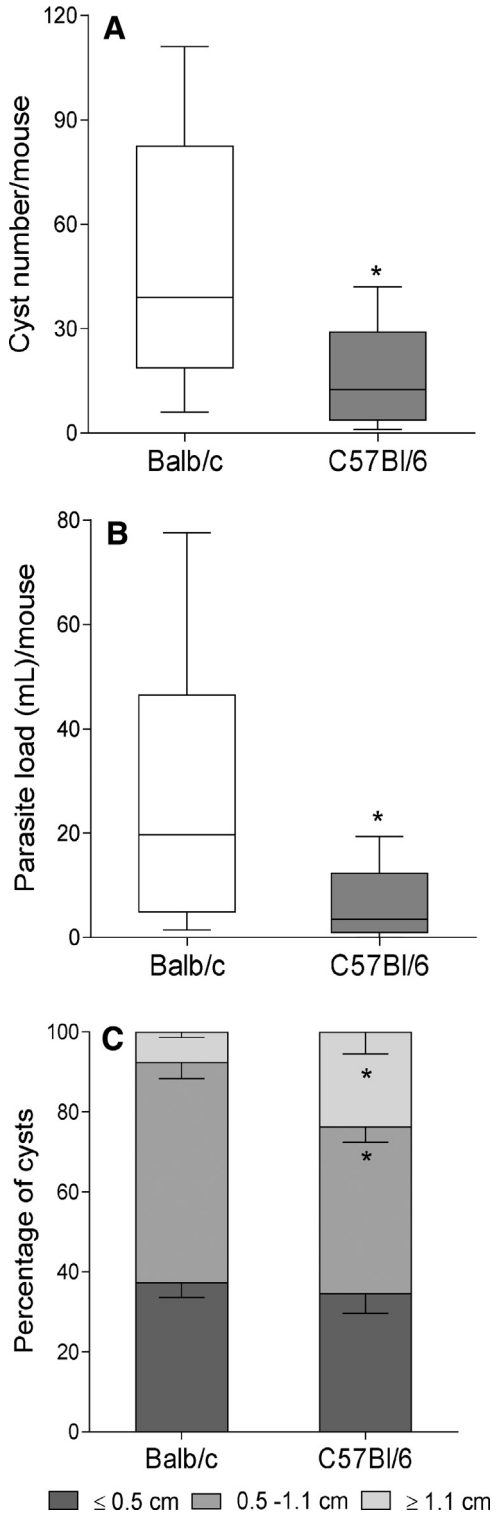

Fig. 1. Balb/c mice are more susceptible to secondary CE than C57Bl/6 mice. Balb/c and C57Bl/6 mice ($n = 8$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. All mice were euthanized 48 weeks pi, and their peritoneal cysts were recovered. Results are shown in box-and-whiskers 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

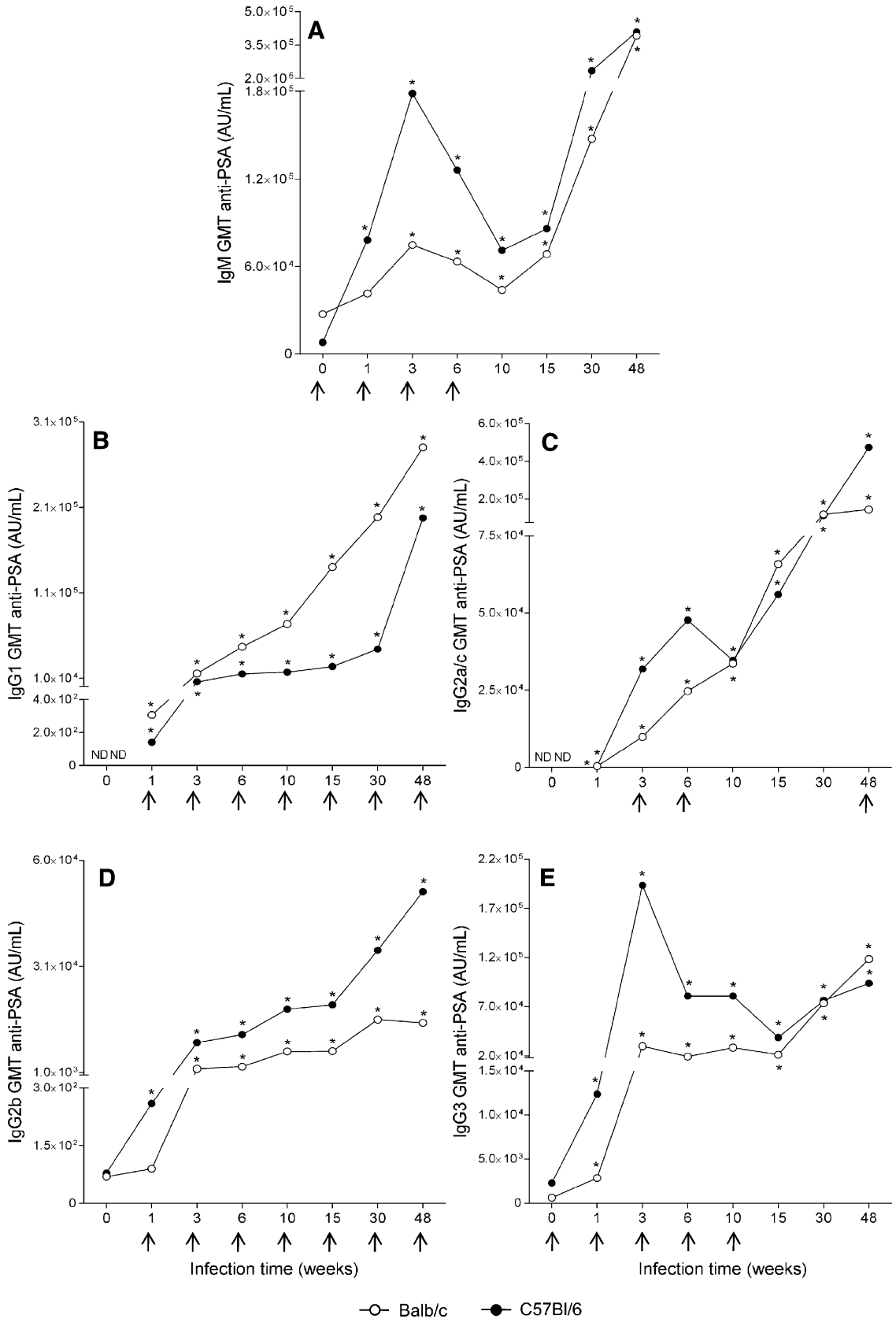

Fig. 2. Kinetics of serum antibody response to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 8$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice from both strains were bled at 0 (pre-infection), 1, 3, 6, 10, 15, 30 and 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.

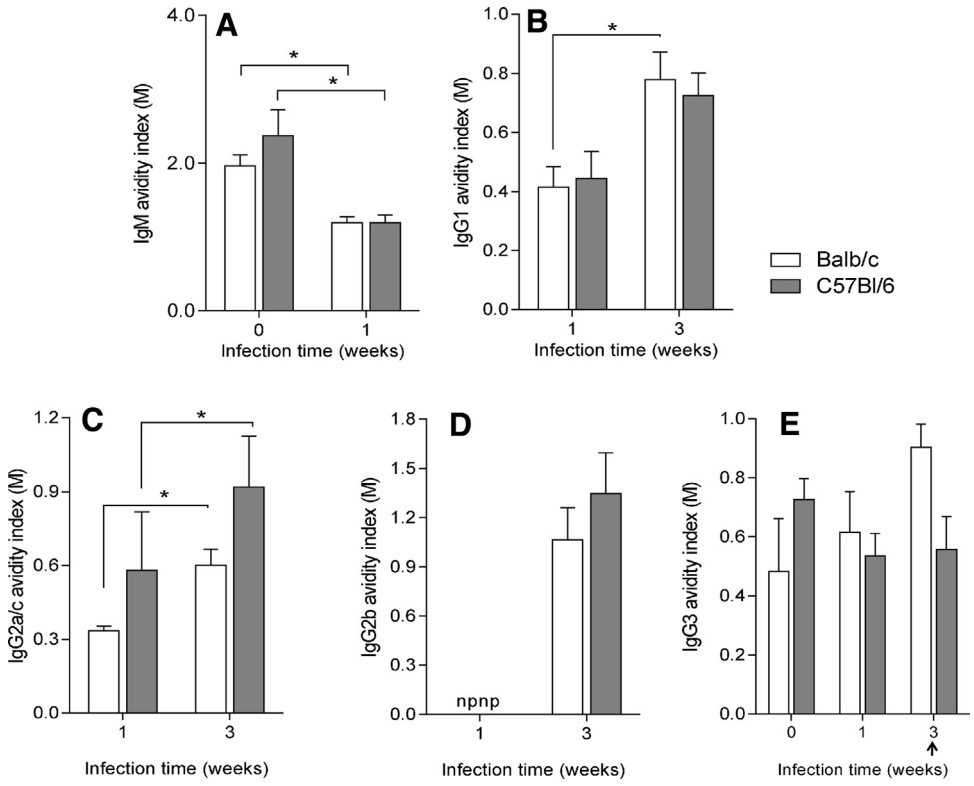

Fig. 3. Avidity analyses of early systemic antibodies to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 8$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice from both strains were bled prior to infection (0 week) and at 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

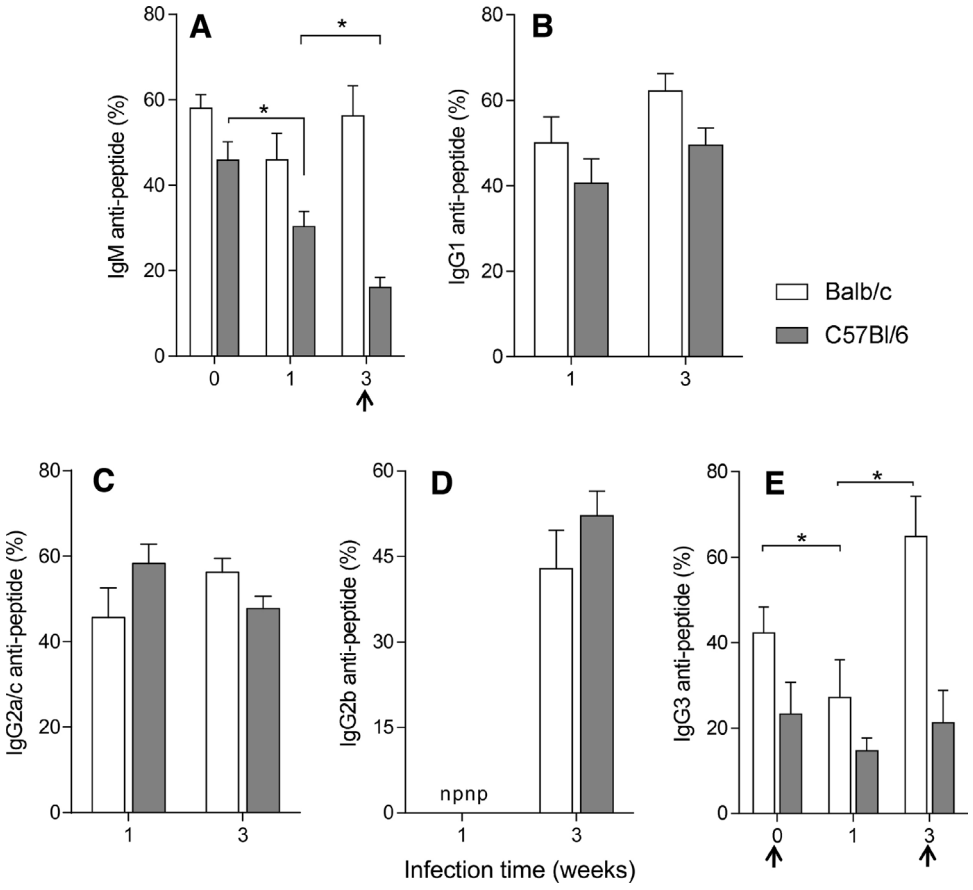

Fig. 4. Peptide epitopes recognition by early systemic antibodies to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 8$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. Infected mice from both strains were bled prior to infection (0 week) 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-

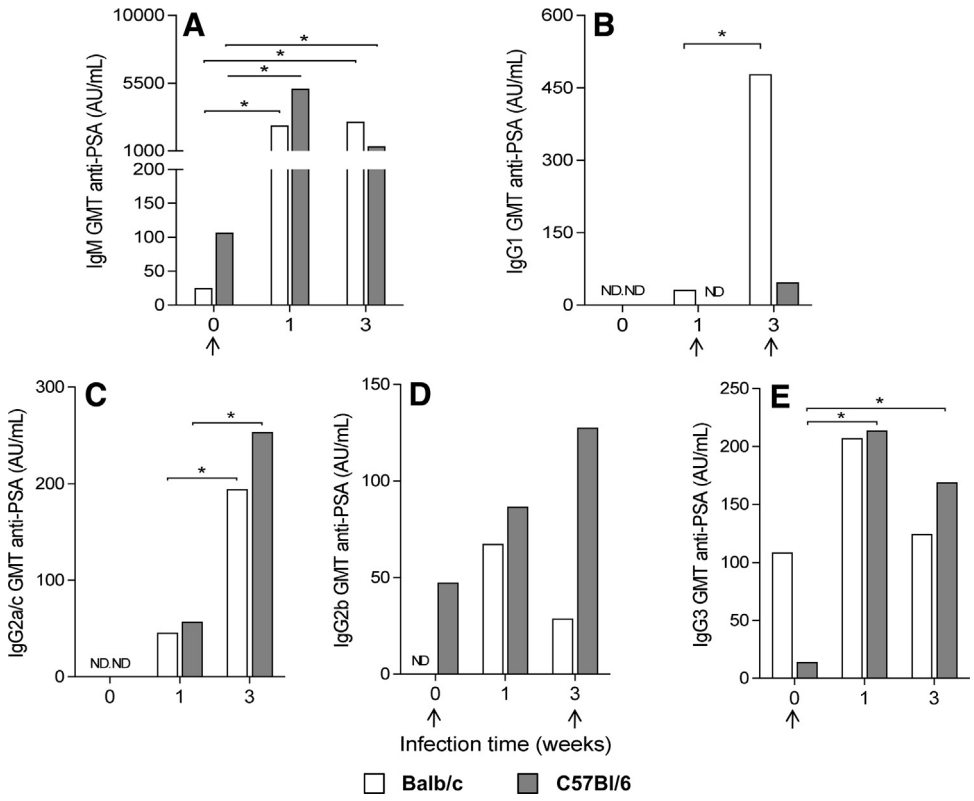

Fig. 5. Kinetics of the early peritoneal antibody response to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 15$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. At 0 (pre-infection), 1 and 3 weeks pi, 5 mice per strain were euthanized 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;

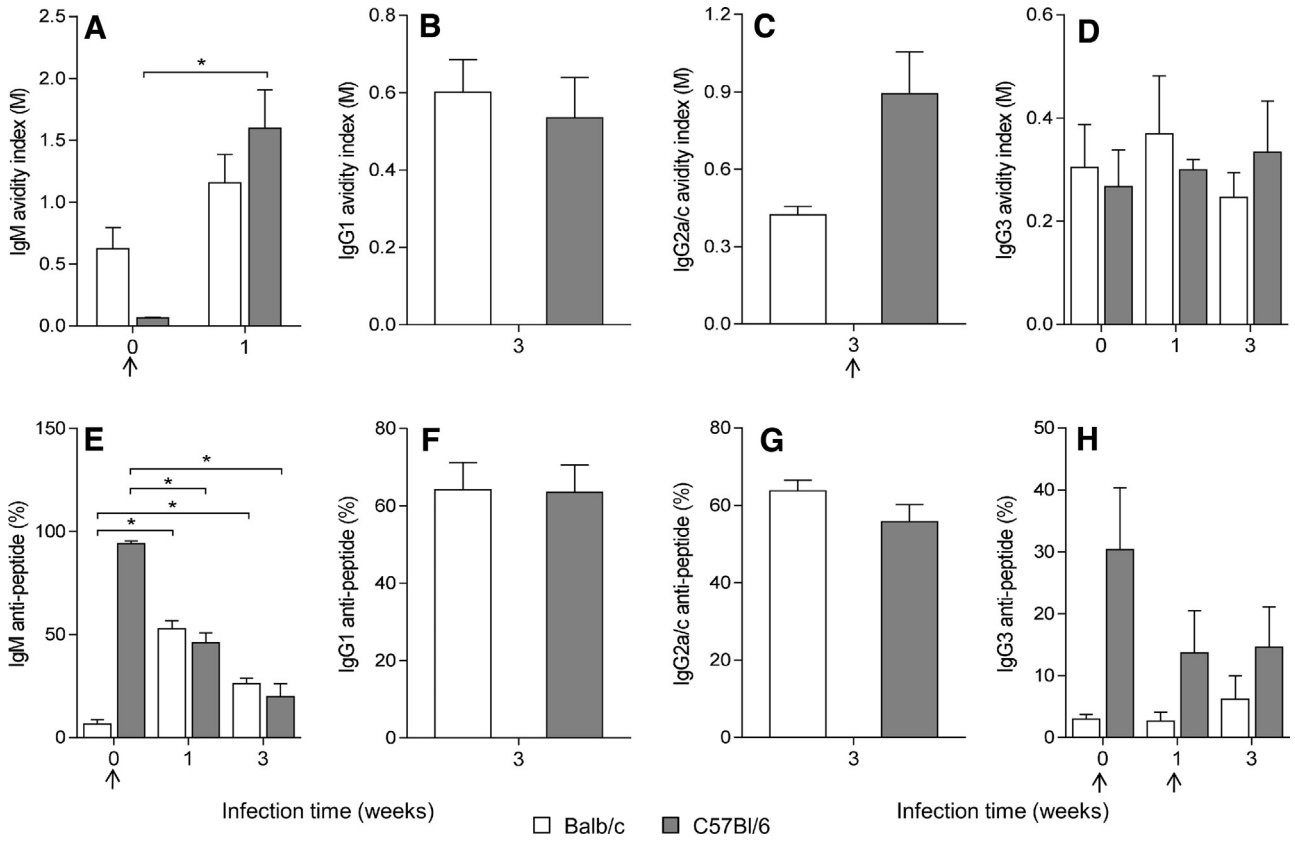

Fig. 6. Quality analyses of early peritoneal antibodies to *E. granulosus* in Balb/c and C57Bl/6 infected mice.
Balb/c and C57Bl/6 mice ($n = 15$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. At 0 (pre-infection), 1 and 3 weeks pi, 5 mice per strain were euthanized 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.

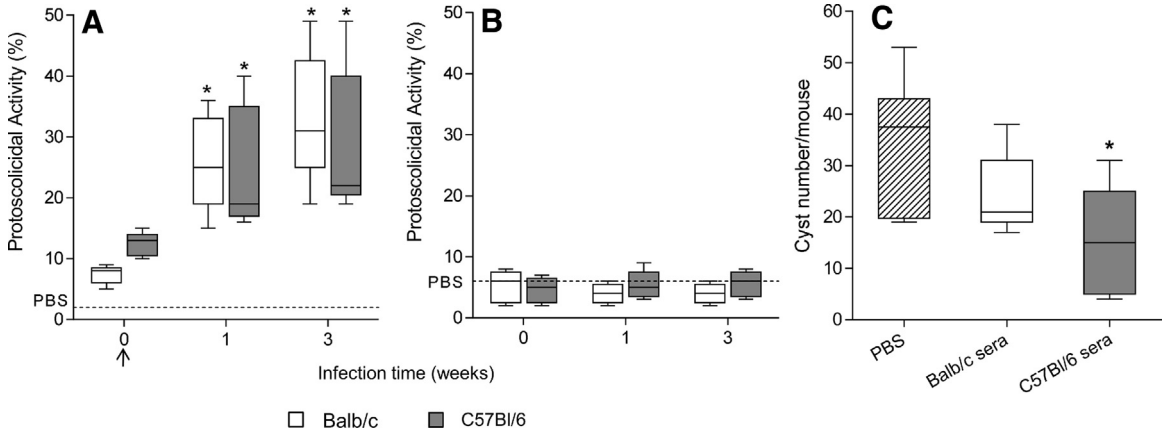

Fig. 7. *In vitro* and *in vivo* anti-parasite activity of Balb/c and C57Bl/6 mice sera.
For *in vitro* serum protoscolicidal activity assessment, Balb/c and C57Bl/6 mice ($n = 15$ per strain) were inoculated ip with 2000 protoscoleces in 200 μL of sterile PBS. At 0 (pre-infection), 1 and 3 weeks pi, 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.

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