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Echinococcus granulosus glycoconjugates induce peritoneal B cell differentiation into antibody-secreting cells and cytokine production

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SUMMARY

Helminth parasite infections are associated with predominant Th2-type cytokine responses, and parasite glycoconjugates have been recognized as partially responsible for such immune bias. It has been proved that Echinococcus granulosus *evokes a Th2-type cytokine pattern characterized by a high production of IL-4, IL-5, IL-6 and IL-10, and no or mild IFN-γ levels in animal models and in patients with cystic echinococcosis, respectively. Here, we show that E4$^+$ (a glycoconjugate-enriched fraction from* E. granulosus protoscolex*) stimulated the secretion of a high concentration of IL-6, followed by IL-10 and TNF-α by normal peritoneal B cells. We determined that E4$^+$ bound to the surface of peritoneal B cells and induced their activation and, also, triggered the differentiation of peritoneal B cells into IgM-, IgG2b- and IgG3-secreting cells in a T-independent way. Interestingly, the IgM released by E4$^+$-stimulated peritoneal B cells from normal mice recognized protoscolex antigens. Results showed that, after the encounter with antigens from* E. granulosus protoscolex*, peritoneal B cells are a source of Th2-type cytokines and polyclonal antibodies, some of which recognize parasite antigens, suggesting that peritoneal B cells can condition the outcome of the infection.*

Keywords *cytokines,* Echinococcus granulosus*, glycoconjugates, peritoneal B cells*

INTRODUCTION

Parasites usually produce long-lasting infections in immunocompetent hosts. Modulation of host immune responses has been proposed as a strategy to ensure parasite survival (1). Host cytokine response determines the outcome of parasite infections (2,3). Most helminth infections bias such responses towards a Th2-type pattern (4–6), which have not been always associated with protective immunity.

Human cystic echinococcosis is a worldwide-distributed cosmopolitan zoonosis caused by the larval stage of the tapeworm *Echinococcus granulosus* (7–10). Human secondary infections are an important medical problem occurring by dissemination of viable protoscoleces after an accidental rupture of fertile cysts and are caused by protoscoleces' ability to develop into new metacestodes (11) evading the host immune responses. Murine experimental cystic echinococcosis is the current model to study *E. granulosus*–host interactions. Experimentally infected mice react against protoscoleces producing local innate and systemic adaptive immune responses that have been well characterized (12–22). We have previously shown that *E. granulosus* induces systemic Th2-type cytokine responses in the early stages of experimental infection (23,24). Because the infection is successfully established despite such an early response, Th2-type responses seem not to be associated with protective immunity. Moreover, results reported by us and others suggest that Th1-type responses may be effective against experimental and natural human cystic echinococcosis (25–28), mainly owing to IFN-γ- and nitric oxide-mediated mechanisms (29–31).

Parasite antigens with immunomodulatory properties have been ascribed as partially responsible for the observed cytokine bias, many of them being glycoconjugates (32–46). Glycoconjugates of *E. granulosus* cysts and protoscoleces are quantitatively important (47,48) and constitute the main antigens of the antibody response seen during the chronic stage of experimental infections (18,19,49). Also, humoral response in early stages of experimental infection shows a strong component of antibodies produced independently of CD4$^+$T cells (50,51), and it was suggested that it can be triggered by carbohydrate-bearing parasite antigens (52–54).

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Previously, we demonstrated that a carbohydrate-enriched antigenic fraction from *E. granulosus* protoscoleces (named $E4^+$) is recognized by antibodies from both $CD4^+$ T cell-depleted and CD4-knockout-infected mice (50,51). $E4^+$ is also recognized by antibodies from patients with cystic echinococcosis (55). Moreover, $E4^+$ induces *in vitro* IL-10 secretion by peritoneal cells from infected and normal mice and by peripheral blood mononuclear cells (PBMC) from patients with cystic echinococcosis and healthy donors (55,56). These results suggest that components present in $E4^+$ would have *in vivo* important biological functions such as modulation of T cell response and antibody production. Peritoneal B lymphocytes are specialized in T-independent antibody secretion (57–59) and have also been shown to be an important source of IL-10 *in vivo* (60,61). Therefore, it is tempting to speculate that antigens present in $E4^+$ and, hence, in protoscoleces could signal peritoneal B cells of normal mice to produce cytokines and antibodies in a T-independent way.

MATERIALS AND METHODS

Mice

Adult female Balb/c mice (6–8 weeks of age) were obtained from DI.LA.VE. (Uruguay) or from C.N.E.A. (Argentina) and were housed in the animal facility at Instituto de Higiene (Montevideo, Uruguay) and Facultad de Ciencias Químicas (U.N.C. – Córdoba, Argentina). Animal experiments were performed in compliance with Comisión Honoraria de Experimentación Animal (CHEA)-Universidad de la República according to the National Uruguayan Legislation No.18.611 (2009) ('Animales en Experimentación e Investigación'), Protocol Number: 090510 (http://csic1.csic.edu.uy/chea).

Protoscolex somatic antigens (PSA)

Echinococcus granulosus protoscoleces were obtained by aseptic puncture of fertile bovine hydatid cysts, and parasite viability was determined according to Baz *et al.* (62). Protoscoleces batches with viability over 90% were pooled together and stored at −20°C until we reached an adequate amount of protoscoleces to prepare enough PSA. Protoscolex somatic antigens were prepared according to Baz *et al.* (50) by ultrasound disruption of viable protoscoleces. The obtained supernatant was filtered through 0·22-μm sterile membranes and stored at −20°C until use. Protoscolex somatic antigens reproducibility has been checked by comparing roughly SDS–PAGE profiles and carbohydrates-to-proteins ratio (usually 1 : 1).

Purification of the carbohydrate-enriched fraction from PSA

Carbohydrate-enriched fraction ($E4^+$) was prepared from PSA by affinity chromatography using E492/G1 MoAb coupled to Sepharose-CNBr (56). $E4^+$ was filtered through 0·22-μm sterile membranes and stored at −20°C until use. Protein concentration was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) following manufacturer's instructions, and carbohydrate concentration was determined by the orcinol–sulphuric acid assay (49). $E4^+$ batches were tested for their carbohydrates-to-proteins ratio (usually 4 : 1) and their enrichment in the epitope recognized by MoAb E492/G1 in comparison with PSA (by ELISA). Endotoxin-free status of $E4^+$ was determined by LAL test using the Endotoxin Detection Kit (Sigma, St. Louis, MO, USA) following manufacturer's instructions. To ensure lipopolysaccharide (LPS) absence, some experiments were performed with the addition of polymyxin B (Sigma) at 10 μg/mL, which is sufficient to completely abrogate the *in vitro* effects of LPS concentrations easily detectable by LAL assay (63).

$E4^+$ binding assay

Peritoneal cells were obtained from normal Balb/c mice by extensive peritoneal cavities washing under sterile conditions with cold RPMI 1640 (Sigma) supplemented with 2% FCS (Gibco, Grand Island, NY, USA). Peritoneal cells from normal Balb/c mice were incubated with Mouse Fc Block (Becton Dickinson, Franklin Lakes, NJ, USA) and then were incubated for 30 min at 4°C with $E4^+$ (50 μg/mL carbohydrates concentration) and appropriately diluted rat anti-mouse CD19-PE (Pharmingen, Becton Dickinson). Subsequently, cells were incubated with an appropriate dilution of MoAb E492/G1-Biotin and were finally incubated with Streptavidin-FITC (Pharmingen, Becton Dickinson). Several controls were used: (i) anti-mouse CD19-PE, E492/G1-Biotin and Streptavidin-FITC; (ii) anti-mouse CD19-PE, $E4^+$ and Streptavidin-FITC; and (iii) anti-mouse CD19-PE and Streptavidin-FITC. Analyses were performed on a FACScalibur flow cytometer (Becton Dickinson) using Cell Quest® software (Becton Dickinson).

Peritoneal B cells isolation

Purification of peritoneal B cells from normal Balb/c mice was performed by negative selection using specific antibodies and magnetic beads. Peritoneal cavities were extensively washed under sterile conditions with cold RPMI 1640 (Sigma) supplemented with 2% FCS (Gibco), and peritoneal cells were suspended in cold RPMI medium, and Fc

receptors were blocked by incubation with Mouse Fc Block (Becton Dickinson) for 20 min. at 4°C. After that, cells were incubated with appropriate dilutions of rat IgG anti-mouse F4/80 MoAb in PBS-FCS 2% for macrophages depletion. Then, stained cells were separated by treatment with magnetic beads coated with goat anti-rat IgG (Dynal Biotech, Invitrogen, Oslo, Norway) following manufacturer's instructions. Subsequently, T cells were removed by treatment with magnetic beads coated with rat IgG anti-mouse Thy1.2 (Dynal Biotech, Invitrogen). Finally, isolated B cells were controlled for their purity by flow cytometry using appropriate fluorochrome-stained monoclonal antibodies and isotype controls, determining in all cases >95% purity in $CD19^+$ cells.

Cell cultures

Total peritoneal cells were seeded in 48-well plates at 1×10^6 cells/well in complete culture medium (RPMI 1640, 10% FCS, 50 μM 2-mercaptoethanol, 100 μg/mL streptomycin and 100 U/mL penicillin, all obtained from Sigma) and incubated with 80 μg/mL of $E4^+$ (carbohydrate concentration) or LPS (5 μg/mL) for 3 or 7 days at 37°C and 5% CO_2. Isolated peritoneal B cells were seeded in 96-well plates at 2×10^5 cells/well in complete culture medium and incubated with 80 μg/mL of $E4^+$ (carbohydrate concentration) or unmethylated cytosine-phosphate-guanine oligodeoxynucleotide (CpG) (1 μg/mL) for 24, 48 or 72 h at 37°C and 5% CO_2. Culture supernatants from isolated B cells were then recovered, and cytokine concentrations were immediately determined.

In both cases (total peritoneal cells and isolated peritoneal B cells cultures), remaining supernatants were stored at −20°C until further antibody analyses.

Phenotypic analyses

Peritoneal cells from normal mice incubated with $E4^+$ or LPS were recovered and incubated with Mouse Fc Block (Becton Dickinson) for 30 min at 4°C. Then, they were incubated for 30 min at 4°C with the following anti-mouse MoAb: CD19-FITC, CD138-PE and CD86-PE, and their specific isotype controls (Pharmingen, Becton Dickinson). Analyses were performed with FACScalibur flow cytometer (Becton Dickinson) using Cell Quest® software (Becton Dickinson).

Cytokines and total antibody determinations

IL-10, IL-6, TNFα and IFNγ concentrations in isolated peritoneal B cells culture supernatants were determined by ELISA using commercial reagents and following the manufacturer's instructions (PharMingen, San Diego, CA, USA). Recombinant mouse cytokines (PharMingen) were used for standard curves.

Total IgM, IgG1, IgG2a, IgG2b and IgG3 antibody concentrations in culture supernatants from peritoneal cells and isolated B cells were also performed by capture ELISA using commercial reagents and following the manufacturer's instructions (Sigma). Purified mouse isotypes (Sigma) were used for standard curves. In all cases, peroxidase activity was determined using O-phenylenediamine (Sigma) and recorded at 492 nm (Titertek Multiscan Plus; Flow Laboratories, Helsinki, Finland).

Specific antibody determination

Anti-PSA-specific antibodies in B cells culture supernatants were measured by ELISA according to Dematteis *et al.* (56). In equally diluted samples, specific IgM and IgG were determined using appropriate goat anti-mouse IgM or anti-mouse IgG antibodies labelled with peroxidase (Sigma). Peroxidase activity was detected using *O*-phenylenediamine as chromophore (Sigma), and absorbance values were recorded at 492 nm (Titertek Multiscan Plus; Flow Laboratories).

Statistical analyses

Statistical analyses were assessed by Student's *t*-test, and differences were regarded as significant with $P < 0{\cdot}05$.

RESULTS

$E4^+$ triggers cytokine production by normal peritoneal B cells

Analysing the appropriate $E4^+$ concentration to use in B cell culture, we observed that $E4^+$ induction of IL-10 and IL-6 secretion by normal peritoneal cells was dose dependent (data not shown). Because of being the minimal concentration able to stimulate high levels of IL-10 and IL-6 secretion, 80 μg/mL (carbohydrate concentration) of $E4^+$ was the selected concentration for further *invitro* assays.

To analyse cytokine production by B cells, including IL-10, we isolated peritoneal B cells from normal Balb/c mice by negative selection and cultured them with $E4^+$ during different time points. IL-10 and other cytokines produced by B cells were determined by ELISA. Results show that peritoneal B cells from normal mice secreted, in culture supernatants, significant levels of IL-10, IL-6 and TNFα in response to $E4^+$ (Fig. 1), while IFN-γ was not detected neither in $E4^+$- nor in CpG-stimulated cultures (data not shown). Interestingly, IL-6 and TNFα

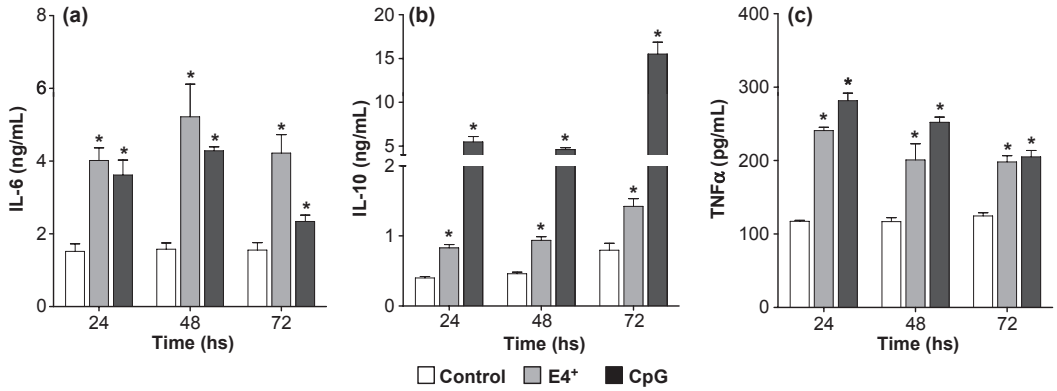

Figure 1 $E4^+$ triggers cytokine production by normal peritoneal B cells. Peritoneal B cells from normal mice were isolated by negative selection and were then incubated with medium, 80 μg/mL $E4^+$ and 1 μg/mL of CpG for 24, 48 and 72 h. IL-10, IL-6 and TNFα concentrations in culture supernatants were determined by ELISA. Data are shown as mean ± SEM. *Statistical significance ($P < 0{\cdot}05$) related to control (B cells cultured with medium alone).

concentrations were similar to those induced by a strong B cell stimulator like CpG (Fig. 1a,c).

$E4^+$ binds to peritoneal $CD19^+$ cells from normal mice

By flow cytometry, we analysed whether components present in $E4^+$ were able to bind to peritoneal B cells from normal Balb/c mice. Contour plots in Fig. 2 show that E492/G1 MoAb recognized *E. granulosus* protoscolex antigens on the surface of $CD19^+$ cells (Fig. 2c) previously incubated with $E4^+$, indicating that $E4^+$ was able to bind to a high percentage of peritoneal B cells (roughly 50%). It is worth noting that no $E4^+$ binding was observed on other cells with lymphoid morphology and nonlymphoid cells (Fig. 2d,e).

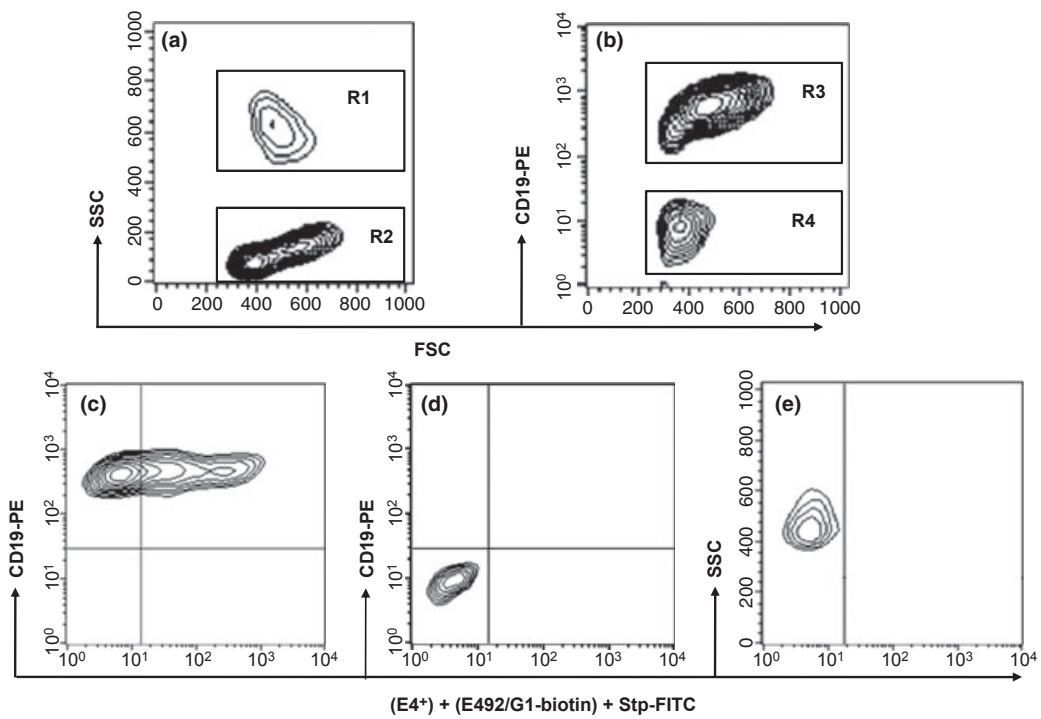

Figure 2 $E4^+$ binds to normal peritoneal B lymphocytes. Peritoneal cells from normal mice were incubated with anti-mouse CD19-PE, $E4^+$, E492/G1-Biotin and Streptavidin-FITC and were then analysed by flow cytometry. Nonlymphoid cells (R1) and lymphocytes (R2) were identified by means of FSC and SSC values (a). $CD19^+$ (R3) and $CD19^-$ (R4) lymphocytes were determined through an R2 subordinated analysis (b). Studies on $E4^+$ binding to $CD19^+$ (c) and $CD19^-$ (d) lymphocytes were performed through R2 and R3 subordinated analyses, respectively. $E4^+$ binding studies to nonlymphoid cells were performed through R1 subordinated analysis (e).

$E4^+$ activates peritoneal B cells from normal mice

Next, we analysed whether $E4^+$ –B cell interaction triggered B cell activation studying the increase in the expression of co-stimulatory molecules such as CD86 on B cell surface (64). Results showed that, although the percentage of $CD19^+$ $CD86^+$ cells was similar in B cells cultured with $E4^+$ or medium alone (Fig. 3a), CD86 surface expression density on $CD19^+$ cells cultured with $E4^+$ significantly increased (Fig. 3b). LPS, a TLR ligand known as a murine B cell activator, was used as positive control that increased the expression of co-stimulatory molecules and cell size. Interestingly, we observed that $E4^+$ increased peritoneal B cell size after 7 days of culture (Fig. 3d),

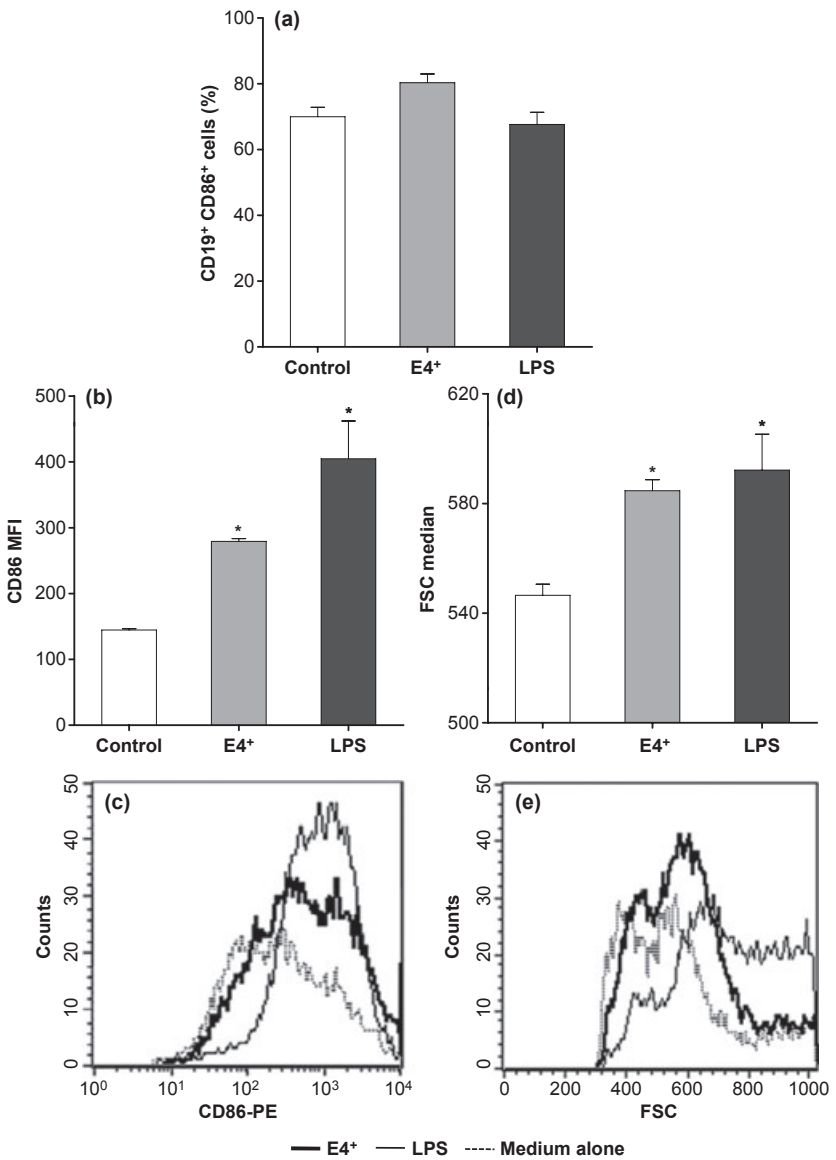

Figure 3 $E4^+$ activates normal peritoneal B cells. Peritoneal cells from normal mice were incubated for 72 h (a–c) or 7 days (d–e) with $E4^+$, LPS or medium alone (control). Then, CD86 surface expression on $CD19^+$ lymphocytes (a–c) and cell size (d–e) was analysed by flow cytometry. (a) Percentage of $CD19^+$ lymphocytes co-expressing CD86. (b) Mean fluorescence intensity (MFI) of CD86 on $CD19^+$ lymphocytes. (c) Representative histogram showing CD86 expression on $CD19^+$ lymphocytes. (d) Median FSC value for $CD19^+$ lymphocytes. (e) Representative histogram showing FSC values for $CD19^+$ lymphocytes. Gating strategies are described in Fig. 2 (all results derive from R3). Dotted lines, solid lines and thick solid lines in histograms represent control, LPS and $E4^+$ conditions, respectively. Data in bar graphs are shown as mean ± SEM. *Statistical significance ($P < 0{\cdot}05$) related to control.

which can be associated with the characteristic morphological modifications occurring during the generation of an efficient secretory machinery. These results suggest that normal peritoneal B cells were activated upon binding to $E4^+$ antigens.

$E4^+$ induces normal B cell differentiation into plasma cells in a T-independent way

Activated B cells are able to differentiate into memory or plasma cells (65,66). To gain insight into the capacity of $E4^+$ to induce terminal differentiation of B cells, we evaluated $CD19^+$ cells cultured with $E4^+$ for the expression of syndecan-1 (a plasma cell marker). We observed that $E4^+$ induced normal B cell differentiation into a $CD19^+$ $CD138^+$ phenotype, with reduced CD19 surface expression (Fig. 4b,d,e,f). It is noteworthy that the phenotypic characteristics of the B cell stage induced by $E4^+$ were very similar to those induced by LPS (Fig. 4c–f). Because $CD19^+$ $CD138^+$ cells represent a phenotype of plasma cells (66), we studied IgM concentration in culture supernatants as a way to confirm the differentiation process into plasma cells. Figure 4g shows that IgM antibodies were secreted by peritoneal B cells from normal mice stimulated with $E4^+$. The IgM production was not affected by polymyxin B treatment, indicating that $E4^+$ effects were not mediated by contaminating LPS (Fig. 4g).

Additionally, we analysed whether $E4^+$ was able to induce IgG secretion by isolated peritoneal B cells from normal mice. Results in Fig. 5a show that total IgG2b and IgG3, in addition to IgM, were induced by $E4^+$ in isolated peritoneal B cells after 72 h of stimulation. When analysing whether $E4^+$-induced antibodies can recognize *E. granulosus* antigens, we observed that IgM was the only isotype able to bind to parasite antigens (Fig. 5b). No anti-PSA-specific IgG was detected in culture supernatants (data not shown). As previously reported (67,68), a high concentration of IgM and all IgG subclasses (data not shown) was detected in the supernatant of B cells cultured with CpG (used as a stimulus that triggers plasma cell differentiation).

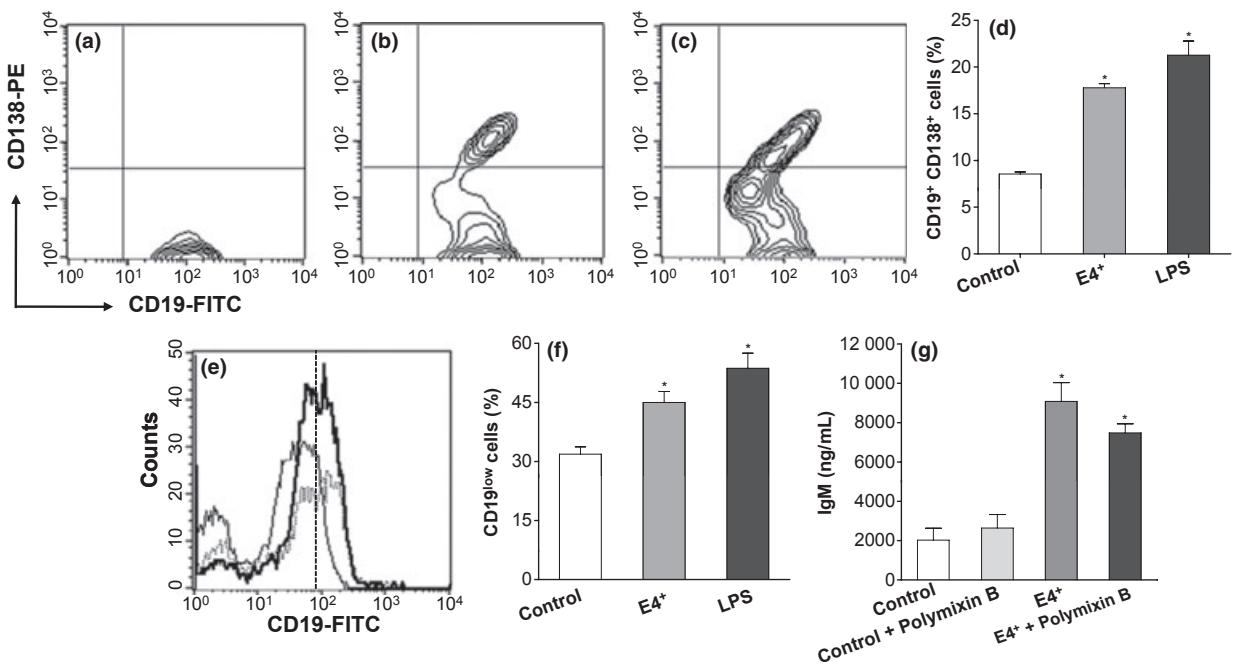

Figure 4 $E4^+$ induces normal peritoneal B cell differentiation into plasma cells. Peritoneal cells from normal mice were incubated with $E4^+$, LPS or medium alone (control) for 7 days. $CD19^+$ $CD138^+$ (a–d) and CD19 fluorescence intensity (e–f) within lymphocytes were analysed by flow cytometry. Contour plots are representative samples of control (a), $E4^+$ (b) and LPS (c) culture conditions. Percentages of $CD19^+$ $CD138^+$ lymphocytes are shown (d). A representative histogram showing CD19 expression within lymphocytes, as well as the criteria used to define $CD19^{low}$ cells (vertical dotted line), is displayed (e). Percentages of $CD19^{low}$ cells within lymphocytes are also shown (f). Gating strategies are described in Fig. 2. Dotted lines, solid lines and thick solid lines in histograms represent control, LPS and $E4^+$ conditions, respectively. Total IgM concentration in culture supernatants was analysed by ELISA (g). Besides negative results in LAL tests, absence of contaminating LPS was ensured by addition of polymyxin B to cell cultures. Data in bar graphs are shown as mean ± SEM. *Statistical significance ($P < 0{\cdot}05$) related to control.

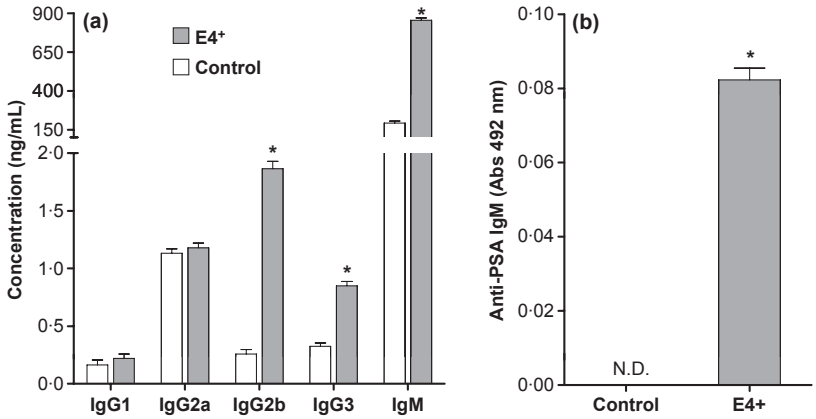

Figure 5 $E4^+$ induces T-independent polyclonal antibody secretion by normal peritoneal B cells. Peritoneal B cells isolated from normal mice were incubated with $E4^+$ for 72 h. Culture supernatants were analysed by ELISA for total IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies concentration (a) and anti-protoscolex somatic antigens-specific IgM (b). Data are shown as mean ± SEM. *Statistical significance ($P < 0{\cdot}05$) related to control (peritoneal B cells cultured with medium alone). ND, not detected.

DISCUSSION

Immunity to pathogens often requires both B cell-dependent humoral and T cell-dependent cellular immune responses that cooperate to clear infectious organisms. $CD4^+$ T cells clearly participate in the antibody responses by providing help to B cells, and they can enhance cellular immunity by producing cytokines; B cells produce antibodies against pathogens, and also, they can secrete cytokines that influence T cell responses (69).

Helminth infections are interesting systems where Th2-type immunomodulation plays a relevant role. In this regard, pathogen antigens have shown to possess relevant biological activities, such as inhibition of both cell proliferation (33) and macrophage effector functions (66). These activities are thought to be partially attributed to IL-10 that indirectly contributes to Th2-type cytokine bias by downregulating Th1-type cytokine secretion (70).

Particularly, helminth parasite glycoconjugates have important roles in driving cytokine response polarization (38,71). In addition to well-known cytokine producers like macrophages and T cells, B cell effector populations able to produce cytokine profiles (e.g. IL-4, IL-10 or IFN-γ) that bias the differentiation of naïve $CD4^+$ T cells into Th1 or Th2 subpopulations have been described (72). Also, B cells have shown to play key roles in immunity against helminth parasites by regulating local and early cytokine responses (73,74).

Here, we have shown that a glycoconjugate-enriched fraction from *E. granulosus* protoscolex ($E4^+$) directly interacted with peritoneal B cells from normal mice and triggered IL-10 as well as IL-6 and TNFα secretion. Interestingly, $E4^+$ was not able to induce detectable levels of IFNγ. Our results agree with those reported by Velupillai and Harn (32) and Velupillai *et al.* (75), which show that carbohydrates from *Schistosoma mansoni* eggs induce IL-10 secretion by B cells, suggesting that this cell population could contribute to the driving of cytokine production towards a Th2-type profile.

In cystic echinococcosis, IL-10 and IL-6 are considered key cytokines in the generation of protective/permissive T cell responses as well as in the monocytes response (30,76,77). It has been suggested that Th2-type cytokines are responsible for disease susceptibility and are associated with chronic stages (76). Also, it has been reported that IL-10 reduces the *in vitro* protoscoleces killing by PBMC from patients with cystic echinococcosis, and this finding correlates with a decrease in NO production (30). Additionally, Rigano *et al.* (77) showed that IL-10 impairs DC maturation and monocytes differentiation, probably contributing to *E. granulosus* evasion from host immune response.

Then, B cells can be considered as an additional source of cytokines related to *E. granulosus* establishment. In this regard, it is interesting to note that the ability of $E4^+$ components to induce IL-10 and IL-6 secretion by peritoneal normal B cells could explain, at least partially, the rapid bias towards Th2-type cytokine pattern observed in *E. granulosus*-infected Balb/c mice (96). The fact that IL-10 and IL-6 production by B cells could condition the profile of T helper cells should be carefully analysed because it has been demonstrated that CpG, as well as $E4^+$ that induces IL-10 and IL-6 production by B cells, is a good inducer of a Th1-type response. Probably, this is attributed to the fact that CpG triggers IL-12 production by dendritic cells and macrophages. While dendritic cells

produce IL-12, IL-6 and IL-10 following TLR-9 ligation, B cells only produce IL-6 and IL-10 in response to CpG (78). Furthermore, in the same work, it has been shown that naïve $CD19^+$ cells, B1 cells and MZ B cells from spleen after CpG stimulation express IL-10 mRNA but not IFN-γ nor IL-12p40 mRNA. Reinforcing this idea, Bamba *et al.* (79) previously showed that following CpG stimulation, peritoneal macrophages and B cells mainly produce IL-12 and IL-10, respectively.

Many pathogens, including parasites, possess immunogenic antigens able to induce T-independent immune responses (54,80,81) and non-specific polyclonal activation (15,82–85). Our results showed that $E4^+$ induces peritoneal B cell differentiation into plasma cells in a T-independent way and, consequently, it induces the production of total IgM, IgG2b and IgG3 antibodies, indicating that components in $E4^+$ may act as polyclonal B cell activators. Interestingly, we have recently observed that *E. granulosus* induces early plasma cell differentiation (96) with active secretion of total IgM and IgG2b antibodies in the peritoneal cavity of infected mice (Mourglia-Ettlin G & Dematteis S, unpublished data).

Polyclonal B cell activation (86,87) and parasite Fc-like receptors (88–91) are thought to be partially responsible for anti-parasite ineffective humoral responses. In diverse infection models of strong polyclonal B cells responses, the most common isotypic distribution pattern is characterized by an increase in IgG2 subclasses (87,92–94). Thus, polyclonal antibodies induction might constitute an evasion strategy exploited by *E. granulosus* to favour its survival. Our results show that components present in $E4^+$ may be responsible for this evasion mechanism.

Interestingly, we observed that $E4^+$ triggers the production of IgM antibodies that bind to *E. granulosus* antigens. This result suggests that antigens present in PSA are recognized by natural IgM antibodies product of polyclonal B cells activation. However, no specific IgG anti-PSA antibodies were detected in culture supernatants. It is important to note that we have previously reported that $E4^+$ accounts for roughly 40% of the epitopes recognized by specific IgG and IgM developed in Balb/c mice immunized with PSA (50). Moreover, we have also reported that IgG2a-, IgG2b- and IgG3-specific antibodies developed in Balb/c mice inoculated with $E4^+$ recognize PSA antigens in almost the same extent as specific IgG subclasses developed following PSA inoculation (56). Therefore, the detection of non-specific IgG might be the result of PSA used as the ELISA coating antigen rather than $E4^+$.

In addition, it has been reported that in the presence of specific antibodies on the protoscoleces surface, the tegumental membrane depolarization is faster and stronger than that observed in the absence of specific antibodies (95). Therefore, it is possible that IgM antibodies that recognize protoscolex antigens, through the activation of the complement system, could be part of the mechanism involved in the elimination of the protoscolex observed in the experimental cystic echinococcosis (18,24). However, this should be demonstrated.

Finally, taking into account that a protective role for antibodies induced by *E. granulosus* is still unknown, results reported here suggest that peritoneal B cells once stimulated with $E4^+$ produce cytokines and antibodies that could condition the outcome of the infection. Currently, we are working on the isolation and characterization of $E4^+$ components responsible for the effects described here, which are, interestingly, quite similar to those induced by live protoscoleces in infected mice.

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