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Short Communication

Fasciola hepatica excretory-secretory products induce CD4+T cell anergy *via* selective up-regulation of PD-L2 expression on macrophages in a Dectin-1 dependent way

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ABSTRACT

Fasciola hepatica excretory-secretory products (FhESP) induce immunomodulatory effects on macrophages. Previously, we demonstrated that these effects are dependent on Dectin-1. Therefore, the aim of this study was to determine how this affects the CD4 T-cells immune response.

We observed that FhESP induce an increased expression of PD-L2 in macrophages *via* Dectin-1. Furthermore, in co-cultures with CD4 T-cell we observed a suppressive effect on proliferative response, down-modulation of IFN-γ and up-modulation of IL-10 *via* Dectin-1 on macrophages. These results suggest that FhESP induce T-cell anergy *via* selective up-regulation of PD-L2 expression on macrophages in a Dectin-1 dependent way.

Introduction

Fasciolosis is a chronic infection in which the parasite is established in the bile ducts, thus demonstrating its ability to evade the host immune system. Similarly to other helminthes, *Fasciola hepatica* infection is characterized by an adaptive immune response associated with a Th2 profile (Finkelman et al., 1991; O'Neill et al., 2000; Maizels and Yazdanbakhsh, 2003; Flynn et al., 2010; Ashrafi et al., 2014). Other features of helminth infections are a down-modulated T cell proliferative response to parasite antigens as well as to unrelated antigens (Maizels et al., 2004), and the presence of two subpopulations of regulatory cells, namely regulatory T cells (Wilson and Maizels, 2006) and alternatively activated macrophages (aaMΦ) (Loke et al., 2002; Rodriguez-Sosa et al., 2002). In this regard, the interaction of FhESP with the innate immune cells, such as peritoneal macrophages (pMΦ), is critical for the establishment of this parasite in the host, and different experimental models have demonstrated the induction of aaMΦ by FhESP (Donnelly et al., 2005; Donnelly et al., 2008; Walsh et al., 2009; Figueroa-Santiago and ESpino, 2014). In a previous work we observed that the *F. hepatica* released products induced a pMΦ population with immunoregulatory properties during the early stages of infection as well as after *in vitro* stimulation, with these effects being dependent on Dectin-1 (Guasconi et al., 2011). Therefore, the aim of this study was to evaluate the participation of Dectin-1 in the immunomodulatory effects induced by FhESP on pMΦ, and to determine how this affects the adaptive immune response.

Materials and methods

Reagents

RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 50 μg/ml gentamicin (Sigma–Aldrich Co., St. Louis, MO, USA) were used. The reagents used and their sources are: 2-mercaptoethanol (Merck, Damstadt, Germany), anti-mouse Dectin-1 antibody (R&D Systems, Minneapolis, MN, USA), PE-anti-mouse F4/80 (Invitrogen, Carlsbad, CA, USA), IL-10 Cytoset and the IFN-γ kit (Biosource, Camarillo, CA, USA; and BD Biosciences, San Jose, CA, USA, respectively). Anti-mouse CD16/CD32, PE-anti-mouse I-A/I-E, anti-mouse CD3e (NA/LE), FITC-anti mouse CD4 and the respective isotype controls (BD Biosciences). Purified anti-mouse CD273 (anti-mouse PD-L2) and anti-mouse CD274 (anti-mouse PD-L1) antibodies (e-Bioscience, San Diego, CA, USA), and FITC anti-mouse IgG (Sigma–Aldrich).

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FhESP preparation

FhESP were prepared according to a procedure described by Diaz et al. (1998) with some variants. Briefly, live adult worms of *F. hepatica* were obtained from the bile ducts of bovine livers, and then washed with phosphate buffered saline (PBS) pH 7.4 before being incubated (1 worm/2 ml of PBS) for 3 h at 37 °C. Then, the supernatant was centrifuged (10,000 rpm, 30 min, 4 °C) before being concentrated using a high-flow YM 10 membrane filter (Millipore-Amicon Corp.), and stored at −20 °C until used. The protein concentration (500 μg/ml) was measured by a Bradford protein assay (Bio-Rad, CA, USA), and the quantity of contaminating LPS present in FhESP was determined using the Limulus amebocyte lysate test (Endosafe Times, Charles River, laboratories, Wilmington, Delaware), resulting in endotoxin levels < 90 units per milliliter of FhESP and <3.6 endotoxin units per milliliter (or 0.36 ng/ml) in the final culture conditions.

Animals

Six- to eight-week-old female BALB/c mice were purchased from the Ezeiza Atomic Center (CNEA, Buenos Aires, Argentina), and housed and cared for in the animal resource facilities of the Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Cordoba, following institutional guidelines. All experimental protocols were approved by the Animal Experimentation Ethics Committee, Faculty of Chemical Sciences, National University of Cordoba.

Purification of macrophages from PECs

To obtain pMΦ, the peritoneal cavity was washed with ice-cold PBS containing 0.1% FBS and 5 mM EDTA. After determination of viability by trypan blue exclusion (cell viability was >95%), the PECs were re-suspended in RPMI supplemented with 10% FBS, 2 mM glutamine and 50 μg/ml gentamicin, and then cultured for 2 ½ h at 37 °C in an atmosphere of 5%CO_2. The remaining adherent cells were highly enriched in macrophages (MΦ), with flow cytometry analysis revealing 90% F4/80$^+$ cells.

Culture of naive pMΦ

Macrophages obtained from non-infected Balb/c mice were stimulated with FhESP at a concentration of 20 μg/ml for 48 h.

To investigate the role of Dectin-1 in the FhESP effects, the cells were pre-incubated with anti-Dectin-1 (0.5 μg/ml) for 30 min at 37 °C in 5% CO_2. Then, after being washed, these cells were stimulated with FhESP at a concentration of 20 μg/ml for 48 h. In some experiments, 5 μg/ml of anti-PD-L1 or anti-PD-L2 were added during the MΦ cultures.

Culture of pMΦ derived from infected animals

Mice were orally infected with 10 metacercariae of *F. hepatica* (Baldwin Aquatics Inc.). Then, 48 h after being infected, peritoneal lavages were carried out and the adherent cells were obtained.

To evaluate the participation of Dectin-1 during the infection, mice were intraperitoneally injected with Laminarin (0.5 mg/ml) or PBS 4 h before being infected (Ozment-Skelton et al., 2006). The blocker concentrations used were based on *in vitro* experiments, and a volume of 2 ml was utilized for the peritoneal cavity.

In vitro antigen presentation by MΦ to naive T cells

Naive mononuclear spleen cells (MSCs) were obtained from untreated BALB/c mice, by pressing spleens through wire-mesh screens to separate the cells. Erythrocytes were lysed with a lysis buffer, pH 7.3, and the MSCs were obtained after a 6 h adherence culture to remove adherent cells. Purified CD4+T cells were obtained by incubating MSCs for 30 min with FITC-labeled anti-CD4, and then for a further 15 min with anti-FITC MicroBeads. By positive selection (MACS; MiltenyiBiotec), more than 97% of pure T cells were obtained with a viability of 98%.

Macrophages were obtained as described above. Then, these cells were washed and fixed in 1% paraformaldehyde to be used during subsequent co-cultures.

Fixed MΦ (1×10^5) were incubated in flat-bottomed 96-well plates containing 3×10^5 naive MSCs or purified CD4+T cells in RPMI-1640 supplemented with 50 μM 2-mercaptoethanol, with these cultures being incubated for 4 days at 37 °C in 5% CO_2 in the presence of anti-CD3 antibody. After the addition of 1 μCi of [^{3}H]thymidine (obtained from Comisión Nacional de Energía Atómica, CNEA) to each well, the incorporation of [^{3}H]thymidine by lymphocytes was determined, 18 h later, using a cell harvester and a liquid scintillation counter. Finally, the production of cytokine by MSCs or purified CD4+T cells was measured in supernatants of 72 h cultures, as described above.

Cytokine assays

Culture supernatants were collected after 72 h *in vitro* as well as *ex vivo* experiments, and assayed for the presence of IL-10 and IFN-γ according to the manufacture's protocol, using a capture enzyme-linked immunosorbent assay (ELISA) kit.

Flow cytometry assay for MΦ surface expression of MHC class II, PD-L1 and PD-L2

Three-hundred thousand cells were placed on a 96-well U-shaped plate FhESP (20 μg/ml) or with medium alone for 48 h. In some experiments, the MΦ were preincubated for 30 min with anti-Dectin-1 (0.5 μg/ml) for 30 min at 37 °C in 5% CO_2. Then, after being washed, the cells were stimulated with FhESP.

The cells were then blocked with anti-mouse CD16/CD32 for 15 min at room temperature and stained with anti-mouse I-A/I-E, anti-mouse PD-L1 and anti-mouse PD-L2 for 30 min under the same conditions. After incubation, the cells were collected by centrifugation and fixed in 1% paraformaldehyde. Then, the cells were analyzed by flow cytometry (Cytoron Absolute; ORTHO Diagnostic Sys-tem, Raritan, NJ), and the percentage of positively labeled cells was determined using logarithmic-scale histograms. Autofluorescence was assessed using untreated cells and control isotypes. In order to discriminate the specific population of pMΦ, we considered the F4/80+ cells, which corresponded to the pMΦ population.

Statistical analysis

Data were expressed as means ± standard errors of the means (SEMs). A two-tailed Student's *t*-test was utilized, and a one-way analysis of variance (ANOVA) with Tukey–Kramer's *post hoc* test was used to determine the statistical significance for all pairwise multiple-comparison procedures. A p value of 0.05 was considered to be significant. All experiments were performed in triplicate and equivalent results were obtained in each experiment.

Results and discussion

Classical activation of T cells involves an interaction between membrane molecules of both, T cells and antigen presenting cells (APCs), where the first signal is provided by engagement of TCR with the antigenic peptide–MHC molecule complex on the APC. In the present study, we analyzed the MHC-II expression in pMΦ

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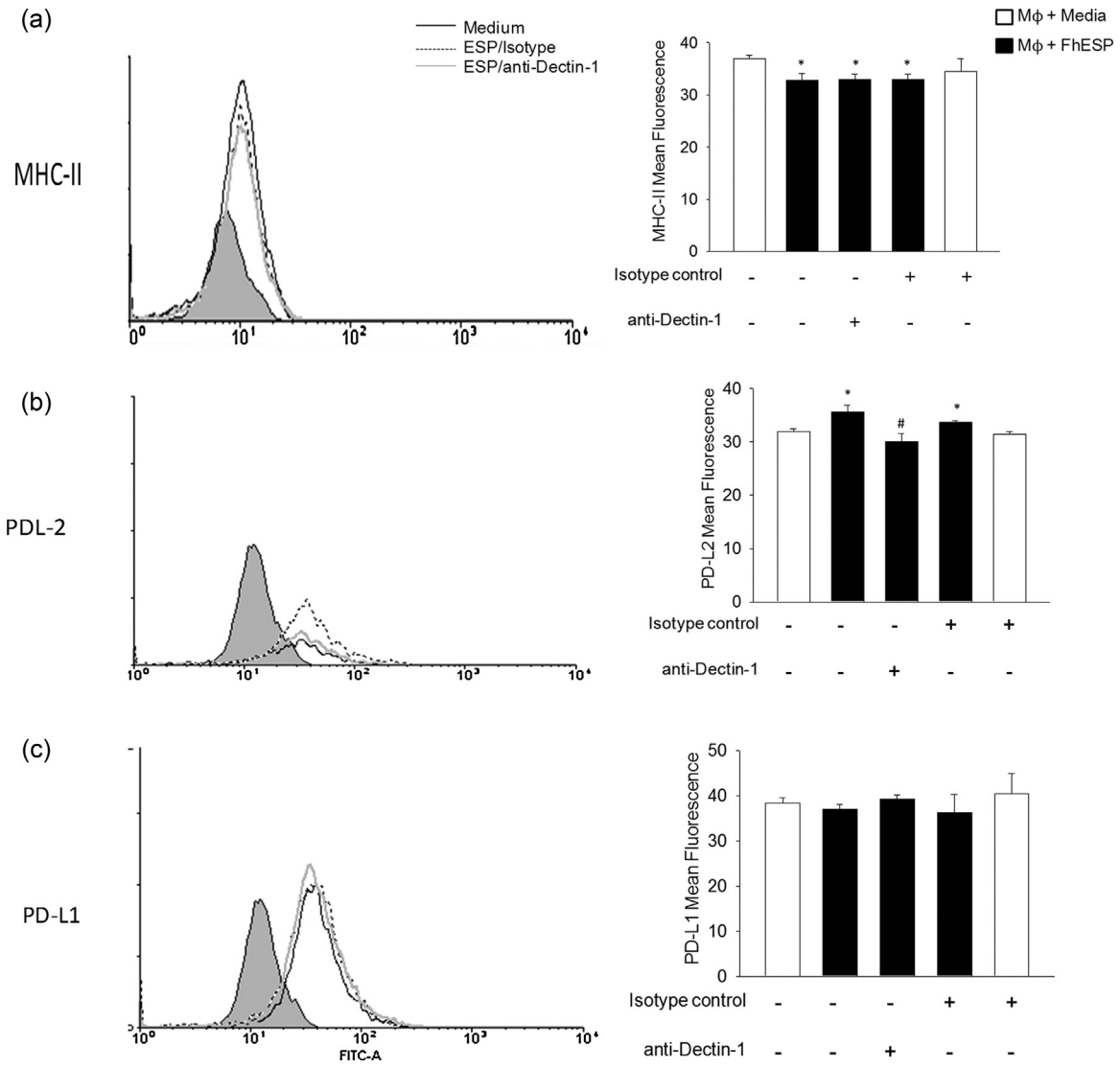

Fig. 1. *F. hepatica* ESP induce a down-modulation of MHC-II and an up-regulation of PD-L2 molecules in pMΦ surface. Peritoneal MΦ were cultured with FhESP or medium alone. In some experiments, the MΦ were pre-incubated for 30 min with anti-Dectin-1 before being stimulated with FhESP. The expression of MHC-II, PD-L1 and PD-L2 was evaluated by flow cytometry in cells gated on F4/80+ cells, with the histograms showing control (solid black line); FhESP stimulated cells and isotype controls (open line); pre-treatment with anti-Dectin-1 (solid gray line). Histograms are representative of two independent experiments. Bars represent the mean ± SEM of two independent experiments, analyzed in triplicate. $^{*}p < 0.05$ respect to pMΦ cultured in medium alone; $\#p < 0.05$ respect to pMΦ stimulated with FhESP.

stimulated with FhESP, and as shown in Fig. 1a, the expression of this molecule was down-modulated ($^{*}p < 0.032$). In this regard, the decreased expression of MHC-II molecules in MΦ could not provide an adequate first signal for activation of T cells during *F. hepatica* infection; and similar effects were observed during *T. gondii* infection, where a defective MHC-II expression by IFN-γ-activated MΦ was accompanied by a severe impairment in the presenting of antigens to CD4+T lymphocytes (Lûder et al., 2001). The TCR–MHC interaction alone is insufficient to induce optimal T-cell activation, with co-stimulatory signals being necessary, which are provided by the binding of specific receptors on T cells with their ligands on APC (Sharpe and Freeman, 2002). When the expression of co-stimulatory molecules (CD80, CD86, CD40) was tested, no significant changes were found respect to the basal group (data not shown). In contrast, other accessory molecules can inhibit T cells, and a more novel pathway that involves the programmed death-1 (PD-1) receptor, which is preferentially expressed on activated T cells and binds the two known ligands PDL-1 and PDL-2 on professional APCs and certain parenchymal cells, as well as on a subpopulation of T and B cells (Freeman et al., 2000; Okazaki et al., 2002). In this regard, we observed that the levels of PD-L2 were up-regulated in pMΦ stimulated with FhESP, with respect to their respective controls (Fig. 3b, $^{*}p < 0.032$) but no significant changes were observed in the PD-L1 levels (Fig. 3c).

Although little is known about the PRRs involved in the recognition of *F. hepatica* PAMPs by MΦ or the subsequent immunoregulatory effects induced on these cells, in a previous study we described the participation of the Mannose receptor (MR) and Dectin-1 in the induction of an alternate activated phenotype in pMΦ by FhESP (Guasconi et al., 2011). Dectin-1 is a signaling PRR that regulates the expression of innate response genes, including those encoding co-stimulatory molecules and pro-inflammatory cytokines and chemokines (Rogers et al., 2005; Brown, 2006; Plato et al., 2013; Dambuza and Brown, 2014). Moreover, a central role in the immunomodulatory activities of β-glucans has also been attributed to this receptor (Rogers et al., 2005; Tsoni and Brown, 2008), which correlates with our previous results (Guasconi et al., 2011). To investigate the role of Dectin-1 in the FhESP effects induced on MHC-II and PD-L2 pMΦ expression, cells were incubated with anti-Dectin-1 and then stimulated with the parasite

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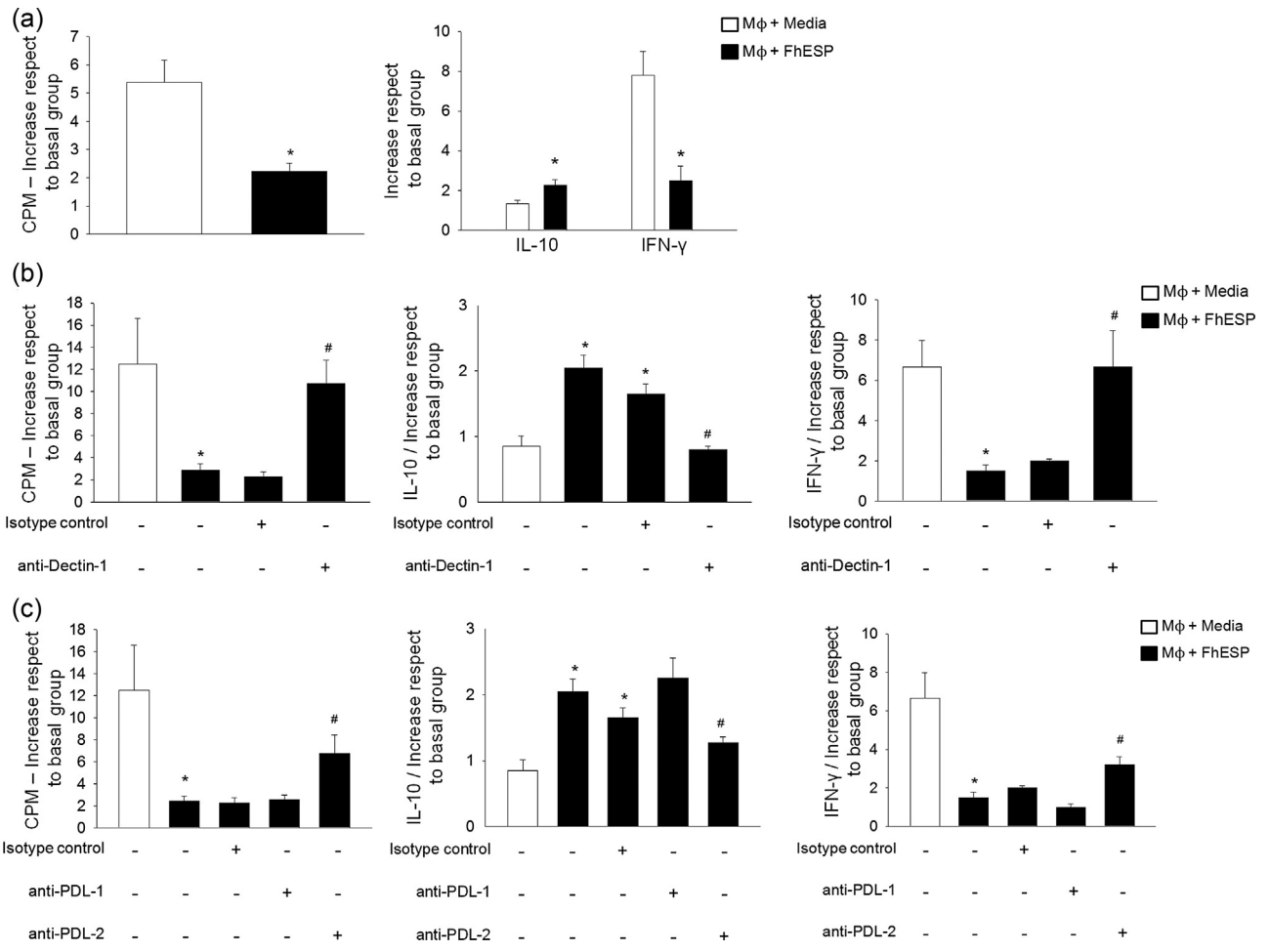

Fig. 2. *F. hepatica* ESP induce T cell anergy *via* selective up-regulation of PD-L2 expression on pMΦ being this effect dependent on Dectin-1. Peritoneal MΦ were incubated with FhESP or medium alone. Then, these cells were co-cultured with purified CD4+T cells for 4 days in the presence of anti-CD3 antibody. Then, the incorporation of [^{3}H]thymidine by lymphocytes was determined using a cell harvester and a liquid scintillation counter (a). To investigate the role of Dectin-1 (b) or PD-L2 (c) in the FhESP effects, the pMΦ were pre-incubated with anti-Dectin-1 or anti-PD-L2 before being stimulated with FhESP. The cytokines produced by CD4+T cells were measured in supernatants of 72 h according to the manufacture's protocol, using a capture enzyme-linked immunosorbent assay (ELISA) kit. Data are shown as mean ± SEM of three independent experiments, analyzed in triplicate. $^*p < 0.05$ respect to CD4+T-cells co-cultured with pMΦ in medium alone; $\#p < 0.05$ respect to CD4+T-cells co-cultured with pMΦ stimulated with FhESP.

products. As shown in Fig. 1, a partial inhibition in the increased levels of PD-L2 was detected in pMΦ pre-incubated with anti-Dectin-1 before being stimulated with FhESP (Fig. 1b, $\#p < 0.043$), compared to the cells stimulated with FhESP, while the down-modulation induced by FhESP in the expression of MHC-II molecules was not Dectin-1-dependent (Fig. 1a).

The propensity of *F. hepatica* infections to modulate the T lymphocyte responses by evoking a Th2 cytokine-biased response is well documented (O'Neill et al., 2000; Flynn et al., 2010; Dalton et al., 2013). In contrast, there are relatively few studies on the mechanism of the less characterized phenomenon of T cell hyporesponsiveness, which is induced during this infection. In the present investigation, in order to evaluate pMΦ functions as APCs, the proliferative response of T-cell derived from naive mice in presence of pMΦ previously stimulated with FhESP was determined, resulting in a suppressive effect in the anti-CD3mAb-stimulated proliferation of CD4+T-cells (Fig. 2a, $^*p < 0.010$) as well as a down-modulation of IFN-γ with an up-modulation of IL-10 levels produced by these cells respect to the basal group (Fig. 2a, $^*p < 0.026$ and $^*p < 0.016$, respectively). Furthermore, in parallel experiments using CD4+T cells from DO11.10 mice in co-cultures with pMΦ stimulated with FhESP, a marked defect in ovalbumin (OVA-peptide) stimulated proliferation was observed (data not shown).

Next, we evaluated the proliferative response of T-cell obtained from naive mice in the presence of pMΦ incubated with anti-Dectin-1 before being stimulated with FhESP, which resulted in a weakening of the suppressive effect on CD4+T-cell proliferation, with respect to the cells cultured with pMΦ stimulated with FhESP (Fig. 2b, $\#p < 0.0071$). Furthermore, an increase of IFN-γ and a decrease of IL-10 levels were produced by CD4+T-cells in culture with pMΦ pre-incubated with anti-Dectin-1 before being stimulated with FhESP, compared to cells cultured with pMΦ stimulated with FhESP (Fig. 2b, $\#p < 0.016$ and $\#p < 0.00039$, respectively). Finally, cultures of CD4+T-cells were carried out with pMΦ incubated with anti-PD-L2 and stimulated with FhESP, and similar results to those obtained with the blocking of Dectin-1 were obtained in the proliferative response (Fig. 2c, $\#p < 0.035$), as well as in the IFN-γ and IL-10 production (Fig. 2c, $\#p < 0.024$ and $\#p < 0.020$, respectively). We have evaluated other cytokines (TNF-α, TGF-β, IL-13), and we observed that only IL-10 and IFN-γ production are Dectin-1 and PDL-2 dependent (data not shown). A similar participation of PD-1/PDL's interactions was observed in experimental murine schistosomiasis (Smith et al., 2004), cysticercosis (Terrazas et al., 2005), *Trypanosoma cruzi* infection (Dulgerian et al., 2011), where the suppressive activity of APC's was attributed to PD-L1, and therefore MΦ-T cell contact dependent. Moreover, here a

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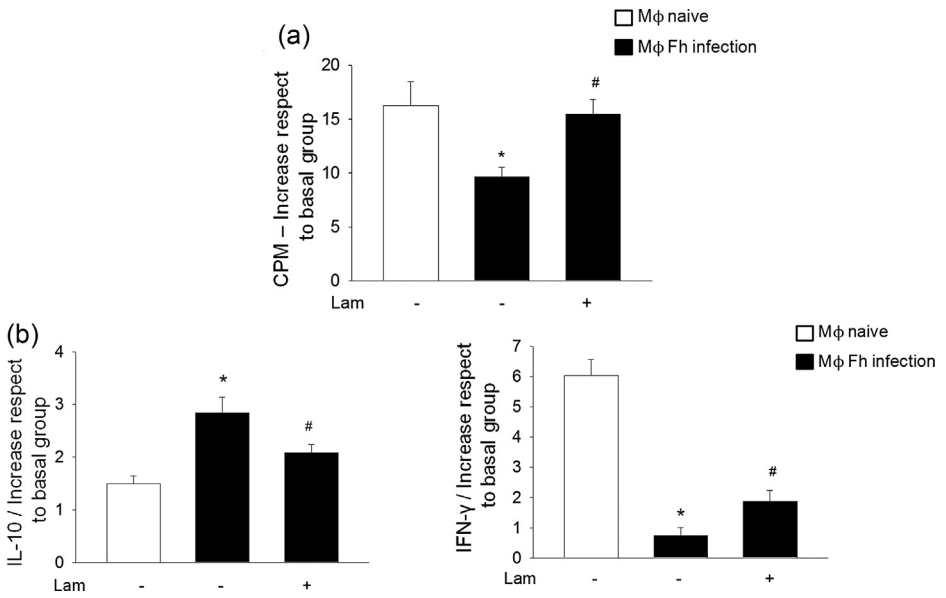

Fig. 3. Peritoneal macrophages obtained from early stages of *F. hepatica*-infected mice are suppressive. BALB/c mice were orally infected with metacercariae of *F. hepatica*. To evaluate the participation of Dectin-1 during the infection, mice were intraperitoneally injected with Laminarin or PBS 4 h before being infected. Forty-eight hours after the infection, pMΦ were obtained and co-cultured with MSCs (mononuclear spleen cells) derived from normal mice for 4 days, in the presence of anti-CD3 antibody. Then, the incorporation of [^{3}H]thymidine by lymphocytes was determined using a cell harvester and a liquid scintillation counter (a). The cytokines produced by lymphocytes were measured in supernatants of 72 h according to the manufacture's protocol, using a capture enzyme-linked immunosorbent assay (ELISA) kit (b). In the infection experiment, four mice/group were analyzed. Data are shown as mean ± SEM of three independent experiments, analyzed in triplicate. $^*p < 0.05$ respect to MSCs co-cultured with pMΦ from non-infected mice; $\#p < 0.05$ respect to MSCs co-cultured with pMΦ from infected mice.

regulatory role of PD-L2 was found on MΦ during a helminthic infection, which could be correlated with previous studies which suggested that PD-L2 may regulate the Th2 responses (Loke and Allison, 2003; Lazar-Molnar et al., 2008).

To determine the role of pMΦ during the early stages of *F. hepatica* infection, MΦ were obtained from infected mice and co-cultured with splenocytes derived from naive mice. As shown in Fig. 3, we could observe a suppressive effect in the anti-CD3mAb-stimulated proliferation of splenocytes (Fig. 3a, $^*p < 0.0026$), as well as a down-modulation of the IFN-γ levels and an up-modulation of IL-10 production respect to the basal group (Fig. 3b, $^*p < 0.0000071$ and $^*p < 0.017$, respectively). Furthermore, a weakening of the suppressive effect occurred on splenocytes proliferation (Fig. 3a, $\#p < 0.00066$), as well as an increase of IFN-γ with a reduction of IL-10 levels produced by these cells (Fig. 3b, $\#p < 0.037$ and $\#p < 0.040$, respectively) were observed in a culture with pMΦ derived from mice that were intraperitoneally injected with Laminarin before being infected. In this regard, although the pMΦ obtained 3 weeks after infection showed a similar decreased function as APC, this effect was Dectin-1-independent (data not shown).

Therefore, based on these results, we can suggest that *F. hepatica* products modulate the immune response through the participation of Dectin-1, and our group has now begun to evaluate the potential signaling pathways involved in Dectin-1 mediated effects. Related to this, the summary of the effects mediated by Dectin-1 may be part of the response that finally prevails over the immunostimulatory signals from other PRRs, which ultimately determines the survival of this parasite in the host. The future identification of the parasite product which interacts with this CLR might help to clarify how *F. hepatica* directs the immune response, which could lead to a better understanding of the parasite pathogenicity mechanisms, and consequently may facilitate the development of novel therapies for fasciolosis control and eradication.

Conflict of interest

None declared.

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