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Short communication

Dectin-1 on macrophages modulates the immune response to *Fasciola hepatica* products through the ERK signaling pathway

Lorena Guasconi*, Verónica L. Burstein, Ignacio Beccacece, Cristian Mena, Laura S. Chiapello, Diana Teresa Masih

Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI - CONICET), Argentina

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ABSTRACT

Fasciolosis is a zoonotic disease of increasing importance due to its worldwide distribution and elevated economic losses. Previously, we demonstrated that *Fasciola hepatica* excretory–secretory products (FhESP) induce immunomodulatory effects on peritoneal macrophages in a Dectin-1 dependent manner.

In this study, we observed that peritoneal macrophages from naive BALB/c mice stimulated in vitro with FhESP presented increased expression levels of phosphorylated extracellular-signal-regulated kinase (ERK), and this effect was dependent on Syk, protein kinase C (PKC) and Dectin-1. In this sense, we observed increased levels of arginase activity, IL-10 and TGF-β in macrophages stimulated with FhESP, which were dependent on PKC and ERK. Furthermore, we observed that the increased arginase activity, as well as in TGF-β and IL-10 levels, was partially dependent on IL-10 receptor signaling in macrophages that were pre-incubated with anti-IL10R before being stimulated with FhESP.

Taken together, these results suggest the participation of Dectin-1 and Syk in FhESP interaction with peritoneal macrophages and the possible role of ERK and IL-10 in downstream signaling pathways involved in the immunomodulatory effects induced by *Fasciola hepatica* products.

1. Introduction

Helminths have evolved potent and diverse immunosuppressive strategies for evading the host immune response and establishing chronic infections. In this sense, these parasites do not kill the host, thus ensuring a niche for their survival and transmission to their next host (Finlay et al., 2014; Maizels, 2016). Helminths excretory-secretory products are able to induce alternatively activated macrophages (aaMΦ, also termed M2) and a Type 2 cell-mediated immune response (Harris and Loke, 2017). M2 macrophages have an anti-inflammatory role characterized by a signature of gene expression, metabolism and protein products that includes arginase-1, RELM-α and the chitinase-like molecule Ym1 (Nair et al., 2005). The immunosuppressive characteristics of M2 macrophages include the inhibition of T cell proliferation, enhancement of Treg cell differentiation and production of immunoregulatory cytokines like IL-10. Furthermore, these cells are instrumental in repair and resolution of tissue damage caused by migratory helminthes (Gause et al., 2013; Maizels and McSorley, 2016).

The helminth *Fasciola hepatica,* also known as liver fluke, is a worldwide economically important pathogen of livestock and is causing an emerging disease in humans (Mas-Coma et al., 2009; Mehmood et al., 2017). Interaction of *F. hepatica* excretory–secretory products (FhESP) with macrophages is crucial for the parasite establishment in the host, and different experimental models have demonstrated the induction of aaMΦ by FhESP (Donnelly et al., 2005, 2008).

Dectin-1 is a C-type lectin receptor (CLR) that mainly recognize microbial β-glucans and effectively function as a pattern recognition receptor (PRR) (Dambuza and Brown, 2015). Upon clustering, Dectin-1 recruits Syk (spleen tyrosine kinase), leading to NF-kβ and immune activation through mechanisms like phagocytosis, respiratory burst killing and production/release of pro-inflammatory cytokines and chemokines (Schnaar, 2016). Nevertheless, some pathogens appear to have developed immunoevasive activities through CLRs (McGreal et al., 2005). In previous works we demonstrated that FhESP induced a Dectin-1-dependent macrophage activation characterized by increased arginase activity, high levels of IL-10 and TGF-β production and PD-L2 expression (Guasconi et al., 2011, 2015).

Other parasites are able to induce ERK 1/2 activation and increased

* Corresponding author at: Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI - CONICET), Medina Allende y Haya de la Torre, Ciudad Universitaria, X5000HUA, Córdoba, Argentina.
E-mail address: lguasconi@fcq.unc.edu.ar (L. Guasconi).

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IL-10 production in macrophages and dendritic cells (Mukherjee et al., 2004; Poncini et al., 2008), which impair an efficient Th1 inflamatory response (Alba Soto et al., 2003, 2010). Furthermore, a downstream pathway of Dectin-1 leads to extracellular signal–regulated kinase (ERK) 1/2 activation, for CARD9 activation and regulation of H-RAs, and through Ras-GRF-1 phosphorylation in BMDCs (Jia et al., 2014). Therefore, in this work we evaluated ERK 1/2 involvement in the signaling pathways downstream FhESP induced-Dectin-1/Syk activation on macrophages.

2. Materials and methods

2.1. Animals

Six- to 8-week-old female BALB/c mice were purchased from the Ezeiza Atomic Centre (CNEA, Buenos Aires, Argentina), and inbreeded in the animal resource facilities of the Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Cordoba. All experimental protocols were approved by the Animal Experimentation Ethics Committee, Faculty of Chemical Sciences, National University of Cordoba.

2.2. Isolation of cells, stimulation and cell analysis

To obtain peritoneal macrophages (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%), and to obtain macrophage monolayers, peritoneal exudate cells were re-suspended in RPMI supplemented with 10% FBS, 2 mM glutamine and 50 μg/ml gentamycin, and added into 24-well (2×10^6 cells/well, in final volume of 500 μl, for Western blot assays) or 48-well (5×10^5 cells/well, in final volume of 1000 μl, for the remaining assays). Then, cells were cultured for $2^{½}$ h at 37 °C in an atmosphere of 5% CO_2. Non-adherent cells were removed by gently washing with warm PBS, and the remaining adherent cells were highly enriched for MΦ, with flow cytometry analysis revealing 90% F4/80$^+$ cells.

Macrophages were stimulated with FhESP at a concentration of 20 μg/ml for 48 h. To investigate the role of different receptors or mediators of intracellular signaling events, we pre-incubated the cells with: Syk inhibitor (20 μM; Calbiochem, Merck, Darmstadt, Germany); anti-Dectin-1 (0.5 μg/ml; R&D Systems, Minneapolis, MN, USA); ERK inhibitor (U0126) (10 μM, InvivoGen, San Diego, CA, USA); staurosporine (1 μM; Sigma-Aldrich Co., St Louis, MO) or anti-mouse IL-10R (10 μg/ml; BD Biosciences, San Jose, CA, USA), for 30 min at 37°C in 5% CO_2. Then, cells were washed and stimulated with FhESP at a concentration of 20 μg/ml.

After 48 h of stimulation with FhESP, arginase activity assay was performed in cell lysates as previously described by Corraliza et al. (1994), and culture supernatants were collected and assayed for the presence of TGF-β (Biosource, Camarillo, CA) and IL-10 (e-Bioscience, San Diego, CA) according to the manufacture's protocol, using ELISA kits. Phosphorylation of ERK was measured by western blot with an ERK specific antibody (Cell Signalling Technology, Danvers, MA, USA).

Controls were performed for drugs dissolved in DMSO (Syk and ERK inhibitors) and DMSO did not affect cell viability.

2.3. FhESP preparation

The FhESP were prepared according to a procedure described by (Diaz et al. (1998). Briefly, live adult worms of *F. hepatica* were obtained from the bile ducts of bovine livers and then washed with PBS, pH 7.4, before incubation (1 worm/2 ml PBS) for 3 h at 37 °C. Then, the supernatant was centrifuged (16,000 *g*, 30 min, 4 °C) before concentration with a high-flow YM 10 membrane filter (Millipore-Amicon Corp., Billeria, MA), and stored at −20 °C until used. The protein concentration was measured using a Bradford protein assay (Bio-Rad, Hercules, CA).

To remove LPS contamination, FhESP preparation was passed through a column containing detoxi-gel endotoxin removing gel (Pierce Biotechnology, Rockford USA). After endotoxin removal, the quantity of LPS present in FhESP was determined by using the Limulus amebocyte lysate test (Endosafe Times Charles River, Laboratories Wilmington, Delaware), whose level was found to be similar to those of the background and complete RPMI 1640 medium.

2.4. Statistical analysis

Data are expressed as means ± SEMs, and all experiments were performed two to three times. Statistical differences were calculated with Student *t*-test or ANOVA. A *P*-value of 0.05 was considered significant.

3. Results and discussion

3.1. FhESP induce dectin-1-mediated ERK 1/2 MAPK phosphorylation

Mitogen-activated protein kinases (MAPKs) are important mediators of diverse intracellular signaling events and regulate important cellular processes such as proliferation, stress responses, apoptosis and immune defense (Arthur and Ley, 2013). Following pathogen infection or tissue damage, the stimulation of PRRs on the cell surface and in the cytoplasm of innate immune cells activates members of each of the major MAPK subfamilies (ERK, JNK and p38) that are responsible to induce the expression of multiple genes that together regulate the inflammatory response (Daley et al., 2017a). In order to examine ERK activation in macrophages by FhPES, pMΦ were stimulated with FhESP at different times and the phosphorylation of ERK 1/2 was measured by western blot. It was observed that high levels of P-ERK 1/2 respect to the basal group were induced, with significant differences at 30 min after FhESP stimulation (Fig. 1, $p < 0.022$).

On the other hand, immunomodulatory properties have been described for carbohydrates present in the excretory-secretory products as

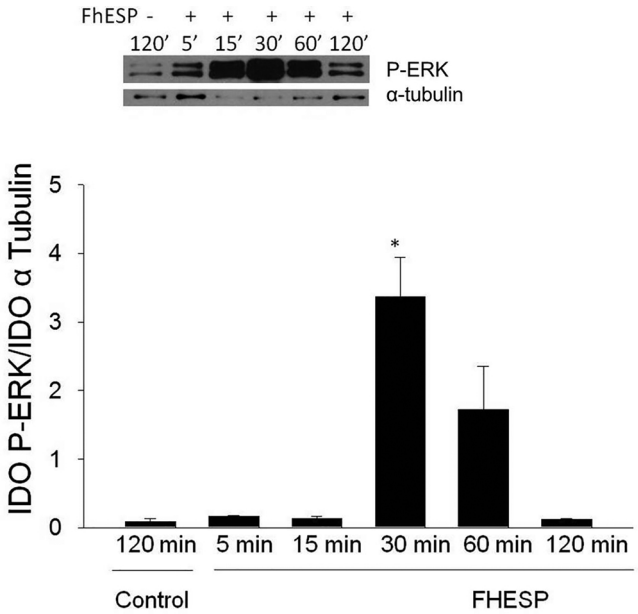

Fig. 1. *Fasciola hepatica* ESP induce high levels of phosphorylated ERK in peritoneal macrophages (pMΦ). Expression levels of P-ERK by western blot were evaluated in pMΦ stimulated with FhESP (20 μg/ml) for 5, 15, 30, 60 and 120 min respectively. Quantification of P-ERK was performed by densitometric analysis. Data are mean ± SEM of two independent experiments, analyzed in triplicate. *$p < 0.05$ respect to pMΦ cultured with medium alone.

well as tegumental antigens from *F. hepatica* (Aldridge and O'Neill, 2016; Rodríguez et al., 2015). Nevertheless, 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.

Dectin-1 has been conclusively demonstrated to function as a signaling PRR, regulating the expression of co-stimulatory molecules and proinflammatory cytokines and chemokines (Dambuza and Brown, 2015; Schnaar, 2016). However, some studies have demonstrated that Dectin- 1 activation also triggers the production of non-protective cytokines, such as IL-23 and IL-10 (Dillon et al., 2006; Daley et al., 2017b). Related to this, in previous works we observed that the *F. hepatica*-released products induced an augmentation in arginase activity and Arginase I expression, high levels of IL-10 and TGF-β, and an increased expression of PD-L2 in pMΦ, and these effects were dependent on Dectin-1/Syk pathway (Guasconi et al., 2011, 2015). These findings demonstrated, for the first time, the participation of Dectin-1 in the innate immune response to any parasite.

Dectin-1 signals via a novel hemITAM motif that becomes phosphorylated by Src family kinases upon receptor engagement (Rogers et al., 2005) thus permitting recruitment and activation of Syk, and subsequent activation of CARD9–Bcl10–Malt1 (CBM) scaffold through PKCδ (Drummond and Brown, 2013). Activation of Dectin-1 by Syk-dependent and other Syk-independent pathways, such as that mediated by Raf-1, results in the activation of several transcription factors including NFAT, IRF1, IRF5, and the canonical and non-canonical subunits of Nf-kβ (p65, RelB, c-Rel, p50 and p52) (Plato et al., 2013). Furthermore, Dectin-1 activation of CARD9 was shown to regulate H-Ras activation, through Ras-GRF-1 phosphorylation, leading to activation of ERK but not NF-kβ (Jia et al., 2014). Related to this, an inhibition of the increased P-ERK expression was observed when pMΦ were pre-incubated with a Dectin-1 blocking antibody before stimulation with FhESP, compared to cells stimulated with FhESP alone (Fig. 2a; $p < 0.036$). Furthermore, as shown in Fig. 2b and c, FhESP-induced ERK phosphorylation was abrogated in the presence of a Syk inhibitor (Fig. 2 b, $p < 0.021$) or staurosporine (an inhibitor for different kinases of the PKC family) (Fig. 2 c $p < 0.046$), respect to cells stimulated with FhESP alone. Therefore, FhESP induce dectin-1-mediated ERK 1/2 MAPK phosphorylation, which is dependent on Dectin-1, Syk and PKC.

3.2. FhESP induce an ERK-mediated increase in arginase activity and production of TGF-β and IL-10 by macrophages

Some pathogens have developed mechanisms to directly modulate MAPK activation (Arthur and Ley, 2013; Soares-Silva et al., 2016). Previously, we observed that pMΦ stimulated with FhESP presented an increased arginase activity and high levels of TGF-β and IL-10, being these effects dependent on Dectin-1 (Guasconi et al., 2011). To provide further insights into the role of ERK1/2 in these phenomena, pMΦ were pre-incubated with an ERK inhibitor for 30 min at 37°C before stimulation with FhESP for 48 h. Fig. 3b shows that ERK inhibitor significantly inhibits macrophage arginase activity as well as TGF-β and IL-10 production induced by FhESP ($p < 0.001$, $p < 0.01$ and $p < 0.01$, respectively), compared with cells stimulated with FhESP. Similar results were observed in the presence of staurosporine, a chemical inhibitor of protein kinases C family (Fig. 3a, $p < 0.05$, $p < 0.0032$, and $p < 0.05$, respectively). In this regard, these results suggest that FhESP induce activation of Dectin-1 and Syk, with the possible participation of a kinase of the PKC family, that leads to the phosphorylation of ERK.

3.3. FhESP-induced arginase activation and TGF-β and IL-10 production depends on IL-10 receptor signaling

Considering that ERK 1/2 pathway modulates the production of IL-10 responsible for the induction of Th cell differentiation into Th2-type, that this type of response is involved in the elimination of extracellular pathogens (Chang et al., 2012; Mosmann et al., 2009), and that IL-10 is a cytokine with important regulatory functions being able to exert autocrine and paracrine effects (Qualls et al., 2010), we evaluated IL-10 participation in the effects induced by FhESP on pMΦ. Fig. 4 shows that arginase activity as well as TGF-β and IL-10 production induced by FhESP in macrophages was significantly reduced in presence of a specific IL-10 receptor (IL-10R) blocking antibody (Fig. 4, $p < 0.001$,

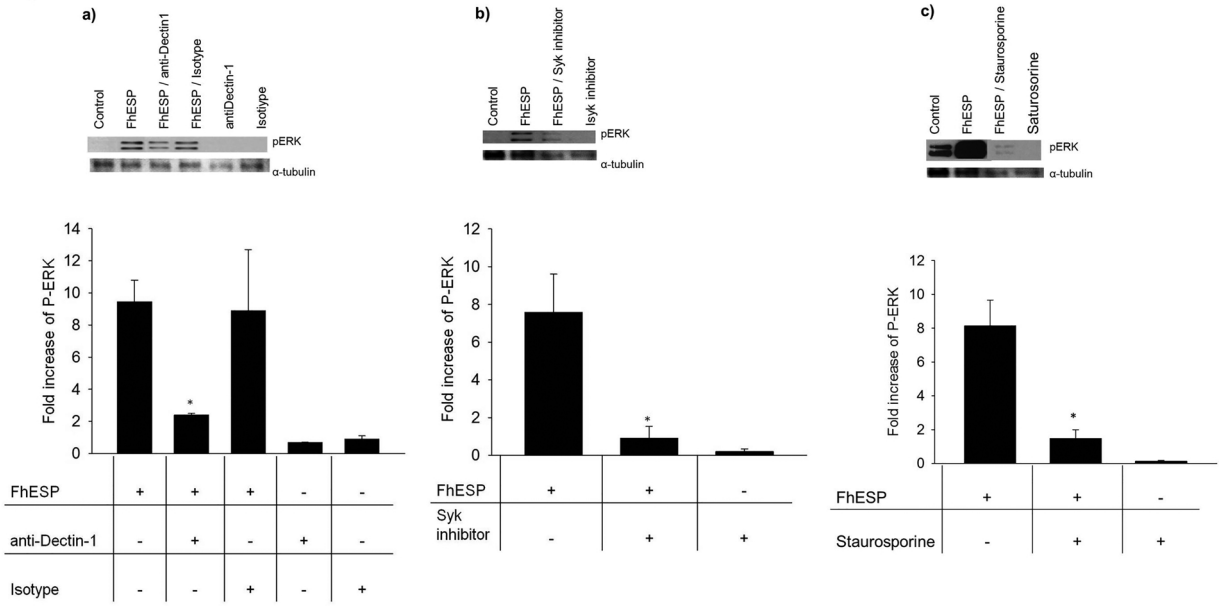

Fig. 2. Dectin-1 participates in the increased phosphorylated extracellular-signal-regulated kinase (ERK) expression induced by *Fasciola hepatica* excretory-secretory products (FhESP) on peritoneal macrophages (pMΦ). P-ERK expression by western blot was determined in pMΦ pre-incubated with anti-Dectin-1 specific antibody (0.5 μg/ml) (a), SyK inhibitor (20μM) (b), or staurosporine (1 μM) (c) for 30 min at 37 °C before being stimulated with FhESP (20 μg/ml) for 30 min. Quantification of P-ERK was performed by densitometric analysis. Data are mean ± SEM of two independent experiments, analyzed in triplicate. *$p < 0.05$ respect to pMΦ stimulated with FhESP.

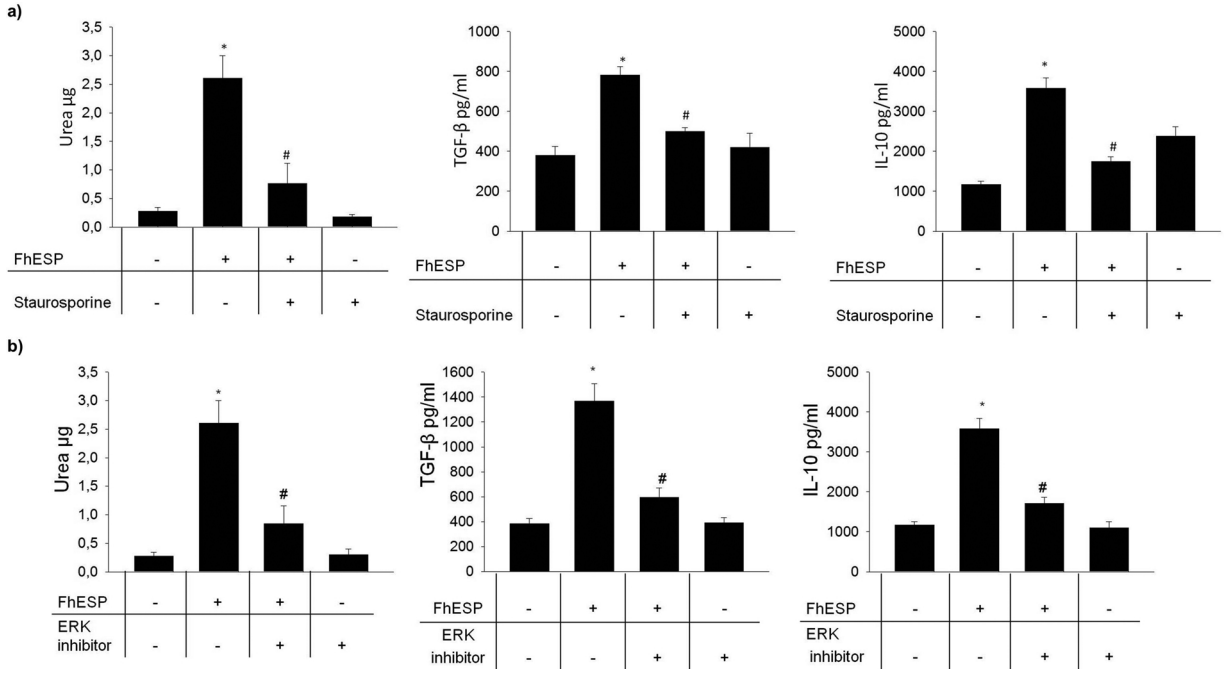

Fig. 3. Immunomodulatory effects induced by *Fasciola hepatica* excretory-secretory products (FhESP) on peritoneal macrophages (pMΦ) are abrogated by inhibitors of protein kinases C family (staurosporine) and ERK (U0126). Arginase activity and TGF-β and IL-10 levels were determined in pMΦ pre-incubated with staurosporine (1 µM, inhibitor for different kinases of the PKC family) (a) or U0126 (10 µM, ERK inhibitor) (b) for 30 min at 37 °C before stimulation with FhESP (20 µg/ml) for 48 h. Data are mean ± SEM of the arginase activity (urea production) and cytokines production by ELISA, of three independent experiments, analyzed in triplicate. *$p < 0.05$ respect to pMΦ stimulated with medium alone; #$p < 0.05$ respect to pMΦ stimulated with FhESP.

$p < 0.05$ and $p < 0.01$, respectively), in comparison with cells stimulated with FhESP alone. Conversely, arginase or TGF-β inhibition by using Nω-Hydroxy-nor-L-arginine or anti-TGF-β, did not modulate IL-10 production by macrophages incubated with FhESP (data not shown).

According with the results described in this work, other authors have shown that parasites like *Trypanosoma cruzi and Leishmania* spp. are able to induce ERK1/2 activation and increase IL-10 production in macrophages (Mukherjee et al., 2004; Poncini et al., 2008), being these effects responsible for impair an efficient Th1 inflammatory response (Alba Soto et al., 2003, 2010) and allow parasite evasion of the host immune response.

Taken together, the results suggest that Dectin-1 and Syk participate in the interaction of FhESP with pMΦ and a possible role of ERK and IL-10 in downstream signaling pathways as important players when defining the mechanisms of immunomodulation induced by *F. hepatica*.

Further studies are required to increase our understanding of the intriguing mechanisms by which *F. hepatica* manipulates the host immune response. Specifically, we will evaluate the possible role of CARD9 and Ras in the immunomodulatory effects induced by FhESP. Ultimately, identification of the molecules which interact with Dectin-1 could help to clarify how this parasite manipulates the immune response and would open new possibilities for exploiting parasite molecules as therapies for inflammatory diseases.

Conflict of interest

The authors declare no conflict of interest

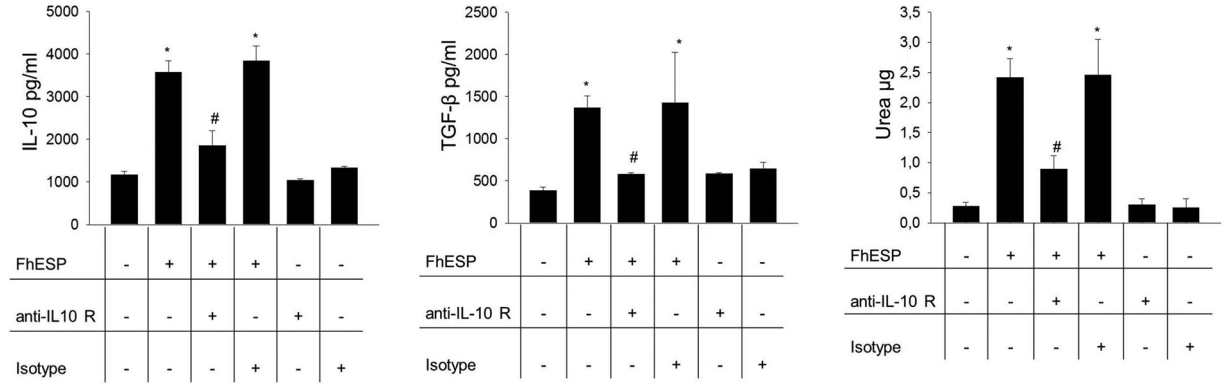

Fig. 4. The increase in arginase activity and in Transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) production induced by *Fasciola hepatica* excretory-secretory products (FhESP) on peritoneal macrophages (pMΦ) was dependent on IL-10. Arginase activity and cytokine production were determined in pMΦ pre-incubated with anti-mouse IL-10R (10 µg/ml) for 30 min at 37 °C before stimulation with FhESP (20 µg/ml) for 48 h. Data are mean ± SEM of the arginase activity (urea production) and cytokines production by ELISA, of three independent experiments, analyzed in triplicate. *$p < 0.05$ respect to pMΦ stimulated with medium alone; #$p < 0.05$ respect to pMΦ stimulated with FhESP.

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