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1 **_Fasciola hepatica_ fatty acid binding protein (Fh12) induces apoptosis and**
2 **tolerogenic properties in murine bone marrow derived dendritic cells**
3
4
5 **Running Head:** *F. hepatica* FABP induces apoptosis of murine DCs
6
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24 **ABSTRACT**
25

26 In a previous study we demonstrated that *Fasciola hepatica* fatty acid binding protein
27 (Fh12) significantly suppress macrophage function by inhibiting IL-6, IL-1B, tumor
28 necrosis factor (TNF) and IL-12 production in TLR4-stimulated murine macrophages, an
29 effect mediated through the signaling of CD14 co-receptor without affecting the viability
30 of these cells. Given that dendritic cells (DCs) are immune cells that play a central role in
31 the initiation of primary immune responses and that are the only antigen-presenting cells
32 capable of stimulating naive T-cells, in the present study we investigated the effect of
33 Fh12 on DCs. We found that Fh12 exerts a strong suppressive effect on activation and
34 function of DCs. However, in contrast to the effect observed on macrophages, Fh12
35 induces early and late apoptosis of DCs being this phenomenon dose-dependent and
36 CD14-coreceptor independent. At low concentration Fh12 modulates the LPS-induced
37 DCs maturation status by suppressing the MHC-II, and co-stimulatory molecules CD40
38 and CD80 surface expression together with the pro-inflammatory cytokines IL-12p70 and
39 IL-6 production whereas increase the IL-10 levels. Besides, Fh12 decreased the ability of
40 LPS-activated DCs to induce IFNg production against allogeneic splenocytes, while
41 increasing IL-4 production. We have described for the first time the ability of Fh12 to
42 modify selectively the viability of DCs by apoptosis induction. The selective diminution
43 in DCs survival could be a *F. hepatica* strategy in order to prevent a host immune
44 response during the earliest phases of infection.

45

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48 **INTRODUCTION**

49 *Fasciola hepatica* is a helminth parasite that causes fascioliasis, a chronic disease that
50 affects around 17 million people worldwide. Fascioliasis also leads to economic losses in
51 livestock, estimated in more than $3 billion annually (Mas-Coma et al., 2005). The
52 survival of helminths in the host over long periods of time is the result of a dynamic co-
53 evolution that results in a predominant Th2/Treg immune response that is only achieved
54 by suppressing the Th1-inflammatory response (Donnelly et al., 2008; O'Neill et al.,
55 2001). Excretory-secretory products (ESPs) and tegumental antigens (FhTeg), which are
56 a complex mixture of antigens, have been largely implicated as responsible for the
57 immune modulation (Anuracpreeda et al., 2006; Brady et al., 1999; Donnelly et al., 2008).
58 These antigens exert a strong influence on the activation status and function of the
59 antigen-presenting cells (APCs) at the early stages of infection. In this regard, ESPs and
60 FhTeg have shown to induce alternative activation of macrophages (Adams et al., 2014;
61 Donnelly et al., 2005; Flynn et al., 2007) and partial activation of dendritic cells (DCs)
62 (Hamilton et al., 2009). Moreover, ESPs have also shown to induce apoptosis of
63 eosinophils (Serradell et al., 2007) and macrophages (Guasconi et al., 2012) during the
64 early stages of parasite infection likely as a mechanism to prevent pro-inflammatory
65 functions of these cells (Adam-Klages et al., 2005).

66 We have demonstrated that *F. hepatica* fatty acid binding protein (FABP), an antioxidant
67 molecule with essential functions for parasite metabolism and that has been many times
68 identified in the FhTeg and ESPs (Hacariz et al., 2012; Morphew et al., 2012; Wilson et
69 al., 2011), possesses powerful anti-inflammatory functions. Native (Fh12) and
70 recombinant (Fh15) variants of FABP have shown to significantly suppress the

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71 production of IL-1β and TNF from bone-marrow derived macrophages (BMDM)
72 stimulated with LPS *in vitro* via toll-like receptor-4 (TLR4) (Martin et al., 2015; Ramos-
73 Benitez et al., 2017). FABP exerts this effect by suppressing the expression of the CD14-
74 coreceptor and the phosphorylation of various kinases downstream TLR4 signaling
75 cascade (Martin et al., 2015). Moreover, Fh12 has also shown to significantly suppress a
76 large number of pro-inflammatory cytokines in a mouse model of septic shock. Also, to
77 impair the murine BMDM function by inhibiting their phagocytic capacity without
78 provoking any toxic or apoptotic effect on these cells (Martin et al., 2015).
79 In the present study we focus on studying the effect of Fh12 on activation and
80 functionality of DCs, which play a relevant role during the initiation of immune response
81 in the recognition of helminth or their products and the subsequent promotion of Th2/
82 Treg development. In contrast to the effect observed on macrophages, we found that Fh12
83 modulate the activation and function of DCs by promoting the early and late apoptosis.
84 The apoptotic effect of Fh12 on DCs was found to be dose-dependent and not mediated
85 by the CD14-coreceptor. Concurrently, we demonstrated that F12 is capable of inducing
86 regulatory features on LPS-activated DCs, thereby impairing their capacity to prime
87 naïve T-cells thus, inducing a phenotype able to promote the development of anti-
88 inflammatory responses.
89

90 **MATERIALS AND METHODS**
91

92 **Animals**
93 Six- to 8-week-old inbred female C57BL/6 and BALB/c mice were indistinctly purchased
94 from Charles River Laboratory (Wilmington, MA) or from the Faculty of Veterinary

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95 Sciences, National University of Litoral (UNL, Argentina). B6.129S4 CD14 knockout
96 (CD14KO) female mice (C57BL/6 background), 6–8-week-old, were purchased from
97 Jackson Laboratory (Bar Harbor, ME). The animal studies were performed at the Animal
98 Resources Center of the Medical Sciences Campus, University of Puerto Rico in
99 accordance with guidelines and protocols approved by the Ethics Institutional Animal
100 Care and Use Committee (MSC-IACUC, Protocol No. 7870215) and Faculty of Chemical
101 Sciences, National University of Córdoba (Approval Number HCD 1637) in strict
102 accordance with the recommendation of the Guide to the Care and Use of Experimental
103 Animals published by the Canadian Council on Animal Care (OLAWAssurance number
104 A5802-01).
105
106 **Fh12 Purification**
107 Fh12 was purified from a whole-fluke extract of adult *F. hepatica* using as previously
108 described (Espino et al., 2001). Briefly, whole fluke extract was first subjected to
109 ultracentrifugation at 30,000 g followed by gel filtration chromatography with Sephadex
110 G-50 (XK 26/1000 column). Fractions containing proteins in the range of 1.5-30kDa
111 were collected, pooled and subjected to two consecutive preparative isoelectric focusing
112 (IEF) at pH 3-10 (first separation) and pH 5-7 (second separation) using a Rotofor Cell
113 (Bio-Rad). The individual IEF fractions were harvested, and their pH value and OD 280
114 nm determined. Each aliquot was subjected to SDS-PAGE and the proteins were
115 visualized by coomasie blue. Fractions from the second IEF run that exhibited a single
116 polypeptide band of around 12-15kDa were manually excised from the gel, washed twice
117 with double-distilled water, digested with sequencing-grade trypsin (Promega, Madison,

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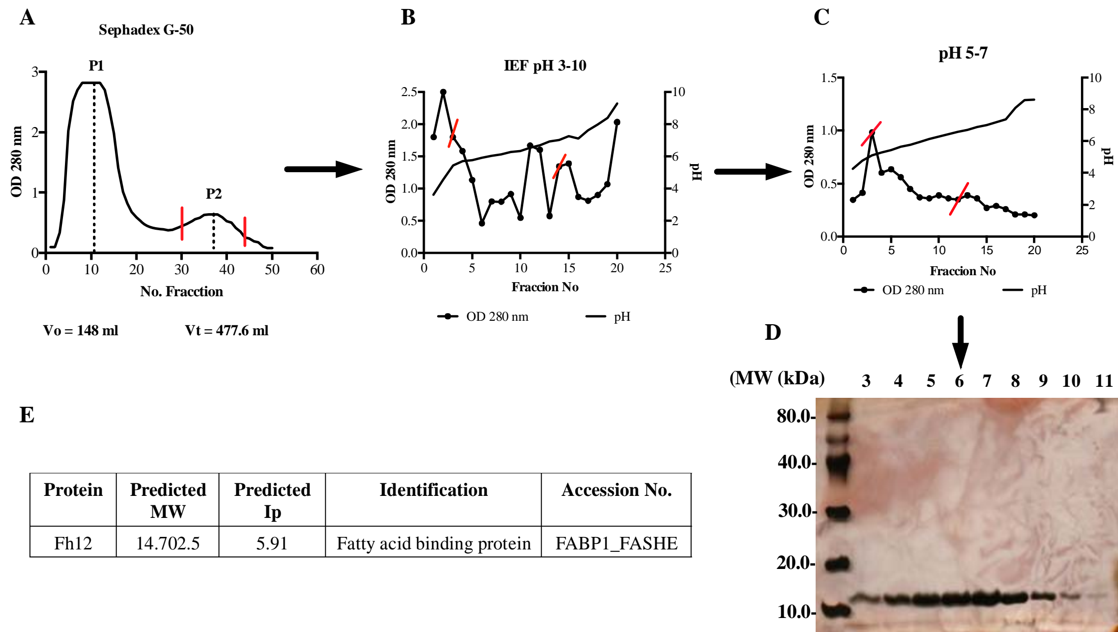
118 WI) and analyzed by MALDI and MS/MS as previously described (Morales and Espino,
119 2012). After confirming the presence of Fh12-FABP as a unique component, these
120 fractions were pooled (**Fig-1S**).



Protein	Predicted MW	Predicted Ip	Identification	Accession No.
Fh12	14.702.5	5.91	Fatty acid binding protein	FABP1_FASHE

121 **Figure-1S. Purification of native *F. hepatica* fatty acid binding protein (Fh12). (A)** *F. hepatica* whole worm extract was loaded onto a gel filtration chromatography with Sephadex G-50 (XK 26/100 column). Fractions eluted in peak-2 (P2) containing proteins among 1.5 to 30kDa were pooled. (**B-C**) P2 was dialyzed against 1% glycine containing 2% ampholytes pH 3-10 (first run) or pH 5-7 (second run) and then loaded onto a liquid isoelectric focusing system (Rotofor, Bio-Rad). Individual fractions were harvested and their pH and absorbance at 280nm were measured. Red lines on figures indicate fractions that were selected for pooling and subsequent purification step. **(D)** Fractions 3 to 11 from the IEF run with pH 5-7, which contain proteins with Ip between 4.61 to 5.9 were analyzed by 15% SDS-PAGE stained with silver stain to corroborate the presence of polypeptide of 12kDa. **(E)** Polypeptide band was excised from gel and analyzed by matrix-assisted laser desorption ionization 9MALDI) and tandem mass spectrometry (MS/MS). Analysis revealed the presence of fatty acid binding protein.

122 **Endotoxin removal**

123 Endotoxins were removed from the Fh12 by using the polymyxin-B (PMB) column
124 according to the manufacturer's instructions, and the levels of endotoxins were measured
125 using the Chromogenic *Limulus* Amebocyte Lysate QCL-1000 Assay (Lonza,
126 Walkersville, MD). Fh12 was considered endotoxin free when endotoxin levels gave
127 similar to background levels (<0.1 EU/ml). Protein concentration was adjusted to 1
128 mg/ml as determined by the bicinchoninic acid (BCA) method using a Pierce protein
129 assay kit (Pierce, Cambridge, NJ).

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130 **Myeloid DC generation and stimulation**

131 DCs were generated as previously described (Falcon et al., 2010), with slight
132 modifications. Briefly, bone marrow was collected from femurs and tibia of BALB/c
133 mice and then seeded into bacteriological Petri dishes at 4 x 10^6 in 10 ml of complete
134 RPMI 1640 medium supplemented with 2mM L-glutamine (Life Technologies,
135 Gaithersburg, MD), 100U/ml penicillin and 100µg/ml streptomycin, 10% heat-inactivated
136 endotoxin-free fetal calf serum (Sigma-Aldrich) and 20ng/ml GM-CSF (R&D System) at
137 37^oC, 5% CO_2. On day 3, an additional 10 ml of medium containing 20ng/ml GM-CSF
138 was added. At day 6, 10 ml of culture supernatant was removed and replaced with fresh
139 culture medium containing GM-CSF. On day 8, the supernatant was removed and
140 replaced with medium without GM-CSF, and cells were harvested 18h later (day 9). After
141 this time, >85% of harvested cells were DCs (MHC class II^+, $CD11c^+$). For stimulation
142 experiments, DCs were seeded into 96-wells plates at $4x10^5$ cells in complete RPMI. DCs
143 were treated for 18h with Fh12 (2.5µg/ml or 1µg/ml LPS (*E. coli* 0111: B4; Sigma-
144 Aldrich). In the inhibition experiments, DCs were treated for 1h with Fh12 (2.5µg/ml)
145 and further stimulated with LPS (1µg/ml). Cells treated with PBS were used as controls
146 in both experiments. Supernatants from cultured DCs were tested for the production of
147 IL-10, IL-12p70 (eBioscience, USA) and IL-6 (BD Pharmingen, USA) by sandwich
148 ELISA.

149

150 **Cell viability and Annexin-V and 7AAD assay**

151 DCs treated with Fh12 (2.5, 5, 10 and 15 µg/ml) in the presence or absence of LPS
152 (1µg/ml) was incubated with 50µl MTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-

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153 tetrazolium]-bis (4- methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent
154 (Roche Life Science, USA) to each well. The absorbance was read at 480 nm. The
155 percentage of viable, necrotic, and apoptotic DCs after treatments were determined by
156 flow cytometry using surface Annexin V detection and 7-Amino-actinomycin D (7-AAD)
157 incorporation (BD Biosciences, USA). DCs were washed with PBS, and then washed
158 twice with annexin-V binding buffer (10mM HEPES, 140 mM NaCl, 2.5 mM CaCl, pH
159 7.4) and resuspended in 100µl of annexin-V binding buffer before being incubated with
160 FITC-conjugated annexin-V (0.5µg / 2 x 10^5 cells) and 0.5µl (7-AAD) solution. After 15
161 min of incubation at room temperature (RT) in the dark, an extra amount of 400µl of
162 annexin-V binding buffer was added to cells, and cells were analyzed by flow cytometry.
163 DCs were gated on the basis of their forward and side light scatter. If there is an alteration
164 in the membrane integrity (due to externalization of phosphatidylserine), annexin-V
165 detects both early- and late- apoptotic cells. Thus, the simultaneous addition of 7-AAD,
166 which does not enter healthy cells with an intact plasma membrane, discriminates
167 between early apoptotic (annexin V-positive and 7AAD-negative), late-apoptotic (both
168 annexin V- and 7AAD-positive), necrotic (annexin V-negative and 7AAD-positive) and
169 live (both annexin V- and 7AAD-negative) cells (Ardestani et al., 2012).
170
171 **DCs Flow Cytometry**
172 Expression of cell surface markers on DCs treated as described above was quantified by
173 two-color flow cytometry using allophycocyanin-, and phycoerythrin-conjugated
174 antibodies specific for CD80, CD40, MHC-II, and CD11c (BD BioSciences).
175 Appropriate labeled isotype-matched antibodies were used as controls. Cell acquisition

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176 was performed using FACS Calibur equipment and MACSQuant Analyzer - Miltenyi
177 equipment, whereas analysis of results was performed using FlowJo software (FlowJo,
178 LLC).
179

180 **Allogeneic mixed lymphocyte reaction (MLR)**

181 DCs from BALB/c mice were previously treated with Fh12 (2.5µg/ml), LPS (1µg/ml), or
182 for 1h with Fh12 (2.5µg/ml) and further stimulated with LPS (1µg/ml) for 18h. Then the
183 cells were cultured in U-bottom 96-well plates with C57BL/6 splenocytes
184 (2×10^5 cells/well) for 5 days at a ratio of 5:1. For cytokine determination, supernatants
185 were collected after 48h for IFN-γ, and 72h for IL-4 and IL-13 detection.

186 **Statistical analysis**

187 All experiments were repeated twice with in different days with three replicates for each
188 determination, and equivalent results were obtained. Data are expressed as mean ±
189 standard error of the mean (S.E.M.) and analyzed statistically using the Student *t*-test.
190 Comparisons of the values for multiple groups were made using one-way ANOVA.
191 Statistical analyses were made using GraphPad Prism software (Prism-6). Statistical
192 significance was assumed at the p-value of <0.05.
193
194

195 **RESULTS**
196
197

198 **Fh12 induce apoptosis of myeloid DCs**

199 Given in our previous studies we had demonstrated that the treatment of murine
200 macrophages with different concentrations of Fh12 alone or combined with LPS
201 suppressed the capacity of these cells to express pro-inflammatory mediators without

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202 affecting the cell viability (Martin et al., 2015); in the present study, we determined
203 whether Fh12 could exert a similar effect on DCs. For this, bone marrow derived DCs
204 were treated for 18h with different concentrations of Fh12 or with Fh12 for 1h and further
205 stimulated with LPS. Results of the MTT-viability test revealed that the treatment of DCs
206 with low Fh12 concentrations (among 2.5 to 5μg/ml) alone or combined with LPS seems
207 to maintain the viability of cells as compared with those cells treated only with PBS or
208 LPS alone. However, at Fh12 concentrations of 10 and 15μg/ml, the absorbance values
209 significantly dropped, which indicates that at these Fh12 concentrations, the viability of
210 cells was seriously compromised ($p<0.0001$) (**Fig. 1**). To determine whether Fh12 could
211 be inducing apoptosis, Fh12-treated DCs were stained with annexin-V and 7AAD and
212 analyzed by flow cytometry. Results demonstrated that ~91% of cells remain viable when
213 are treated with medium alone for 18h. However, when cells are treated with Fh12 the
214 viability of cells drop significantly. At Fh12 concentrations of 2.5 or 5μg/ml ~ 52% and
215 41.9% of DCs remained viable, whereas that 46.5% (23.6% early apoptosis and 22.9%
216 late apoptosis) and 52.9% (18.8% early apoptosis and 34.1% late apoptosis) was in
217 apoptosis, respectively. The number of apoptotic cells increased significantly by 80.7%
218 (22.1% early apoptosis and 58.6% late apoptosis) and 88.7% (18.3% early apoptosis and
219 70.4% late apoptosis) when the Fh12 concentration increased to 10μg/ml and 15μg/ml,
220 respectively (**Fig. 2**). These results clearly demonstrate that Fh12 promotes the late
221 apoptosis of DCs in a dose-dependent manner.

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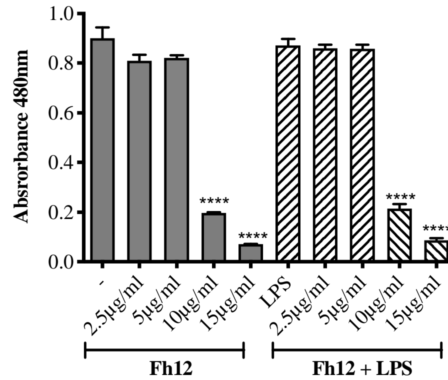

Figure-1. MTT-viability assay of Fh12 treated DCs. Murine DCs were seeded into 96-wells plates at $4x10^5$ cells in complete RPMI plus 20ng/ml GM-CSF and then treated with Fh12 (2.5, 5, 10, 15µg/ml) in the presence or absence of 1µg/ml LPS (*E. coli* 0111:B4). Control cells were treated with Fh12 or LPS alone. After 18h of incubation at 37C, 5% CO_2 50µl MTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4- methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent was added to each well and the absorbance was read at 480 nm. Viability of cells significantly dropped (**** p<0.0001) at Fh12 concentrations ≥10µg/ml.

222

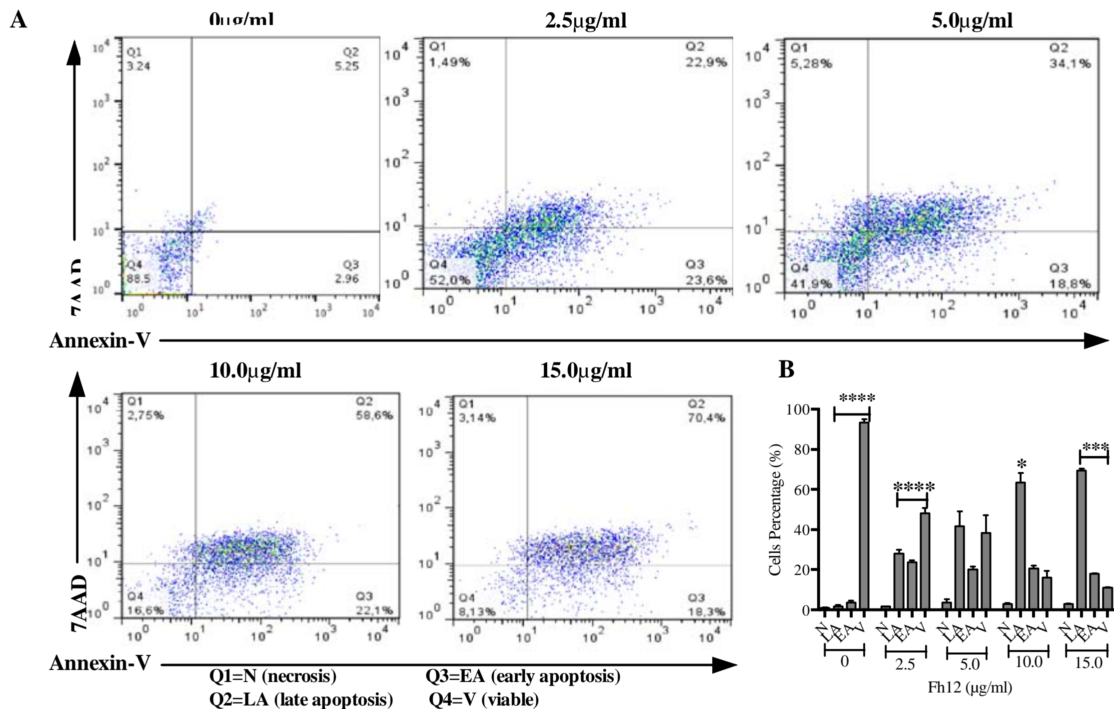

Figure-2. Fh12 induces apoptosis of DCs, as measured by Annexin-V binding to externalized phosphatidylserine. Myeloid DCs from C57BL/6 mice were treated with Fh12 (2.5, 5, 10, and 15 µg/ml) or remain untreated for 18 hours. After incubation, the cells were harvested and analyzed by two-color flow cytometry for Annexin V and 7-AAD. Cells were gated based on the CD11c expression. **(A)** Histogram representative of an experiment showing that the apoptosis of DCs induced by Fh12 is dose-dependent. **(B)** Average percentages and their SEM of at least three independent experiments showing that the percentage of DCs viable (V) at Fh12 concentration of 2.5µg/ml is significantly high (****p<0.0001) compared to the percentage of cells in early (EA) or late apoptosis (LA). In contrast, the percentage of DCs in apoptosis significantly increased at Fh12 concentration of 10µg/ml. (*p= 0.0146) and 15µg/ml (***p<0.0008).

223

224

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225 **Fh12 induces apoptosis of DCs in absence of CD14 co-receptor**

226 Since in our previous studies with murine macrophages we had demonstrated that Fh12
227 targeted the CD14 coreceptor as a mechanism to suppress the expression of pro-
228 inflammatory cytokines and prevent the phagocytic capacity of macrophages (Martin et
229 al., 2015), and given that CD14 has been involved in the regulation of programmed cell
230 death (apoptosis) in immune and no-immune cells (Frey and Finlay, 1998; Heidenreich et
231 al., 1997), we investigate whether Fh12 could require CD14 to induce apoptosis of DCs.
232 Myeloid DCs were collected from CD14 KO mice and fully differentiated *in vitro.*
233 Further, cells were cultured and treated with 15µg/ml Fh12. Results demonstrated that in
234 the absence of CD14 Fh12 induces similar apoptosis levels that those observed in DCs of
235 wild type animals (more than 70% **Fig. 3**). This result indicates that CD14 signaling is
236 not involved in the apoptosis mechanism induced by Fh12.

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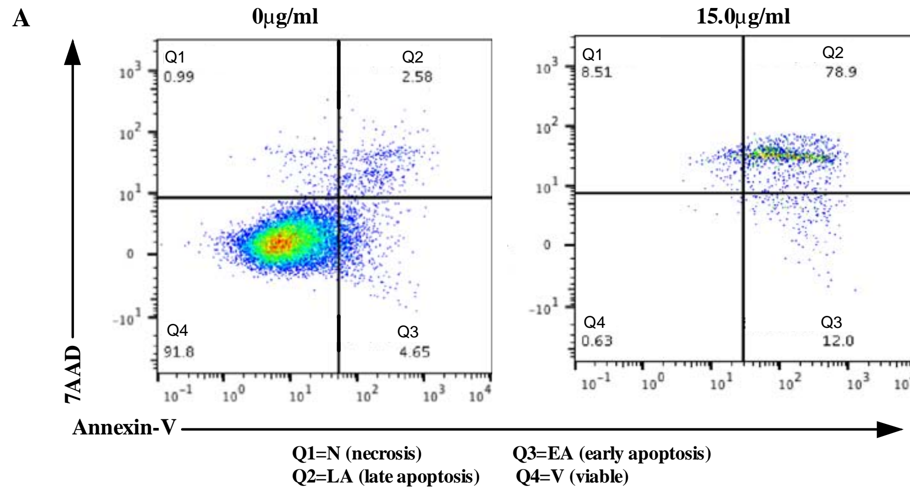

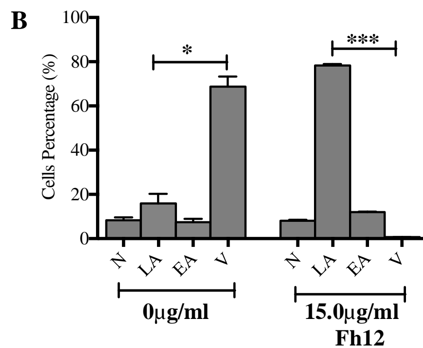

Figure-3. Fh12 induces apoptosis of DCs in absence of CD14 coreceptor. Myeloid DCs from CD14 KO C57BL/6 mice were treated with Fh12 (15μg/ml) for 18 hours or remain untreated. After incubation, the cells were harvested and analyzed by two-color flow cytometry for Annexin V and 7-AAD. Cells were gated based on the CD11c expression. **(A)** Histogram representative of an experiment showing the apoptosis of DCs induced by Fh12. **(B)** Average percentages and their SEM of at least three independent experiments showing that the percentage of DCs viable (V) compared to apoptotic (EA or LA) or necrotic cells (N) in untreated CD14 KO cells is significantly high (*p=0.144) whereas the number of DCs in late apoptosis (LA) compared to viable (V) cells is significantly high (****p<0.0001).

237

238 **Fh12 did not induce DCs classical maturation but affected LPS-induced DCs**

239 **activation**

240 Having observed that after treatment with low Fh12 concentrations (2.5μg/ml) near 52%

241 of DCs still remain viable, we investigated whether at this experimental conditions Fh12

242 could modify the activation status of LPS-activated DCs. The cytokines and the

243 expression of surface proteins involved in the T-cell activation and polarization were

244 analyzed in DCs cultured with medium alone, Fh12 (2.5μg/ml), LPS (1μg/ml) or for 1h

245 with Fh12 and further stimulated with LPS. As expected, DCs stimulated with LPS

246 produced significantly more IL-12p70 (p=0.0006) and IL-6 (p=0.0003) than untreated

247 DCs and did not produce IL-10. However, although Fh12 alone did not induce the

248 production of IL-12p70, IL-6, or IL-10, it significantly suppressed the LPS-induced IL-

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249 12p70 (p<0.031) and IL-6 (p<0.0458) production. In addition, in the presence of Fh12,
250 LPS-stimulated cells produced significantly more IL-10 (p=0.0039) than untreated cells
251 (**Fig. 4**). Moreover, the expression of the MHC-II and co-stimulatory molecules CD40
252 and CD80 induced by LPS-stimulation was found significantly downregulated by the
253 Fh12 treatment (*p*=0.0072, *p*=0.0023, and *p*=0.0398, respectively) (**Fig. 5**). Together, our
254 data showed that Fh12 was not able to induce classical DC maturation but exerted control
255 over the DC activation induced by LPS. These not only by virtue of its ability to inhibit
256 expression of co-stimulatory molecules essential for T-cell activation, but also by directly
257 suppressing the secretion of cytokines critical for the Th1 cell polarization.

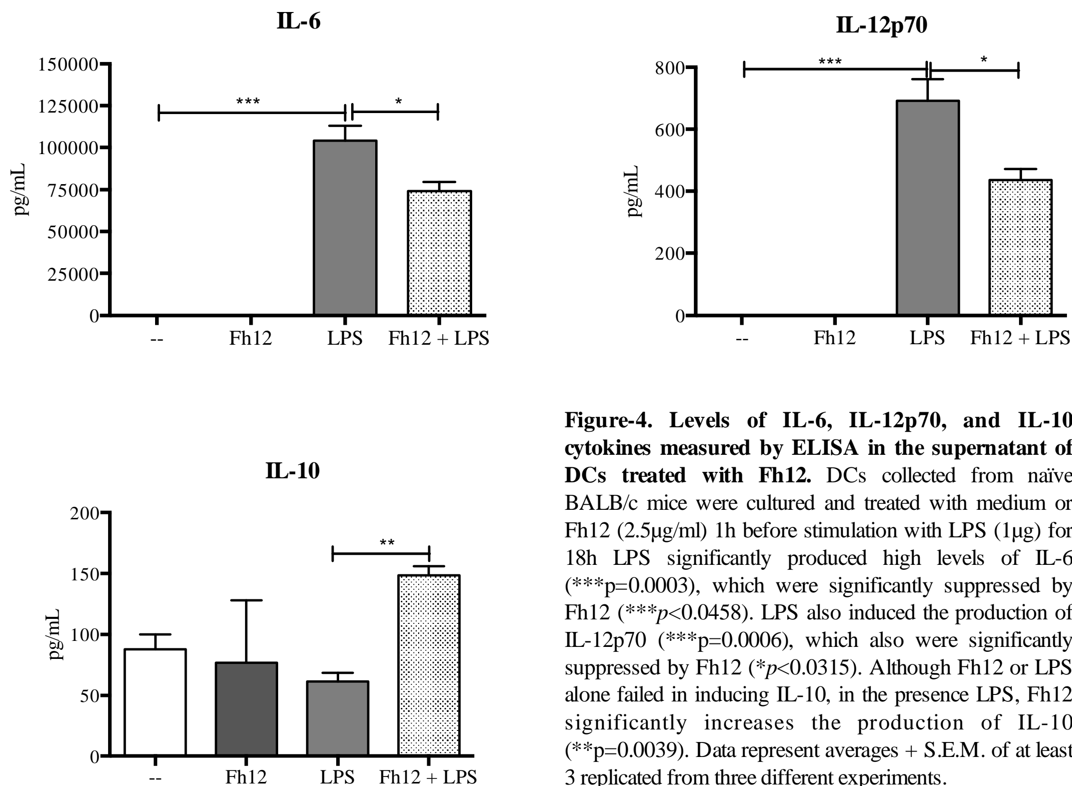

Figure-4. Levels of IL-6, IL-12p70, and IL-10 cytokines measured by ELISA in the supernatant of DCs treated with Fh12. DCs collected from naïve BALB/c mice were cultured and treated with medium or Fh12 (2.5µg/ml) 1h before stimulation with LPS (1µg) for 18h LPS significantly produced high levels of IL-6 (***p=0.0003), which were significantly suppressed by Fh12 (****p*<0.0458). LPS also induced the production of IL-12p70 (***p=0.0006), which also were significantly suppressed by Fh12 (**p*<0.0315). Although Fh12 or LPS alone failed in inducing IL-10, in the presence LPS, Fh12 significantly increases the production of IL-10 (**p=0.0039). Data represent averages + S.E.M. of at least 3 replicated from three different experiments.

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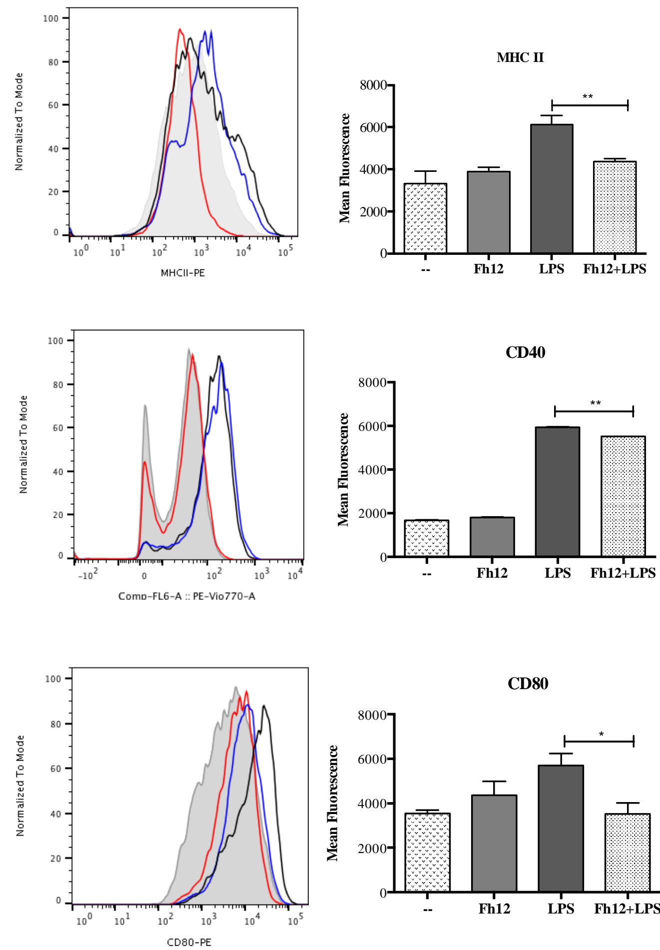

Figure-5. Fh12 inhibits MHC II and co-stimulatory molecules expression on LPS-activated DCs. DCs from BALB/c mice were treated for 1h with Fh12 (2.5 μg/ml) and further stimulated with LPS (1μg) for 18h. After incubation, the cells were harvested and analyzed by two-color flow cytometry for MHC-II, CD40 and CD80. Cells were gated based on the expression of CD11c. Histograms (left panel) show LPS-stimulated cells (black line), Fh12-treated cells (red line), Fh12+LPS-stimulated cells (blue line) and untreated cells (--) (gray shaded histogram). Right panel represents mean fluorescence intensity of untreated DCs or treated with Fh12, LPS or Fh12/LPS, for MHC-II, CD40 and CD80, respectively (**p=0.0072, **p=0.0023, *p=0.0398).

259

260 **Fh12-treated DCs showed reduced ability to induce allogeneic responses**

261 Given our data showing that Fh12 inhibits the maturation of LPS-treated DCs, we
262 reasoned that the ability of these cells to prime allospecific T-cell responses could be
263 impaired. Then, we wanted to determine which T helper profile was promoted by Fh12-
264 treated DCs in a mixed lymphocyte reaction. To this end, immature or LPS-treated DCs

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265 or treated for 1h with Fh12 and further stimulated with LPS from BALB/c mice were
266 then cultured with allogeneic splenocytes from C57BL6. Cultures with untreated DCs
267 were used as control. As expected, DCs stimulated with LPS and co-cultured with
268 allogeneic splenocytes induced the secretion of significantly higher amounts of IFN-γ
269 (p=0.0003) compared to untreated cells and failed to induce allogeneic IL-4 or IL-13
270 production. In contrast, Fh12-treated DCs induced significant amounts of IL-4
271 (*p*=0.0022) and IL-13 (*p*<0.0001) compared to untreated cells. Importantly, LPS-
272 stimulated DCs that were pre-treated with Fh12 and then co-cultured with allogeneic
273 splenocytes lack the ability to induce INF-γ production (p=0.0003) whereas significantly
274 augmented their capacity to produce IL-4 (p=0.0073). Fh12 also showed a tendency to
275 increase the production of IL-13 in DCs stimulated with LPS and then co-cultured with
276 splenocytes, but these increases were not found significant **(Fig. 6)**. These results suggest
277 that Fh12 could be able to promote the activation of T-helper type-2 (Th2) responses of
278 immature or LPS-mature DCs, whereas concurrently suppress the ability of these cells to
279 promote the Th1-response.
280
281

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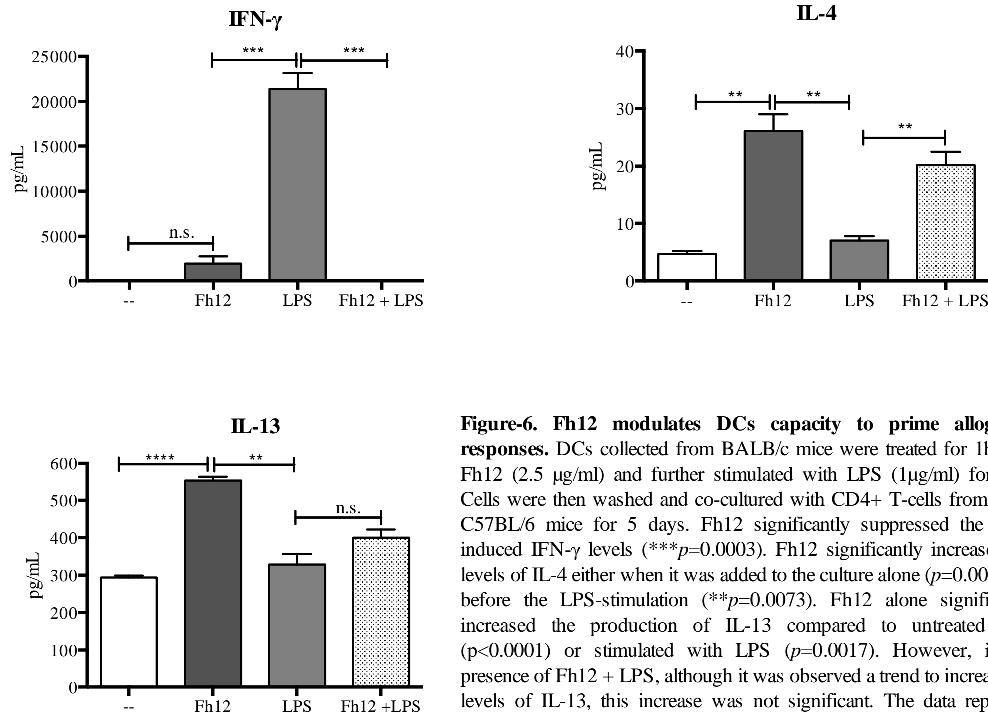

Figure-6. Fh12 modulates DCs capacity to prime allogeneic responses. DCs collected from BALB/c mice were treated for 1h with Fh12 (2.5 μg/ml) and further stimulated with LPS (1μg/ml) for 18h. Cells were then washed and co-cultured with CD4+ T-cells from naïve C57BL/6 mice for 5 days. Fh12 significantly suppressed the LPS-induced IFN-γ levels (****p*=0.0003). Fh12 significantly increased the levels of IL-4 either when it was added to the culture alone (*p*=0.0022) or before the LPS-stimulation (***p*=0.0073). Fh12 alone significantly increased the production of IL-13 compared to untreated cells (p<0.0001) or stimulated with LPS (*p*=0.0017). However, in the presence of Fh12 + LPS, although it was observed a trend to increase the levels of IL-13, this increase was not significant. The data represent averages with their SEM of at least 3 replicates from three independent experiments.

DISCUSSION

F. hepatica can be considered a successful parasite because of its ability to migrate through the host tissues without suffering damage to finally allocate in the bile ducts, where it can survive for years. To achieve this survival, *F. hepatica* has co-evolved with the host and is able to induce a type of response that is not harmful to it. During its migration, the parasite excretes and/or secretes many products capable of modulating the immune response (Molina-Hernandez et al., 2015). Soon after the infection, the juvenile stage of *F. hepatica* crosses the intestinal wall and reaches the peritoneum inducing the recruitment of alternatively activated macrophages (Adams et al., 2014; Donnelly et al., 2005; Donnelly et al., 2008). Different reports have shown the ability of various products such as ESPs (Guasconi et al., 2011), and FhTeg (Hacariz et al., 2011) and different

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297 enzymes such as thioredoxin peroxidase (Donnelly et al., 2005), 2-Cys peroxiredoxin
298 (Donnelly et al., 2008), fatty acid binding protein (Fh12) (Figueroa-Santiago, 2014) and
299 more recently heme-oxygenase-1 (Carasi et al., 2017) to modulate macrophage activation
300 toward an alternative profile. This phenotype promotes the secretion of anti-inflammatory
301 factors, enhancing the differentiation of Th2 and Treg cells (Kreider et al., 2007). In
302 addition to the modulation of macrophages activation, *F. hepatica* derived products such
303 as ESPs (Falcon et al., 2010), FhTeg (Hamilton et al., 2009) and secreted proteins such as
304 Kunitz type molecule (KTM) (Falcon et al., 2014) has been related to a down-modulation
305 in DCs activation. It is reasonable to assume that distinct molecules, secreted or
306 expressed by the parasite in its tegument, may participate during its migration in the
307 prevention of an appropriate activation of DCs, contributing to an anti-inflammatory
308 control.
309 Given that Fh12 is an immunogenic protein that plays an important role in nutrient
310 acquisition and survival of the parasite and is present in ESPs and FhTeg, its interaction
311 with DCs might be relevant. In this work, we investigate the effect of Fh12 on maturation
312 and function of DCs. Unexpectedly, our results showed that Fh12 induces the early and
313 late apoptosis of DCs in a dose dependent manner. Additionally, Fh12 inhibited the
314 ability of DCs to mature in the presence of LPS. Considering that Fh12 inhibits
315 inflammatory cytokines in LPS matured macrophages through a mechanism that involves
316 CD14, we hypothesize that this molecule might be involved in the apoptosis. However,
317 our results are against this hypothesis since DCs from CD14 deficient mice in the
318 presence of Fh12 showed similar apoptosis levels of those observed in DCs from normal
319 animals, suggesting the independence of this phenomenon from CD14.

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320 Being a CD14 a coreceptor that participates along with the toll-like receptor-4 (TLR4)
321 and MD-2 in the recognition of bacterial LPS (Dowling, 2018; Kitchens, 2000) its
322 importance in the recognition of Fh12 by DCs seems to be relative. In contrast, it has
323 been shown that CD14 is involved in the apoptotic death of LPS-stimulated DCs via
324 NFAT activation has been demonstrated (Zanoni et al., 2011). However, unlike what
325 happens with DCs, macrophages do not die after LPS activation. The authors argue that
326 after activation by LPS, significant differences are generated in the signal transduction
327 pathways in DCs and macrophages. These last cells were unable to mobilize Ca, a crucial
328 event to induce apoptosis. Interestingly tissue-resident macrophage survival after
329 activation is a crucial event for inflammation resolution (Zanoni et al., 2009).
330 Interestingly, in previous reports, the induction of apoptosis in eosinophils by ES
331 products of *F. hepatica* through the induction of reactive oxygen species (ROS) has been
332 shown (Serradell et al., 2007). Since Fh12 is part of the *F. hepatica* ESPs, a similar
333 mechanism of apoptosis induced by Fh12 in DCs might be occurring. Experiments using
334 ROS inhibitors or other apoptosis mediators should be used to dissect the mechanism by
335 which Fh12 exerts this effect on DCs.
336 Based on our results showing that the lowest concentration of Fh12 induce approximately
337 47% of the cells in apoptosis, while low concentration of LPS (100ng/ml) induced only
338 17.5% of the apoptotic cells (**Fig. 2S**) and both stimuli induce different activation status
339 in DCs, we hypothesize that the apoptosis and the modulation of DCs activation might be
340 independent events. Data supporting this notion show that the signaling involved in
341 survival and LPS-induced activation in DCs would be different, dependent on ERK and
342 NF-κB, respectively (Rescigno et al., 1998). Both phenomena could occur simultaneously,

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343 and it would be difficult to determine whether they are dependent or independent events.
344 During the allogenic responses, a mismatch between the MHC-II molecules presents in
345 the DCs and the TCR on T lymphocytes promotes the proliferation and production of
346 cytokines, mainly IFN-γ. This phenomenon is exacerbated when the microenvironment
347 generated during DCs stimulation is inflammatory (Herrera et al., 2004). Our results
348 indicate that Fh12 exerts a negative regulation on DCs maturation induced by LPS
349 inhibiting MHCII and co-stimulatory expression generating defective activated DCs with
350 low production of proinflammatory molecules or cytokines. The poor maturation status of
351 DCs might explain the impaired ability of these cells to induce allogeneic responses. This
352 effect exerted by Fh12 does not appear to be redundant since other molecules derived
353 from *F. hepatica* such as cathepsin L1 or glutathione S-transferase inhibit IL-23 secretion
354 from LPS-matured DCs, with the consequence of a reduction in the inflammatory Th17
355 response. Taken together, these results suggest that different antigens derived from *F.*
356 *hepatica* migtht attenuate the DCs activation, which in turn is responsible for the decrease
357 of both type Th1 and Th17 inflammatory responses, what otherwise it would be harmful
358 to the parasite. The control of DCs activation, together with the apoptosis of these cells,
359 might be useful to reduce the impact of bacterial ligands translocation during the larvae
360 passage through the intestinal wall. Thus, our data suggest an immunomodulatory role for
361 Fh12 on DCs function and its possible involvement in immunoevasion mechanisms.

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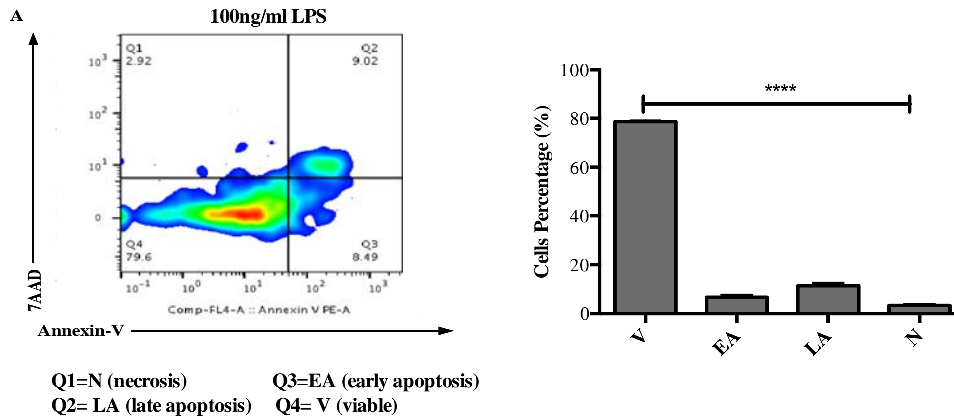

Figure-2S. LPS does not promote significant apoptosis to DCs. Myeloid DCs from C57BL/6 mice were treated with LPS (100ng/ml) or remain untreated for 18 hours. After incubation, the cells were harvested and analyzed by two-color flow cytometry for Annexin V and 7-AAD. Cells were gated based on the CD11c expression. **(A)** Histogram representative of an experiment showing that at this experimental conditions most of cells (79.6%) remain viable and that only the 17% of cells are apoptotic. **(B)** Average percentages and their SEM of at least three independent experiments showing that the percentage of DCs viable (V) at LPS concentration of 100ng/ml is significantly high (****$p<0.0001$) compared to the percentage of cells in early (EA) or late apoptosis (LA).

362

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498 **LEGENDS OF FIGURES**
499
500
501 **Figure-1**. **MTT-viability assay of Fh12 treated DCs**. Murine DCs were seeded into 96-
502 wells plates at $4x10^5$ cells in complete RPMI plus 20ng/ml GM-CSF and then treated
503 with Fh12 (2.5, 5, 10, 15µg/ml) in the presence or absence of 1µg/ml LPS (*E. coli*
504 0111:B4). Control cells were treated with Fh12 or LPS alone. After 18h of incubation at
505 37^oC, 5% CO_2 50µl MTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-
506 methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent was added to each well
507 and the absorbance was read at 480 nm. Viability of cells significantly dropped (****
508 p<0.0001) at Fh12 concentrations ≥10µg/ml.
509
510 **Figure-2. Fh12 induces apoptosis of DCs, as measured by Annexin-V binding to**
511 **externalized phosphatidylserine.** Myeloid DCs from C57BL/6 mice were treated with
512 Fh12 (2.5, 5, 10, and 15 µg/ml) or remain untreated for 18 hours. After incubation, the cells
513 were harvested and analyzed by two-color flow cytometry for Annexin V and 7-AAD. Cells
514 were gated based on the CD11c expression. **(A)** Histogram representative of an experiment
515 showing that the apoptosis of DCs induced by Fh12 is dose-dependent. **(B)** Average
516 percentages and their SEM of at least three independent experiments showing that the
517 percentage of DCs viable (V) at Fh12 concentration of 2.5µg/ml is significantly high
518 (****p<0.0001) compared to the percentage of cells in early (EA) or late apoptosis (LA). In
519 contrast, the percentage of DCs in apoptosis significantly increased at Fh12 concentration of
520 10µg/ml. (*p= 0.0146) and 15µg/ml (***p<0.0008).
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522 **Figure-3. Fh12 induces apoptosis of DCs in absence of CD14 coreceptor.** Myeloid DCs
523 from CD14 KO C57BL/6 mice were treated with Fh12 (15μg/ml) for 18 hours or remain
524 untreated. After incubation, the cells were harvested and analyzed by two-color flow
525 cytometry for Annexin V and 7-AAD. Cells were gated based on the CD11c expression. **(A)**
526 Histogram representative of an experiment showing the apoptosis of DCs induced by Fh12.
527 **(B)** Average percentages and their SEM of at least three independent experiments showing
528 that the percentage of DCs viable (V) compared to apoptotic (EA or LA) or necrotic cells (N)
529 in untreated CD14 KO cells is significantly high (*p=0.144) whereas the number of DCs in
530 late apoptosis (LA) compared to viable (V) cells is significantly high (****p<0.0001).
531

532 **Figure-4. Levels of IL-6, IL-12p70, and IL-10 cytokines measured by ELISA in the**
533 **supernatant of DCs treated with Fh12.** DCs collected from naïve BALB/c mice were
534 cultured and treated with medium or Fh12 (2.5μg/ml) 1h before stimulation with LPS (1μg)
535 for 18h LPS significantly produced high levels of IL-6 (***p=0.0003), which were
536 significantly suppressed by Fh12 (***p<0.0458). LPS also induced the production of IL-
537 12p70 (***p=0.0006), which also were significantly suppressed by Fh12 (*p<0.0315).
538 Although Fh12 or LPS alone failed in inducing IL-10, in the presence LPS, Fh12
539 significantly increases the production of IL-10 (**p=0.0039). Data represent averages +
540 S.E.M. of at least 3 replicated from three different experiments.
541

542 **Figure-5. Fh12 inhibits MHC II and co-stimulatory molecules expression on LPS-**
543 **activated DCs**. DCs from BALB/c mice were treated for 1h with Fh12 (2.5 μg/ml) and
544 further stimulated with LPS (1μg) for 18h. After incubation, the cells were harvested and

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545 analyzed by two-color flow cytometry for MHC-II, CD40 and CD80. Cells were gated based
546 on the expression of CD11c. Histograms (left panel) show LPS-stimulated cells (black line),
547 Fh12-treated cells (red line), Fh12+LPS-stimulated cells (blue line) and untreated cells (--)
548 (gray shaded histogram). Right panel represents mean fluorescence intensity of untreated
549 DCs or treated with Fh12, LPS or Fh12/LPS, for MHC-II, CD40 and CD80, respectively
550 (***p*=0.0072, ***p*=0.0023, **p*=0.0398).
551
552 **Figure-6. Fh12 modulates DCs capacity to prime allogeneic responses.** DCs collected
553 from BALB/c mice were treated for 1h with Fh12 (2.5 μg/ml) and further stimulated with
554 LPS (1μg/ml) for 18h. Cells were then washed and co-cultured with CD4+ T-cells from
555 naïve C57BL/6 mice for 5 days. Fh12 significantly suppressed the LPS-induced IFN-γ levels
556 (****p*=0.0003). Fh12 significantly increased the levels of IL-4 either when it was added to
557 the culture alone (*p*=0.0022) or before the LPS-stimulation (***p*=0.0073). Fh12 alone
558 significantly increased the production of IL-13 compared to untreated cells ($p<0.0001$) or
559 stimulated with LPS (*p*=0.0017). However, in the presence of Fh12 + LPS, although it was
560 observed a trend to increase the levels of IL-13, this increase was not significant. The data
561 represent averages with their SEM of at least 3 replicates from three independent experiments.
562
563
564 **Figure-1S. Purification of native *F. hepatica* fatty acid binding protein (Fh12). (A)** *F. hepatica*
565 whole worm extract was loaded onto a gel filtration chromatography with Sephadex G-50 (XK 26/100
566 column). Fractions eluted in peak-2 (P2) containing proteins among 1.5 to 30kDa were pooled. (**B-C**)
567 P2 was dialyzed against 1% glycine containing 2% ampholytes pH 3-10 (first run) or pH 5-7 (second
568 run) and then loaded onto a liquid isoelectric focusing system (Rotofor, Bio-Rad). Individual fractions

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569 were harvested and their pH and absorbance at 280nm were measured. Red lines on figures indicate
570 fractions that were selected for pooling and subsequent purification step. **(D)** Fractions 3 to 11 from the
571 IEF run with pH 5-7, which contain proteins with Ip between 4.61 to 5.9 were analyzed by 15% SDS-
572 PAGE stained with silver stain to corroborate the presence of polypeptide of 12kDa. **(E)** Fractions 3 to
573 11 containing the 12kDa polypeptide were pooled, dialyzed against PBS, concentrated to 1mg/ml and
574 reanalyzed by SDS-PAGE (line-1) and western-blot (line-2) using a specific rabbit polyclonal antibody
575 against Fh12 (17). **(F)** Polypeptide band was excised from gel and analyzed by matrix-assisted laser
576 desorption ionization 9MALDI) and tandem mass spectrometry (MS/MS). Analysis revealed the
577 presence of fatty acid binding protein.
578
579 **Figure-2S. LPS does not promote significant apoptosis to DCs.** Myeloid DCs from C57BL/6 mice
580 were treated with LPS (100ng/ml) or remain untreated for 18 hours. After incubation, the cells were
581 harvested and analyzed by two-color flow cytometry for Annexin V and 7-AAD. Cells were gated
582 based on the CD11c expression. **(A)** Histogram representative of an experiment showing that at this
583 experimental conditions most of cells (79.6%) remain viable and that only the 17% of cells are
584 apoptotic. **(B)** Average percentages and their SEM of at least three independent experiments showing
585 that the percentage of DCs viable (V) at LPS concentration of 100ng/ml is significantly high
586 (****p<0.0001) compared to the percentage of cells in early (EA) or late apoptosis (LA).