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Anisakis simplex: Immunomodulatory effects of larval antigens on the activation of Toll like Receptors

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ABSTRACT

Aims: The objective of this investigation is to evaluate the mechanisms *Anisakis simplex* employs to modify its host immune system, regarding the larval antigens interactions with Toll-Like-Receptors (TLRs).

Methods and Results: In a previous study, we described that the stimulation of bone marrow derived dendritic cells (BMDCs) with *A. simplex* larval antigens drive an acute inflammatory response in BALB/c mice, but a more discrete and longer response in C57BL/6J. Moreover, when A. simplex larval antigens were combined with TLR agonists (TLR 1/2–9), they modified mainly TLR2, TLR4 and TLR9 agonists responses in both mice strains, and also TLR3, TLR5 and TLR7 in BALB/c. Antigen-presenting ability was analyzed by the detection of CD11c + cells expressing surface markers (CD80-86, MHC I-II), intracellular cytokines (IL-10, IL-12, TNF-α) and intracellular proteins (Myd88, NF-κβ) by Flow Cytometry. Secreted IL-10 was measured by ELISA.

Conclusion: Our findings confirm not only that the host genetic basis plays a role in the development of a Th2/Th1/Treg response, but also it states *A. simplex* larval antigens present specific mechanisms to modify the innate response of the host. As allergies share common pathways with the immune response against this particular helminth, our results provide a better understanding into the specific mechanisms of *A. simplex* allergy related diseases.

Keywords: *Anisakis simplex*, Dendritic Cell, Cytokine, Immunoregulation, Flow cytometry, Innate Immunity

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1. Introduction

Anisakiasis is acquired by the accidental ingestion of third stage *Anisakis* larvae (L3) in uncooked infected fish, which can persist for two or three weeks in the gastric and intestinal mucosa. During infection, a range of different severe symptoms develop from gastric or intestinal anisakiasis depending on the localization of the larva [1,2], to a sensitization to parasite allergens [3,4]. After subsequent exposure, they can result in a variety of systemic reactions [5]. *A. simplex* triggers an acute immune reaction, that leads to its own elimination. However, even after the expulsion of the larvae, some patients develop chronic symptoms, like urticaria. Thus, the immune response against this parasite, like it happens with *Ascaris lumbricoides*, shares common pathways with allergic phenomena [67].

Helminths, in particular nematodes, have evolved to regulate their host's immune system in their favour [8]. Furthermore, helminth antigens activate dendritic cells (DCs) in a particular way: instead of reaching a mature state significantly increasing the co-stimulation molecules and the pro-inflammatory cytokines, its antigens promote a semi-mature phenotype, with low expression or even absence of the aforementioned activation markers [9]. Afterwards, these DCs lead to the development of Th2 and Treg responses [10,11]. Helminths' ability to modulate the immune system usually originates chronic and long parasitations, most of the time asymptomatic, like bilharziasis by *Schistosoma* [8]. However, as humans beings are an accidental host for this parasite, *A. simplex* has not develop specific paths to immunomodulate its immune system. Instead, it causes a short-term and intermittent infection whose effects can persist even after the elimination of the parasite. Whereas only the live larva produces the acute allergy reaction, patients are re-exposed to other antigens when continuing to eat parasitized fish. Besides, *Anisakis* larval antigens also promote regulatory DCs and so, their continuous stimulus could drive the upregulation of the immunoregulatory axis [12,13,14].

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According to the location of the larva in intestinal anisakiasis, larval antigens of *A. simplex* would be recognized by the Toll Like Receptors (TLRs) of the local DCs on the lamina propia, like in *Trichinella spiralis* and other helminths [15]. These receptors are essential for the innate response: 10 different TLRs (TLR 1–10) have been described in human beings and 12 in mice (TLR 1–9, TLR 11–13), each one with its own ligands and functions [16]. Every TLR presents a unique intracellular domain Toll/Interleukin-1 Receptor (TIR)-like, that captures Myd88 after its activation and begins an intracellular chain reaction which ends with NF-κβ translocation to the nucleus and the expression of inflammatory cytokines and type-I IFN [17]. However, they all have very different expression profiles over human intestinal mucosa [18]

Likewise, DCs are professional antigen-presenting cells of the immune system with an extraordinary ability to stimulate naive T lymphocytes and initiate immune responses. They are the point of connection between innate and adaptive immune responses. In addition, they are in charge of the central and peripheral tolerance, resulting in resolution of ongoing immune responses and prevention of autoimmunity [19,20]. DCs are very abundant in the intestinal mucosa (lamina propria) and in lymphoid organs associated with the intestine such as the Peyer's Plates, where they act as sentinels [21]. Therefore, as TLRs bind to *A. simplex* antigens, they turn on DCs, which are in charge of the activation of T and B lymphocytes, starting and running the adaptative immune response [22,23].

Due to the particular type of infection that characterizes *A. simplex* in humans' beings, the identification of the specific mechanisms, which *A. simplex* larval antigens employ to lead to a Th1//Treg/Th2 response, would contribute to clarify how allergies work, and how they can be cope with better and new treatments. Our experimental hypothesis of the present work is to determine how *Anisakis* larval antigens interact with the TLRs of the DCs, to set more light on the mechanisms that lead to an acute or chronic immune response after *A. simplex* infection, and how this could be related to allergies. Two mice

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strains were selected for this purpose: BALB/c and C57BL/6J, as the main prototype of Th2 and Th1responses, respectively. These two strains are known to display dramatically different mucosal immune responses [24,25], which would allow us to compare two immunological backgrounds, and correlate them with the different clinical profiles (chronic versus acute anisakiosis) that patients can develop when they are infected by *A. simplex*, depending on their immunological profile (Th1/Th2) [26,27].

2. Materials and methods

2.1. Animals

Female 8–12 weeks old BALB/C and C57BL/6J mice (20–25 gr) were purchased from Charles River Laboratories (L'Arbresle, France). Mice were housed in cages (6 individulas/cage), under established and regulated conditions of temperature, humidity, light, feeding, ventilation, and hygiene. All procedures were carried out in accordance with Royal Decree 53/2013, of 1 February, which establishes the basic rules applicable for the protection of animals used in experimentation and other scientific purposes. The project was approved by the Ethics Committee of the UCM and evaluated and approved by an authorized body authorized by the Community of Madrid for the evaluation of Projects. A total of 30 mice were used (15 mice/strain). Although in the last few years it is encouraged the use of both sexs, we chose female because they are underrepresented in animal research, as well as they are easier to take care of than males in small cages.

2.2. In-vitro bone-marrow-derived dendritic cells stimulation assays

BMDCs were obtained from mice femurs and tibia, and then cultured in 30 ml RPMI 1640 (SIGMA) (Flasks 225 m2) with 20 ng/ml GM-CSF (PeproTech) (5x105 cells/ml) in a stove (37 ◦C, 5% CO2) for 6 days. They were collected with a cell scraper and added to a 12 wells plate (2 ml/well, 1x106 cells/ml). Before final incubation in a stove (37 ◦C,

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5% CO2), the different antigens were supplemented in duplicate wells: excretory-secretory (ES) (0.012 μg/ml) or crude extract (CE) (50 μg/ml) either alone, or combined with TLR agonists [TLR1/2 agonist: Pam3CSK4 (0.1 μg/ml); TLR2 agonist: HKLM (107 cells/ml); TLR3 agonist: Poly (I:C) (1 μg/ml); TLR3 agonist: Poly (I:C) LMW (1 μg/ml);

TLR4 agonist: LPS E. coli K12 (1 μg/ml); TLR4 agonist: LPS E. coli 026B6 (1 μg/ml); TLR5 agonist: ST-FLA (0.1 μg/ml); TLR2/6 agonist: FSL-1 (0.1 μg/ml); TLR7 agonist: ssRNA 40 (0.25 μg/ml), and TLR9 agonist: CpG (ODN1826) (50 μg/ml) (Invitrogen)]. TLR agonists work concentrations were provide by the fabricant instructions, and ES/CE work concentrations were set and used in a previous research [11]. In the case of C57BL/6J strain, neither FSL-1 nor ssRNA 40 were tested (lack of resources). To measure intracellular cytokines, we added brefeldin A (Penicilium brefeldianum, Life Technologies) (10 μg/ml) after 6 h of stimulation. We used different exposure times for each marker studied: 24 h for surface molecules (CD80, CD86, MHC I, MHC II); 12 h for intracellular molecules (IL-10, IL-12, TNF-α, Myd88, NF-κβ) in CD11c + cells by Flow Cytometry. We studied IL-10 levels in supernatants after 48 h of stimulation by ELISA. A control of DCs cultured in medium without antigens was included in all the experiments. All experiments were performed in duplicate wells for each condition and repeated with 3 mice per strain.

2.3. Obtention of Anisakis simplex larval antigens

Both ES and CE were obtained from third stage alive larvae (L3), which were found in the muscle or entrails of *Micromesistius poutassou* (blue whiting). For the extraction of ES, we followed Perteguer 1996 [28]: viable *A. simplex* larvae were introduced for 1 h in NaCl (0.85%) containing gentamicin (4 g/L). One larva per millimetre was placed in each of 24-well plates (1 ml Minimum Essential Medium Eagle with Earle's salt) and maintained at 28 ◦C. Supernatants were collected weekly and dialyzed in PBS. In the case of CE, alive larvae were manually washed, fragmented, and homogenized until obtaining

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a paste. Followed by sonication (10 s/pulse, 70% power), extraction in PBS (4 ◦C, overnight) and delipitation with n-hexane (7:3 v/v). Then, centrifuge (8497 g, 30 min, 4 ◦ C) and dialyze the supernatant in PBS overnight at 4 ◦C to remove salts. Equally, ES and CE protein content was assessed by Bradford technique (1976) [29]. Before storage, *A. simplex* larval antigens were sterilized by a 25 μm diameter filter (Seringue filter Ac 30 MM 0.2 μm, Single). We used the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Rockford, IL) for the detection of gram-negative bacterial endotoxins. The amounts of endotoxin present in our antigenic samples were negligible compared to the doses of 1 μg/mL of LPS used as positive control. Stock concentrations were 500 μg/ml (CE) and 0.12 μg/ml (ES), stored at - 20◦ C. Work concentrations used for the stimuli were been previously evaluated in the same murine in vitro experimental mode [12].

2.4. Flow Cytometry

Prior to staining, cells were collected and fixed in 4% paraformaldehyde (15 min, 4◦ C), then incubated with an anti-Fcγ III/II receptor. After incubation, cells were stained with specific antibodies to detect surface molecules: CD11c [PE/FITC Hamster Anti-Mouse CD11c (BD PHarmingen)], CD80 [FITC Hamster Anti-Mouse CD80 (BD PHarmingen)], CD86 [FITC Rat Anti-Mouse CD86 (BD PHarmingen)], MHC Class I-II [FITC Mouse Anti-Mouse H-2D[b], FITC Mouse Anti-Mouse H-2 K[d], or FITC Rat Anti-Mouse 1A/1E (BD Biosciences)]. For intracellular molecules, cells were stained in a saponin tampon to allow the entrance of the antibodies: IL- 12p40/p70 [Anti-Mouse IL-12/IL-23p40 PE (eBioscience)], IL-10 [PE Rat Anti-Mouse IL-10 (BD Biosciences)], TNF-α [Anti-Mouse TNF alpha APC (eBioscience)], NF-κβ p65 [NF-κβ p65 (D14E12) XP Rabbit mAb (Cell Signalling)] and Myd88 [Rabbit anti-Myd88 (Biolegend), Anti-Rabbit IgG (whole molecule) FITC (SIGMA)]. Incubations were carried out in ice for 30–40 min. Data were collected using FACSCan flow cytometer and CELLQuest software (Becton Dickinson) in the Cytometry and Microscopy Center of the Complutense

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University of Madrid. For each sample, at least 20,000 cells were analyzed. Data were analyzed in Flowing software.

2.5. Il-10 levels by ELISA

BMDCs of both strains were stimulated at 6th day of maturation with ES/CE or TLRs agonists alone or combined (in C57BL/6 strain neither FSL-1 nor ssRNA 40 were tested) for 48 h in 96 wells plates (200 µl/well, 1x106 cells/ml). Supernatants were collected and IL-10 (pg/ml) was analyzed by the KIT Mouse IL-10 ELISA Ready-SET-Go! (2nd Generation) eBioscience. Fabricant instructions were followed (450 nm, ELX808, BIOTEK).

2.6. Statistics

Flow cytometry. All experiments were conducted in triplicate. Data are presented as mean ± standard deviation (SD). Nonparametric tests were performed (Wilcoxon and Monte Carlo Tests) to compare the means (SPSS 19, Inc., Chicago, IL, USA). A P value<0.05 was used as the threshold for significance (*). Near significance (P value near to 0.05) are tagged with an alpha (α) and meet the following criteria: absence of standard deviation overlapping of the groups mean.

ELISA. All data were collected and analyzed with GraphPad Prism 6.0 software. Data are presented as mean ± SD. A one-way ANOVA test was performed. A P value<0.05 was used as the threshold for significance.

3. Results

3.1. Effects of ES/CE did not modify BMDCs response compared to the negative control

A.simplex larval antigens slightly changed BMDCs surface markers compared to the control after 24 h of stimulation in both mice strains. CD11C + CD80 + cells were

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significantly increased by ES in C57BL/6J mice. Besides, the percentage of CD11c + IL-10 + cells was higher in C57BL/6J mice than in BALB/c, while the opposite was found for CD11c + IL-12 + population in untreated or stimulated cells after 12 h. Regarding CD11c + Myd88+, it was significantly decreased by ES in BALB/c mice (Table 1).

3.2. Effects of Anisakis simplex larval antigens on TLRs agonists response

ES/CE mediated inhibition of Pam3CSK4 (TLR1/2 agonist) induced IL-12 and IL-10. When BMDCs were pulsed simultaneously with ES or CE and Pam3CSK4, CD11c + IL-12 + tended to decline in both strains (Fig. 1A/1B). CE caused a reduction near to significance of CD11c + IL-10 + population compared to Pam3CSK4 alone in BALB/c.

ES/CE inhibited HMLK (TLR2 agonist) Th1 response in BALB/c mice but promoted in 57BL/6J. Both CD11c + NF-κβ + and CD11c + Myd88 + tended to decrease in BALB/c mice by ES, but not significant. In C57BL/6J, ES decreased significantly CD11c + MHC II+, and ES/CE caused a reduction close to significance of CD11c + TNF-α+, but increased CD11 + IL-12 + and CD11c + IL-10 + caused by HKLM (Fig. 1C/1D).

ES/CE tended to modify Poly (I:C)/Poly (I:C) LMW (TLR3 agonist) cytokines profiles. In BALB/c, both TLR agonists caused an inflammatory response characterized by the enhance of CD11c + TNF-α + . In addition, CD11c + CD80 + was significantly increased by ES compared to Poly (I:C) alone (Fig. 2A). Regarding Poly (I:C) LMW, ES significantly increased CD11c + IL10 + whereas CE caused a reduction close to significance in CD11c + IL12 + compared to Poly (I:C) LMW alone (Fig. 2C). In both mice strains, CE showed a promotion near to significance of CD11c + TNF-α + when combined with Poly (I:C) LMW (Fig. 2C/2D).

ES/CE modulated LPS E. coli K12 and LPS E. coli 026B6 (TLR4 agonist) Th1 response. In the case of LPS E. coli K12, ES caused an increased near to significance of CD11c + IL-10 + and CD11c + TNF-α+, and tended to block CD11c + IL-12 + compared to LPS E. coli K12 alone in BALB/c. CE showed the opposite effect (Fig. 3A). In C57BL/6J, the

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association of LPS E. coli K12 with ES significantly enhanced CD11c + CD80+, but both ES and CE diminished the CD11c + IL-10 + population near to significance (Fig. 3B). The association of ES or CE with LPS E. coli 026B6 turned out to slightly promote the agonist response in both mice strains. In BALB/c, ES significantly improved CD11c + MHC I + when combined with LPS E. coli 026B6, and CE caused a reduction close to significance of CD11c + IL-10+ (Fig. 3C). In C57BL/6J, CE promoted significantly CD11c + TNFα + and showed an increment close to significance of CD11c + IL-10 + compared to LPS E. coli 026B6 alone (Fig. 3D).

CE increased ST-FLA (TLR5 agonist) IL-10 production. After cells were pulsed simultaneously with CE and ST-FLA, CD11c + IL-10 + exhibited an increased close to significance in both mice strains (Fig. 4A/4B). In the case of C57BL/6J, CD11c + IL-10 + improvement with CE took place according to an increased near to significance of CD11c + IL-12 + compared to ST-FLA alone (Fig. 4B).

ES/CE blocked CpG (ODN1826) (TLR9 agonist) Th1 response in C57BL/6J mice but promoted it in BALB/c. In BALB/c mice, ES and CE caused a promotion close to significance of CD11c + TNF-α + compared to CpG (ODN1826) alone. CE significantly increased CD11c + NF-κβ + but tended to inhibit CD11c + IL10 + population. On the other hand, ES significantly decreased the upregulation of CD11c + IL-12 + caused by CpG (ODN1826) (Fig. 4C). In C57BL/6J, a significant decreased was observed for CD11c + CD86 + when ES was combined with CpG (ODN1826), and for CD11c + MHC II + and CD11c + MHC-I + when associated with CE. Besides, CE caused a reduction close to significance CD11c + Myd88 + and decreased significantly CD11c + NFκβ + compared to CpG (ODN1826) alone (Fig. 4D).

ES/CE did not modify FSL-1 (TLR2/6 agonist) response in BALB/c mice. This agonist was only tested in BALB/c mice. The association of *A. simplex* larval antigens with FSL-

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1 did not modify the inflammatory response caused by FSL-1 alone, but a decrease close to significance of CD11c + CD80 + population was observed with CE (Fig. 5A).

ES/CE tended to inhibit ssRNA40 (TLR7 agonist) Th1 response in BALB/c mice. This agonist was only tested in BALB/c mice. First, ES and CE decreased significantly CD11c + Myd88+, compared to ssRNA40 alone. It was also observed that CE reduced CD11c + CD86, CD11c + MHC I+, CD11c + NF-κβ + and CD11c + IL10 + populations compared to ssRNA40 alone near to significance (Fig. 5B).

3.3. Effects of Anisakis simplex larval antigens on IL-10 levels

Although not significant, ES or CE originated a decreased on the production of IL-10 compared to the control in BALB/c, while it was improved in C57BL/6J after 48 h of stimulation. In most cases, the levels of IL-10 were much higher in C57BL/6J than in BALB/c mice. Besides, ES inhibited HKLM, Poly (I:C) and FSL-1 upregulation of this cytokine significantly in BALB/c (Table 2), while in C57BL/6J, ES significantly increased IL-10 levels when combined with LPS E. coli 026B6 and CpG (ODN1826). With regard to CE, it inhibited the production of IL-10 previously originated by most of the agonists studied in BALB/c. This decrease was significant when CE was combined with HKLM or FSL-1. However, CE significantly upregulated the levels of IL-10 when combined with Poly (I:C) or ST-FLA (Table 2). In the case of C57BL/6J mice, both CE and ES showed similar effects when combined with LPS E. coli 026B6 and CpG (ODN1826), which significantly increased IL-10 levels, especially ES. On the contrary, ES and CE caused opposite effects when associated with Pam3CSK4, Poly (I:C) and LPS E. coli K12, that decreased IL-10 levels (Table 2).

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4. Discussion

TLRs are essential in the defense mechanisms of the innate immune responses for bacteria, virus, and helminths [30]. They represent one of the main connections between the innate and adaptive response of the host, via their activation through helminths secretory-excretory products [31,32,33]. In particular, *A. simplex* releases its secretory-excretory products during the infection of the digestive system. Later, when the larva is destroyed by the granuloma in longer infection, it releases its somatic antigens [34,35,36]. Nevertheless, only a few works have described the contribution of *Anisakis* larval products to TLRs activation [12,37,38]. Our previous results confirmed that ES and CE gave rise to DCs with a "semi-mature" phenotype that increase the frequency of regulatory T lymphocytes in vitro, which in turn produce IL-10 and IFN-γ. These cytokines could suppress the development of Th1 and/or Th2 responses at the beginning of the infection. However, our results also pointed out the promotion of inflammatory T lymphocytes, which produced Il-12 [12,39]. Hence, *A. simplex* parasitism promotes a dual response, acute and intermittent par excellence, that may explain the lack of enough regulatory mechanisms to establish itself chronically in its accidental. Thus, an allergic phenotype predominates, which is related to the Hygiene Hypothesis: the more regulatory mechanisms, the less allergic response, and the better chronic infection [40,41]. Here, we show that *A. simplex* larval antigens perform their immunostimulatory properties through TLRs, mainly TLR2, TLR4 and TLR9.

First, ES and CE alone promoted IL-10 production only in C57BL/6J. Perhaps these antigens lack a successful ability to immunoregulate acute inflammatory responses (Th2), which are more likely develop by BALB/c, whereas C57BL/6J mice are predisposed to more resistant but discrete reactions (Th1) [12,42]. Similar results were observed in splenocytes from C57BL/6J [43,44]. Taken together, these results demonstrate significantly higher anti-inflammatory responses in C57BL/6J animals compared to BALB/c mice.

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Second, both TLR1 and TLR2 play a crucial role in developing immunity against protozoan infections in humans [45,46], and its agonists Pam3CSK4 and HKLM, are potent activators of NF-κβ [46,47,48]. Depending on the mice strain, ES and CE tended to modify the cytokine profile of both agonists, by mainly decreasing CD11c + IL-12 + population. This reduction was also observed in *Schistosoma mansoni* and *Ascaris lumbricoides*, by high stimulation of the ERK pathway through TLR2 [49]. In addition, IL-10 levels were dismissed after 12 h and 24 h of stimulation, except when ES or CE were combined with HKLM in C57BL/6J mice: they promoted the inflammatory response originated by HKLM (↑CD11c + IL-12+, ↑IL-10 in supernatants). Even though IL-10 plays an important role in the Th2 response, low levels of this cytokine also correlate to less inflammation [50]. These observations suggest that Anisakis larval antigens, in most of the cases, antagonized the inflammatory action of TLR1 and TLR2 agonists, promoting less inflammation (↓IL-12), no longer requiring high levels of IL-10. On the contrary, in C57BL/6J mice, the promotion of HKLM Th1 response precisely increased CD11c + IL-10 + as an autocrine regulation of inflammation [51]. Thus, *A. simplex*, as other helminths, may lead the Th1 response to a Th2 response through TLR2, depending on the genetic background of the host [30].

As an intracellular receptor, TLR3 also activates NF-κB [52,53]. We observed that the inflammatory response caused by Poly (I:C)/Poly (I:C) LMW was slightly modified by ES or CE, mainly what refers to the cytokines profile in BALB/c: CE inhibited CD11c + IL-12 + population when combined with [Poly (I:C)], but it promoted CD11c + TNF-α + DCs. Also, ES caused an increment in IL-10 production after 12 h and 24 h of stimulation when combined with Poly (I:C) LMW. Hence, *A. simplex* larval antigens might conduct the initial Th1 response originated by TLR3 agonists to a Th2 balance through the depletion of IL-12 and promotion of IL-10, as previously described for *Schistosoma* or *T. spiralis* [54,55,56].

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Similarly, TLR4 is known for leading strong inflammatory responses through the activation of NF-κβ signaling pathway [57,58]. In fact, several helminths use this receptor to modulate the immune system of the host in its favor [59]. Our results show that *A. simplex* larval antigens regulated LPS response mainly through IL-10 levels. In C57BL/6J mice, where the inflammation lasts longer, we found higher levels of this cytokine. However, in BALB/c where there is an acute response, IL-10 levels were lower. In addition, ES provoked a significant increase in IL-10 levels compared to LPS alone, mainly in BALB/c, whereas CE tended significantly to inhibit this cytokine mostly. As an immunosuppressive cytokine, IL-10 is essential for the autocrine regulation of macrophages/monocytes when facing LPS. The higher the inflammatory signals are, the higher IL-10 will be, with the goal of avoiding the exacerbation of this signal [60]. Previous in vivo experiments with mice infected with *Nippostrongylus brasiliensis* (short, sharp infection, low levels of IL-10) or *Heligmosomoides polygyrus* (chronic infection, high levels of IL-10) showed similar results [61]. Likewise, ES seemed to stimulate more CD11c + IL-10 + than CE when combined with LPS, which might be related to the fact that ES antigens are released at the first state of the parasitation (acute), while CE would be released when the larvae die inside the granuloma (chronic) [62].

On the other hand, *A. simplex* larval antigens also caused the improvement of MHC I in BALB/c, when combined with LPS E. coli 026B6. This could be understood as a promotion to a Th1 response or to a Th2 response. Some studies have shown that T CD8 + can turn into CD4 + lymphocytes depending on MHC I without losing their antigenic specificity. These T CD4 + lymphocytes can lead to Treg with Foxp3 expression [63,64]. Therefore, the increase in expression of MHC I could also indicate an inhibition of Th1 response. The suppression of effects unleashed by E. coli LPS has also been described with other helminths that also parasite the intestinal system like *Anisakis*: in vitro, *T. spiralis* and *N. brasiliensis* secretory-excretory products [32,60]; in vivo, for *Ascaris suum* [65].

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As TLR5 is a driver to Th1 response, this receptor could be related to a larger susceptibility to helminths [66]. Neither ES nor CE modified meaningfully ST-FLA response, though CE increased IL-10 levels significantly after 12 h and 48 h of stimulation, when associated with STFLA in both mice strains. Thus, CE may encourage Th2 response through the regulation of TLR5 response by promoting the production of IL-10.

TLR9 leads to the activation of DCs and IL-12 release [67]. In BALB/c mice, even when ES reduced CD11c + IL-12 + significantly compared to CpG (ODN 1826) alone, and CE diminished IL-10 levels in supernatants, *A. simplex* larval products showed an insufficient ability to block the inflammatory response. On their behalf, in C57BL/6, ES and CE showed a clear tendency to block the CpG (ODN1826) inflammatory response and promoted significantly higher IL-10 levels in supernatants compared to CpG (ODN826) alone, particularly after 48 h. The increase of IL-10 was progressive and delayed in time, along with CD11c + NF-κβ + DCs depletion. Similar results were obtained for *T. spiralis*: ES declined NF-κβ expression previously produced by LPS, which led to IL-12 reduction and IL-10 rose [68]. The fact that ES and CE promote CpG (ODN1826) response in BALB/c mice, but downregulate it in C57BL/6J, reinforces the importance of the genetic bases of the host when it comes to the development of a Th1 or Th2 response.

TLR6 is a partner of TLR2 and induces inflammation through the activation of NF-κβ [69]. The combination of ES or CE with FSL-1, which was only determined in BALB/c mice, did not modified FSL-1 response. However, after 48 h, IL-10 levels in supernatants showed a clear tendency to significance to diminished by ES and CE, compared to FSL-1 alone (48 h). The same suppression occurred with HKLM (TLR1/2) when combined with ES or CE. As both HKLM and FSL-1 are TLR2 agonists, IL-10 reduction could be a consequence of a reduced inflammatory response due to ES or CE suppressing activity. According to our research, ES and CE tend to inhibit HKLM response, but not FSL-1 in

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BALB/c mice. Deeper investigation is needed to elucidate the real role that ES and CE are playing over the regulation of TLR2.

Finally, TLR7 is intracellular and recognizes retroviruses singlestranded RNA (ssRNA) [70]. The association of ES or CE with ssRNA 40 inhibited significantly CD11c + Myd88 + cells regarding ssRNA 40 alone. Some surface molecules were near to significance when reduced too, like CD86 and MHC I. It is known that TLR agonists recruit Myd88 to activate intracellular cascades that become a major expression of MHC I/II (signal 1), co-stimulating molecules (signal 2) and production of IL-12 (signal 3) (Th1 response). In the case of Th2 response, signals 2 and 3 are not clear. In fact, the lack of these signals was taken to be the “Hypothesis by default” [10]. Therefore, even when *A. simplex* larval antigens obstruct ssRNA intracellular cascade, it is not clear how it influences the development of Th2 or Th1 response.

To sum up, ES and CE seem to alter the response unleashed by TLR2, 4, and 9 agonists, which are the main TLRs involved in helminths recognition [30] and are related to the Th1 and Th2 balance [30]. Thus, *A. simplex* infection is narrowly linked to TLRs, as it has been described for other helminths [71,72]. This study highlights the dual character of the immunomodulatory proprieties of *A. simplex* larval antigens [12,14], which also happened for *Anisakis pegreffii* and *Anisakis typica* when human DCs were stimulated with ES/CE antigens [73,74]. However, our main limitation was a small mice sample which originated results with a high standard deviation variability that complicated the determination of statistical differences among treatments. Besides, the study of other secreted cytokines apart from IL to 10, like IFN-γ, TNF-α or IL-4, it would have provided a better understanding on the immune response developed by the two mice strains. Even when further studies are needed to deep in how *A. simplex* modifies the immune response of its host, our results confirm *A. simplex* larval antigens develop a mixed pattern between immunosuppression (mainly ES) and inflammation (mainly CE), and verify that this

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parasite leads its atypical immune response through several mechanisms which are closely correlated with TLRs.

5. Conclusions

A. simplex larval products promoted the response originated by TLR3 agonist, but inhibited TLR1/2, TLR2, TLR6/2, TLR5, and TLR7, mainly acting over cytokine's levels. In the case of TLR4 and TLR9, a dual effect (inhibition/promotion) over the response generated was noted. Overall, the main changes were detected in TLR2, TLR4 and TLR9. These observations highlight a lack of specific mechanisms by *A. simplex* for the immune system of the host (mice), as *A. simplex* is not properly adapted to it, like in human beings. Due to the preliminary character of this work and the lack of similar studies with *A. simplex* to compare with, a more exhaustive analysis is necessary to determine how exactly ES and CE antigens are involved in the host immune system, which will be essential to understand allergic diseases and to improve its treatments. Besides, in vivo studies are required to confirm that these results can be comparable to the infection model.

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7. Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical statement

All animal studies were approved by the Animal Experimentation Committee of the Complutense University of Madrid and were performed according with RD 53/2013 (Directive 2010/63/EU).

CRediT authorship contribution statement

Vega Zamora: Software, Investigation, Data curation, Writing – original draft, Visualization, Formal analysis. Juan Carlos Andreu-Ballester: Software, Formal analysis, Writing – review & editing. Marta Rodero: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. Carmen Cuéllar: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1.- Mean and Standard Deviation (SD) of CD11c + cells (%) after 12–24 h of stimulation with ES/CE in BALB/c or C57BL/6J mice.

%Mean (SD)	BALB/c			C57BL/6J		
	Control	ES	CE	Control	ES	CE
CD11c + CD80+	42 (17)	56 (15)	54 (27)	45 (22)	58 (17) *	54 (21)
CD11c + CD86+	51 (17)	58 (15)	55 (22)	45 (17)	49 (14)	38 (28)
CD11c + MHC I+	32 (26)	32 (24)	47 (26)	24 (20)	22 (20)	30 (23)
CD11c + MHC II+	67 (23)	70 (22)	68 (20)	71 (21)	76 (22)	71 (23)
CD11c + IL-10+	7 (5)	7 (3)	7 (4)	14 (11)	18 (18)	8 (3)
CD11c + IL-12+	14 (8)	13 (9)	14 (7)	9 (4)	10 (7)	11 (8)
CD11c + TNF-α+	14 (16)	12 (14)	15 (18)	25 (42)	16 (19)	22 (15)
CD11c + Myd88+	79 (24)	72 (24) *	73 (28)	85 (14)	90 (14)	90 (8)
CD11c + NF-κβ+	40 (17)	37 (21)	38 (23)	52 (19)	46 (10)	51 (15)

Significant differences between % pos cells untreated or incubated with ES/CE alone are indicated with asterisk (*p < 0.05).

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Table 2. Mean and Standard Deviation (SD) of IL-10 levels (pg/ml) in supernatants of BMDCs after 48 h of stimulation in BALB/c or C57BL6/J mice.

	IL-10 (pg/ml)	
	BALB/c Mean (SD)	C57BL/6J Mean (SD)
Control	103 (26)	47 (11)
ES	61 (14)	78 (19)
CE	94 (42)	101 (33)
Pam3CSK4 (TLR1/2 agonist)	136 (12)	136 (54)
Pam3CSK4 + ES	133 (7)	9 (2) $^{\alpha}$
Pam3CSK4 + CE	101 (5) $^{\alpha}$	63 (2) $^{\alpha}$
HKLM (TLR2 agonist)	831 (94)	13 (7)
HKLM + ES	156 (12)***	23 (26)
HKLM + CE	146 (17) ***	83 (40)
Poly (I:C) (TLR3 agonist)	118 (33)	48 (19)
Poly (I:C) + ES	86 (7) ***	143 (135)
Poly (I:C) + CE	476 (144) ***	13 (7)
Poly (I:C) LMW (TLR3 agonist)	126 (7)	38 (14)
Poly (I:C) LMW + ES	149 (35) $^{\alpha}$	6 (2)
Poly (I:C) LMW + CE	94 (19) $^{\alpha}$	33 (2)
LPS $_{E. Coli\ K12}$ (TLR4 agonist)	303 (26)	2799 (91)
LPS $_{E. Coli\ K12}$ + ES	333 (16)	2793 (40)
LPS $_{E. Coli\ K12}$ + CE	219 (59)	2508 (118) $^{\alpha}$
LPS $_{E.coli\ 026B6}$ (TLR4 agonist)	498 (42)	3649 (535)
LPS $_{E.coli\ 026B6}$ + ES	524 (38)$^{\alpha}$	5136 (290) ***
LPS $_{E.coli\ 026B6}$ + CE	406 (191) $^{\alpha}$	3954 (297) $^{\alpha}$
ST-FLA (TLR5 agonist)	128 (14)	14 (5)
ST-FLA + ES	139 (44)	33 (21)
ST-FLA + CE	369 (87) *	24 (9)
CpG (ODN1826) (TLR9 agonist)	104 (24)	573 (309)
CpG (ODN1826) + ES	83 (68)	2589 (196) ***
CpG (ODN1826) + CE	66 (2) $^{\alpha}$	979 (73) $^{\alpha}$
FSL-1 (TLR2/6 agonist)	469 (82)	–
FSL-1 + ES	134 (23) ***	–
FSL-1 + CE	116 (8) ***	–
ssRNA40 (TLR7 agonist)	121 (23)	–
ssRNA40 + ES	224 (90) $^{\alpha}$	–
ssRNA40 + CE	119 (12)	–

SD: Standard Deviation. Significant differences between IL and 10 levels in cells incubated with TLR agonists alone, or together with ES/CE, are indicated with asterisk (*p < 0.05; **p < 0.005; ***p < 0.001) (N = 3). Near significance (P value near to 0.05) ere indicated with α.

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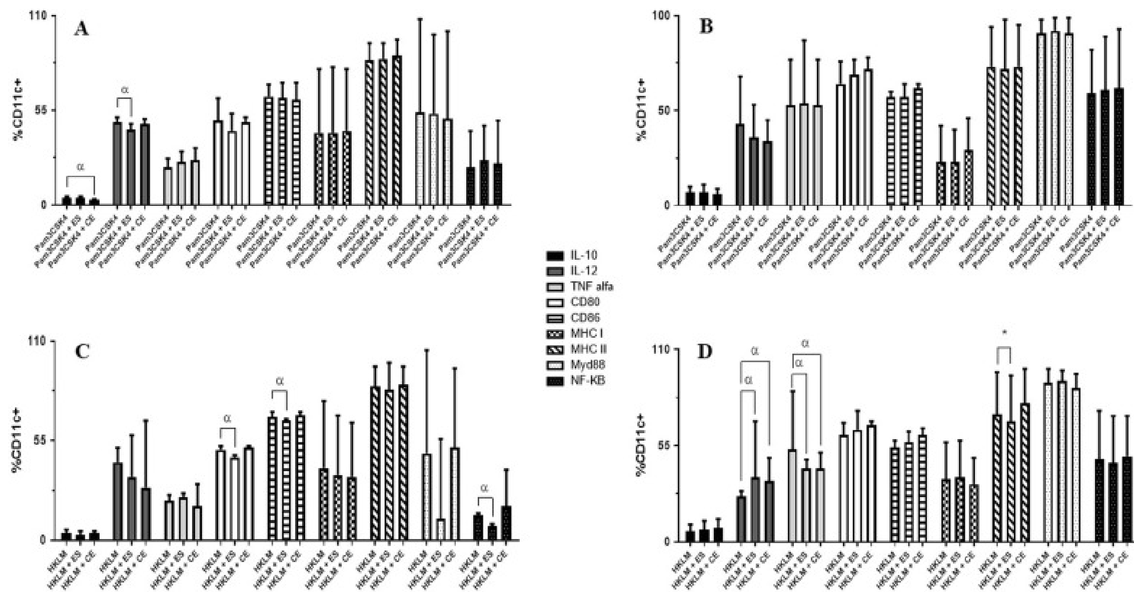

Fig. 1. Mean and Standard Deviation of CD11c + cells positive (% pos) for CD80, CD86, HC I and MHC II (24 h); IL-10, IL-12 and TNF-α (12 h), and Myd88 or NF-κβ (12 h) in BALB/c or C57BL/6 mice. Significant differences between % pos cells incubated with Pam3CSK4 (TLR1/2 agonist)/HKLM (TLR2 agonist) alone or combined with ES/CE are indicated with asterisk (*p < 0.05). Near significance (P value near to 0.05) were indicated with α. N = 3. A. BALB/c (Pam3CSK4) B. C57BL/6J (Pam3CSK4) C. BALB/c (HKLM) D. C57BL/6J (HKLM).

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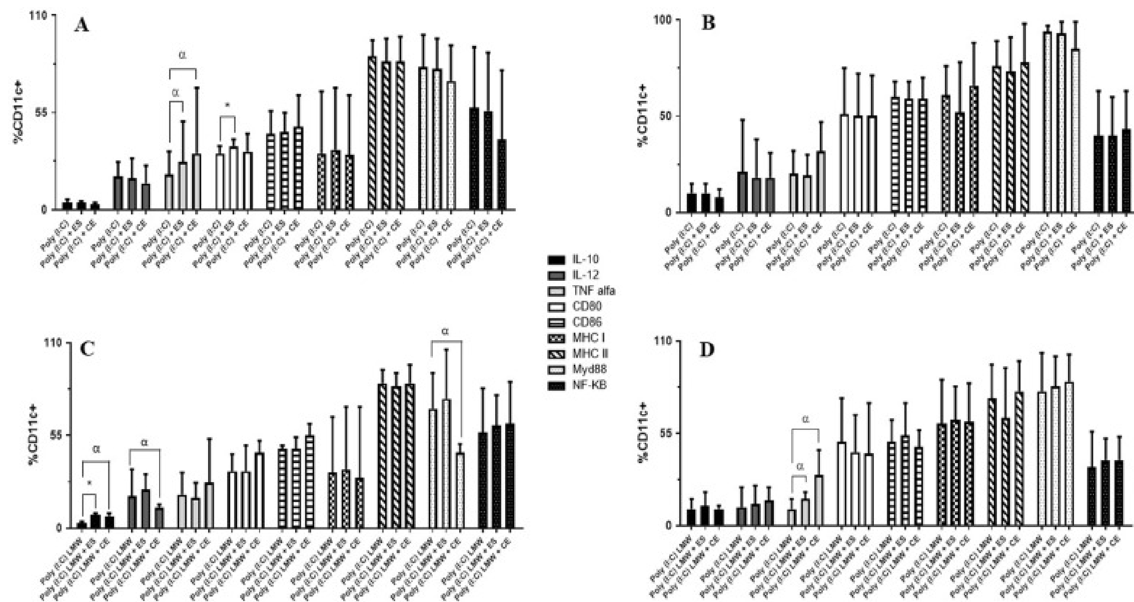

Fig. 2. Mean and Standard Deviation of CD11c + cells positive (% pos) for CD80, CD86, MHC I and MHC II (24 h); IL-10, IL-12 and TNF-α (12 h), and Myd88 or NF-κβ (12 h) in BALB/c or C57BL/6 mice. Significant differences between % pos cells incubated with Poly (I:C) (TLR3 agonist)/Poly (I:C) LMW (TLR3 agonist) alone or combined with ES/CE are indicated with asterisk (*$p < 0.05$). Near significance (P value near to 0.05) were indicated with α. N = 3. A. BALB/c (Poly (I:C)) B. C57BL/6J (Poly (I:C)) C. BALB/c (Poly (I:C) LMW) D. C57BL/6J (Poly (I:C) LMW).

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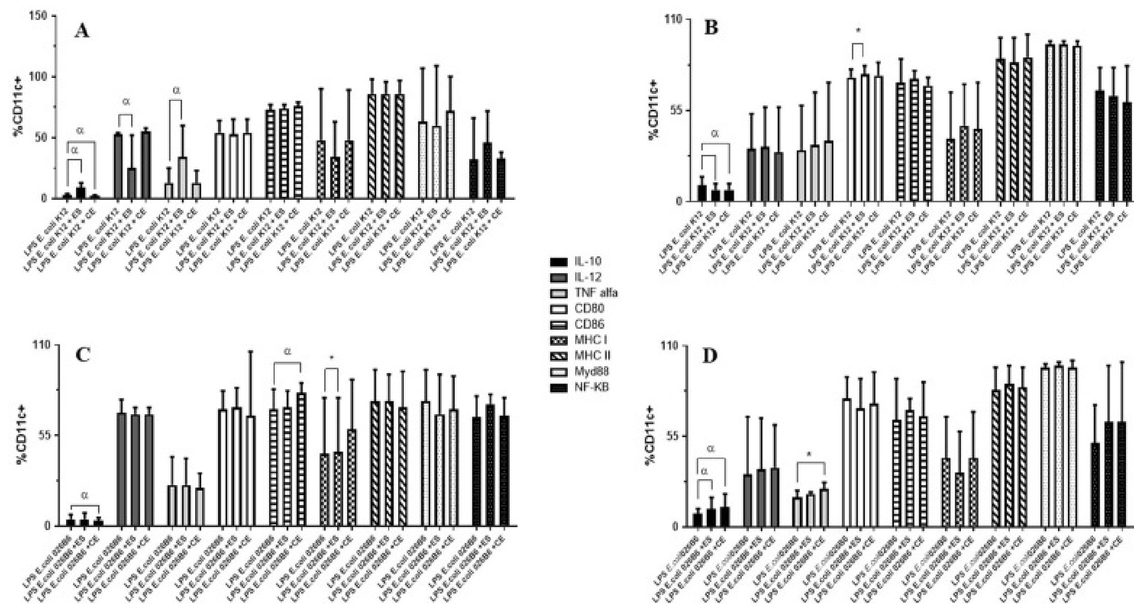

Fig. 3. Mean and Standard Deviation of CD11c + cells positive (% pos) for CD80, CD86, MHC I and MHC II (24 h); IL-10, IL-12 and TNF-α (12 h), and Myd88 or NF-κβ (12 h) in BALB/c or C57BL/6 mice. Significant differences between % pos cells incubated with LPS E. coli K12 (TLR4 agonist)/LPS E. coli 026B6 (TLR4 agonist) alone or combined with ES/CE are indicated with asterisk (*p < 0.05). Near significance (P value near to 0.05) were indicated with α. N = 3. A. BALB/c (LPS E. coli K12) B. C57BL/6J (LPS E. coli K12) C. BALB/c (LPS E. coli 026B6) D. C57BL/6J (LPS E. coli 026B6).

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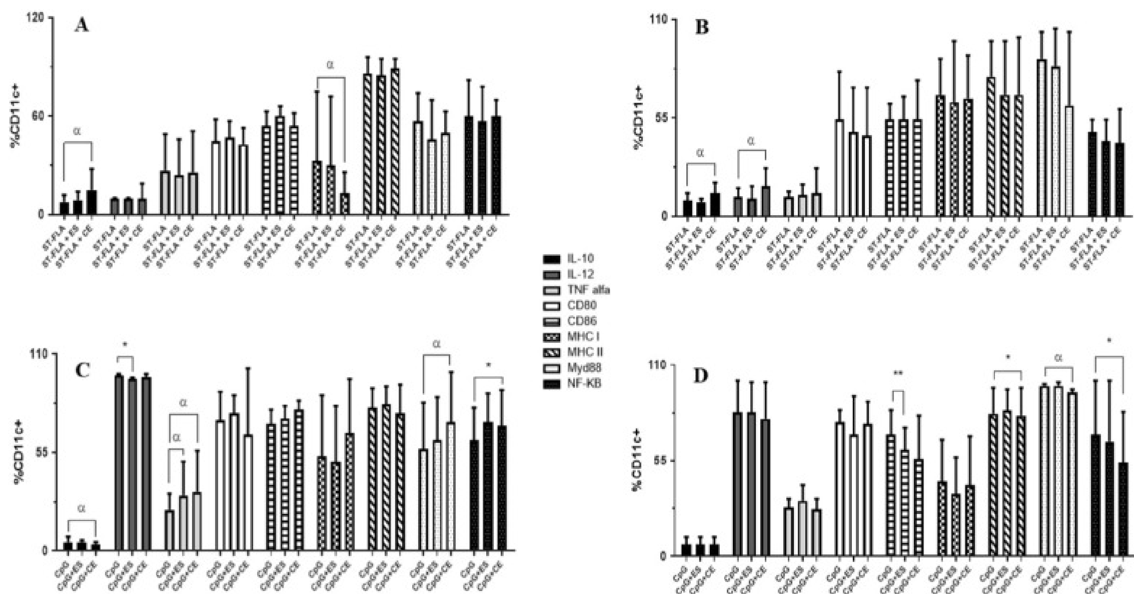

Fig. 4. Mean and Standard Deviation of CD11c + cells positive (% pos) for CD80, CD86, MHC I and MHC II (24 h); IL-10, IL-12 and TNF-α (12 h), and Myd88 or NF-κβ (12 h) in BALB/c or C57BL/6 mice. Significant differences between % pos cells incubated with ST-FLA/CpG (ODN 1826) alone or combined with ES/CE are indicated with asterisk (*p < 0.05). Near significance (P value near to 0.05) were indicated with α. N = 3. A. BALB/c (ST-FLA) B. C57BL/6J (ST-FLA) C. BALB/c (CpG (ODN 1826) D. C57BL/6J (CpG (ODN 1826).

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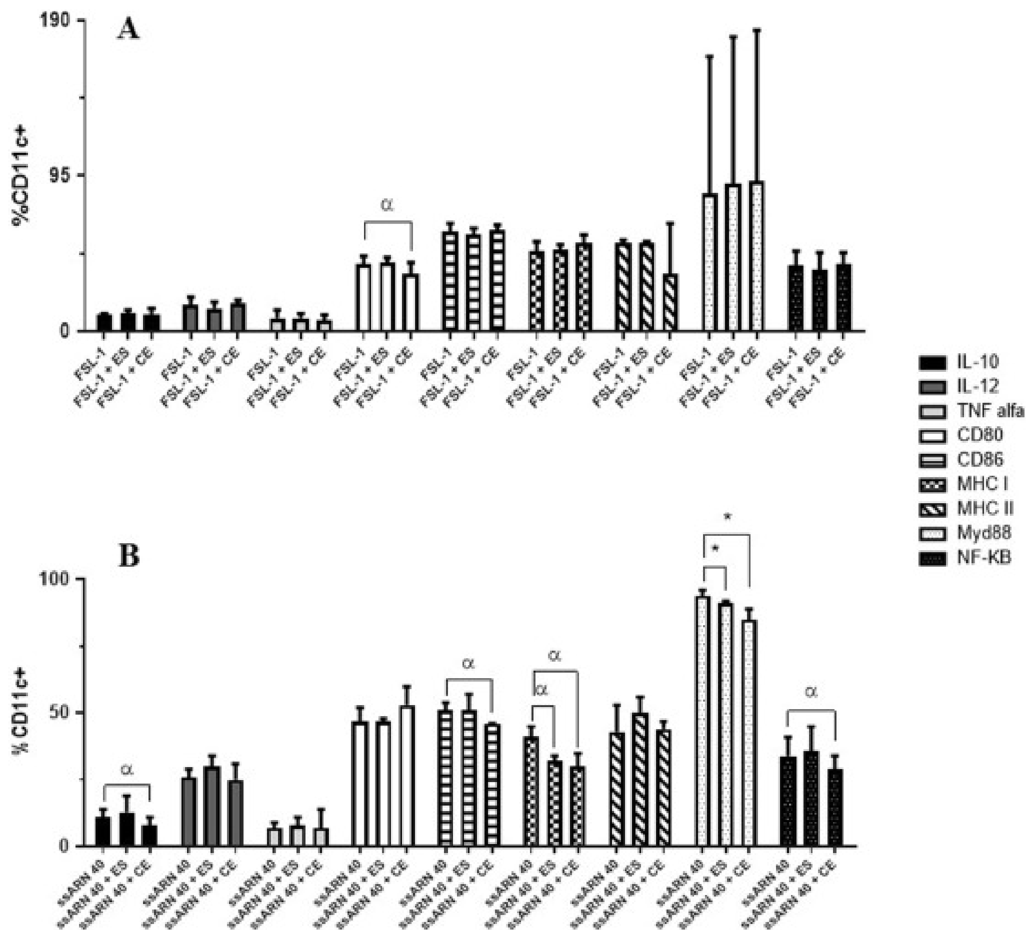

Fig. 5. Mean and Standard Deviation of CD11c + cells positive (% pos) for CD80, CD86, MHC I and MHC II (24 h); IL-10, IL-12 and TNF-α (12 h), and Myd88 or NF-κβ (12 h) in BALB/c. Significant differences between % pos cells incubated with FSL-1 (TLR2/6 agonist)/ssRNA 40 (TLR7 agonist) alone or combined with ES/CE are indicated with asterisk (*p < 0.05). Near significance (P value near to 0.05) were indicated with α. N = 3. A. FSL-1B. ssRNA 40.