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**Secreted products of *Fasciola hepatica* inhibit the induction of T cell responses
that mediate allergy**

Running Title: *F. hepatica* products in allergy

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Summary

There is evidence from epidemiology studies of a negative association between infection with helminth parasites and the development of allergy and asthma. Here we demonstrate that the excretory/secretory products of the helminth *Fasciola hepatica* (FHES) protected mice against ovalbumin (OVA)-induced allergic asthma when administered at time of allergen sensitisation. FHES reduced the accumulation of mucus, eosinophils and lymphocytes into the airways of allergen-challenged mice. Furthermore, FHES treatment suppressed Th2 responses in the airways. Interestingly, systemic administration of FHES at allergen challenge had no effect on airway inflammation, demonstrating that alum-induced Th2 response are set following initial allergen sensitisation. Our findings highlight the immunomodulatory potential of molecules secreted by *F. hepatica*.

Key words: Allergy/atopy, Immune modulation, Asthma, *Fasciola hepatica*

Introduction

There is now convincing epidemiological evidence to suggest that helminth infections attenuate allergy.¹ Helminths exert immunoregulatory mechanisms as an immune evasion strategy to suppress the generation of protective Th2 responses. The host may also exert its own regulatory mechanisms to prevent immune-mediated pathology.² The combination of host and parasite-induced immune regulation may explain how helminth infections can protect against allergy.

Infection with *Nippostrongylus brasiliensis*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Litomosoides sigmodontis* and *Heligmosomoides polygyrus* have all been shown to suppress the development of experimental allergic asthma.³⁻⁶ The protective effect of *H. polygyrus* and *L. sigmodontis* infection against allergic airway inflammation were both reversed upon depletion of Treg cells, highlighting Treg cells as a mediator of helminth-induced immunoregulation.^{3,5} These experimental and epidemiological studies have led to exploratory clinical trials with helminth-based therapy in humans. However, to date these trials have not demonstrated a protective effect against allergic disease,^{7,8} and are complicated by logistic and safety issues around to use of live parasites/eggs as a therapeutic approach for human diseases. An alternative approach is to use helminth-derived products, which can mimic the immunomodulatory effect of infection. The secreted products of *H. polygyrus*, and *N. brasiliensis* and the total antigen of *Ascaris suum* have been shown to suppress allergic airway inflammation.⁹⁻¹² Individual recombinant helminth proteins have also been shown to be beneficial against experimental allergic asthma.^{11,13}

We have previously demonstrated infection with the trematode fluke *Fasciola hepatica*, or administration of the excretory/secretory products of *F. hepatica* (FHES), protected mice against experimental autoimmune encephalomyelitis (EAE).^{14,15} Here, we investigate the effect of FHES on allergic responses in mice.

Material and Methods

Mice

BALB/c Mice (6-12 weeks old, Harlan UK.) were housed under SPF conditions. Animal experiments were conducted in accordance with the recommendations and guidelines of the Health Products Regulatory Authority (HPRA), the competent authority in Ireland responsible for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes in accordance with the requirements of the S.I No 543 of 2012. Animal experiments were carried out under license (B100/2412) approved by the HPRA and in accordance with protocols approved by Trinity College Dublin Animal Research Ethics Committee.

Preparation of FHES

Live adult flukes were collected from infected bovine livers at a local abattoir, washed with PBS containing 100 µg/ml penicillin and streptomycin. Live flukes were incubated overnight in PBS at 37°C and 5% CO₂. The supernatant was harvested, centrifuged at 13,000 g for 30 min, filtrated through 0.22 µm filter, and stored at -80°C. The endotoxin (LPS) levels in FHES were determined using a commercially available kinetic chromogenic limulus amebocyte lysate (LAL) assay according to manufacturer's instructions and found to be < 50 pg/mg equating to an in vivo dose of < 2 pg /mouse.

Induction of OVA-induced allergic asthma

BALB/c mice were sensitised by i.p. administration of ovalbumin (OVA; 50 µg, Sigma) emulsified with a alhydrogel (alum, 1 mg) and boosted 14 days later. Mice, treated at sensitisation, were injected with PBS or FHES (40 µg) on day 0 and day 14. On days 24-26, mice were challenged for 30 minutes with OVA (6%, in water) aerosolised via a nebuliser. Mice were killed on day 28 to assess airway inflammation. In some experiments mice were injected i.p. with PBS or FHES (40 µg) on days 24-27. Bronchialalveolar lavage (BAL) was performed using three 1 ml administrations of PBS using a 23-gauge IV catheter (Angiocath™, BD).

Results

FHES ameliorates OVA-induced allergic asthma when given at time of allergen sensitisation

We investigated the effect of FHES on allergic responses using the OVA-induced allergic asthma model, where mice were sensitised to OVA by immunisation with OVA adjuvanted with alum followed by challenge with aerosolised OVA (Fig. S1A). Challenged mice developed airway inflammation, associated with eosinophilic inflammation in the lung, production of anti-allergen IgE and pulmonary Th2 cytokine production (Fig. 1-2 & Fig. S2). FHES, given at the time of sensitisation, significantly reduced the infiltration of cells into the BAL fluid of OVA-challenged mice (Fig. 1A). There was a significant reduction in the numbers of eosinophils and lymphocytes in the BAL (Fig. 1A). In contrast, FHES had no suppressive effect on OVA-specific IgG₁ or IgE in the BAL (Fig. S2A) or serum (Fig. S2B), or on total IgG₁ or IgE in the serum (Fig. S2C). Administration of FHES enhanced OVA-specific IgE in the BAL (Fig. S2A) and total IgE in the serum (Fig. S2C). We were unable to detect IL-4 or IL-5 in the BAL fluid (data not shown), however, FHES, given at sensitisation, markedly reduced the production of IL-4 and IL-13 by CD4⁺ T cell from the lungs of OVA-challenged mice, as determined by flow cytometry (Fig. 1C). Lung cells from FHES-treated mice also produced less IL-4, IL-5 and IL-13 upon restimulation with OVA *ex vivo* (Fig. 1D). In contrast, FHES treatment had no effect on the frequency of FOXP3⁺ Treg cells in lung T cells after OVA challenge (Fig. S3). While we did not detect the presence of IL-33 or eotaxin in BALF fluid from any group of mice (data not shown), there was a decrease in the concentrations of IL-25 and TSLP in the BAL fluid of OVA-challenged mice that were sensitised in the presence of FHES (Fig. S4)

An examination of HE stained lung sections of mice sensitised with alum + OVA and challenged with allergen revealed a marked peribronchiolar and luminal inflammatory infiltrate comprised of lymphocytes, macrophages and eosinophils/neutrophils. Furthermore, epithelial hypertrophy and epithelial cell shedding and smooth muscle hypertrophy was evident in/around the bronchioles (Fig. 2A). The inflammation/remodelling of airways was markedly reduced in the mice treated with FHES during sensitisation (Fig. 2A). Analysis of AB-PAS stained lung sections revealed a reduction of goblet cells in the epithelium and lack of mucin in the lumen of airways in FHES treated mice at sensitisation compared with PBS-treated mice

sensitised with alum + OVA following allergen challenge (Fig. 2B). These data demonstrate that although FHES given at sensitisation did not inhibit OVA-specific antibody, it did inhibit lung inflammation, Th2 cytokines and eosinophilic infiltration into the lung following allergen challenge.

FHES inhibits dendritic cell function, but has no effect on inflammasome-dependent immune responses

Alum activates the NLRP3 inflammasome, which mediates some of its adjuvant properties.¹⁷⁻¹⁹ IL-1 signalling has been shown to be important for induction of airway inflammation in the alum-OVA allergy model.²⁰ This raises the possibility that FHES might directly inhibit inflammasome-dependent IL-1 β release. However, we found that FHES had no effect on inflammasome-dependent IL-1 β production from macrophages induced by alum or MSU in combination with LPS (Fig S5A). Furthermore, we found that FHES did not inhibit neutrophil or macrophage recruitment in an inflammasome-dependent sterile peritonitis model induced by injection with MSU²¹ (Fig S6A).

FHES has previously been shown to inhibit the maturation and APC function of dendritic cells (DC).²²⁻²⁴ We found that FHES inhibited LPS-induced production of IL-12p70 and IL-23 by bone marrow-derived DC (BMDC) (Fig S6A). Moreover, FHES reduced LPS-induced expression of CD40, CD80 and MHC-II on DCs (Fig S6B). Since FHES inhibited DC maturation, we tested its effect on APC function. BMDC, loaded with OVA as an antigen, and incubated with either PBS, FHES, LPS or FHES and LPS were adoptively transferred into BALB/c mice. We found that treating LPS-activated BMDC with FHES, prior to adoptive transfer, reduced the ability of these cells to induce cytokine production by OVA-specific T cells following ex vivo antigen restimulation of LN cells on day 7 post-transfer. Interestingly, there was a significant reduction in the production the Th2 effector cytokines IL-5 and IL-13 as well as IFN- γ .

FHES has no effect on OVA-induced allergic asthma when given during allergen challenge

Several studies that have demonstrated a beneficial effect of helminth products in experimental allergy administered these products only at the initial allergen sensitisation.^{6,9,16}

Evidence of a protective effect of helminth products given at allergen challenge is less clear. Thus, we investigated the efficacy of FHES given systemically during allergen challenge, as a positive treatment control, another group were given FHES at sensitisation only (Fig. S1B). Administration of FHES at time of allergen challenge did not affect OVA-induced lung inflammation in sensitised mice as determined by H&E staining (Fig. 2A). Moreover, AB-PAS staining revealed that FHES at challenge, did not suppress the build-up of mucin in airways of allergen-challenged mice (Fig. 2B). Furthermore, FHES, given at challenge, did not affect the frequency or total numbers of eosinophils in the BAL of sensitised and allergen-challenged mice (Fig. 2C-D) or the production of OVA-specific IL-4 or IL-13 by lung T cells (Fig. 2E). In contrast, in the same experiment, FHES administered at allergen sensitisation significantly reduced airways inflammation, eosinophil recruitment and Th2 cytokine production (Fig 2A-E).

Discussion

This study demonstrates that the soluble products of *F. hepatica* blocked the development of OVA-induced allergic asthma. Administration of FHES at sensitisation, but not at allergen challenge, suppressed lung inflammation, blocked the induction of Th2 cells and reduced the influx of eosinophils into the airways of challenged mice. Unlike the use of *N. brasiliensis* ES in the same model, FHES had no effect on allergen-specific IgE and IgG₁. This suggests that FHES only ablates the development of cellular, but not humoral allergic responses. However, this model is not dependent on antibody with disease being maintained in B and mast cell-deficient mice.^{25,26}

The role of Treg cells in mediating helminth-induced immunomodulation has received much attention.^{3,5} Indeed, we have previously demonstrated that live infection of mice with *F. hepatica* induces expansion of Treg cells¹⁴. However, here we found that FHES did not enhance the numbers of FOXP3⁺ Treg cells in the lungs of allergic mice. Consistent with our previous report on FHES suppressing autoimmunity¹⁵ and with the report from McSorley et al on *H. polygyrus*¹⁶, it appears that certain helminth products have less capacity than infection to expand Treg cells.

The fact that FHES failed to attenuate allergic responses when given at challenge is a limitation of our study. Indeed, this lack of efficacy after the allergen-sensitisation phase

appears to be a common feature of other helminth/helminth products; McSorley et al reported that *H. polygyrus* ES had a very limited effect on AHR and type 2 responses when given at challenge.¹⁶ Likewise, *N. brasiliensis* ES inhibited the induction of AHR when given at sensitisation but not when given 7 days later.⁹ Furthermore, infection with *N. brasiliensis* prior to, but not after allergen sensitisation blocked lung eosinophilia.⁶ These studies suggest that alum-induced allergic responses are largely set following initial sensitisation and that prior exposure to helminth products is required for effective helminth therapy. Many published studies have examined the effect of helminth infection/products on AHR at allergen sensitisation only. Thus, these studies have assessed the effect of helminth products on the alum-mediated induction of Th2 cells that drive allergy rather than established allergic responses.

In our previous study of the effect of FHES on autoimmunity we demonstrated that FHES promoted *F. hepatica*-specific Th2 responses *in vivo*, and on the face of it this appears to be difficult to reconcile with its ability to inhibit allergic sensitisation.¹⁵ However, similar to our findings, the ES products of *N. brasiliensis* which inhibited OVA-alum-induced allergic inflammation, also induced Th2 responses to its own antigens,⁹ suggesting that induction of a parasite-specific Th2 response and concurrent suppression of alum-induced Th2 cells is not mutually exclusive. Our demonstration that FHES blocks Th2-mediated allergic responses might be explained by the fact that Th2 responses induced by FHES or alum are mediated by different mechanisms. Helminths primarily induce Th2 responses via IL-4/IL-13/STAT6 signalling. In contrast alum-induced Th2 responses are independent of IL-4 or IL-13 signalling.²⁷ Alum-OVA-induced airway hypersensitivity (AHR) is much diminished in IL-1-deficient mice.²⁰ Given the evidence that the inflammasome plays a role in alum adjuvanticity we hypothesised that FHES would inhibit inflammasome activation. In contrast, we found that FHES did not inhibit inflammasome-dependent IL-1 β production *in vitro*, or neutrophil recruitment *in vivo*. The specific role of the inflammasome in mediating alum adjuvanticity is complex, for instance it is required for the production of IgE,¹⁷ but dispensable for the induction of Th2 cells.^{18,19} Nevertheless, our findings still suggest that FHES impacts on early innate cell activation by alum. The effect of FHES on alum-activated dendritic cells may be key here since FHES has been shown to exert inhibitory effects on the APC function of DC, including suppression of DC cytokine production, maturation and APC function.^{22,23} FHES-derived cathepsins have been shown to augment macrophage activation through degradation of

TLR3.²⁴ We added to these findings by showing that FHES inhibits DC cytokine production, maturation and APC function. Interestingly we showed that FHES inhibited the ability of LPS-activated DC to promote the production of IL-5 and IL-13 from LN cells, suggesting that FHES impacts the ability of DC to expand Th2 cells, despite the fact that FHES promotes Th2 responses to its own antigens in vivo¹⁵. Thus, the inhibitory effect of FHES on DC may represent a mechanism by which FHES inhibits allergic sensitisation.

We have previously shown that FHES induces the release of IL-33 in vivo in C57BL/6 mice, which promotes eosinophilia that was required for its protective effect in EAE.¹⁵ In contrast, the present study showed that FHES blocked allergic eosinophilia. However, in the OVA-induced allergic asthma model, eosinophils are essentially a readout of Th2 cell activity in the allergen-challenged lung, thus we have shown that FHES blocks alum-induced Th2 cell induction rather than directly inhibiting eosinophils. In fact, it is likely that FHES recruits eosinophils to the injection site during allergen sensitisation. The effect of FHES-recruited eosinophils on allergic sensitisation has not been examined. IL-33 is widely considered to be an exacerbating factor in allergy and asthma,²⁸ where it plays activates type 2 responses during early allergen sensitisation.²⁹ Indeed, alum induces IL-33 production in vivo³⁰. Oboki et al demonstrated that IL-33-deficient mice are resistant to OVA-induced allergic asthma.³¹ However, that study also found that C57BL/6 mice are more responsive to IL-33 than BALB/c mice, the mice we used in the present study.³¹ Therefore, it is possible that FHES-induced IL-33 may play less of a role in BALB/c mice. The method of allergen sensitisation appears to have an effect on the requirement of IL-33 in this model; genetic ablation of ST2 (IL33R) did not diminish allergic responses in mice that were sensitised twice with alum and OVA.^{32,33} In contrast, ST2 was shown to play a role in allergic responses in mice that were immunised once.³⁴ Since we immunised twice, this may explain the lack of an effect of FHES-induced IL-33 on allergic sensitisation. This suggests that IL-33 has a less potent effect on established antigen-specific immune responses than it does upon early development of antigen-specificity. Consistent with this view, alum-induced IL-33 was found to be required for the early alum-induced cytokine responses, but ultimately had no impact on later alum-induced antigen-specific T cells/antibody responses.^{30,31}

In Summary, our study shows that the secreted products *F. hepatica* inhibits allergic sensitisation induced by alum and thus may be a potential source of immunodulatory molecules.

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Figure legends

Figure 1. FHES administration at sensitisation inhibits OVA-induced allergic asthma.

BALB/c mice were sensitized with either alum, alum+OVA or alum+OVA+FHES on days 0 and 14 and challenged with aerosolised OVA on days 24-27. A, from top down, total BAL cell count on day 28, total BAL CD3⁺ and CD19⁺ lymphocyte counts and number of BAL Siglec-F⁺ CD11c⁻ CD11b⁺ SSc^{high} eosinophils. B, Representative flow cytometric plots depicting eosinophil gates, gated on live BAL cells. C, Expression of IL-4 and IL-13 by lung CD3⁺ CD4⁺ T cells. E, The concentrations of IL-4, IL-5 and IL-13 in the supernatant at 72 h of lung cells restimulated with OVA *ex vivo*. Results are representative of 3 separate experiments, with data in A being the mean of those experiments. Data presented are means +/- SEM. Statistical assessment was performed by unpaired Student's t-test. *p<0.05 **p<0.01 ***p<0.001

Figure 2. Administration of FHES at challenge does not affect OVA-induced allergic asthma.

BALB/c mice were sensitized with either alum, alum+OVA or alum+OVA+FHES (sensitisation) on days 0 and 14 and intra-tracheally challenged with OVA on days 24-27. On days 23-27 mice that were sensitised to alum+OVA were treated with either PBS (PBS) or FHES (challenge). A, Representative photomicrographs (magnification, x10) of lung HE-stained sections. B, Representative photomicrographs sections stained with Alican Blue-PAS. Photomicrographs are representative of 3 sections taken from 4 mice per group. C, Proportion and number of Siglec-F⁺ CD11c⁻ CD11b⁺ eosinophils in the day 28 BAL (n=4). E, The concentrations of IL-4 and IL-13 in the supernatant at 72 h of lung cells restimulated with OVA *ex vivo*. Data presented are means +/- SEM. Statistical assessment was performed by unpaired Student's t-test. *p<0.05 **p<0.01 ***p<0.001.

Figure 1

Figure 2

Supporting information for:

Secreted products of *Fasciola hepatica* inhibit the induction of T cell responses that mediate allergy

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Supplemental Material and Methods

Flow Cytometry

Suspensions of BAL or whole lung cells were blocked with Fc γ blocker (BD Pharmingen; 1 μ g/ml) and stained with LIVE/DEAD Aqua (Life technologies). In order to discriminate eosinophils Siglec-F⁺ CD11b⁺ CD11c⁻ SSC^{high} eosinophils and Fsc^{low} SSC^{low} CD3⁺ or CD19⁺ lymphocytes cells were surface stained with CD11b (M1/70), CD11c (N418), CD3 (145-2C11), CD4 (GK1.5), CD19 (1D3) and Siglec-F (E50-2480). To assess T cell cytokine production cells were stimulated for 5 h with PMA (25 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (5 μ g/ml) for the final 4 h. Cells were then fixed and permeabilized (Fix and Perm cell permeabilization kit; Caltag Laboratories) and stained for intracellular IL-4 (BVD6-24G2), IL-5 (TRFK5) and IL-13 (eBio13A). To Identify Treg cells, T cells were surface stained as before and permabilised using FOXP3 staining buffers (ebioscience) before staining with anti-FOXP3 (FJK-16A). Cells were acquired using a Cyan ADP (Dako Cytomotion) or FACS Canto II (BD). Analysis was done using FlowJo v9.2 (Tree Star Inc). For identification of cells in the peritoneal cavity, peritoneal lavage cells were surface stained with CD11b (M1/70), CD11c (N418), Ly6G (1A8), Ly6C (HK1.4) and Siglec-F (E50-2480). For analysis of BMDC, BMDC were stained with antibodies against CD40 (HM40-3) , CD80 (16-10A1) and MHC-II (I-A/I-E, M5/114.15.2)

Lung Histopathology

From each mouse the left lung lobe was fixed in 10% neutral buffered formalin. Following fixation, tissues were embedded in paraffin wax and sectioned at 5 μ m. Sections were stained with Gill®-2 Haematoxylin and Eosin (HE) to assess inflammatory cell infiltration in and around small airways and airway remodelling, goblet cell hyperplasia/metaplasia, smooth muscle thickening. Sections were also stained with Aican-Blue-Periodic Acid Schiff (AB-PAS) for mucin in airway lumens and goblet cells.

Bone marrow-derived dendritic cells and macrophages

Bone marrow-derived immature DCs were prepared by culturing bone marrow cells from C57BL/6 or Balb/c mice for 10 days in J558-conditioned medium containing GM-CSF to a final concentration of 20 ng/ml, semi adherent cells were transferred to a new flask on day 6 of culture and reseeded with J558-conditioned medium. Non-adherent dendritic cells were isolated on day 10 of culture. Bone marrow-derived macrophages were prepared by culturing C57BL/6 bone marrow cells in DMEM supplemented with 15% of the MCSF-containing L929 cell culture supernatant for 6 days. On day 6 macrophages were collected by removing non-adherent cells by washing in warm medium and collecting adherent cells via cell scraping.

A

B

Supplemental figure 1. Experimental design of airway hypersensitivity experiments. A-B, BALB/c mice were immunized by i.p. administration of either alum; alum with OVA; or alum, OVA and FHES on day 0 and day 14. Mice were challenged for 30 m with aerosolised OVA on days 24, 25 and 26, and rested for one day before tissue isolation on day 28. B, in separate experiments, mice were treated at challenge by i.p. injection with PBS or FHES on days 24, 25 and 26.

Supplemental figure 2. Co-administration of FHES at sensitisation does not inhibit IgE/IgG₁ antibody production. BALB/c mice were sensitized with either alum, alum+OVA or alum+OVA+FHES and intra-tracheally challenged with OVA as in Fig 1. On day 28 the presence of OVA-specific IgE and IgG₁ in A, neat BAL fluid or B, a 1 in 50 dilution of blood serum was determined by ELISA. C, Total unspecific IgE and IgG₁ in a 1/50 dilution of serum as determined by ELISA. Results are the combined results of 2 experiments. n=8. Data presented are means +/- SEM. Statistical assessment was performed by an unpaired Student t-test vs mice sensitised with alum alone. **p<0.01.

Supplemental Figure 3. Co-administration of FHES at sensitisation does not affect frequency of FOXP3 expression by lung T cells in the lungs of challenged mice BALB/c mice were sensitized with either alum, alum+OVA or alum+OVA+FHES and intra-tracheally challenged with OVA as in Fig 1. On day 28, the expression of FOXP3 gated on live, CD3⁺CD4⁺ T cells from the homogenised lung. Data are representative of 2 experiments.

Supplemental Figure 4 Co-administration of FHES at sensitisation reduces TSLP and IL-25 concentrations in the BAL fluid, but does not reduce serum eotaxin concentrations. BALB/c mice were sensitized with either alum, alum+OVA or alum+OVA+FHES on days 0 and 14 and challenged with aerosolised OVA on days 24-27. On day 28 mice were sacrificed and the concentration of IL-25 and TSLP in the BAL fluid (BALF) in the serum was determined by ELISA.

Supplemental Figure 5. FHE does not block alum or MSU-induced IL-1 β or PGE₂. Bone marrow-derived macrophages were primed with LPS (100 ng/ml) for 2 h with and without FHES for the final 1 h prior to stimulation with either Alum (200, 400 μ g/ml) or MSU crystals (40, 80 μ g/ml). A, The concentration of IL-1 β at 24 hours determined by ELISA. Data presented are means $\pm$ SEM of triplicate assays. Results are representative of 2 separate experiments.

Supplemental Figure 6. FHES does not inhibit the neutrophil and macrophage recruitment to the peritoneal cavity in response to MSU or alum.

In separate experiments C57 BL/6 Mice were injected i.p. with 0.5 mg of MSU crystals (A) or 1 mg of alum (B) with and without 40 μ g of FHES. Peritoneal cells were isolated after 24 hours and analysed by flow cytometry. A, representative flow cytometric plots with neutrophil gates, gated on total live cells. B, Percentage Ly6G⁺CD11b⁺ neutrophils, F4/80^{low}CD11b⁺MHC-II⁺ recruited macrophages in the peritoneal cavity as determined by flow cytometry

A

B

C

Supplemental figure 7. FHES suppresses DC maturation and cytokine production and APC function. A-B, BMDC expanded from C57BL/6 mice ($1 \times 10^6/\text{ml}$) were treated with either PBS, FHES (increasing to 5% v/v ($100 \mu\text{g}/\text{ml}$)), LPS ($100 \text{ ng}/\text{ml}$) or FHES + LPS. A, Supernatant concentrations of IL-12p70, and IL-23 in the supernatant at 24 h were determined by ELISA. B, At 24 h BMDC were stained with antibodies against CD11c and expression of CD40, CD80, CD86 and MHC-II was analysed by flow cytometry. C, BMDC ($1 \times 10^6/\text{ml}$) expanded from BALB/c mice were incubated with either PBS, OVA ($50 \mu\text{g}/\text{ml}$), OVA + FHES (5% v/v ($100 \mu\text{g}/\text{ml}$)), OVA + LPS ($100 \text{ ng}/\text{ml}$) or OVA + FHES + LPS. OVA or PBS was added at time 0, FHES at time 2 hours and LPS at time 2.5 hours. After 24 hours, BMDC (1×10^6) were removed from culture and transferred into the footpads of naive C57BL/6 ($0.5 \times 10^6/\text{hind footpad}$) mice by s.c. injection, $n=3$. 7 days after adoptive transfer the popliteal LN of recipient mice were excised and pooled from each group. LN cells were restimulated *ex vivo* with OVA ($100 \mu\text{g}/\text{ml}$) for 86 hours. The concentrations of IFN- γ , IL-17, IL-4, IL5, IL-10 and IL-13 in the supernatant after 86 hours was determined by ELISA. Data presented are means $\pm$ SEM of triplicate assays. Statistical assessment was performed as indicated using Student's T test *** $P<0.001$, ** $P<0.01$, * $P<0.05$. Results are representative of 2 separate experiments.