

# *Trichinella spiralis*-secreted products modulate DC functionality and expand regulatory T cells *in vitro*

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## SUMMARY

*Helminths and their products can suppress the host immune response which may benefit parasite survival. Trichinella spiralis can establish chronic infections in a wide range of mammalian hosts including humans and mice. Here, we aim at studying the effect of T. spiralis muscle larvae excretory/secretory products (TspES) on the functionality of DC and T cell activation. We found that TspES suppress in vitro DC maturation induced by both S- and R-form lipopolysaccharide (LPS) from enterobacteria. Using different toll-like receptor (TLR) agonists, we show that the suppressive effect of TspES on DC maturation is restricted to TLR4. These helminth products also interfere with the expression of several genes related to the TLR-mediated signal transduction pathways. To investigate the effect of TspES on T cell activation, we used splenocytes derived from OVA-TCR transgenic D011.10 that were incubated with OVA and TspES-pulsed DC. Results indicate that the presence of TspES resulted in the expansion of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells. These regulatory T (Treg) cells were shown to have suppressive activity and to produce TGF- $\beta$ . Together these results suggest that T. spiralis secretion products can suppress DC maturation and induce the expansion of functional Treg cells in vitro.*

**Keywords** DC, Helminths, Immunoregulation, LPS, Regulatory T cells, TLR, Trichinella spiralis

## INTRODUCTION

Helminths and their products have been shown to suppress the host's immune response by inducing a regulatory network, for example, regulatory T (Treg) cells that dampen parasite-specific immune responses and favour parasite survival (1). This anti-inflammatory property of a number of helminths is not restricted to the parasite itself but may also affect other immune responses and immunopathological disorders of the host (2). In this regulatory network, dendritic cells (DC) play a pivotal role. DC through several types of pattern recognition receptors such as toll-like receptors (TLR), C-type lectin receptors, RIG-I like receptors and Nod-like receptors may recognize various pathogen-associated molecular patterns. Upon stimulation, DC undergo phenotypic and functional changes that allow them to migrate to lymph nodes and prime appropriate T cell responses (3,4). DC can prevent, inhibit or modulate T cell-mediated effector responses by the production of anti-inflammatory factors that can result in induction of Treg cells (5).

After ingestion of *T. spiralis* infected meat, the larvae are released in the stomach, migrate to the small intestine where they mature into adult worms and release newborn larvae that rapidly disseminate throughout the host, and eventually enter skeletal muscle to remain for many years (6,7). The host-mediated immune response against *T. spiralis* depends on CD4<sup>+</sup> T cells and mast cells that have an essential role in worm expulsion (8). Interleukin-4 and IL-13 are involved in this protective immune response, and there is evidence that when the effects of both of these cytokines are inhibited, worm survival is extended (9). Other findings indicate that IL-10 and TGF- $\beta$  control the level of inflammation during the muscle stage of infection (10). Few studies have suggested that *T. spiralis* can modulate the host immune response, protecting it against other immune pathologies (11–13). However, little is known about the role of Treg cells induced by this helminth and their products.

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Modulation of the immune response by helminths involves the excretory/secretory (ES) products released by these parasites. These products include proteases, protease inhibitors, venom allergen homologues, glycolytic enzymes, lectins, lipids and glycans, which together or individually could be potential immunomodulators (14,15). There are only few studies on *T. spiralis* products and their effect on the immune response (16,17). We have previously shown that ES products from *T. spiralis* (TspES) suppress DC maturation induced by the S-form of *Escherichia coli* LPS but not by the R-form of *Neisseria meningitidis* LPS (16). Because the S-form of LPS requires CD14 for TLR4 activation whereas the R-form does not (18–20), a possible role for CD14 in this suppression was suggested.

Here, we aim at studying further the effect of TspES on DC maturation and on T cell activation. We found that the suppressive effect of TspES on DC maturation does not depend on the form of the LPS used, and therefore, it is independent of CD14. In addition, we show that the suppressive effect of TspES on DC maturation is restricted to TLR4 and that these helminth products interfere with the expression of several genes related to the TLR-mediated signal transduction pathways. Using splenocytes derived from OVA-TCR transgenic D011.10 mice, we show that TspES induce *in vitro* the expansion of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells in a TGF- $\beta$ -dependent manner. These findings contribute to our understanding on the mechanisms involved in the immunoregulation induced by *T. spiralis*.

## MATERIALS AND METHODS

### Animals

Six- to 10-week-old BALB/c mice (Harlan, Zeist, The Netherlands) and OVA-TCR transgenic (DO11.10) mice on BALB/c background were housed under specific pathogen-free conditions at the animal care facility of the National Institute for Public Health and the Environment (RIVM). The transgenic mice were kindly provided by Prof. Dr. Willem van Eden from the Utrecht University, the Netherlands. This study was agreed upon by the Committee on Animal Experimentation of the RIVM (Bilthoven, the Netherlands) under permit number 200900205 and 200900192. Animal handling in this study was carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation.

### Preparation of parasite antigen

Preparation of TspES was performed as previously described by Gamble (21). Briefly, Wister rats (Harlan,

Horst, The Netherlands) were infected with 3000 *T. spiralis* RIVM-strain. At 42 days post-infection, the muscle larvae were recovered by acid-pepsin digestion, washed and incubated at a concentration of 10<sup>5</sup> larvae per ml, for 19 h at 37°C in 5% CO<sub>2</sub> in RPMI medium supplemented with 1% penicillin/streptomycin. After incubation, the medium was centrifuged and the supernatant containing the secreted products (TspES) was dialysed and concentrated. The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL, USA). The endotoxin level was below the detection limit (0.2 EU/mL) of the QCL-1000 chromogenic LAL Endpoint Assay (Lonza, Basel, Switzerland).

### TLR ligands

The TLR ligands: *E. coli* LPS S-form (TLR4), Pam3CSK4 (TLR1/2), FSL-1 (TLR2/6), Poly I:C (TLR3), flagellin derived from *Salmonella typhimurium* (TLR5), ssRNA40 (TLR7) and ODN1826 (TLR9) were purchased from Invivogen (San Diego, CA, USA). *E. coli* LPS serotype R15 (R-form) and *S. minnesota* S-form LPS were purchased from Alexis Biochemicals (San Diego, CA, USA). *Neisseria meningitidis* LPS, which in nature exists as a R-form only, was purified from wild-type strain H44/76 by a modified hot phenol-water extraction (22).

### Bone marrow dendritic cell culture

Culture of bone marrow-derived dendritic cells (BM-DC) was performed according to the adapted method of Lutz *et al.* (23). Briefly, bone marrow cells were collected from euthanized naive mice by flushing femurs and tibiae with sterile phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA). Cells were resuspended at 2.5 × 10<sup>5</sup> cells/mL in complete RPMI-1640 medium (Invitrogen) containing 1% penicillin/streptomycin, 1% glutamine (Gibco-Invitrogen, Grand Island, NY, USA), 50  $\mu$ M  $\beta$ -mercaptoethanol (Invitrogen) and 10% foetal bovine serum (FBS) (Gibco, Invitrogen). Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Cytocen, Utrecht, The Netherlands) at 20 ng/mL was added and the cells were grown in 1 mL per well in 12-well plates (Costar, Corning, NY, USA) (day 0). On day 2, equal amounts of fresh medium (1 mL) and 20 ng/mL GM-CSF were added, and on day 4, only 20 ng/mL GM-CSF was added. On day 7, cells were incubated with TspES (5  $\mu$ g/mL) alone or together with the different LPS forms (1 ng/mL) or other TLR ligands (concentrations are indicated in the legends of Figure 3) for 24 h. After incubation, nonadherent cells were harvested for FACS analysis and the supernatants stored at –20°C for cytokine determination.

## Culture of TLR4/MD2-CD14 HEK293 cells

Human Embryonic Kidney 293 (HEK) cells stably transfected with mouse TLR4/MD2-CD14 (Invivogen) were cultured in DMEM (Gibco-Invitrogen) containing 10% FBS, 10 µg/mL Blasticidin (Invivogen) and 50 µg/mL Hygromycin B (HygroGold; Invivogen). These cells were incubated with the different LPS forms (1 ng/mL) in the presence or absence of TspES (5 µg/mL). Culture supernatants were collected to measure IL-8 production.

## Cytokine gene expression

Total RNA (10 ng) was extracted from DC incubated either with the different LPS forms (1 ng/mL) alone or together with TspES (5 µg/mL) for 6 h using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Total RNA was used as a template for the kit Quantifast SYBR Green RT-PCR One-Step (Qiagen). This is a one-step reaction where reverse transcription (cDNA) and PCR take place. The expression of *Il1a*, *Il6*, *Il10*, *Il12a* and *Tnf* genes was measured relative to the housekeeping genes *Gapdh*, *Gusb* and *Hprt1*. Primers for RT-PCR analysis were from Qiagen (QuantiTect Primer Assays). Light Cycler 480 (Roche, Almere, The Netherlands) was used for detection. All reactions were performed according to the manufacturer's instructions.

## PCR array for TLR signalling pathways

Total RNA (10 ng) was extracted from DC incubated with either *E. coli* LPS S-form (1 ng/mL) alone or together with TspES (5 µg/mL) for 6 h using the RNeasy Plus Mini Kit (Qiagen). For cDNA synthesis, a Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (SABiosciences, Frederick, MD, USA) was used. The cDNA was analysed using the mouse TLR signalling pathway RT<sup>2</sup> Profiler PCR Array (For more information cat. num. PAMM-18F, SABiosciences) that measures the expression of 84 genes related to TLR-mediated signal transduction (TLRs, effectors and members of the NFκB, JNK/p38, NF/IL6 and IRF signalling pathways downstream of TLR signalling) and five housekeeping genes. This PCR array also includes mouse genomic DNA, positive PCR controls and three reverse transcription controls. Procedures were performed according to manufacturer's instructions. Briefly, samples were run in the Light Cycler 480, using the following protocol, heat activation 95°C for 10 min, PCR cycles 45 of 15 s at 95°C, and 1 min at 60°C and melting curves of 15 s at 60°C and 1 min at 95°C. The normalized threshold cycle ( $C_t$ ) value was obtained for each gene, and the  $\Delta C_t$  was determined by subtracting the  $C_t$  value of the housekeeping gene, from the  $C_t$  value of the gene of interest. The change in the  $C_t$  value

between control and treated samples for each gene of interest,  $\Delta\Delta C_t$ , was then calculated, and the fold change was determined using the formula  $2^{-\Delta\Delta C_t}$ . Up-regulation of gene expression was considered for values above 2 and down-regulation for values below -2 fold change of gene expression.

A complete list of genes analysed by the array can be found at: [http://www.sabiosciences.com/rt\\_pcr\\_product/HTML/PAMM-018A.html](http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-018A.html).

## Culture of spleen cells and TGF-β assay

Splenocytes derived from OVA-TCR transgenic (tg) DO11.10 mice were cultured in 96-well round-bottom plates (Costar) at  $5 \times 10^5$  cells/well in complete RPMI-1640 (prepared as mentioned earlier). TspES-pulsed DC (pulsed for 24 h) (TspES-DC) or DC in medium only were added to the spleen cells together with 20 µg/mL OVA protein (Endograde, Hyglos GmbH, Germany). After 4 days, supernatants were stored for cytokine analysis, and cells were harvested for flow cytometric analysis.

For the TGF-β assay, 100 µg/mL anti-TGF-β1 (Sigma, St Louis, MO, USA) and 100 µg/mL IgG1 isotype control (Sigma) were added to splenocytes incubated with OVA and TspES-DC or DC. Recombinant hTGF-β1 (R&D systems, Minneapolis, MN, USA) 2 ng/mL was added to splenocytes incubated with DC + OVA as positive control and in the presence of anti-TGF-β1 as negative control. After 4 days, supernatants were stored for cytokine analysis, and cells were harvested for flow cytometric analysis.

## Treg suppression assay

To determine whether the Treg cells induced by the *T. spiralis* products have suppressive activity, an *in vitro* suppression assay previously described by Finney *et al.* (24) was carried out. Briefly,  $5 \times 10^4$  CD4<sup>+</sup> CD25<sup>+</sup> T cells isolated from OVA-TCR tg DO11.10 splenocytes, which were previously cultured with TspES-DC + OVA or DC + OVA, were added to 96-well round-bottom plates (Costar) together with  $5 \times 10^4$  CFSE-labelled effector (CD4<sup>+</sup> CD25<sup>-</sup>) T cells derived from the spleen of naïve BALB/c mice and naïve BM-DC ( $1 \times 10^5$ ) in the presence of 2.5 µg/mL Concavalin-A (Con-A) during 4 days. CD4<sup>+</sup> CD25<sup>+</sup> or CD4<sup>+</sup> CD25<sup>-</sup> T cells were isolated by Magnetic-activated cell sorting (MACS) using the Regulatory T Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer instructions. The effector cells were labelled at  $10^6$  cells/mL in PBS containing CFSE at a concentration of 2 µM for 10 min at 37°C. Cells were then washed repeatedly in complete RPMI 1640 before addition to suppression assays. Proliferation of effector cells was measured by flow cytometry using a FACSCanto II (BD

Biosciences, Erembodegem, Belgium). The percentage of cell division was calculated using the FLOWJO software (version 7.6; Tree Star Inc., Ashland, OR, USA).

### Flow cytometric analysis

For flow cytometric analysis using BM-DC, the cells were washed in FACS buffer (PBS containing 5% FBS and 5 mM sodium azide) and stained for 30 min at 4°C with the fluorescent-labelled antibodies: anti-CD40-FITC, anti-CD80-PE, anti-CD86-PE, anti-MHCII-FITC and anti-CD11c-APC (eBioscience, San Diego, CA, USA). Cells ( $10^5$  events) were acquired and analysed on a FACSCanto II. FACS analysis was performed on CD11c positive cells. Data were analysed using the FLOWJO software.

For splenocytes, the cells were first washed in PBS followed by staining for 30 min at 4°C with LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen). Next, cells were blocked with an unconjugated anti-CD32/CD16 for 10 min at 4°C. Then, anti-CD4-APCH7 and anti-CD25-PerCPCy5.5 (BD Pharmingen, San Diego, CA, USA) were added to the cells and incubated for 30 min at 4°C. For intracellular staining, anti-FOXP3-APC (Bioscience, San Diego, CA, USA), anti-IL-10-FITC (BD Pharmingen) and anti-TGF- $\beta$ 1-2-3-PE (R&D systems) were added for 30 min at 4°C after fixation and permeabilization of the cells according to the manufacturer's recommendation. Cells ( $5 \times 10^5$  events) were acquired and analysed on a FACSCanto II. Data were analysed using the FLOWJO software.

### Cytokine determination

The levels of IL-1 $\alpha$ , IL-6, IL-10, IL-12p70 and TNF- $\alpha$  in DC culture supernatants and IL-2, IL-4, IL-5, IL-10, IL-17 and IFN- $\gamma$  in supernatants from spleen cell cultures were measured using Bio-Plex assays (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. The samples were analysed on a Luminex 100 (Luminex, Austin, TX, USA). The levels of IL-8 in the TLR4/MD2-CD14 HEK293 cell culture supernatants were measured using ELISA PeliPair reagent set (Sanquin, Amsterdam, The Netherlands). The levels of free active TGF- $\beta$ 1 in spleen cell culture were measured using ELISA kit Legend Max (Biolegend, San Diego, CA, USA).

### Statistical analyses

One-way analysis of variance (ANOVA) was performed followed by the Bonferroni's multiple comparison test to analyse differences in means between different groups of treated cells (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA). Data are pre-

sented as means  $\pm$  SEM, and differences were considered significant at  $P$  values of  $\leq 0.05$ .

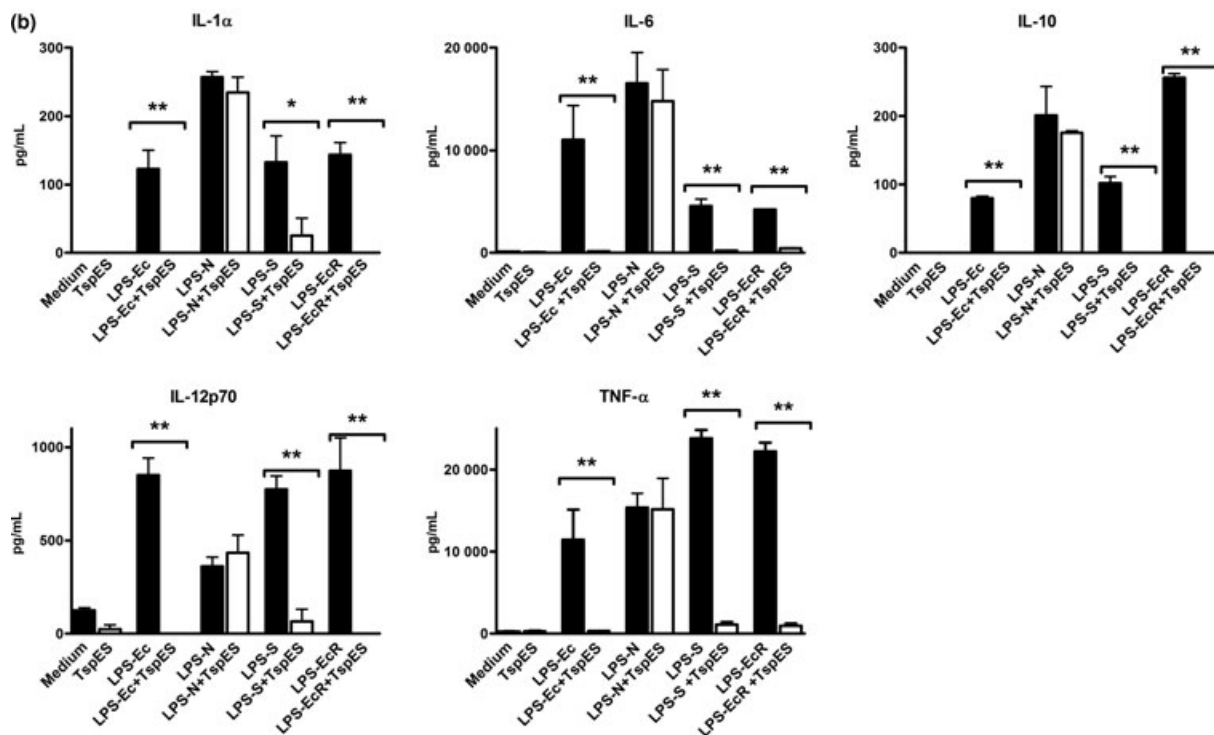
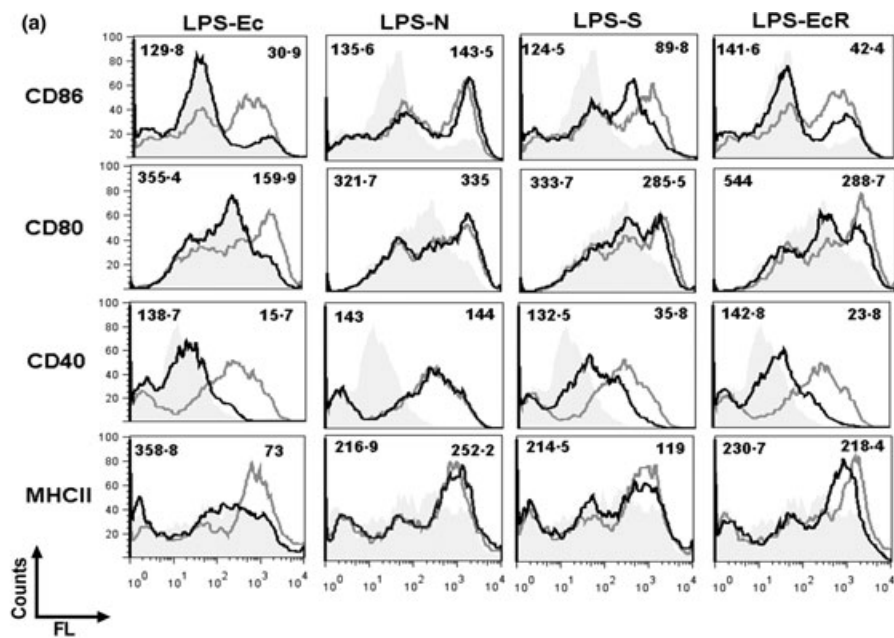
## RESULTS

### TspES suppress DC maturation induced by S- and R-form LPS

As TspES suppress DC maturation induced by *E. coli* LPS (S-form) but not by *N. meningitidis* LPS (R-form), we investigated whether this effect was dependent on the form of LPS used implying a role for CD14. For this purpose, the S-form of *S. minnesota* LPS and the R-form of *E. coli* LPS were also tested. Dendritic cells were cultured for 24 h with the different LPS forms, TspES alone or the combination of both. The expression of DC maturation markers, cytokine production and gene expression were measured. Consistent with our previous findings, TspES suppressed the expression of the maturation markers and cytokine production induced by *E. coli* LPS S-form but not *N. meningitidis* LPS R-form (Figure 1a,b). These differences were also observed at the cytokine gene expression level (Table 1). The expression of DC maturation markers, cytokine production and cytokine gene expression induced by *S. minnesota* LPS S-form were also suppressed by TspES (Figure 1a,b; Table 1). Reduced expression of the maturation markers due to the presence of TspES was stronger when the concentration of *S. minnesota* LPS S-form was reduced to 0.1 ng/mL (data not shown). Interestingly, the expression of DC maturation markers, cytokine production and gene expression induced by *E. coli* R-form LPS were also reduced by TspES (Figure 1a,b; Table 1). The reduced expression of MHCII was stronger when a lower concentration (0.1 ng/mL) of *E. coli* R-form LPS was used (data not shown). These results were confirmed by using HEK cells transfected with the mouse TLR4 receptor complex (TLR4/MD2-CD14). These cells were incubated with the different LPS forms, TspES or the combination of both. TspES significantly suppressed the production of IL-8 induced by the S- and R-form of *E. coli* and the S-form of *S. minnesota* S-form, but it did not suppress the production of IL-8 induced by *N. meningitidis* LPS R-form (Figure 2). These results suggest that TspES inhibit the response to most LPS variants tested. Remarkably, the response to *Neisseria* LPS was not affected even when LPS concentrations as low as 0.01 ng/mL were used (data not shown).

### Suppression of DC maturation by TspES is restricted to TLR4

As TspES suppress TLR4-mediated activation of DCs, we investigated whether TspES suppress DC activation induced by other TLRs. For this purpose, DC were incubated with

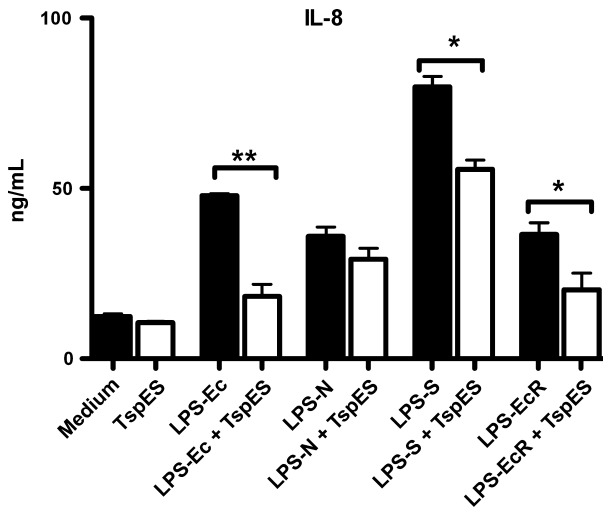


**Figure 1** Effect of TspES on DC maturation induced by different S- and R-form LPS. DC were incubated with TspES (5  $\mu$ g/mL) alone or in combination with different LPS (1 ng/mL) for 24 h. (a) Expression of surface markers on DC incubated in medium only (filled histogram), with LPS (grey line) or with LPS + TspES (black line). Numbers inside each histogram are the MFI (median fluorescence intensity) of DC with LPS alone (left) and LPS + TspES (right). (b) Production of cytokines by DC is represented as mean of triplicate values (pg/mL  $\pm$  SEM). The data are representative of three independent experiments. Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: \* $P$  < 0.05, \*\* $P$  < 0.01. LPS-Ec, *E. coli* LPS S-form; LPS-S, *S. minnesota* S-form LPS; LPS-EcR, *E. coli* R-form LPS; LPS-N, *N. meningitidis* R-form LPS; FL, fluorescence; TspES, excretory/secretory products from *T. spiralis*.

**Table 1** Fold increase cytokine gene expression of DC stimulated with different types of LPS and TspES. Dendritic cells were incubated with different types of LPS (1 ng/mL) either alone or in combination with TspES (5 µg/mL) for 24 h. The data are shown as fold-increase expression over unstimulated cells. The data are representative of two independent experiments

	<i>Il1a</i>	<i>Il6</i>	<i>Il10</i>	<i>IL12p35</i>	<i>Tnf</i>
LPS-Ec	657.87	758.32	4.49	128.15	20.21
LPS-Ec + TspES	10.47	6.96	1.52	4.4	1.47
LPS-N	513.18	767.13	10.24	116.84	12.41
LPS-N + TspES	756.57	1241.9	12.50	84.06	18.04
LPS-S	360.37	277.88	3.85	29.21	38.19
LPS-S + TspES	35.88	8.60	1.07	1.43	6.67
LPS-EcR	303.38	177.91	7.08	16.55	15.96
LPS-EcR + TspES	40.13	12.79	2.46	4.62	4.90

LPS-Ec, *E. coli* LPS S-form; LPS-S, *S. minnesota* S-form LPS; LPS-EcR, *E. coli* R-form LPS; LPS-N, *N. meningitidis* R-form LPS; TspES, excretory/secretory products from *T. spiralis*.



**Figure 2** Effect of TspES on IL-8 production by HEK-TLR4/MD2-CD14 cells stimulated with different LPS. HEK cells transfected with mouse TLR4/MD2-CD14 were incubated with TspES (5 µg/mL) alone or in combination with different types of LPS (1 ng/mL) for 24 h. The data represent the mean of triplicate values (ng/mL ± SEM) and are representative of three independent experiments. Statistical analysis was performed by one-way ANOVA with the Bonferoni's test: \* $P < 0.05$ , \*\* $P < 0.01$ . LPS-Ec, *E. coli* LPS S-form; LPS-S, *S. minnesota* S-form LPS; LPS-EcR, *E. coli* R-form LPS; LPS-N, *N. meningitidis* R-form LPS; TspES, excretory/secretory products from *T. spiralis*.

the following TLR ligands: Pam3CSK4 (TLR1/2), FSL-1 (TLR2/6), Poly I:C (TLR3), ST-FLA (TLR5), ssRNA40 (TLR7) and ODN1826 (TLR9) in the presence or absence of TspES. Results show that TspES had no significant effect on the expression of CD80, CD86, CD40 and MHCII on

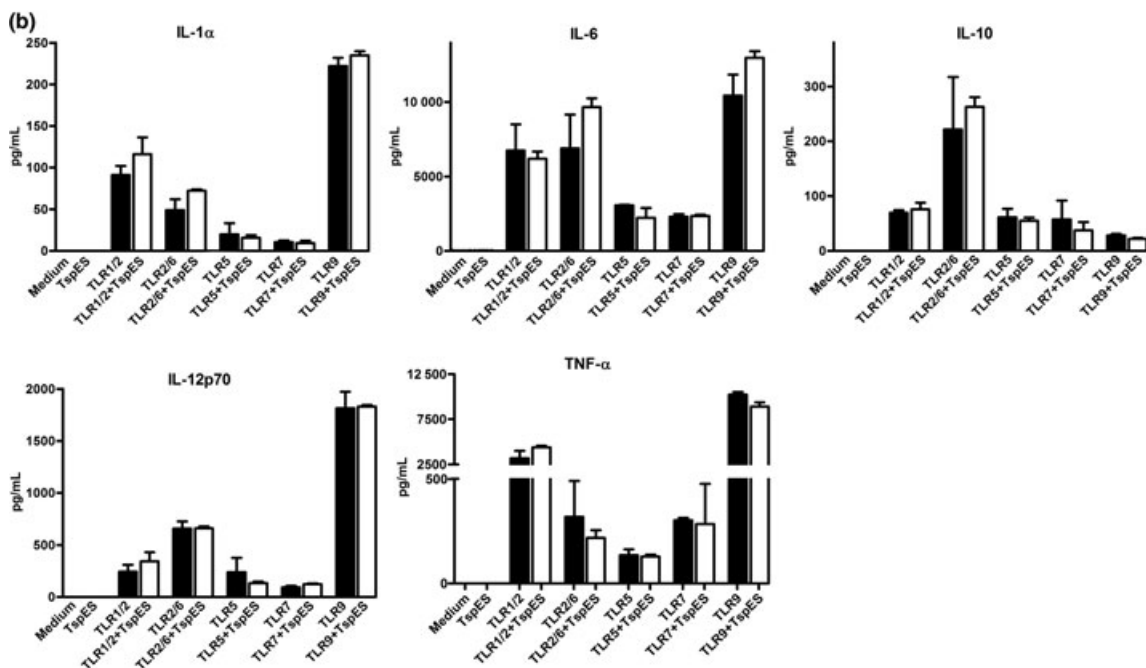
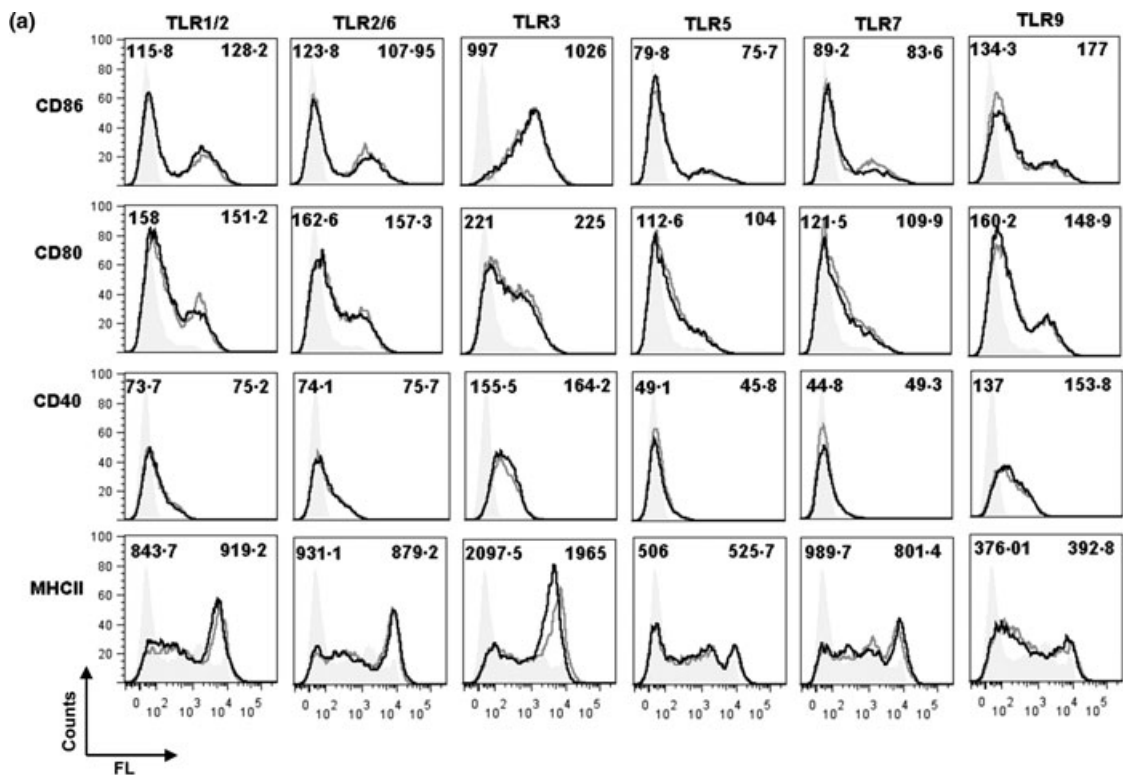
DC after incubation with any of the TLR ligands mentioned above (Figure 3a). Similarly, no effect on the production of IL-1 $\alpha$ , IL-6, IL-10, IL-12p70 and TNF- $\alpha$  (Figure 3b) was observed. In this experiment, *E. coli* LPS was also tested as a positive control. As expected, TspES suppressed the expression of surface molecules and cytokine production induced by *E. coli* LPS S-form (data not shown). For Poly I:C, only the expression of surface markers is shown because this TLR agonist does not induce measurable amounts of any of the tested cytokines. Titration of the different TLR ligands in combination with TspES was performed. TspES did not suppress DC maturation induced by concentrations of TLR agonist 10 fold lower or higher than the ones indicated in Figure 3 (data not shown). These results indicate that the suppressive effect of TspES on DC maturation is restricted to TLR4.

### TspES alter LPS-induced gene expression involved in the TLR signalling pathway

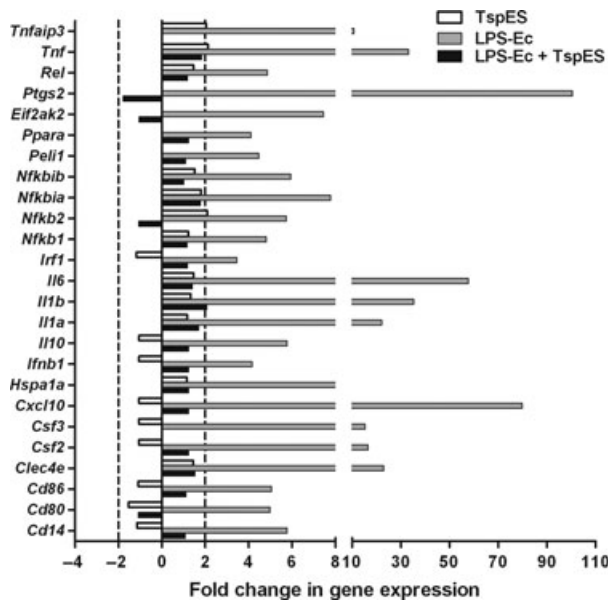
To investigate the effect of TspES on the expression of different genes associated with the TLR signalling pathway, we used a PCR array that measures the gene expression of the different TLRs; the adaptors and proteins that interact with the TLRs and effectors; and members of the NFKB, JNK/p38, NF/IL6 and IRF family. For this experiment, DC were incubated for 6 h with TspES, *E. coli* LPS S-form or the combination of both. No major changes in the expression of genes in DC incubated with TspES only were observed. Of the 84 genes included in this array, 25 were affected by *E. coli* LPS S-form (Figure 4). The presence of 5 µg/mL TspES interfered with the LPS-mediated expression of genes of the MyD88-dependent and independent signalling pathway as well as genes from the NFKB, JNK/p38, NF/IL6 and IRF downstream pathway. The expression of all of these genes was reduced to background levels in the presence of TspES. As expected, reduced expression was observed for genes encoding for different surface molecules and cytokines. Together these results indicate that TspES interfere with the expression of different LPS-induced genes involved in the TLR signalling pathway.

### TspES induce expansion of Treg cells and increase TGF- $\beta$ production

In addition to determining the effect of TspES on the functionality of DC, we were interested in investigating the effect of these helminth products on T cell activation. For this purpose, DC previously incubated with TspES (TspES-DC) for 24 h were cultured with splenocytes from OVA-TCR *tg* mice in the presence or absence of the OVA



**Figure 3** Effect of TspES on DC maturation induced by different TLR ligands. DC were incubated for 24 h with TspES (excretory/secretory products from *T. spiralis*) 5 µg/mL alone or in combination with 100 ng/mL of TLR ligand Pam3CSK4 (TLR1/2), 100 ng/mL FSL-1 (TLR2/6), 10 µg/mL Poly I:C (TLR3), 1 µg/mL, flagellin (TLR5), 5 µg/mL ssRNA 40 (TLR7) and 5 µM ODN 1826 (TLR9). (a) Expression of surface markers on DC incubated in medium only (filled histogram), with TLR ligand (grey line) or TLR ligand + TspES (black line). Numbers inside each histogram are the MFI of TLR ligand alone (left) and TLR ligand + TspES (right). (b) Production of cytokines by DC is represented as mean of triplicate values (pg/mL ± SEM). The data shown are representative of three independent experiments.



**Figure 4** Effect of TspES on *E. coli* LPS S-form-induced expression of TLR signaling pathway genes. DC were incubated with 5  $\mu\text{g}/\text{mL}$  TspES (excretory/secretory products from *T. spiralis*), 1 ng/mL LPSEc or the combination of both for 6 h. The expression of 84 genes involved in the TLR signaling pathway ([http://www.sabiosciences.com/rt\\_pcr\\_product/HTML/PAMM-018A.html](http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-018A.html)) was assessed by real-time quantitative RT-PCR array and calculated relative to five housekeeping genes. Only the genes affected by either TspES, LPS-Ec or LPS-Ec + TspES are shown in the graph. The data is shown as fold-change expression over unstimulated cells and are representative of three independent experiments. Up-regulation or down-regulation of gene expression was considered for values above or below 2 or  $-2$  units of fold change of gene expression, respectively. LPS-Ec, *E. coli* LPS S-form.

protein. T cell responses were measured after 4 days. Results show that in the  $\text{CD4}^+ \text{CD25}^+$  T cell population derived from splenocytes cultured with DC + OVA compared to splenocytes cultured with TspES-DC + OVA, the Foxp3 expression increased in average from 7 to 15.5% and the TGF- $\beta$  expression from 1.5 to 8.6% (Figure 5a–b). The intracellular expression of IL-10 by the  $\text{CD4}^+ \text{CD25}^+$  T cell population was lower when splenocytes were cultured with TspES-DC + OVA, but the difference was not significant (Figure 5c). The cytokine production of splenocytes incubated with TspES-DC + OVA was different compared to cells incubated with DC + OVA. The levels of IL-2, IL-4, IL-5, IL-13 and IL-17 production were comparable, while the production of IFN- $\gamma$ , IL-10 and TGF- $\beta$  varied (Figure 6). The production of IFN- $\gamma$  as well as IL-10 decreased in supernatants of splenocytes incubated with TspES-DC, while the TGF- $\beta$  production increased (Figure 6). To determine whether the Treg population

expanded by TspES-primed DC is because of the TGF- $\beta$  produced, a blocking experiment was performed. Figure 7(a) shows that by adding an antibody against TGF- $\beta$ , the expanded  $\text{CD4}^+ \text{CD25}^+ \text{FOXP3}^+$  T cell population was blocked. As a positive control, rhTGF- $\beta$  was added to the D011.10 splenocytes incubated with OVA and DC only (without TspES) which resulted in an increase from 9% to 13% of the  $\text{CD4}^+ \text{CD25}^+ \text{FOXP3}^+$  T cell population. This expansion was blocked by anti-TGF- $\beta$  (data not shown). Cytokine analysis of the supernatants indicates that addition of anti-TGF- $\beta$  abrogates the decreased IFN- $\gamma$  and IL-10 production induced by the presence of TspES-primed DC. The levels of IL-10 were however, not significantly different from cells without this antibody (Figure 7b). The presence of anti-TGF- $\beta$  did not affect the level of the other cytokines mentioned earlier (data not shown).

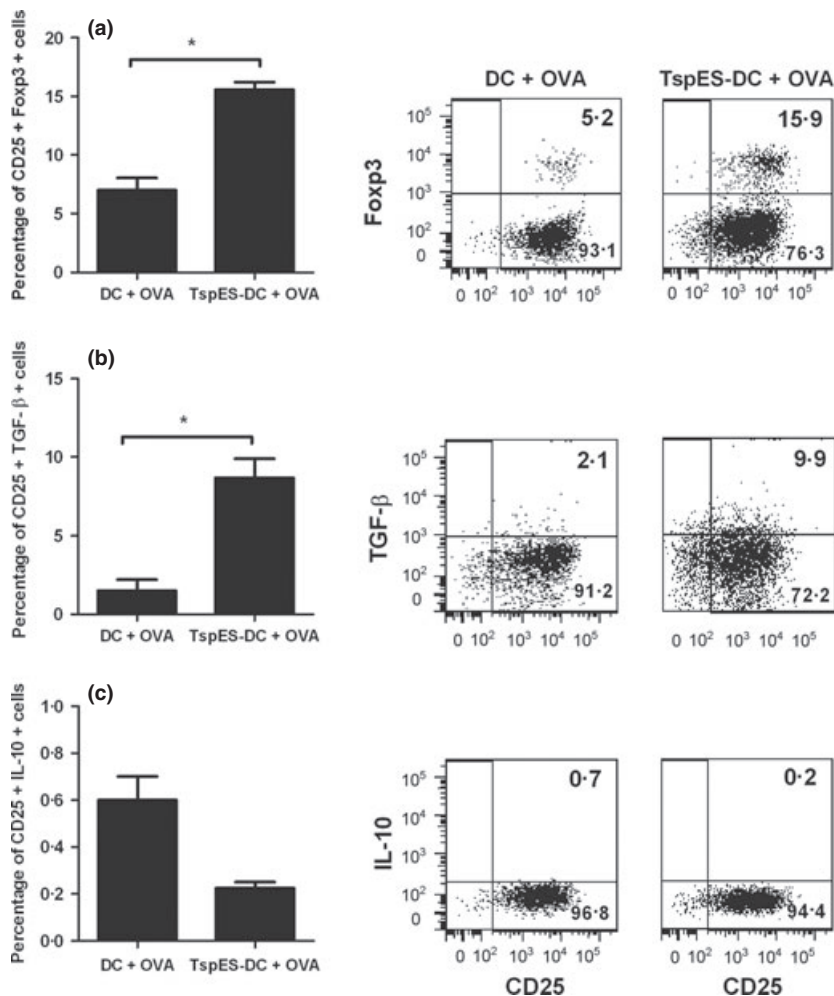
### TspES induce functional Treg cells with suppressive activity

To determine whether the  $\text{CD4}^+ \text{CD25}^+$  T cell population induced by TspES-primed DC are functional Treg cells in terms of suppressive capacity, functional assays were performed. Figure 8 shows suppression of the effector cell proliferation induced by Treg cells isolated from spleen of OVA-TCR tg mice cultured with DC + OVA in the presence or absence of TspES. Results indicate that the percentage of proliferating effector cells decreased from 81 to 67.3% when Treg isolated from the splenocytes incubated with OVA and TspES-primed DC were used, indicating that this  $\text{CD4}^+ \text{CD25}^+$  T cell population has suppressive activity *in vitro*.

## DISCUSSION

We have previously shown that TspES can suppress *in vitro* the maturation of DC induced by the S-form of *E. coli* LPS, but not by the R-form of *N. meningitidis* LPS (16). These two different LPS forms have been shown to activate TLR4 differentially. In contrast to the S-form, the R-form of LPS does not depend on CD14 for TLR4 activation (18,19). This led us to investigate whether suppression by ES from *T. spiralis* was dependent on the form of LPS used, implying a role for CD14. Using now the R-form LPS from *E. coli* and the S-form LPS from *S. minnesota*, we show that this suppression does not depend on the LPS form because TspES can suppress DC maturation induced by both LPS. Therefore, the suppressive effect of TspES on LPS-induced DC maturation is CD14 independent. This suppressive effect depends most likely on the bacterial source of LPS. The

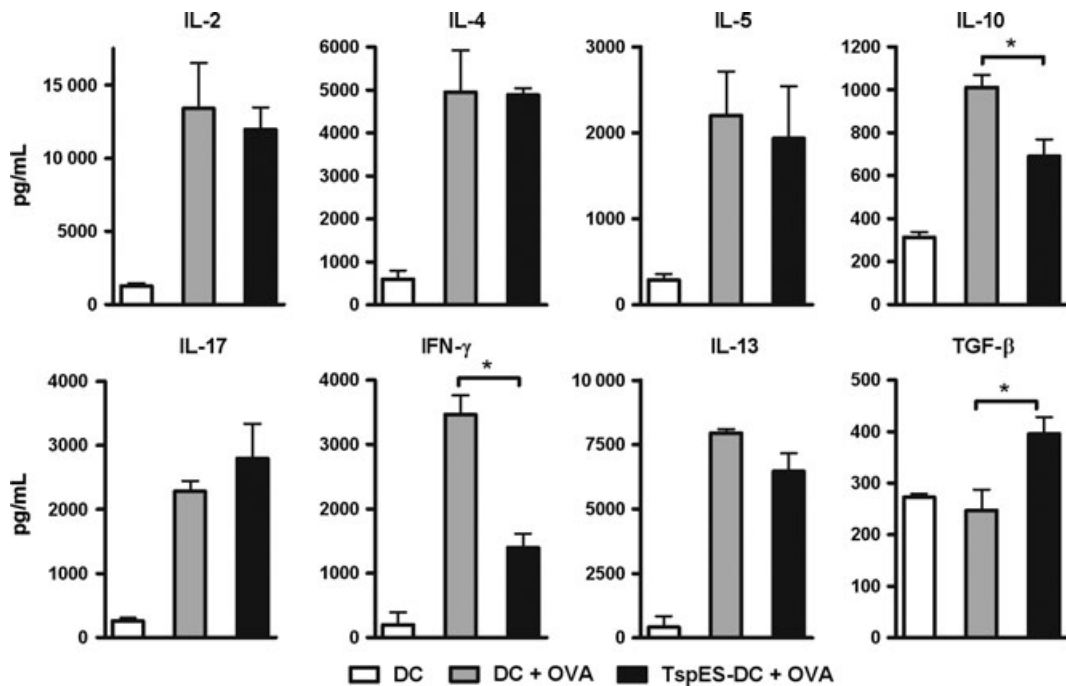




**Figure 5** TspES-pulsed DC increase the expression of Foxp3+ and TGF-β in the CD4+ CD25+ splenocyte cell population. Splenocytes from D011.10 mice were cultured for 4 days in the presence of DC, DC + OVA or TspES-DC + OVA. (a) Percentage of CD25+ cells expressing Foxp3+ gated on CD4+ T cells and representative plots (b) Percentage of CD25+ cells expressing TGF-β gated on CD4+ T cells and representative plots. (c) Percentage of CD25+ cells expressing IL-10 gated on CD4+ T cells and representative plots. Percentages are represented as mean values (% ± SEM). Numbers within the plots represent the percentage of cells in the corresponding quadrant. Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: \* $P < 0.05$ . Data are representative of three independent experiments. TspES, excretory/secretory products from *T. spiralis*.

LPS structure of different enterobacteria such as *Escherichia coli* and *S. minnesota* is similar (25), whereas there is a clear difference between LPS from enterobacteria and *N. meningitidis* LPS. In the lipid A structure of *N. meningitidis* LPS, the localization of the secondary acyl chain is symmetrical, while in enterobacteria LPS is asymmetrical (26). Lipid A represents the conserved molecular pattern of LPS and is the main inducer of immunological responses to LPS. Its composition and number of acyl chains are important in the activation of TLR4/MD2. Different lipid A structures vary considerably in potency to activate DC and can differentially use signal transduc-

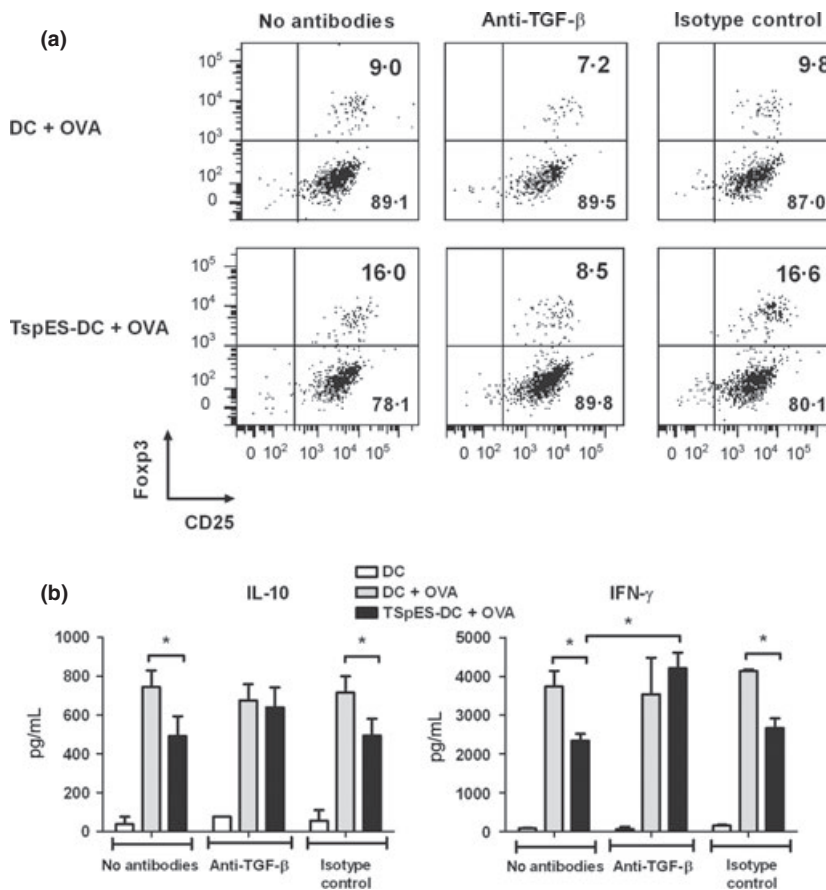
tion pathways leading to diverse patterns of inflammatory responses (18,27,28). During migration from intestine to muscle, *T. spiralis* larvae may drag enterobacteria (29) which could lead to sepsis. Nevertheless, the number of patients with trichinellosis that develop sepsis is very low, 2.11% (30). Perhaps, one of the strategies used by this helminth to prevent the induction of sepsis and ensuring host survival is to inhibit the immune response against enterobacteria LPS. Further studies should be carried out to determine whether *Trichinella*-secreted products suppress DC maturation induced only by LPS derived from enterobacteria.



**Figure 6** TspES-pulsed DC increase TGF- $\beta$  production by splenocytes from D011.10 mice and decreases IFN- $\gamma$  and IL-10 production. Splenocytes from D011.10 mice were cultured for 4 days in the presence of DC, DC + OVA or TspES-DC + OVA. Production of cytokines is represented as mean of triplicate values (pg/mL  $\pm$  SEM). The data shown are representative of three independent experiments. Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: \* $P < 0.05$ . TspES, excretory/secretory products from *T. spiralis*.

In this study, we also show that inhibition of LPS-induced DC maturation is restricted to the TLR4 signalling pathway because TspES does not suppress DC maturation induced by other TLR ligands. This finding is different to the one reported by Hamilton *et al.* (31), who have shown that *Fasciola hepatica* tegumental products suppress cytokine production and expression of co-stimulatory markers on DC induced not only by TLR4 but also by a range of other TLRs. In another study, Kane *et al.* (32) showed that *Schistosoma mansoni* soluble egg products (SEA) suppress IL-12p40 production induced by the engagement of TLR3, TLR4 or TLR9. These findings suggest that the effect of helminth products on TLR-induced DC maturation differs among helminths. TLR4 signalling involves two main intracellular pathways, namely the 'MyD88 (myeloid differentiation primary-response gene 88)-dependent' pathway that mediates the production of pro-inflammatory cytokines and 'MyD88-independent or TRIF' pathway that mediates the up-regulation of co-stimulatory and MHCII molecules by DC. The MyD88-dependent pathway is shared by the other TLRs with the exception of TLR3, which signals through TRIF (33). TspES inhibit the outcome of both, MyD88-dependent (cytokine production) and independent (expression of surface molecules) signalling pathway via TLR4 but not via other TLRs. It is, therefore, likely that

the suppressive effect of these helminth products involves molecules of the TLR4 signalling pathway only and that it occurs at an initial stage of the cascade. Using PCR array, we examined the effect of TspES on the expression of genes involved in the TLR signalling pathways. Although TspES on its own did not induce major changes, these helminth products impaired already after 6 h, the expression of all genes downstream of the TLR pathway induced by LPS. Harnett *et al.* have recently shown that the glycoprotein ES-62 from the nematode *Acanthocheilonema viteae* suppresses TLR-mediated pro-inflammatory responses and that this process occurs as an early event (34). These authors showed that ES-62 induces autophagy on antigen-presenting cells which results in down-regulation of TLR4 and MyD88 expression. Other mechanism by which this helminth could interfere with the TLR signalling pathways is through an indirect effect via interaction with other pattern-recognition receptors, such as C-type lectins (15). For instance, the mycobacterial cell wall component ManLAM has been shown upon binding with DC-SIGN to interfere with TLR4-mediated signals down-regulating in this way the DC-mediated immune responses (35). Products secreted by helminths including those produced by *T. spiralis* are a complex mixture that may modulate the immune response in several ways. The possibility that enzymatic activity in

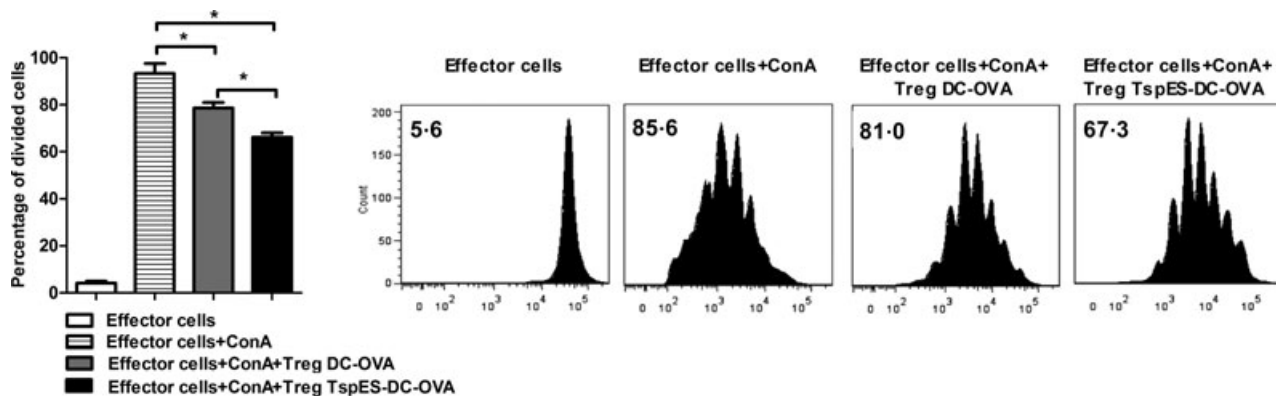


**Figure 7** Anti-TGF- $\beta$  abrogates the expansion of Fcγ3+ expression and the inhibition of IFN- $\gamma$  and IL-10 production induced by TspES. Anti-TGF- $\beta$  and isotype controls (IgG1) were added to splenocytes from D011.10 mice cultured for 4 days in the presence of DC, DC + OVA or TspES-DC + OVA. (a) Percentage of CD25+ cells expressing Fcγ3+ gated on CD4+ T cells. Numbers within the plots represent the percentage of cells in the corresponding quadrant. (b) Production of cytokines is represented as mean values (pg/mL  $\pm$  SEM). Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: \* $P < 0.05$ . Data are representative of two independent experiments. TspES, excretory/secretory products from *T. spiralis*.

the TspES is responsible for suppressing LPS-induced DC could be excluded because heat inactivation did not affect the suppressive activity of this helminth antigen (data not shown). Preservation of the suppressive activity after heat inactivation has been also reported for secretion products from *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* indicating that the products responsible for the suppressive effect are either of a nonprotein nature or denatured proteins that are still functional (36). Other components in the TspES such as lipids or glycans could also be responsible for the observed suppressive activity as has been reported for other helminths (14,15). Identification and characterization of the TspES components with immunomodulatory properties is currently being investigated.

Studies with *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis*, *Schistosoma mansoni* and *Litomosoides*

*sigmodontis* among others have shown that these helminths can modulate the host immune response by inducing suppressive Treg cells that produce TGF- $\beta$  and/or IL-10 (1,37–42). *Trichinella spiralis* has evolved to allow the larvae to complete their cycle in a single host, where the larvae migrate from the gut to the muscle, remaining alive for many years (6). Immunoregulation by this helminth may, therefore, allow the larvae to invade and remain in the muscle, preserving the integrity of the occupied cells and thus keeping their host alive. Local inflammation against the muscle stage of this parasite has been shown to be limited by IL-10 during infection (43). *In vivo* studies have shown that *T. spiralis* infection protects against experimental autoimmune encephalomyelitis and suppresses airway hyperresponsiveness of OVA-challenged mice (10,11). These findings indicate that this helminth can modulate the host immune response.



**Figure 8** TspES-pulsed DC induce CD4<sup>+</sup> CD25<sup>-</sup> T cells with suppressive activity. The proliferative response of effector CD4<sup>+</sup> CD25<sup>-</sup> CFSE-labeled T cells cultured for 4 days with DC and ConA with or without CD4<sup>+</sup> CD25<sup>+</sup> Treg cells isolated from splenocytes of D011.10 mice was measured by flow cytometry. Bars represent the percentages of the cells that have divided. These percentages are expressed as mean values (% ± SEM). Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: \**P* < 0.05. The data shown are representative of three independent experiments. Treg DC-OVA: CD4<sup>+</sup> CD25<sup>+</sup> T cells isolated from D011.10 spleen cells cultured with DC + OVA. Treg TspES-DC-OVA: CD4<sup>+</sup> CD25<sup>+</sup> T cells isolated from D011.10 spleen cells cultured with TspES-DC + OVA. TspES, excretory/secretory products from *T. spiralis*.

However, the role of Treg during infection with this helminth has not yet been well investigated. Here, we show that secreted products from *T. spiralis* muscle larvae expand suppressive Treg cells *in vitro*, as indicated by an increase in the CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells population after splenocytes from D011.10 mice were co-cultured with OVA and TspES-pulsed DC. Unlike our findings, Ilic *et al.* (44) have recently reported that *T. spiralis* antigens do not induce de novo generation of Foxp3<sup>+</sup> T cells *in vitro*. Two major differences could explain the contrasting results. First of all, we used in our *in vitro* cultures OVA protein, whereas these authors used an OVA peptide. *Trichinella spiralis*-secreted products may affect DC antigen processing and presentation, which has consequences for T cell activation. These effects of TspES cannot be evaluated when adding the peptide. In fact, in early studies, we used an OVA peptide, and indeed we did not observe expansion of Foxp3<sup>+</sup> T cells *in vitro* (data not shown). Another important difference is the concentration of *Trichinella* antigen used to prime the DC. Ilic *et al.* used 50 µg/mL that resulted in DC maturation, whereas we used only 5 µg/mL. Although we did not observe increased expression of DC surface markers or cytokine production, we did observe expansion of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells. Studies have shown that low antigen concentrations induce tolerogenic DC (45) and these DC can induce Treg cells (5). Whether that is the case for low *Trichinella* antigen concentrations, and whether higher concentrations result instead in the expansion of effector T cells remains to be investigated. Our study also shows that splenocytes derived from D011.10 mice produce TGF-β when co-cultured with

OVA and TspES-pulsed DC. This cytokine has been shown both *in vitro* and *in vivo*, to be required for the generation of Foxp3<sup>+</sup> inducible Treg cells (46, 47). By blocking TGF-β, we observed that the expansion of the CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cell population is abrogated and the production of IFN-γ and IL-10 is restored. Perhaps, the *in vitro* conditions used in this study may simulate natural conditions during chronic infection in which muscle larvae-secreted products induce Treg cells in a TGF-β-dependent manner. This regulatory mechanism may favour parasite survival and could also benefit the host, as it has been shown for other helminths. For instance, infection with *H. polygyrus* has been reported to suppress the outcome of experimentally airway allergy induced by OVA and house dust mite. Here, the CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells producing TGF-β and IL-10 were shown to down-regulate allergen-induced lung pathology *in vivo* (1). These findings demonstrate that helminth infections can elicit Treg cells able to modulate the response to bystander antigens. Currently, we are investigating whether *in vivo* *T. spiralis* induces Treg cells and what is the effect of infection with this helminth on other immunopathologies.

In this study, we show that *T. spiralis* products have immunomodulatory properties as indicated by the suppression of DC maturation and expansion of functional Treg cells *in vitro*. While the exact mechanisms by which *T. spiralis* antigens mediate immune suppression remains to be elucidate, these findings are relevant because they could contribute to the development of new strategies to treat or prevent inflammatory diseases.

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