



Short communication

Effects of *Ascaris* and *Trichuris* antigens on cytokine production in porcine blood mononuclear and epithelial cells

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A B S T R A C T

Helminth parasites are highly prevalent in swine production, causing chronic infections and considerable morbidity due to growth retardation. Moreover, helminths actively modulate host immune responses to other pathogens and/or vaccines. Here, we investigated the modulatory effects of *Ascaris suum* adult body fluid (ABF) and *Trichuris suis* Soluble Products (TsSP) on the cytokine response in porcine peripheral blood mononuclear cells (PBMCs) and the intestinal epithelial cell line IPEC-J2. In PBMCs, TsSP induced the secretion of IL-6, IL-10 and IL-1 β , but not TNF- α . Moreover, TsSP significantly enhanced the production of bacterial lipopolysaccharide (LPS)-induced IL-6 and IL-10 but suppressed the production of LPS-induced TNF- α . ABF did not induce cytokine secretion from PBMC, but suppressed LPS-induced secretion of TNF- α and IL-6. ABF did not have any effect on cytokine production in IPEC-J2 cells. In contrast, TsSP selectively induced the secretion of IL-6, and enhanced the IL-6 response induced by LPS. The IL-6 response appeared to be a conserved response to *T. suis* products, as significant secretion was also observed in alveolar macrophages. Thus, *T. suis* products have diverse modulatory effects on cytokine secretion in vitro, with IL-6 production a consistent feature of the innate host response.

1. Introduction

Helminth parasites are highly prevalent in swine production and can cause chronic infections and considerable morbidity due to growth retardation. *Ascaris suum*, the large roundworm of pigs, is found worldwide, and along with the whipworm, *Trichuris suis*, is thought to be responsible for significant economic losses (Hale and Stewart, 1979; Haugegaard, 2010; Stewart and Hale, 1988). In addition to direct effects on growth performance, helminth infections are also known to decrease resistance to viral and bacterial co-infections and can diminish the efficacy of vaccines (Adedjei et al., 2009; Steenhard et al., 2009; Vlamincck et al., 2015). Recent research has shown that antigens from *A. suum* and *T. suis* dampen the inflammatory responses of human dendritic cells and macrophages following toll-like receptor stimulation, suggesting that these parasites actively modulate host immune function (Almeida et al., 2018; Laan et al., 2017; Ottow et al., 2014), but their effect on immune cells from their natural porcine host has been examined in less detail.

Here, we explored the effects of antigens from *A. suum* and *T. suis* on cytokine production in porcine cells. We used peripheral blood mononuclear cells (PBMC) and the epithelial cell line IPEC-J2 as a model for the innate interactions between helminths and their host. We quantified cytokine responses to helminth products alone and in combination with lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria that serves as a model for inflammatory responses

induced by prokaryotic pathogens. Our results may have implications for understanding the complex immune-modulation observed during helminth infection in pigs.

2. Methods and materials

2.1. Isolation of peripheral blood mononuclear cells and alveolar macrophages

Heparinized blood was obtained from healthy pigs (Landrace/Yorkshire/Duroc, females and castrated males, aged 12–18 weeks), with the approval of the Danish Animal Experimentation Inspectorate (License number 2015-15-0201-00760). PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). Cells were counted using Trypan Blue staining and suspended in RPMI 1640-medium (Life Technologies), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/mL penicillin and 100 μ g/mL streptomycin. The cells were stimulated immediately after isolation. Alveolar macrophages were isolated as described by Liu et al. (2012). Briefly, healthy pigs (Landrace/Yorkshire/Duroc, females aged 4–5 weeks) were euthanized and the lungs immediately excised. Approximately 50 mL PBS was introduced into the lungs, after which the lungs were massaged for approximately 2 min in order to collect the macrophages. The bronchoalveolar lavage fluid was then filtered and centrifuged at 400 g for 15 min. Cell pellets were washed with Hank's

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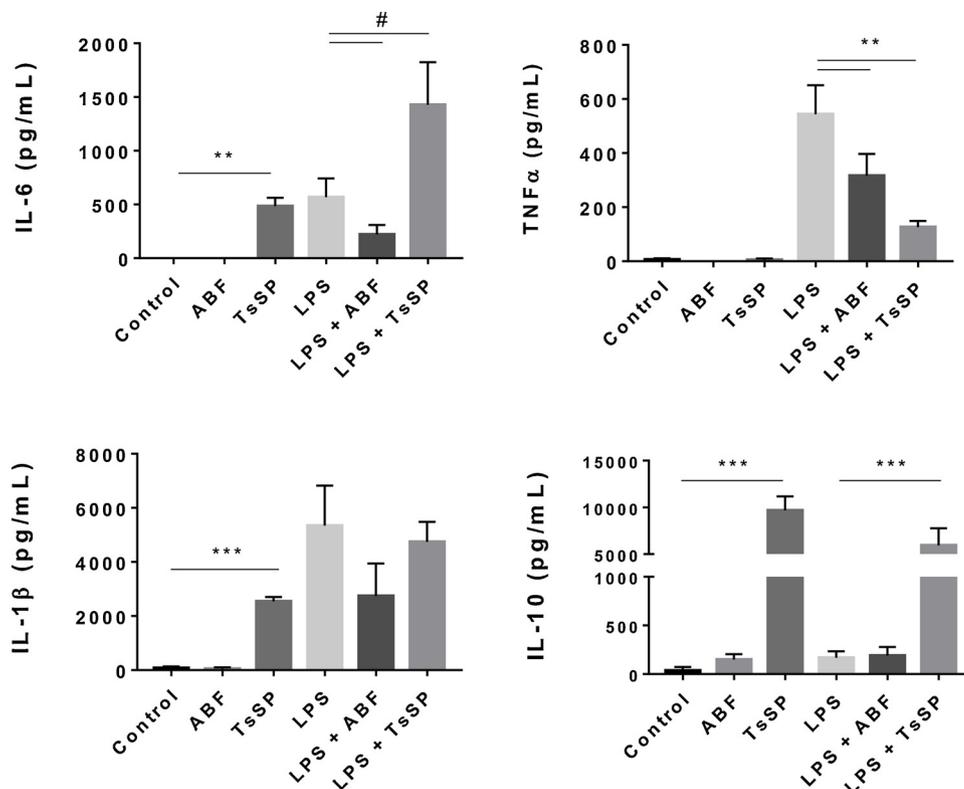


Fig. 1. Concentrations of A) IL-6, B) TNF- α , C) IL-1 β and D) IL-10 released from porcine peripheral blood mononuclear cells after 24 h exposure to either *Ascaris suum* body fluid (ABF) or *Trichuris suis* soluble products (TsSP), with or without current stimulation with lipopolysaccharide (LPS). Shown is the mean \pm S.E.M. of separate experiments, each time using blood from different donor pigs (n = 8 with LPS, and n = 5 without LPS). *** $P < 0.005$; ** $P < 0.01$; # $P = 0.06$ by one-way ANOVA and Fisher's LSD.

balanced saline solution (Sigma-Aldrich), counted and then suspended in supplemented RPMI-1640 media as described above. Non-adherent cells were removed, and the resulting adherent population was examined morphologically and found to consist exclusively consist of macrophage-like cells, which were termed alveolar macrophages (Dickie et al., 2009; Liu et al., 2012).

2.2. IPEC-J2 cell culture

The IPEC-J2 cell line (ACC 701) was grown in T75 tissue culture flasks (Greiner Bio-One) in Advanced DMEM:F12 (1:1) medium (Life Technologies) supplemented with 5% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin and 1% Glutamax (Life Technologies) at 37 $^{\circ}$ C in a humidified atmosphere at 5% CO₂. Cells were sub-cultured by trypsinization.

2.3. Helminth antigens

T. suis worms were harvested from the caecum and colon of infected pigs at necropsy, washed with 0.9% saline and stored at -80° C until needed. *Trichuris suis* Soluble Products (TsSP) were prepared by placing the worms in liquid nitrogen and ground into a powder. The powder was dissolved in PBS, sterile filtered, and protein content determined by BCA assay (Thermo Fisher). *Ascaris suum* adult body fluid (ABF) was obtained by collecting the pseudocoelomic fluid of fresh worms by decapitation. The fluid was centrifuged for 15 min at 10,000 g and the supernatant sterile filtered. Protein concentration was determined as above. ABF was collected from male and female worms in equal ratio. *T. suis* worms were collected in bulk and the gender was not noted, but males and females were expected to be present in approximately equal numbers. Endotoxin contamination in both antigen preparations was measured by LSL assay and found to be below detection limits. Both antigens are known to be strongly recognized by cells and/or antibodies from infected hosts, and were selected on this basis for investigation here of their effects on innate responses (Williams et al., 2017a).

2.4. Cell stimulation

IPEC-J2 cells were stimulated with LPS and/or helminth products similarly to the protocol described by Sargeant et al. (2011). Cells were seeded into 6-well tissue culture plates at a concentration of 5×10^5 cells per well and cultured for 48 h to reach confluence. The medium was then replaced, and the cells stimulated by adding 1 μ g/mL LPS (*Escherichia coli* O26:B6, Sigma-Aldrich) and/or ABF or TsSP at a concentration of 100 μ g/mL. The concentrations of parasite antigens was determined by preliminary experiments which showed that lower concentrations did not reliably induce activity in PBMCs. Unstimulated wells were used as negative controls, and each experiment performed with duplicate wells. Supernatant was harvested after 24 h and stored at -80° C until use. PBMCs were challenged similarly to IPEC-J2 cells but were prepared in 24-well tissue culture plates in concentrations of 5×10^6 cells/mL. Supernatant was harvested after 24 h as above. Alveolar macrophages were seeded into 48-well culture plates at a final concentration of 6×10^4 cells/well, and incubated at 37 $^{\circ}$ C, 5% CO₂ overnight to allow attachment. The following day, the medium was replaced, and the cells cultured in triplicate with TsSP at a final concentration of 50 μ g/mL or were unstimulated. Supernatant was harvested after 24 h as above.

2.5. ELISA

Concentrations of secreted IL-1 β and IL-6 (R&D Systems), and IL-10 and TNF- α (Life Technologies) were determined by sandwich-ELISA on Maxisorb ELISA plates (Nunc, Denmark). using appropriate antibody pairs according to the manufacturers' instructions. The plates were read at 450 nm on a MultiSkan FC Microplate Photometer and the concentrations of the samples determined with SkanIt software (Thermo Fisher Scientific) using a 4-parameter logistic standard curve.

2.6. Statistical analysis

The effect of helminth antigens on cytokine production in non-LPS

or LPS-treated cells was assessed by one-way ANOVA, or Friedman's test where data were non-normally distributed. Where significant ANOVA effects were observed, post-hoc testing was conducted with Fisher's LSD. The effect of TsSP on IL-6 production in alveolar macrophages was assessed by paired t-test. Analyses were performed using Graphpad Prism (Graphpad Software, San Diego, CA) and significance taken at $P < 0.05$.

3. Results and discussion

We first assessed the effect of *A. suum* and *T. suis* products on cytokine production by porcine PBMCs, and whether responses to LPS were modulated by concurrent exposure to the helminth antigens. TsSP induced marked secretion of IL-6, IL-10 and IL-1 β (all $P < 0.005$; Fig. 1), but not TNF- α , while ABF did not induce secretion of any of the tested cytokines. When co-cultured with LPS, significant modulatory effects on the LPS-induced responses were apparent. TsSP enhanced the LPS-induced production of IL-6 ($P = 0.06$; Fig. 1A) and IL-10 ($P < 0.05$; Fig. 1D), and reduced LPS-induced TNF- α secretion ($P < 0.05$; Fig. 1B). ABF reduced LPS-induced IL-6 ($P = 0.06$; Fig. 1A) and TNF- α secretion ($P < 0.05$; Fig. 1B). ABF did not significantly affect LPS-induced secretion of IL-10. LPS-induced IL-1 β was unaffected by exposure to the helminth products.

We next explored whether the helminth products influenced cytokine production by epithelial cells. IL-10, TNF- α and IL-1 β secretion were not consistently detected in response to stimulation with either antigen. In contrast, IL-6 was significantly secreted in response to TsSP (Fig. 2A). The amount of IL-6 induced by co-exposure to LPS and TsSP was higher than that induced by LPS alone ($P < 0.05$). TsSP and LPS did not additively increase IL-6 production, as had been observed for PBMC, with levels similar to that of TsSP stimulation alone. ABF did not induce IL-6 production, either alone or in combination with LPS (Fig. 2A).

Given that IL-6 appeared to be specifically produced in response to TsSP stimulation, we sought to assess the breadth of this response by assessing TsSP-induced IL-6 secretion in alveolar macrophages, which represent a convenient model of an innate myeloid immune cell residing at the mucosal surface. Cells exposed to TsSP secreted significant amounts of IL-6, indicating an apparently conserved host innate response (Fig. 2B).

Our results indicate that *T. suis* products induce the secretion of a diverse set of cytokines from peripheral immune cells. IL-6 was also specifically induced in epithelial cells and secreted in large amounts by alveolar macrophages. In agreement with this, *T. suis* excretory-secretory products have previously been shown to induce the secretion of IL-6 in the porcine intestinal cell line IPEC-1 (Parthasarathy and Mansfield, 2005). We further observed that IL-6 was induced in PBMCs in large amounts by LPS, with an additive effect between LPS and TsSP,

suggesting that in some circumstances *T. suis* may increase bacterial-induced inflammatory responses, consistent with reports that *T. suis* infection synergizes with some bacteria to induce colonic inflammation in pigs (Mansfield et al., 2003). IL-6 is a multifaceted cytokine which may play a key role in both type-1 and type-2 immune responses, as well as maintaining epithelial cell homeostasis (Nguyen et al., 2015). In mice, IL-6 can regulate susceptibility to the helminth *Heligmosomoides polygyrus*, where IL-6 deficient mice show significantly reduced worm burdens after 28 days of infection, as well as increased clearance of *Schistosoma mansoni* (Angeli et al., 2001; Smith and Maizels, 2014). IL-6 is produced in large amounts by monocytes and dendritic cells following exposure to bacterial and viral antigens, and its role in pro-inflammatory responses is well-known (Tanaka et al., 2016). However, it also plays key roles in wound-healing and resolution of tissue damage, and can promote enterocyte survival and barrier function in the intestine (Jin et al., 2010). Notably, *IL6* gene expression is increased in the colon of pigs infected with *T. suis* at both the larval and adult stage, which suggests that it might play a key role in host-*Trichuris* interactions (Myhill et al., 2018; Wu et al., 2012). The precise role that IL-6 plays in *T. suis* infections is not yet clear, but given the diverse biological functions mentioned above, may be involved in initiation of innate responses to infection as well as potentially regulating homeostasis as the infection progresses.

We also noted that TsSP suppressed TNF- α production, and increased IL-10 secretion, in PBMCs stimulated with LPS. This would suggest a regulatory effect, in agreement with reports that *T. suis* can dampen autoimmune responses in human patients (Elliott and Weinstock, 2017). Whilst it is not possible to directly compare results between a mixed PBMC population, as we used here, and purified cell populations, it is noteworthy that human dendritic cells and macrophages also secrete more IL-10 and less TNF- α in response to LPS when co-cultured with TsSP (Williams et al., 2017b). This is suggestive of a marked ability of TsSP to alter the cytokine balance of host immune cells. The functional implications of this for porcine health clearly need further exploration, as does the identification of the specific cells within porcine PBMCs that respond to *T. suis* antigen.

In contrast to the results with TsSP, ABF was shown to be non-immunogenic, eliciting no cytokine responses by itself and tending to have suppressive effects on LPS-induced cytokine production. This is in agreement with results in human immune cells where ABF strongly suppresses cytokine production following LPS treatment (Midttun et al., 2018). The lack of response in epithelial cells is perhaps consistent with recent reports that live *A. suum* larvae induce minimal transcription of immune genes in IPEC-J2 cells, suggesting a degree of immune quiescence to this parasite (Ebner et al., 2018). Despite this, it is known that *A. suum* infections in pigs do induce a robust expulsion of worms and protective immunological memory responses (Jungersen et al., 2002), however the host-parasite cellular interactions that induce this

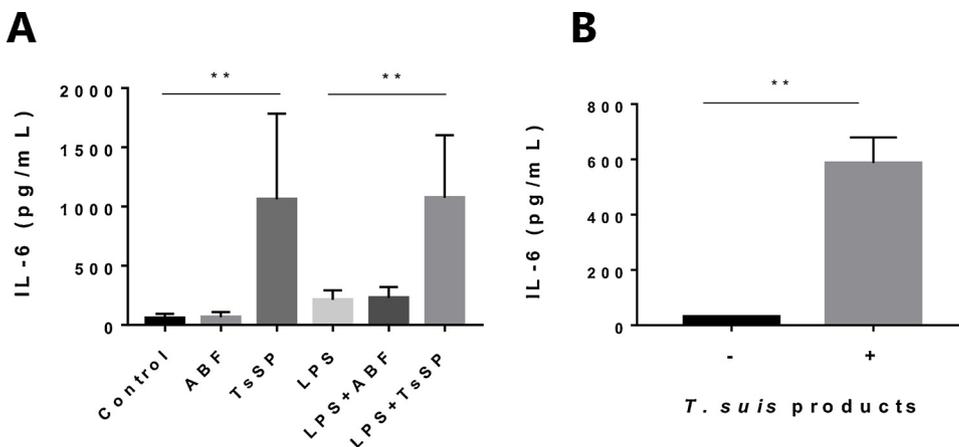


Fig. 2. A) Concentration of IL-6 released from IPEC-J2 cells after 24 h exposure to either *Ascaris suum* body fluid (ABF) or *Trichuris suis* soluble products (TsSP), with or without current stimulation with lipopolysaccharide (LPS). Shown is the mean \pm S.E.M. of separate experiments ($n = 5$). $**P < 0.01$ by one-way ANOVA and Fisher's LSD. B) Concentration of IL-6 released from porcine alveolar macrophages after 24 h exposure to *Trichuris suis* soluble products (TsSP). Shown is the mean \pm S.E.M. of separate experiments ($n = 3$) each time using cells from a different donor pig. $**P < 0.01$ by paired t-test.

immunity remain undefined.

In conclusion, we have shown that *T. suis* antigens induce secretion of IL-6 specifically from porcine epithelial cells and have a broad immunomodulatory activity in PBMCs. This may aid our understanding of the complex host-parasite relationship of *T. suis* in its natural host. In contrast, the *A. suum* antigens used in this study induced little response in the host cells, but had a tendency to suppress cytokine production by PBMCs. Further work is needed to understand how these parasites interact with the host innate immune system, in order to guide rational immunological-based control strategies (e.g. vaccines), and to better understand how they modulate responses to other pathogens.

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