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Cytokine 156 (2022) 155919

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Antigens from the parasitic nematode *Trichuris suis* induce metabolic reprogramming and trained immunity to constrain inflammatory responses in macrophages

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ARTICLE INFO

Keywords:
Bone marrow-derived macrophages (BMDMs)
Inflammation
Toll-like receptors (TLRs)
Trichuris suis soluble products (TSPs)

ABSTRACT

Regulation of macrophage (Mϕ) function can maintain tissue homeostasis and control inflammation. Parasitic worms (helminths) are potent modulators of host immune and inflammatory responses. They have evolved various strategies to promote immunosuppression, including redirecting phagocytic cells toward a regulatory phenotype. Although soluble products from the whipworm *Trichuris suis* (TSPs) have shown significant effects on Mϕ function, the mechanisms underlying these modulatory effects are still not well understood. In this study, we find that TSPs suppressed inflammatory cytokines (TNF and IL-6) in Mϕs stimulated with a broad panel of TLR agonists, whilst inducing IL-10. Moreover, M1 markers such as MHCII, CD86, iNOS, and TNF were down-regulated in TSP-treated Mϕs, without polarizing them towards an M2-like phenotype. We showed that TSPs could establish a suppressed activation state of Mϕs lasting at least for 72 h, indicating an anti-inflammatory innate training. Moreover, we found that TSPs, via repression of intracellular TNF generation, decreased its secretion rather than interfering with the release of surface-bound TNF. Metabolic analysis showed that TSPs promote oxidative phosphorylation (OXPHOS) without affecting glycolytic rate. Collectively, these findings expand our knowledge on helminth-induced immune modulation and support future investigations into the anti-inflammatory properties of TSPs for therapeutic purposes.

1. Introduction

Helminths have co-evolved with their human and animal hosts and developed an array of molecules that modify the host immune system supporting their long-term survival [1]. To this end, helminths tend to suppress inflammation via releasing molecules and promote the activation and recruitment of immune cells with an immunoregulatory phenotype, such as alternatively activated macrophages (M2 Mϕs) [2]. It is well-established that Mϕs play a key role in activating the adaptive immune response (Th1 or Th2). Thus, regulation of Mϕ function in the inflammatory context is essential for tissue homeostasis [3]. M1 Mϕs (pro-inflammatory) dominate during inflammatory conditions, while M2 Mϕs are mainly associated with the resolution of inflammation and tissue repair [4]. Dysregulation of the M1/M2 balance and its consequences are central, but understudied, to our understanding and treatment of autoinflammatory disorders, tumors, and infections [3].

Helminths can redirect inflammatory Mϕs and dendritic cells (DCs) towards an anti-inflammatory phenotype. Parasite antigens can mitigate toll-like receptor (TLR)-mediated inflammatory signals [5], and thereby partly compromise host immune responses [6,7]. For instance, DCs and Mϕs treated with parasite antigens show hyporesponsiveness to inflammatory agents such as TLR agonists [8,9]. In this regard, pre-treatment of DCs with soluble products of *Schistosoma mansoni* egg antigens (SEA) and antigen B (a hydatid cyst-derived antigen) from *Echinococcus granulosus* suppressed the expression of activation markers such as MHCII, CD80, CD86, and pro-inflammatory cytokines like TNF

Abbreviations: BMDMs, Bone marrow-derived macrophages; TLRs, Toll-like receptors; TSPs, *Trichuris suis* soluble products; NO, Nitric oxide; Mϕs, Macrophages; OCR, Oxygen consumption rate; ECAR, Extracellular acidification rate; OXPHOS, Oxidative Phosphorylation; TNF, Tumor Necrosis Factor; DCs, Dendritic cells; ROS, Reactive oxygen species.

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https://doi.org/10.1016/j.cyto.2022.155919
Received 2 March 2022; Received in revised form 13 May 2022; Accepted 17 May 2022
Available online 29 May 2022

[8,10]. Moreover, SEA have been found to release molecules such as lysophosphatidylserine, lacto-N-fucopentaose III, and double-stranded RNA that reduce LPS-induced inflammatory responses via inhibition of TLR signalling and by polarizing DCs toward an anti-inflammatory phenotype [5]. Likewise, the phosphorylcholine-containing glycoprotein ES-62, secreted by the filaria worm *Acanthocheilonema viteae,* interrupts TLR4 signalling and thus reduces the production of inflammatory cytokines [11,12]. Hartmann and colleagues also observed that a single adoptive transfer of AvCystatin (derived from *A. viteae*)-treated Mϕs to mice before allergen challenge not only downregulates local and systemic Th2 responses (allergen-specific IgE, the influx of eosinophils into the airways, IL-4, and IL-13) and mucus production in lung bronchioles but also elevates local and systemic IL-10 production by $CD4^+$ T cells [13]. In addition, several studies showed that *Trichuris* spp. releases immunosuppressive components that interfere with TLR signalling [14,15]. *T. muris* was shown to induce anti-inflammatory responses by conditioning immune cells to release IL-10 and TGFβ and suppression of inflammatory cytokines (TNF and IL-6) [16]. Prostaglandin E2 (PGE2) is released by *T. suis* and interferes with TLR signalling by impairing signalling downstream of MyD88 and the TIR-domain-containing adaptor-inducing IFNβ [17,18]. Molecular dissection revealed that *T. suis* soluble products (TSPs) induce overexpression of Rab7b, resulting in TLR4 downregulation in Mϕs [18,19].

Emerging findings have highlighted the central role of cellular metabolism in immune cells differentiation and function. Pathogens have shown an extensive ability to modulate host Mϕs metabolism [20]. Since helminths redirect Mϕs toward M2 phenotype, their metabolic signature displays enhanced oxidative phosphorylation, lipid oxidation, and amino acid metabolism while suppressed glycolysis [21]. In this way, SEA has shown a profound metabolic effect on host Mϕs supporting lipid oxidation [22]. SEA affects the expression level of genes associated with fatty acid oxidation [23] and glycolysis in Mϕs, leading to activation of the AMPK, AKT, and mTORC1 pathways [24]. However, the duration of the immunomodulatory effects of TSPs is still unknown nor is it known whether TSPs alter the metabolic signature of Mϕs.

Here, we show that TSPs suppress inflammatory mediators, including TNF, IL-6, IL-12, IL-1β, and CCL5, while inducing IL-10 in Mϕs stimulated with a panel of TLR agonists. Moreover, M1 markers such as MHCII, CD86, and iNOS were downregulated in TSP-treated BMDMs while no polarization toward the M2 phenotype was observed. TSPs impaired reactive oxygen species (ROS) and nitric oxide (NO) generation that was further corroborated by downregulation of iNOS expression in TSP-stimulated BMDMs. We showed that TSPs could establish a state of immunosuppression in BMDMs by promoting persistent anti-inflammatory conditions. Moreover, we demonstrated that TSPs repress the intracellular TNF but do not prevent the release of transmembrane TNF (tmTNF). Finally, we observed that TSPs support OXPHOS activity while not affecting the glycolysis rate. Collectively, this study offers new insights into the regulatory mechanisms of TSPs.

2. Materials & methods

2.1. Preparation of helminth soluble product

TSPs were prepared as previously described by Kuijk *et al.* [25]. Briefly, adult *T. suis* were collected from the caecum and proximal colon of infected pigs and washed thoroughly with saline three times and then 7 times with RPMI 1640 medium supplemented with antibiotics over a period of 4 h, all at 37 °C, 5% CO_2. TSPs were prepared by homogenizing whole worms in phosphate-buffered saline (PBS) as reported by Kuijk *et al.* [25]. Protein concentration was measured using bicinchoninic acid assay (BCA) (Thermo Fisher) and endotoxin level was evaluated by LAL assay (Thermo Fisher), which was below detection limits.

2.2. BMDMs cell culture

Murine bone-marrow-derived macrophages (BMDMs) were obtained as described previously [26]. Briefly, to generate BMDMs, 8-week-old C57BL/6 mice were sacrificed, then femurs and tibias were separated and washed with PBS and disinfected with 70 % ethanol. Subsequently, bone marrow cells were harvested in a sterile 50 ml conical centrifuge tube on ice. Cells were centrifuged for 5 min at 300g, and after removing the supernatant, the cells were resuspended in RBC lysis buffer for 1 min at room temperature. After that, RPMI 1640 (Gibco/Life Technologies) containing 10% fetal bovine serum (FBS) (GIBCO BRL, Life Technologies, Inc.) and 100 g/ml penicillin–streptomycin (100 U mL-1: 100 U mL-1; Sigma–Aldrich) was added and centrifuged for 5 min at 300g. The supernatant was removed, and the cell pellet was resuspended in RPMI 1640 and transferred to sterile Petri dishes. To generate BMDMs, the BM cells were cultured in RPMI 1640 containing 10% FBS supplemented with 20% L929 cell supernatant (containing macrophage colony-stimulating factor) [27]. Exposure of BM cells to granulocyte–macrophage colony-stimulating factor (GM-CSF 20 ng/ml) was led to the generation of a type of pro-inflammatory BMDMs [28].

The BM cells were cultured in a humidified incubator with 5% CO_2 at 37 °C for 7 days with media exchange every 2 days. The purity of BMDMs was evaluated using two surface markers with fluorochrome-conjugated anti-mouse antibodies, including labelled mouse CD11b & F4/80. More than 95% of the differentiated cells were positive for corresponding markers.

2.3. ROS measurement

ROS measurement was carried out using 2′,7′–dichlorofluorescein diacetate-based DCFDA/H2DCFDA-Cellular ROS Assay (Invitrogen) according to the manufacturer's instructions. DCFDA is passively absorbed by cells and cleaved by intracellular esterases, and oxidized by intracellular ROS, generating a highly fluorescent compound, DCF (2′,7′–dichlorofluorescein). Briefly, 100,000 BMDMs were plated in 96-well flat-bottom microplates in RPMI 1640 supplemented with FBS. BMDMs were pre-treated with TSPs or PBS for 30 min before stimulation with LPS for 5 h. BMDMs supernatants were collected for ELISA and media was replaced with 100ul fresh RPMI 1640 containing DCFDA. BMDMs were treated with 4 μM DCFDA probe and incubated in the dark for 40 min (37 °C, 5% CO2). Next, BMDMs were centrifuged for 4 min at 500 g and the supernatant was discarded. The cell pellet was resuspended in FACS buffer and the DCF level was detected in the green channel (FITC spectrum) using BD LSRII Flow Cytometer (BD, Biosciences).

2.4. Nitric oxide measurement

Nitrite, a stable NO metabolite, was measured in cell-free supernatants using the Griess assay (Promega, Denmark) and was performed as described by the manufacturer. Briefly, supernatants were mixed with an equal volume of Griess reagent. The absorbance was measured at 540 nm using a microplate reader and a sodium nitrite calibration curve was used to measure the nitrite concentrations.

2.5. Cell stimulation

BMDMs were seeded into 48-well culture plates (Sarstedt, Germany) at a density of 100,000 cells/well. Cells were incubated for 2 h at 37 °C and 5% CO2 to allow attachment. Thirty minutes before stimulation with TLR agonists, cells were pre-treated with the TSPs (20 μg/ml, otherwise indicated) or PBS. The BMDMs were stimulated with LPS (10 ng/ml, 100 ng/ml, 1,000 ng/ml), IFNγ (50 ng/ml), CpG-ODN (7.5 μM), Poly I:C (10 μg/ml), R848 (10 μg/ml), zymosan (50 μg/ml), Pam3Csk4 (100 ng/ml), IL-4 (50 ng/ml), or PBS. After 24 h, the supernatants were collected for cytokine measurement, and cells were detached for flow

cytometry. For assessment of long-term effects, BMDMs were stimulated with TSPs (20 μg/ml) for 24 h and subsequently, cells were thoroughly washed with PBS and after refreshment of media, cells were incubated (37 °C and 5% CO2). After 72 h incubation BMDMs were stimulated with aforementioned TLR agonists for 24 h.

2.6. Flow cytometry on BMDMs

To block Fc binding and to discriminate dead cells, BMDMs were incubated with anti-mouse FcR antibody (eBioscience), Aqua live/dead staining (Invitrogen), and anti-surface TNF (Biolegend, Cat# 506324) for 30 min at 4 °C in FACS buffer. Subsequently, cells were washed three times with FACS buffer. Next, cells were washed and fixed with 2% paraformaldehyde for 10 min at room temperature before intracellular staining. Cells were permeabilized with 1X eBioscience permeabilization buffer for 5 min at room temperature. Lastly, cells were stained in a final volume of 50 μl FACS buffer containing labelled antibodies in two different panels for 30 min at 4 °C. The antibodies used for staining includes: anti-CD11b-PE-Cy7 (eBio, Cat# 25–0112-82); anti-F4/80-APC-Cy7 (eBio, Cat# 47–4801-82); anti-MHCII-APC (eBio, Cat# 17–5321-82); anti-CD86-PE (BD Biosciences, Cat# 553692); anti-PDL-2-Percp-Cy5.5 (eBio, Cat# 46–9972-80); anti-NOS2-PE (eBio Cat# 12–5920-80), anti-YM-1-Percp-Cy5.5 (Biolegend, Cat# 405214); anti-RELMα-APC (Invitrogen, Cat# A21244); anti-CD206-FITC (Biolegen, Cat# 141703); anti-CD301-FITC (Biorad, Cat# MCA2392A488); anti-CD200-APC (Biolegend, Cat# 123809); anti-CD80-APC (e-Bio, Cat# 17–0801-81); anti-CCR7-BV605 (Biolegend, Cat# 120125); anti-TNF-PE-Cy7 (Biolegend, Cat# 506324), anti-IL-6-APC (Biolegend, Cat# 504507). To calibrate the machine, unstained cells and single fluorochrome stained BD™CompBeads Compensation Particles (BD Biosciences) were included. The BD FACSCanto II or BD LSRII Flow Cytometer (BD, Biosciences) was used, and results were analyzed with the FlowJo software version 7.6.5 (Tree Star). Results are reported as geometric mean fluorescence intensity (MFI). Fluorescence-minus-one (FMO) controls were used for gating positive cells. BMDMs were identified as $F4/80^+$ $CD11b^+$ cells.

2.7. ELISA

The concentration of TNF, IL-10, CCL5, and IL-6 released by BMDMs was measured by ELISA (R&D Systems, Minneapolis, Minn), as per the manufacturer's protocol and the concentrations were determined against a standard curve.

2.8. Seahorse extracellular flux analysis

BMDMs were plated at $7x10^4$ cells/well into XFe96 cell culture microplates (Agilent) and were allowed to adhere during 4 h incubation at 37 °C and 5% CO2. BMDMs were stimulated with PBS, LPS and TSPs for 24 h. Sensor cartridge (Agilent Technology) was pre-incubated the day before running the XF Assay with 200 μl/well XF96 calibrant solution on one utility plate overnight at 37 °C in a non-CO_2 humidified incubator. Cell culture media was replaced with RPMI 1640 (GIBCO) supplemented with 1% L-glutamine + 5% FCS and incubated for 1 h at 37 °C without CO_2. To measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), different components were prepared, including 10 mM glucose, 1 μM Oligomycin, 2 μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and 1 μM Rotenone/Antimycin A (R/AA) with the Seahorse XFe-96 Bioanalyzer (Agilent) and were analyzed using XFe Wave software. Each measurement cycle consisted of 3 min mix, 0 min wait, and 3 min measure. After the measurement, cell numbers were counted, and all the results were normalized to the number of cells.

2.9. Statistical analysis

Student's *t*-test was used to compare two groups and one-way ANOVA was used for more than two groups using Bonferroni correction to adjust for multiple testing. Data were analyzed with Graphpad version 9, and p-values < 0.05 were considered significant. Each experiment was conducted two or three times and represented as mean ± SEM.

3. Results

3.1. TSPs suppress inflammatory cytokines while inducing IL-10 in BMDMs

TSPs did not cause cell death in BMDMs at the tested concentrations (data not shown). To explore dose–response effects of TSPs on BMDMs, BM cells were differentiated into Mϕ (M0) and stimulated with different concentrations of TSPs (1, 5, 10, 20, 40, 80 μg/ml) for 0.5 h and subsequently stimulated for 24 h with LPS (10 ng/ml). We observed that TSPs suppress LPS-induced TNF and IL-6 in a dose-dependent manner (Fig. 1A). In addition, we showed that TSPs also suppressed LPS-induced IL-6 and TNF when used at higher concentrations (100 ng/ml and 1 μg/ml) whilst increasing IL-10 production (Fig. 1B). Thus, TSPs showed a potent ability to suppress LPS-induced pro-inflammatory cytokine production by BMDMs. LPS induces a high level of NO and ROS in inflammatory Mϕs [29,30] and we observed that TSPs reduced this production in LPS stimulated BMDMs (Fig. 2A). As a positive control, the suppressive effects of TSPs on ROS production were compared with the potent ROS scavenger N-acetylcysteine (NAC).

To examine whether TSPs engaged with receptors other than TLR4, we included a panel of both surface and intracellular located TLR agonists such as PAM3CSK4 1 μg/ml (TLR1/2 agonist), Zymosan 50 μg/ml (TLR2/6), Poly I:C 10 μg/ml (TLR3), R848 10 μg/ml (TLR7/8), CpG-ODN 10 μg/ml (TLR9). TNF and IL-6 production in response to all tested TLR agonists was suppressed by TSP exposure (except for IL-6 following zymosan stimulation). However, TSPs induced higher IL-10 production in response to zymosan and Pam3CSK4 but suppressed this cytokine in the presence of intracellular TLR agonists (Poly I:C, R848, and CpG-ODN) (Fig. 2B).

Thus far, we pre-treated M0 Mϕs (non-polarized or naive phenotype) with TSPs and subsequently exposed them to TLR agonists. M1 Mϕs (treated with LPS + IFNγ) and also Mϕs differentiated from GM-CSF-treated BM cells represent an inflammatory phenotype [31,32]. To evaluate whether TSPs can also suppress Mϕs differentiated from GM-CSF-generated BM cells, they were exposed to TSPs followed by TLR stimulation. TSPs reduced TNF and increased IL-10 production in GM-CSF generated BMDMs in response to TLR stimuli, while no difference was observed for CCL5 (Supp Fig. 1A). Similarly, TSPs downregulated TLR induced TNF and IL-6 in Mϕs polarized by LPS + IFNγ (Supp Fig. 1B).

Finally, we altered the order of stimulation and pre-treated the BMDMs with TLR agonists, and subsequently exposed the cells to parasite antigens. TSPs could dampen LPS and the combination of R848 and Poly I:C-induced TNF secretion from BMDMs while no effect on IL-6 was observed (Supp Fig. 2).

3.2. TSPs control intracellular TNF production but not transmembrane TNF in response to LPS

To investigate whether TSPs immediately inhibit the release of inflammatory cytokines or over time, we performed a time-course experiment in which BMDMs were exposed to LPS with or without TSPs for 1, 3, 5, 7, 9, 12 or 24 h followed by quantification of TNF, IL-6, IL-10, and CCL5. We observed that TNF secretion remained low and constant in TSP-treated BMDMs after 3 h stimulation and onwards (Supp Fig. 3A). While TSPs initially synergized with LPS to induce IL6, we observed a

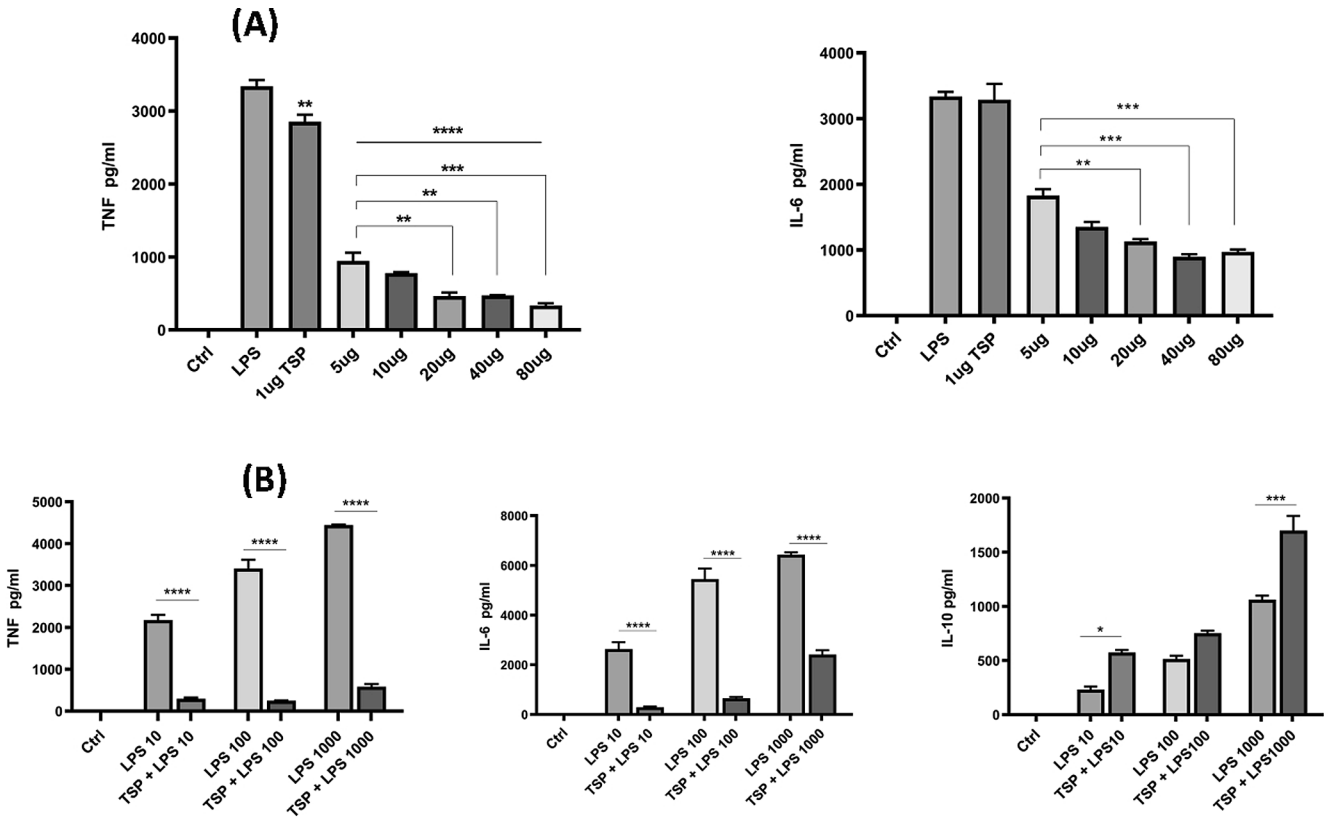

Fig. 1. ***Trichuris suis*** **soluble products (TSPs) suppress lipopolysaccharide (LPS)-induced inflammatory cytokines in macrophages (Mϕs).** (A) Mϕs were pre-incubated with TSPs (ranging from 1 to 80 ug/ml) for 0.5 h and stimulated with LPS (10 ng/ml) for 24 h. (B) Mϕs were pre-treated with TSPs (20ug/ml) and exposed to different LPS concentrations (10, 100, 1000 ng/ml) for 24 h. TNF, IL-6, and IL-10 released in the culture medium was measured by ELISA. TSPs and LPS alone was included as control. All values are expressed as mean ± SEM of three independent experiments *P < 0.05, **P < 0.01, and ***P < 0.001.

significant suppression after 9 h (Supp Fig. 3B). TSPs induced production of IL-10 over time, whilst CCL5 as an inflammatory chemokine remained unaffected (Supp Fig. 3C, D). The intracellular level of IL-6 was also evaluated in TSP- and PBS-treated BMDMs after 5 h in response to inflammatory stimuli and consistent with the extracellular IL-6 level, we did not observe suppression in early hours (Supp Fig. 4). Altogether, we found that TSPs rapidly suppressed TNF released from LPS-treated BMDMs while slowly downregulated IL-6.

TNF is a potent pro-inflammatory cytokine that is synthesized in a precursor form known as tmTNF. tmTNF is cleaved by metalloproteinases and released in a soluble form to be circulated and exert biological activities [33]. To explore whether TSPs can confine TNF generation intracellularly or suppress tmTNF cleavage, we analyzed the expression of the intracellular and transmembrane forms of TNF by flow cytometry. We observed that TSPs strongly reduced TNF production intracellularly in response to all tested inflammatory stimuli (Fig. 3A). However, while no differences were seen in the tmTNF level between TSP-treated and control in response to LPS (Fig. 3B), tmTNF was upregulated in TSP-treated BMDMs exposed to Poly I:C and R848, suggesting that in these conditions, TSPs can suppress TNF release or interfere with membrane cleavage (Fig. 3B). Together these findings suggest, TSPs suppress TNF release in Poly I:C and R848-treated BMDMs via both inhibition of intracellular production and tmTNF cleavage while downregulating only intracellular TNF in LPS-treated BMDMs.

3.3. TSPs decrease the expression of classical M1, but not M2 surface markers in BMDMs

Next, we used flow cytometry to determine if the suppressive effects of TSPs on pro-inflammatory cytokine production were coupled to changes in M1 and M2 marker expression. TSP-treated BMDMs showed downregulation of the M1 markers iNOS, MHCII, and CD86 (Fig. 4A) but not CD80 and CCR7 in response to R848, Poly I:C, LPS, and LPS + IFNγ (Supp Fig. 5).

Since most helminth antigens induce a Th2 response and support M2 Mϕ polarization [34], we next asked whether TSPs could polarize BMDMs toward M2 phenotype. To this end, we evaluated the expression of M2 markers including CD301, PDL-2, YM-1, RELMα, and CD206. However, none of these markers were upregulated in TSP-treated BMDMs in response to TLR agonists (data not shown). Interestingly, TSPs in combination with IL-4 showed a synergistic effect and upregulated CD301, whilst suppressing RELMα in the presence of IL-4 (Fig. 4B). In summary, TSPs did not polarize BMDMs toward M2 but suppress MHCII and classical M1 markers such as CD86 and iNOS consistent with the downregulation of released inflammatory cytokines.

3.4. TSPs shape the metabolic activity toward oxidative phosphorylation (OXPHOS) in naive Mϕs but not inflammatory Mϕs

The changes observed in the M1 markers and inflammatory cytokines induced by TSP prompted us to explore the metabolic characteristics of TSP-treated Mϕs by extracellular flux analysis, as recent evidence suggests that Mϕs metabolic reprogramming can affect their functionality. To characterize the metabolic capacity of TSP-treated and untreated BMDMs, we collected the cells after 24 h of stimulation and performed metabolic flux analyses, including LPS-stimulated BMDMs as control. TSP-stimulated BMDMs showed a higher mitochondrial oxygen consumption rate (OCR) (Fig. 5A, B), which indicates increased mitochondrial oxidative phosphorylation (OXPHOS). In contrast, LPS-treated BMDMs showed a reduced ability to increase their OCR in response to FCCP. At baseline, TSP-treated and untreated BMDMs displayed similar extracellular acidification rates (ECARs), a measure for

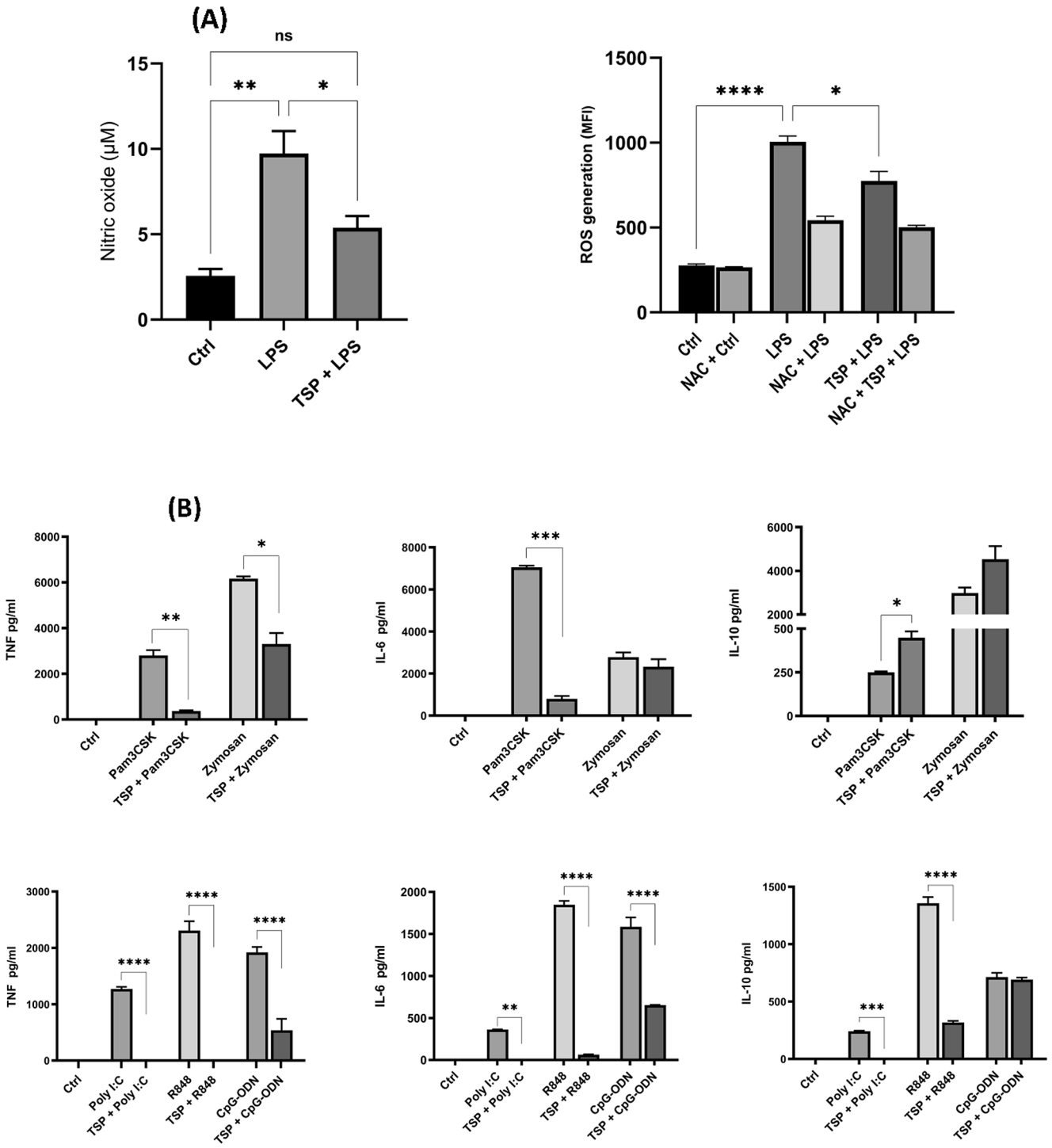

Fig. 2. *Trichuris suis* soluble products (TSPs) inhibit TLR-induced inflammatory cytokines, nitric oxide (NO), and reactive oxygen species (ROS) in macrophages (Mϕs). (A) Mϕs were pre-treated with TSPs (20ug/ml) and exposed to LPS (100 ng/ml) for 24 h. The level of NO and ROS was measured in the culture media. NAC was included as standard ROS inhibitor. (B) Mϕs were pre-incubated with TSPs (20ug/ml) for 0.5 h and exposed to different TLR agonists including Pam3csk4 (TLR1/2), Zymosan (TLR6), Poly I:C (TLR3), R848 (TLR7), and CpG (TLR9) for 24 h. TNF, IL-6, and IL-10 released in the culture medium was measured by ELISA. All values are expressed as mean ± SEM of two independent experiments. *P < 0.05, and ***P < 0.001.

aerobic glycolysis (Fig. 5C, D). Moreover, TSP-treated BMDMs increased their OCR in response to the mitochondrial uncoupler carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP), implying a high spare respiratory capacity (SRC), calculated as the difference between basal and maximal OCR (Fig. 5E). However, stimulation of TSP-treated BMDMs with LPS showed that TSPs fail to elevate OCR or OXPHOS activity, implying that the suppressive effects of TSPs are likely independent of metabolic modulation (Fig. 5F).

In summary, TSPs increased OCR in naive Mϕs but not inflammatory Mϕs, indicating that OXPHOS might not be the active metabolic pathway in TSP-treated inflammatory Mϕs.

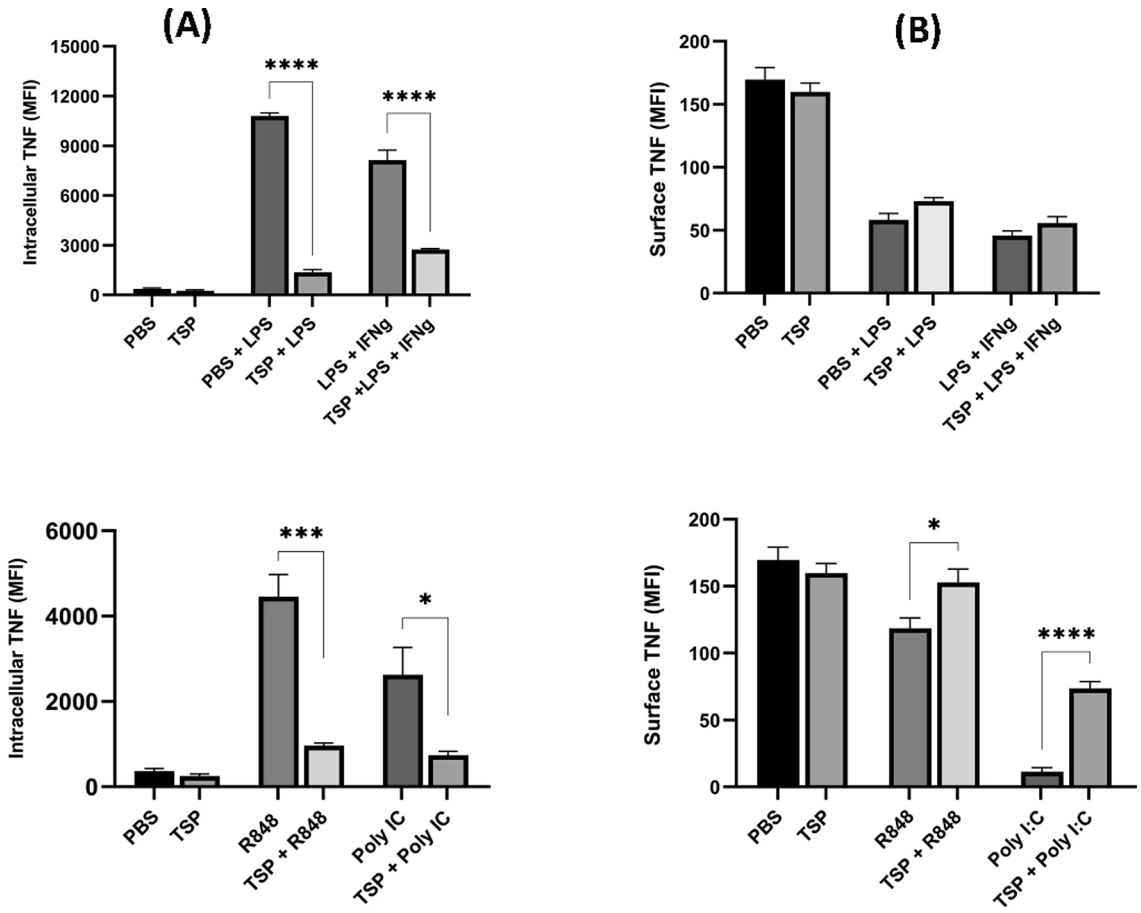

Fig. 3. *Trichuris suis* soluble products (TSPs) suppress intracellular TNF but not surface TNF in macrophages. Mϕs were pre-incubated with TSPs (20ug/ml) for 0.5 h and exposed to LPS (10 ng/ml) with or without IFNγ (50 ng/ml), Poly I:C (TLR3), and R848 (TLR7) for 6 h. (A) Intracellular and (B) surface TNF measured by flow cytometry. TSPs and PBS were included as control. All values are expressed as mean ± SEM of two independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.

3.5. TSPs induce long-term effects on BMDMs

Having shown that TSPs can suppress inflammatory responses to classical TLR activation while inducing IL10 secretion, we next investigated if the immunomodulatory effects of TSP were sustained over a longer time period.

BMDMs were incubated with either TSPs or PBS for 24 h followed by thoroughly washing, media replacement and the cells were incubated for 48 h and subsequently stimulated with TLR agonists for 24 h (Supp Fig. 6). BMDMs previously exposed to TSP showed increased IL-10 production in response to stimulation with all agonists. In contrast, the secretion of the inflammatory cytokine IL-6 was reduced in response to all TLR agonists except for zymosan (Fig. 6). Intriguingly, in contrast to what we found earlier in some of the experiments, only stimulation with a TLR1/2 (Pam3Csk4) and a TLR7/8 agonist (R848) resulted in the suppression of TNF, while zymosan (TLR2/6) and CpG-ODN (TLR9) elevated TNF in TSP-pulsed BMDMs (Fig. 6). Furthermore, to evaluate whether TSPs could polarize BMDMs, the expression of M1 and M2 markers was assessed using flow cytometry. Poly I:C downregulated MHCII and CCR7 in TSP-pulsed BMDMs relative to PBS control. Also, LPS increased CD86 in TSP-pulsed BMDMs (Supp Fig. 7) and CD206 was upregulated in all TSP-pulsed BMDMs (Fig. 7). Collectively, these data show that TSPs induce a long-term cellular reprogramming characterized bymodulated inflammatory cytokine production in response to agonist stimulation 72 h later.

4. Discussion

Helminths have been shown to direct the host immune responses toward tolerance or hyporesponsiveness, of which modulation of Mϕs function is an important component. Mϕs are among the first responders to invasive pathogens and play a central role in initiating and amplifying inflammatory responses against pathogens. Previous studies have shown that TSPs suppress LPS-induced inflammatory responses but remained unknown for how long, nor was it clear whether this effect was associated with metabolic changes in Mϕs. This study set out to extend our understanding of the immunomodulatory properties of TSPs on Mϕs by addressing these questions. Further to the potent suppressive effects of TSPs on TNF, we showed that this was mediated by repression of intracellular TNF and not tmTNF.

It has previously been shown that TSP impairs the production of TNF and IL-6 in response to LPS in Mϕs and DCs [35], but here we showed that TSPs downregulate the same inflammatory cytokines in BMDMs stimulated with a panel of extra- and intracellular TLR agonists including TLR1/2, TLR3, TLR2/6, TLR7/8, and TLR9. However, despite this general suppression involving multiple TLRs, CCL5 and IFNγ release was not affected, indicating that TSPs might target specific transcription factors and inflammatory signals. For instance, several transcription factors including NFkB, AP-1, C/EBP, Ets-1 have been suggested to be involved in CCL5 gene expression [36] and LPS can induce CCL5 transcription in humans via activation of transcription factors ATF and Jun, through a CRE/AP-1 binding site [36]. As several studies have reported that helminth-derived antigens can degrade or occupy TLRs and

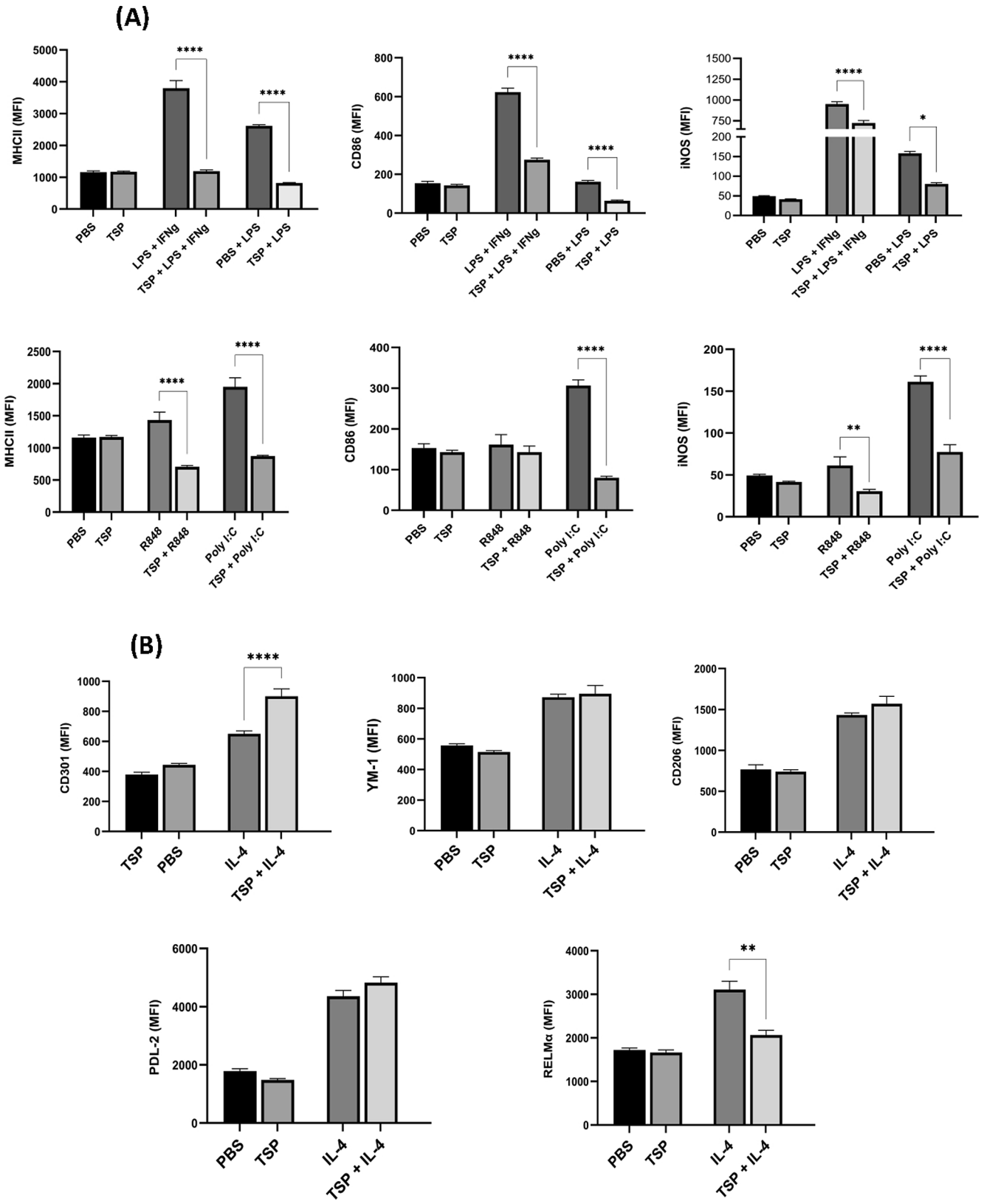

Fig. 4. ***Trichuris suis*** **soluble products (TSPs) alter the expression of some M1 and M2 markers in BMDMs.** (A) Mϕs were pre-incubated with TSPs (20ug/ml) for 0.5 h and exposed to LPS (10 ng/ml) with or without IFNγ (50 ng/ml), Poly I:C (TLR3), and R848 (TLR7) for 6 h. The expression of markers associated with M1 Mϕ including MHCII, CD86, and iNOS was measured by flow cytometry. (B) Mϕs were pre-incubated with TSPs (20ug/ml) for 0.5 h and exposed to IL-4 (50 ng/ml) for 24 h. The expression of M2 Mϕ markers such as CD301 and RELMα was measured by flow cytometry. TSPs and PBS were included as control. All values are expressed as mean ± SEM of two independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.

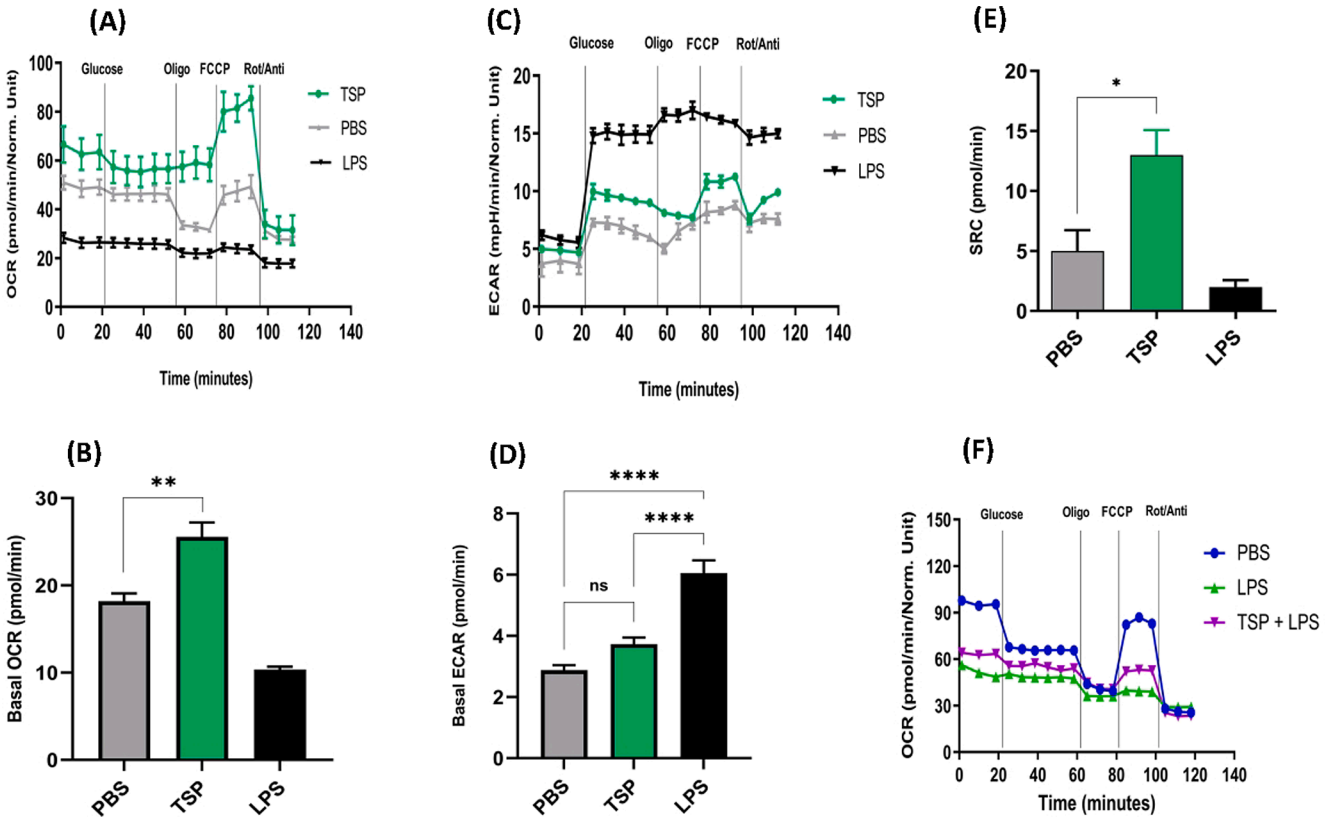

Fig. 5. ***Trichuris suis*** **soluble products (TSPs) upregulate OXPHOS in naive Mϕs but not in inflammatory Mϕs.** (A-D) Mϕs were pre-incubated with either TSPs (20ug/ml) or PBS for 24 h and the level of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) was measured by seahorse assay. (E) Spare respiratory activity of Mϕs was also calculated based on OCR data. (F) Mϕs were pre-treated with either TSPs (20ug/ml) or PBS for 0.5 h then stimulated with LPS (10 ng/ml) for 24 h. OCR was measured by seahorse assay. All values are expressed as mean ± SEM of two independent experiments. *P < 0.05 and **P < 0.01.

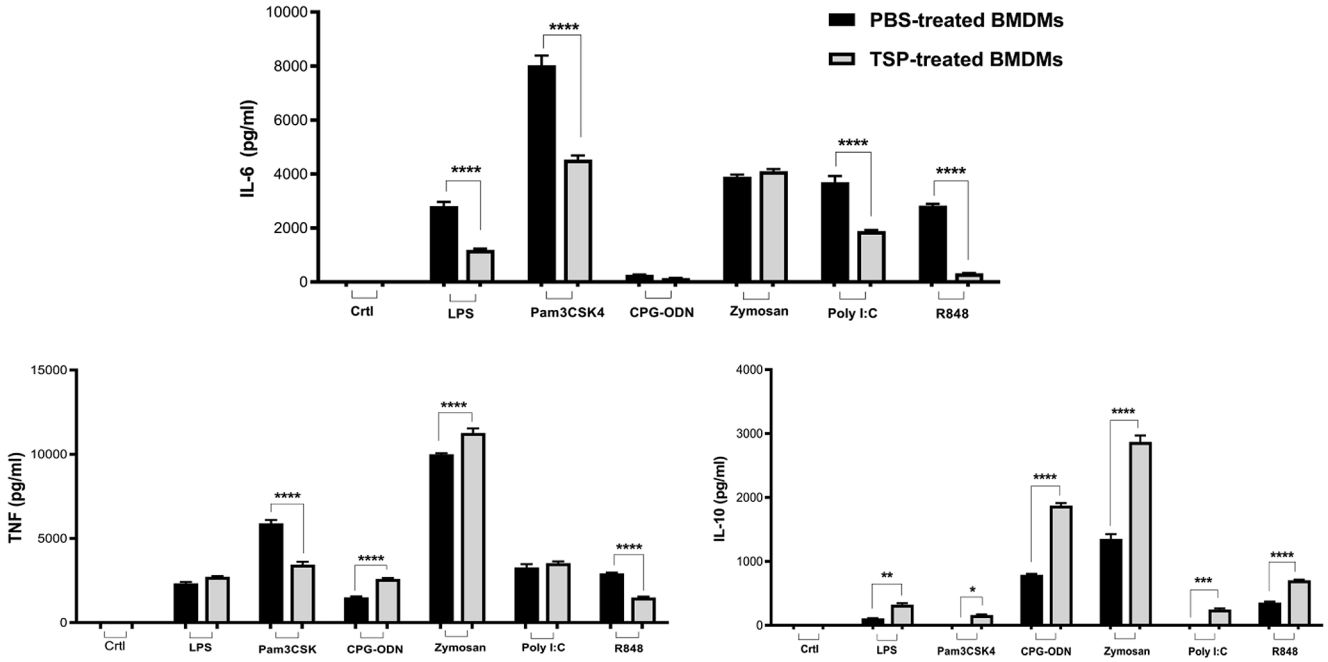

Fig. 6. ***Trichuris suis*** **soluble products (TSPs) induce long-term effects on Mϕs**. Mϕs were pre-treated with TSPs (20ug/ml) for 24 h then washed and media refreshed. After 72 h, TSP-treated Mϕs were exposed to different TLR agonists including Pam3csk4 (100 ng/ml), Zymosan (50ug/ml), Poly I:C (10ug/ml), R848 (10ug/ml), and CpG (7.5uM) for 24 h. TNF, IL-6, and IL-10 released in the culture medium was measured by ELISA. All values are expressed as mean ± SEM of two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.01, and ****P < 0.0001.

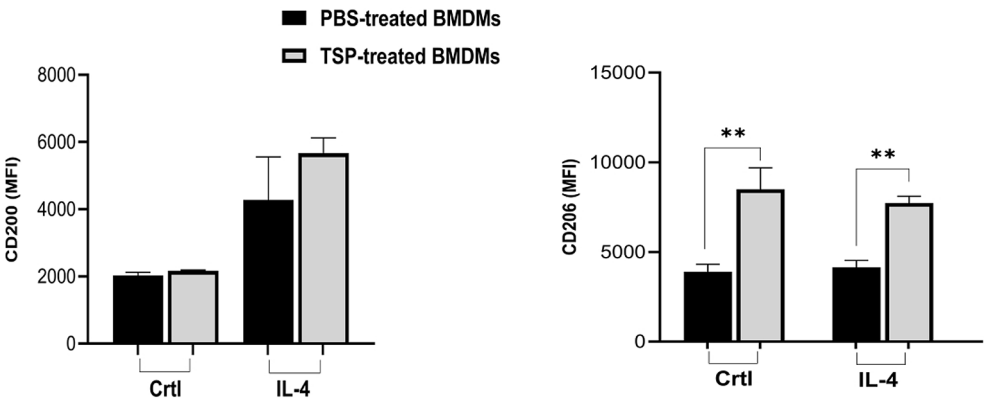

Fig. 7. *Trichuris suis* soluble products (TSPs) induce long-term effects on M2 Mϕs markers. Mϕs were pre-treated with TSPs (20ug/ml) for 24 h then washed and media refreshed. After 72 h, TSP-treated Mϕs were exposed to IL-4 (50 ng/ml) or PBS for 24 h. The expression of markers associated with M2 Mϕs including CD206 and CD200 was measured by flow cytometry. TSPs and PBS were included as control. All values are expressed as mean ± SEM of two independent experiments (triplicate) **P < 0.01.

associated signalling molecules [37–39], we pre-treated BMDMs with TLR agonists followed by worm stimulation. As TSPs still suppressed TNF (though not IL-6) we suggest that the main suppressive effects of TSPs are likely not via degrading or involving the TLRs and/or preventing physical interaction with corresponding agonists, but further studies are needed to illuminate the specific function of TSPs.

We showed that, in addition to M0 Mϕs (M−CSF generated BMDM), exposure of inflammatory Mϕs (M1 and GM-CSF-generated BMDMs) to TSPs led to downregulation of inflammatory cytokines, suggesting that TSPs can affect Mϕs independent of their activation status and possibly target a conserved pathway such as a shared transcription factor or other downstream molecules. Following that, TSPs also dampen key pro-inflammatory M1 Mϕ markers, including iNOS, MHCII, and CD86, in the presence of TLR agonists. TNF has been shown to induce ROS generation via activation of ROS modulator 1 protein [40], while IL-6 stimulates ROS production via activation of NADPH oxidase 2 as a major oxidase system for ROS production [41]. Hence, suppression of ROS and NO in TSP-treated BMDMs might be attributed to the downregulation of inflammatory cytokines and iNOS inhibition, respectively.

The inhibition of pro-inflammatory markers along with inflammatory cytokines, implying that TSPs limit M1 Mϕ polarization, appeared not to be a consequence or repolarization towards an M2 Mϕ phenotype, as none of the expression of YM-1, CD206, and PDL-2 was affected by TSPs. However, TSPs upregulated CD301 and synergistically downregulate RELMα in the presence of IL-4. It seems that exposure of TSPs to M2 supportive conditions such as exogenous IL-4 can activate its function. The disparate expression patterns of CD301 and RELMα might be due to the difference in the regulation of gene expression. Similarly, Meester *et al.* [42] also observed that despite induction of YM-1 and IL-13 by the intracellular pathogen *Nocardia brasiliensis,* the expression of RELMα was downregulated in BMDMs. In addition, RELMα has been found to have some pro-inflammatory activities [43,44]; for instance, injury-initiated RELMα production in Mϕs can lead to the generation of DAMPs like HMGB1 [45]. Laan et al. (2017) suggested that PGE2 is one of the main components in the TSPs that contribute to the suppression of inflammatory mediators in LPS-treated human DCs [17]. In this regard, the suppressive effects of TSPs on RELMα in the presence of IL-4 can be attributed to PGE2, as Sanin et al. (2018) also showed that Mϕs co-stimulated with IL-4 plus PGE2 exhibited downregulation of RELMα and upregulation of CD301, consistent with our data [46]. Therefore, future studies should evaluate the contribution of PGE2 in mediating the modulatory effects of TSPs on Mϕs.

Previous studies have only measured the secretory form of TNF and suggested that TSPs affect signalling and downstream transcription factors in LPS-stimulated Mϕs [35]. TNF is primarily bound to the cell surface and is cleaved off by sheddase enzymes such as TNF converting enzyme (TACE) thereby releasing the cytokine to the extracellular environment [33]. We, therefore, assessed the intracellular, transmembrane, and extracellular levels of TNF in TSP-treated BMDMs in the presence of TLR agonists. We found that TSPs mainly confine LPS-induced TNF at the intracellular level, probably via suppression of gene expression while no effect on tmTNF was observed. In addition, TSPs have shown no inhibitory effect on TACE, which is in accordance with its partial effect on tmTNF (Unpublished data). TSPs only inhibited the release of tmTNF in BMDMs in response to the TLR3 agonist (Poly I:C) and TLR7/8 (R848) and it might be one of the mechanisms by which TSPs decrease the secretory form of TNF in response to these agonists. However, it is unknown how TSPs can suppress the release of TLR3 and TLR7/8-induced tmTNF and therefore warrants further investigations.

The metabolic activity of Mϕs can significantly affect their functionality. M1 Mϕs tend to support glycolysis and generation of acid lactic, while M2 Mϕs shape metabolic signatures toward oxidative phosphorylation for ATP production [20]. We evaluated the rate of ECAR and OCR in BMDMs as a measure of glycolysis and OXPHOS, respectively. TSP-stimulated Mϕs displayed higher oxidative activity (higher basal OCR) but no difference in glycolytic rate (lower basal ECAR) compared with PBS-treated BMDMs. In contrast, stimulation of TSP-treated Mϕs with LPS could dampen OCR, highlighting the suppressive functions of TSPs are not due to modulation of core metabolic signatures at least OXPHOS. However, Bossche et al. (2016) showed that NO could blunt mitochondrial respiration and prevent IL-4 mediated M2 redirection in inflammatory Mϕs [47], but suppression of NO by TSPs did not lead to upregulation of OXPHOS. In accordance with Bossche et al. (2016), we also find that inflammatory (M1) Mϕs fail to convert into M2 Mϕs even in the presence of M2-supporting factors such as IL-4 and TSPs, while M2 Mϕs are more plastic and can readily be polarized to M1 phenotype.

The increase of OXPHOS by TSPs might not be due to PGE2 contents as Sanin et al. (2018) showed that co-stimulation of BMDMs with PGE2 and IL-4 suppresses OXPHOS [46], while we observed a synergistic effect by TSPs and IL-4 on OXPHOS (data not shown).

The long-term effects of worm products on host progenitor and immune cells remain poorly understood. Cunningham et al. (2021) recently demonstrated that *Fasciola hepatica* excretory-secretory products could imprint an anti-inflammatory phenotype on long-term hematopoietic stem cells and monocyte precursor populations, rendering anti-inflammatory monocytes [48]. We observed that TSPs could establish a state of immunosuppression in BMDMs by promoting persistent anti-inflammatory conditions lasting for at least 72 h, as measured by reduced secretion of inflammatory cytokine (IL-6), an increase of IL-10, and a trend for up-regulation of CD206 expression (M2 marker) in response to a panel of inflammatory stimuli targeting surface and endosomal TLR ligands. Surprisingly, despite the potent suppressive effects of TSPs on BMDMs during 24 h, we found that TSP-treated BMDMs can recover TNF generation after 72 h. This difference might be due to the fast generation of TNF, as an early response cytokine, relative to other inflammatory cytokines [49,50] upon removing TSPs and also its faster turnover on the cell surface.

Quinn et al. (2019) have shown that total extract from *Fasciola hepatica* (FHTE) can train innate immunity by induction of epigenetic modification on Mϕs that subsequently affect their response to inflammatory stimuli [51]. Consistent with our observation of TSP-stimulated BMDMs, they showed that Mϕs that had been stimulated *in vitro* with FHTE elevated IL-10 production and expression of M2 markers after exposure to TLR ligands. The FHTE effects were abrogated by inhibiting DNA methylation, indicating epigenetic imprinting in the immune cells [51]. In addition, IL-6 and TNF production were downregulated in trained BMDMs after exposure to TLR ligands [51]. Similarly, we observed suppression of IL-6 in TSP-trained BMDMs in response to TLR ligands. But in contrast to Quinn et al. (2019), we did only observe TNF reduction in TSP-treated BMDMs for Pam3CSK and R848, which might relate to helminth species-specific differences [51]. Hoeksema et al. (2016) showed that blocking histone deacetylases could completely abrogate the inhibitory functions of TSPs on human M1 Mϕs owing to impaired histone acetylation at the TNF and IL-6 promoters [52], and our findings are therefore somewhat similar to this study. We showed that treating BMDMs with TSPs can result in'innate training' and durable effects, but further investigations are needed to probe the mechanistic pathways, including epigenetic modulations.

CRediT authorship contribution statement

Amin Zakeri: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **Bart Everts:** Formal analysis, Writing – review & editing, Supervision. **Andrew R. Williams:** Methodology, Writing – review & editing. **Peter Nejsum:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Amin Zakeri reports financial support was provided by Lundbeck Foundation. Amin Zakeri reports a relationship with Lundbeck Foundation that includes: funding grants.

Acknowledgments

This work was supported by the Novo Nordisk Foundation (NNF18OC0053231) and Aarhus University, Denmark. A.Z. was supported by the Lundbeck Foundation (R249-2017-763) and P.N. was supported by Independent Research Fund Denmark (DFF-6111- 00521).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2022.155919.

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