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Reduced *Trichomonas vaginalis* viability in mice pretreated with parasite DNA

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Research Article

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Abstract

Trichomonas vaginalis is an extracellular parasite that colonizes the human urogenital tract leading to trichomoniasis, the most common sexually-transmitted non-viral disease worldwide. The immune response plays a critical role in the host defense against this parasite. Trichomonas' DNA contains unmethylated CpG motifs (CpGDNA) that in other microorganisms act as modulators of the immune response. However, the molecular mechanisms responsible for CpGDNA immune modulation are still unclear. As macrophages participate in the first line of defense against infection, we investigated the type of immune response of murine macrophages to *T. vaginalis* DNA (*Tv*DNA). We observed high expression of the proinflammatory cytokines IL-6 and IL-12p40 in macrophages stimulated with *Tv*DNA. In contrast, the anti-inflammatory response, assessed by IL-10 and IL-13 mRNA expression was delayed. This suggests that the immune response induced by *Tv*DNA is modulated through cytokine production, mediated partly by NADPH-oxidase activity, as *Tv*DNA induced reactive species of oxygen production and a rounded morphology in macrophages indicative of an M1 phenotype. Furthermore, infected mice pretreated with *Tv*DNA displayed persistent vulvar inflammation and decreased parasite viability consistent with higher proinflammatory cytokine levels during infection compared to untreated mice. Overall, our findings suggest that *Tv*DNA pretreatment modulates the immune response favouring parasite elimination.

Introduction

Trichomonas vaginalis is a flagellated parasite that infects the genito-urinary tract of humans. It is the causal agent of trichomoniasis, one of the most common non-viral sexually transmitted diseases worldwide with an estimated 276 million cases per year according to the World Health Organization (WHO) (Newman *et al.*, 2015). Currently, trichomoniasis is associated with a high risk of acquiring human immunodeficiency virus (HIV) and human papillomavirus (HPV) (Lazenby *et al.*, 2014; Ghosh *et al.*, 2017), and may leads to adverse pregnancy outcomes (Silver *et al.*, 2014; Mielczarek and Blaszkowska, 2016). Furthermore, resistance to 5-nitroimidazoles, the conventional clinical treatment for this infection, has been observed in some *T. vaginalis* strains (Dunne *et al.*, 2003), making difficult their use in symptomatic patients.

Trichomonas vaginalis adheres to epithelial cells and survives in the urogenital tract of the host (da Costa *et al.*, 2005; Pereira-Neves and Benchimol, 2007), hence the mucosal immune response is the first line of defense against this pathogen. Particularly, the innate immune system plays a critical role in the containment and elimination of the parasites through the action of specialized cells including neutrophils, dendritic cells and macrophages (Iwasaki and Medzhitov, 2015; Sica *et al.*, 2015). Apart from their phagocytic function, macrophages' plasticity allows them to acquire different phenotypes with distinct biological functions depending on the microenvironment or metabolic state of the host (Martinez and Gordon, 2014; Fraternale *et al.*, 2015; Tan *et al.*, 2016; Murray, 2017). For example, macrophages activated by either a TLR agonist as LPS or microbial products, M(LPS + IFNγ), display an M1 phenotype, critical for elimination of pathogens. Macrophages activated by IL-4, M(IL-4), show an M2 phenotype to promote wound healing and inflammation resolution (Martinez and Gordon, 2014; Murray *et al.*, 2014).

Macrophages recognize Pathogen-Associated Molecular Patterns (PAMP's) through Receptor Recognition Patterns (RRPs) (Takeuchi and Akira, 2010; Broz and Monack, 2013). Upon activation of RRPs, a complex signalling pathway is triggered that culminates with the activation of transcription factors inducing the expression of inflammatory genes (Takeuchi and Akira, 2010; Kumar *et al.*, 2011; Broz and Monack, 2013), reactive oxygen species (ROS) like superoxide anion, and nitric oxide (NO) production (Forman and Torres, 2002; Bogdan, 2015), all of which will contribute to pathogen elimination. Toll-like receptor 9 (TLR9) is one member of the TLRs family expressed predominantly in dendritic cells, B lymphocytes and macrophages, and is localized in the endoplasmic reticulum, endosomes and lysosomes (Hemmi *et al.*, 2000; Latz *et al.*, 2004; Farrokhi *et al.*, 2017). TLR9 recognizes unmethylated CpG dinucleotides (CpG DNA), which are common in bacterial, viral and protozoan genomes (Shoda *et al.*, 2001;

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Ahmad-Nejad *et al.*, 2002; Krieg, 2002) but are rarely found (~1%) in mammalian genomes (Jones and Takai, 2001; Krieg, 2002). Several reports have demonstrated that CpG DNA has immunostimulatory properties, inducing both maturation and activation of professional phagocytes as well as promoting a Th1 immune response (Messina *et al.*, 1991; Stacey *et al.*, 1996; Krieg, 2000, 2002), all of which is important to resolve infections. It has also been shown, both *in vitro* and *in vivo*, that parasite CpG DNA displays immunostimulatory effects on diverse immune cells *via* TLR activation and pro-inflammatory factor production, leading to improved infection resolution (Zimmermann *et al.*, 1998; Shoda *et al.*, 2001; Das *et al.*, 2015; Gong *et al.*, 2016). Furthermore, CpG DNA induces an enhanced mucosal immunity, which has resulted in its safe use in vaccines for humans as co-adjuvant (McGhee *et al.*, 1992; Stacey and Blackwell, 1999; Holmgren and Czerkinsky, 2005; Iho *et al.*, 2015). For example, the licensed Hepatitis B vaccine Engerix-B® (Halperin *et al.*, 2003) and the licensed Anthrax vaccine Bio Thrax® (Rynkiewicz *et al.*, 2011) both contain CpG ODN as an adjuvant. In this study, we hypothesized that *T. vaginalis* DNA (*Tv*DNA) can have immunomodulatory functions. To address this, we evaluated the effect of *Tv*DNA on the immune response *in vitro* (using RAW264.7 cells), in addition to an *in vivo* trichomoniasis murine model. Our data show that *Tv*DNA elicits an increase of pro-inflammatory cytokines and induces a local inflammatory response. Furthermore, *Tv*DNA is able to modulate the immune response favouring the host and contributing to parasite infection resolution.

Materials and methods

Cell lines and strains

RAW264.7 cells were maintained in DMEM GlutaMAX medium (10566-016, Gibco) supplemented with 10% foetal bovine serum (FBS) (16000-044, Gibco) at 37 °C in a 5% CO_2 atmosphere. *Trichomonas vaginalis* GT21 strain (Olmos-Ortiz *et al.*, 2017) was maintained in TYI-S-33 medium supplemented with 6% of adult bovine serum (ABS) (SU-120 microLab) and incubated at 37 °C.

Animals

Female BALB/c mice (6–8 weeks of age) were maintained under specifications from the Mexican Official Norm NOM-062-ZOO-1999 and fed *ad libitum*.

Trichomonas vaginalis *DNA isolation*

Parasites (3.8×10^6) were grown in 120 mL of TYS-33 medium supplemented with 6% of ABS at 37 °C for 24 h. The trophozoites were harvested by spinning down at 500×***g*** for 5 min and the pellet was washed three times with phosphate solution buffer – Tris EDTA (PBS-EDTA) pH 7.2. *Trichomonas vaginalis* DNA (*Tv*DNA) was isolated by two methods. In the first method, *Tv*DNA was isolated using the Wizard Genomic DNA purification kit (A1120, Promega) with some modifications and additional phenol-chloroform isolation. In the second method, freshly isolated *Tv*DNA using the Wizard Genomic DNA purification kit was additionally purified by affinity chromatography using the system DetoxiGel Endotoxin Removing Gel (20344, ThermoScientific) under the manufacturer's specification. Finally, *Tv*DNA was quantified in a Gene-Quant spectrophotometer and stored at −20 °C until used.

Bacterial and mammalian DNA isolation

Escherichia coli DNA and mouse spleen DNA were isolated using the Wizard genomic DNA purification kit (Promega) following the manufacturer's instructions. The bacterial and mammalian genomic DNA were quantified and stored as described above.

Limulus amoebocyte lysate-QCL and differential SDS-PAGE assay

To verify the purity of *Tv*DNA, the endotoxin level from *Tv*DNA was tested by limulus amoebocyte lysate (LAL)-QCL-1000 (5064-7U, LONZA) following the manufacturer's specifications. Additionally, 50 μg of *Tv*DNA were analysed in SDS-PAGE and stained using three methods: Coomassie, silver and periodic acid-shift (PAS), to rule out any possible contamination of proteins and/or saccharides coming from the parasite. Moreover, 50 μg of *Tv*DNA were used for electrophoresis on a 2% agarose gel and visualized under ethidium bromide staining to verify the integrity of *Tv*DNA.

Nitric oxide production assay

The nitric oxide (NO) production in culture supernatants was indirectly determined by a modified Griess assay (Arias-Negrete *et al.*, 2004), evaluating the nitrite concentration released by RAW264.7 cells. Briefly, 2.5×10^5 macrophages were placed in 96-well plates and stimulated with *Tv*DNA (50 μg mL^{-1}). Bacterial lipopolysaccharide (LPS, L3012 Sigma Aldrich) was used as a conventional positive control (Su *et al.*, 2011). Unstimulated macrophages and DNA from BALB/c mice spleen (50 μg mL^{-1}) were used as negative controls. To block LPS, cells were treated in the presence or absence of polymyxin B (25 mg mL^{-1}). The cells were incubated at 37 °C in a 5% CO_2 atmosphere for 36 h. The Griess assay was performed using 100 μL of macrophage supernatants, adding 50 μL of sulphanilamide solution and 50 μL of N-1-naphthyl-ethylenediamine. The reaction was performed at room temperature in the absence of light for 40 min. Nitrite accumulation was determined by spectrophotometry at 540 nm using a standard calibration curve of nitrite.

Semiquantitative RT-PCR

Expression of both pro-inflammatory cytokines (IL-6, IL-12p40, TNF) and anti-inflammatory cytokines (IL-10 and IL-13) in RAW264.7 cells was evaluated at different times of exposure to *Tv*DNA (0.5, 1, 2, 3, 6, 9, 12, 15, 18 h). Briefly, total RNA was isolated from RAW264.7 cells incubated with *Tv*DNA by TRIzol™ Reagent according to the manufacturer's instructions. Two micrograms of total RNA were treated with DNase I, amplification grade system to remove any trace of genomic DNA. Retrotranscriptase assays were performed using SuperScript™ III Reverse Transcriptase system according to the manufacturer's instructions. Expression level analysis for IL-6, IL-12p40, TNF, IL-10, IL-13 and GAPDH (as control), was performed by Platinum® PCR SuperMix using the specific oligonucleotides (Table S2). The amplification conditions for IL-6, TNF and GAPDH were: first denaturalization at 94 °C/3 min (1 cycle), second denaturation at 94 °C/30 s, followed by alignment at 51 °C/30 s and extension at 72 °C/1 min (35 cycles) and a final extension of 72 °C/10 min (1 cycle). The band sizes observed were 159 bp for IL-6, 203 bp for TNF and 300 bp for GAPDH. The amplification conditions for IL-10, IL-13 and IL-12p40 were: first denaturation at 94 °C/3 min (1 cycle), second denaturation at 94 °C/30 s, followed by alignment at 55 °C/30 s, extension at 72 °C/1 min (35 cycles) and a final extension at 72 °C/10 min (1 cycle). The observed band sizes were 563 bp for IL-10, 110 bp for IL-13 and 125 bp for IL-12p40. All amplification products were analysed in 2% agarose gels using ChemiDoc MP

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Imaging Systems and the densitometric analysis was performed using software ImageLab4.0.

Macrophage polarization assay

Macrophages' morphological changes induced by *Tv*DNA were determined by confocal microscopy. Briefly, 1×10^5 RAW264.7 macrophages were seeded on 12 mm coverslips placed inside the wells of 24-well tissue culture plates in DMEM without foetal bovine serum but containing the following stimuli: lipopolysaccharide (LPS) (10 ng mL^{-1}) plus interferon gamma (IFN-γ) (10 ng mL^{-1}) as positive control for M1 phenotype induction; IL-4 (20 ng mL^{-1}) as positive control for M2 phenotype induction and *Tv*DNA (50 μg mL^{-1}). Cells were incubated for 24 h with the different stimuli at 37 °C in 5% CO_2. Next, cells were washed three times with PBS. Macrophages were fixed with 2% paraformaldehyde and then washed twice with PBS-glycine. Finally, the cells were mounted with 10 μL of ProLong™ Gold Antifade Mountant. Images were taken by confocal microscopy and were edited using ZEN 2 lite software. The morphology of 100 cells per field was evaluated. The axial ratio was calculated using ImageJ software (https://imagej.nih.gov/ij/) and reported as the aspect ratio.

Nitro blue tetrazolium assay

Reactive Oxygen Species (ROS) released from activated macrophages under different stimuli were determined by nitro blue tetrazolium (NBT) assay (Choi *et al.*, 2006). Macrophages (1×10^5 cells) were seeded into 96-well culture plates in DMEM without foetal bovine serum in the presence of lipopolysaccharide (LPS) (500 ng mL^{-1}) plus interferon gamma (IFN-γ) (10 ng mL^{-1}) as control for M1 phenotype induction, IL-4 (20 ng mL^{-1}) as control for M2 phenotype induction, *Tv*DNA (50 μg mL^{-1}) or diphenyleneiodonium (DPI) (30 μM), a NADPH oxidase (Nox2) inhibitor. NBT (1 mg mL^{-1}) was added along with each different stimulus at the start of the assay to determine all released ROS. The plate was centrifuged at 800×***g*** for 1 min at room temperature and incubated at 37 °C in a 5% CO_2 atmosphere for 40 min. Next, the supernatant was discarded and the cells were quickly washed with 70% methanol to remove any trace of non-reduced NBT. Formazan particles formed inside macrophages' phagosomes were solubilized with 2 mM KOH (110 μL) and DMSO (140 μL). Finally, released ROS was measured at 620 nm.

Trichomonas vaginalis *infection in female BALB/c mice*

Female BALB/c mice (6–8 weeks old) were used to establish *T. vaginalis* infection according to the protocol reported by Olmos-Ortiz *et al.* (2017). Five different groups, with five female BALB/c mice per group, were established. Four out of five groups were treated subcutaneously with 50 μL of oestradiol valerate/norethisterone enanthate (MESIGYNA, BAYER) at 5 and 50 mg mL^{-1}, respectively (Fig. 4A). To explore the effect of *Tv*DNA, two groups were treated with 50 μg mL^{-1} of *Tv*DNA, vaginally administered two days before infection. Two groups of mice were vaginally inoculated with 1×10^5 parasites in 50 μL TYI-S-33 medium. From the second day until the tenth day post-infection, vaginal washes were collected. One group of five uninfected mice was used as a control. The *in vivo* experiments were conducted twice.

Vulvar inflammation evaluation

The parameters to define vulvar inflammation due to *T. vaginalis* infection, were vulvar irritation (redness), the presence of purulent secretions and vulvar size increase. The vulvar size increase was evaluated by the length (in a number of pixels) of vulvar diameter over the rear width of each mouse on photos taken at each post-infection day. The vulvar size values were acquired in a double-blind fashion.

Trichomonas vaginalis *viability in female BALB/c mice pretreated with* TvDNA

To determine the effect of *Tv*DNA pretreatment on the murine model of infection with live trophozoites, the parasites' viability at 4, 6 and 10 days post-infection was quantified using the LIVE/DEAD™ Viability/Cytotoxicity Kit system according to the manufacturer's instructions. Vaginal washes from female mice treated and untreated with *Tv*DNA and later infected with the parasite were centrifuged at 400×***g*** for 10 min, the pellet was resuspended in 100 μL of TYI-S-33 medium and deposited in culture tubes with 5 mL of TYI-S-33. The cultures were incubated at 37 °C for 3 days. A culture tube with viable trophozoites from a regular culture and another with non-viable trophozoites (killed with methanol 70% at 37 °C for 30 min) were incubated along with the culture tubes from the vaginal washes being tested. Next, the culture tubes were centrifuged at 500×***g*** for 5 min and the pellet was resuspended in 50 μL of PBS to which 50 μL of calcein 10 μM (to distinguish live cells) and 100 μL of ethidium homodimer 4 μM (to identify dead cells) were added. The cell suspension was gently mixed and incubated for 30 min at room temperature in darkness. Afterwards, samples were centrifuged at 500×***g*** for 5 min, the pellet was washed twice with PBS and the trophozoites were fixed with 2% paraformaldehyde. Then, trophozoites were washed twice with PBS-glycine and resuspended in PBS. The trophozoites were mounted with 10 μL of ProLong™ Gold Antifade Mountant. Images were taken by confocal microscopy and were edited using de software ZEN 2 lite. The ratio of calcein-AM (green-fluorescence) over ethidium-homodimer-1 (red fluorescence) parasite number was plotted as viability percentage.

Quantification of cytokines in vaginal washes of infected female mice

The effect of *Tv*DNA on cytokine secretion was evaluated through changes in IL-6, IL-10 and IL-17 cytokine levels. These cytokines were quantified by ELISA kits (Invitrogen, San Diego, CA, USA: 88-7064-22 for IL-6, 88-7105-22 for IL-10 and eBiosciencie, Waltham, MA, USA: BMS6001 for IL-17) according to the manufacturer's guidelines. Cytokines were measured at 0, 4, 8, 10 and 14 days post-infection in mice pretreated with or without *Tv*DNA. Mice treated with oestradiol valerate/norethisterone enanthate and untreated were used as control. We analysed the cytokine secretion in vaginal washes from *T. vaginalis*-infected mice from two independent experiments, collecting 150 μL from each vaginal wash (in PBS at pH 7) at each day post-infection. Samples for each cytokine determination were performed using 50 μL from the vaginal washes recovered. Data plotted correspond to the average from 10 mice per condition ($n = 10 \pm$ standard error).

Statistical analysis

The aspect ratio of the cells, nitrite and ROS production data were analysed by the Kruskal–Wallis test with subsequent Dun's test (three independent experiments). The parasite viability and the cytokine production *in vivo* experiments data were analysed by the Kruskal–Wallis test with subsequent Dun's test (two independent experiments). Vulvar inflammation data were analysed by the Kruskal–Wallis test and *post hoc* test. All data are presented as the mean ± standard error.

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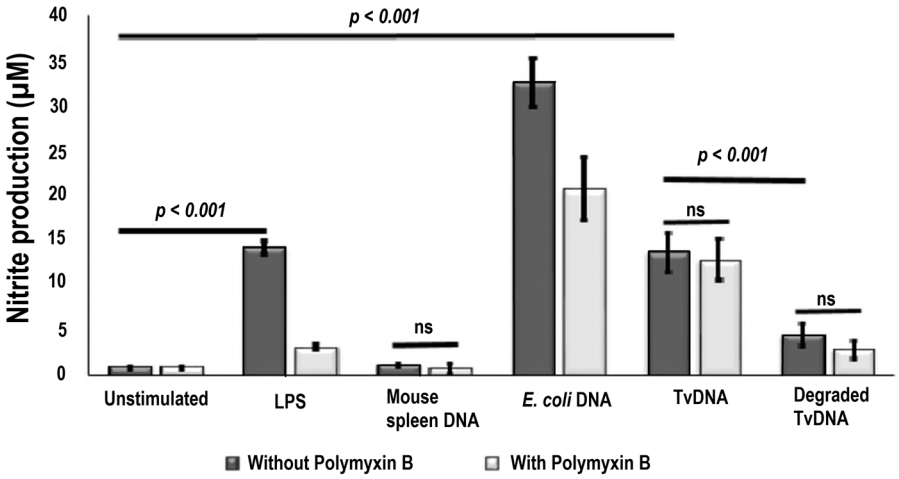

Fig. 1. *Tv*DNA induces NO production in RAW264.7 cells. NO production was determined in culture supernatants of untreated (black bars) and polymyxin B-treated (25 mg mL^{-1}, grey bars) RAW264.7 cells by the Griess modified assay after 36 h of incubation with LPS (10 ng mL^{-1}), mouse spleen DNA (50 μg mL^{-1}), *E. coli* DNA (50 μg mL^{-1}) or *Tv*DNA (50 μg mL^{-1}). Degraded *Tv*DNA was obtained after incubation with DNAse I. Unstimulated macrophages were used as negative control. Data are representative of three independent experiments expressed as mean ± S.D. Significance was determined by Kruskal–Wallis analysis and Dunn's test ($n = 18$, $^{***}P < 0.001$). ns = non-significant.

Results

Tv*DNA induces NO production in RAW264.7 cells*

Nitric oxide (NO) is a small inorganic radical that is implicated in the modulation of several physiological functions in mammals. In immune cells, NO is produced by inducible nitric oxide synthase (iNOS) and has beneficial microbicidal, antiparasitic, antiviral and antitumoral effects (Pautz *et al.*, 2010; Bogdan, 2015). External components of *T. vaginalis* activate human macrophages (Han *et al.*, 2009) as it has been reported that attenuated parasites induce NO production. Therefore, we tested whether *Tv*DNA, an internal molecule of the parasite, could induce activation in RAW264.7 cells. Upon exposure to *Tv*DNA (50 μg mL^{-1}) murine macrophages released nitrite at similar levels as bacterial LPS (10 ng mL^{-1}), the main component of the cell wall of Gram-negative bacteria (Fig. 1). In order to confirm that the activation was due to *Tv*DNA and not to residual bacterial LPS contamination from the *Tv*DNA isolation procedure, we added polymyxin B (which binds to LPS) (Galanos *et al.*, 1985; Schletter *et al.*, 1995; Raetz and Whitfield, 2002) to macrophages stimulated with *Tv*DNA. The addition of polymyxin B did not abolish the NO production of macrophages stimulated with *Tv*DNA as in those macrophages that were treated only with bacterial LPS or stimulated with *E. coli* DNA (Fig. 1). Importanlty, there was no significant NO production by RAW264.7 cells stimulated with either DNase I-degraded *Tv*DNA nor DNA obtained from mouse spleen (Fig. 1). Furthermore, the purity of *Tv*DNA was assessed by endotoxin test (Table S1) and differential SDS-PAGE assays (Fig. S1A). Additionally, we evaluated the efficiency of two methods to obtain the parasite nucleic acid by determining the NO production in RAW264.7 cells treated with *Tv*DNA (Fig. S1B). Our findings indicate that both 'isolated' and 'isolated and purified' *Tv*DNA can activate macrophages for an efficient immune response starting with high NO production. Moreover, our data show that the integrity of *Tv*DNA is required to induce macrophage activation (Fig. 1 and Fig. S1C). Henceforth, 'isolated' *Tv*DNA was used in the following *in vitro* and *in vivo* experiments to further evaluate its effect.

Tv*DNA induces an early pro-inflammatory profile in murine macrophages*

Our results indicated that *Tv*DNA is a macrophage activator similar to bacterial LPS (Fig. 1). Next, we investigated the type of response induced by *Tv*DNA through cytokine mRNA expression in RAW264.7 cells. Significant mRNA expression levels of IL-6, a pro-inflammatory cytokine, were induced within 30 min and a further increase on IL-6 mRNA was observed after up to 2 h of

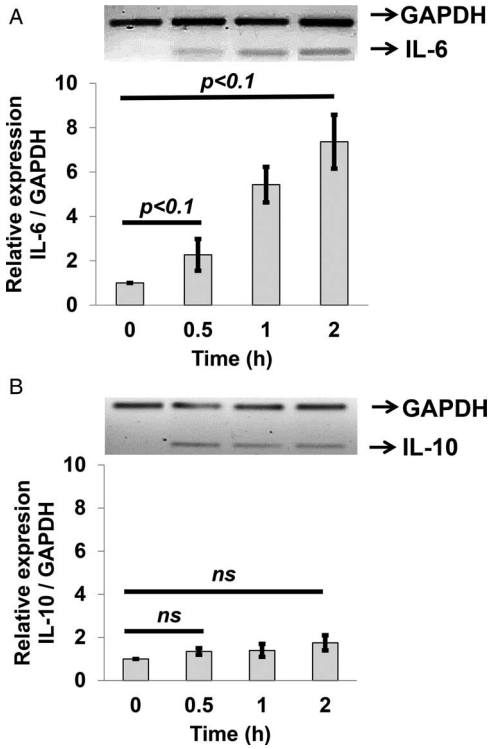

Fig. 2. Early cytokine mRNA expression in RAW264.7 cells stimulated with *Tv*DNA. Relative expression of IL-6 mRNA (A) and IL-10 mRNA (B) were evaluated by RT-PCR. GAPDH was used as a constitutive expression control. The densitometry analysis was performed in ImageLab6.0 software. Data are representative of three independent experiments expressed as the mean ± S.E. Significance was determined by Kruskal–Wallis analysis and Dunn's test ($n = 9$, $^{*}P < 0.1$).

*Tv*DNA treatment (Fig. 2A). In contrast, there was no significant increase in the anti-inflammatory cytokine IL-10 mRNA when RAW264.7 cells were stimulated with *Tv*DNA for up to 2 h (Fig. 2B). We next looked at the effect of longer *Tv*DNA exposure times on the mRNA levels of these cytokines. Interestingly, the highest IL-6 mRNA expression level was observed at 3 h followed by a gradual decrease after 18 h of *Tv*DNA exposure (Fig. 3A). Although different in magnitude, a similar temporal effect was observed for the mRNA levels of pro-inflammatory cytokines TNF and IL-12p40 (Fig. 3B and C). The RAW264.7 macrophages required three hours to show a significant increase in IL-10 mRNA expression that reached the highest levels until 6 h of treatment. These increased IL-10 mRNA levels were sustained up to 15 h of *Tv*DNA exposure and started to decrease by 18 h (Fig. 3D). On the other hand, the expression levels of IL-13,

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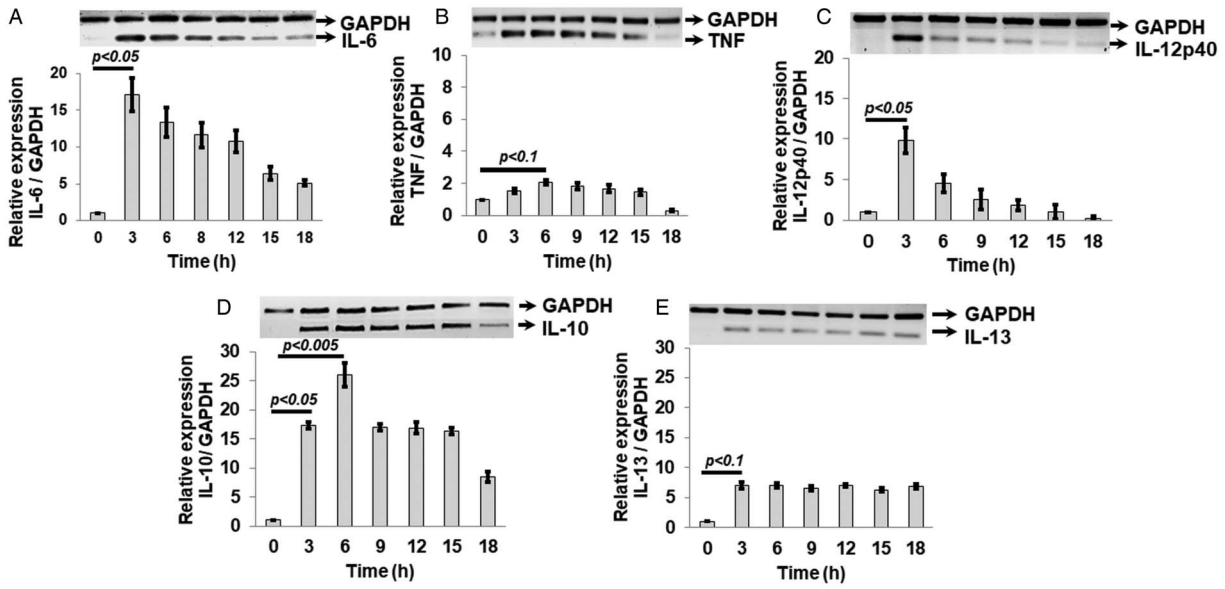

Fig. 3. *Tv*DNA induces an early proinflammatory and delayed anti-inflammatory profile in RAW264.7 cells. Relative expression of IL-6 (A), TNF (B), IL-12p40 (C), IL-10 (D) or IL-13 (E); mRNA levels were evaluated by RT-PCR. GAPDH was used as a control for a constitutively expressed gene. Data are representative of three independent experiments expressed as mean ± S.E. Significance was determined by Kruskal–Wallis analysis and Dunn's test ($n = 9$, $^{+}P < 0.1$, $^{**}P < 0.05$, $^{***}P < 0.005$).

another anti-inflammatory cytokine, showed a significant increase 3 h after *Tv*DNA treatment that was sustained throughout 18 h (Fig. 3E). Taken together, these findings suggest that *Tv*DNA modulates macrophages' response *via* a robust cytokine production through an early pro-inflammatory phase, followed by an anti-inflammatory profile to avoid an exacerbated inflammatory stage.

Tv*DNA induces NADPH oxidase (Nox2) activity and morphology changes in murine macrophages*

Macrophages are professional phagocytes in which NO is a pro-inflammatory mediator that contributes to the elimination of pathogens (Bogdan, 2015). However, ROS production is also part of the microbicide machinery of these cells through NADPH oxidase activity (Nox2) (Warnatsch *et al.*, 2017). ROS, particularly superoxide anion, are involved in M1 and M2 macrophage differentiation (Martinez and Gordon, 2014; Murray, 2017). Thus, we first evaluated superoxide anion production using the nitro blue tetrazolium colorimetric assay (Choi *et al.*, 2006). Because ROS release occurs within minutes after stimulation (Choi *et al.*, 2006), we exposed RAW264.7 macrophages to *Tv*DNA (50 μg mL^{-1}) for 40 min. As shown in Fig. 4A, macrophages displayed increased levels of ROS upon *Tv*DNA treatment that were similar to those induced by a combination of LPS (500 ng mL^{-1}) plus IFNγ (10 ng mL^{-1}). Importantly, ROS production was inhibited when macrophages were treated with *Tv*DNA plus DPI (30 μM), a selective inhibitor of Nox2 (Kowluru and Kowluru, 2014; Zhu *et al.*, 2017). These results suggest that *Tv*DNA promotes ROS production *via* Nox2 activity. Interestingly, the inhibition of ROS levels with DPI was comparable to the low levels produced by macrophages that were treated with the anti-inflammatory cytokine IL-4, that promotes M2 phenotype in macrophages (Gordon, 2003; Martinez and Gordon, 2014). Because the role of M1 or M2 macrophages during *T. vaginalis* infection remains unknown, we next investigated whether *Tv*DNA induced any morphological changes in RAW264.7 cells that characterize the functionally distinct M1/M2 phenotypes. Therefore, RAW264.7 macrophages were co-stimulated for 24 h with LPS (10 ng mL^{-1}) plus IFNγ (10 ng mL^{-1}). As expected, these macrophages displayed a 'fried egg'-like shape, the characteristic morphology of M1 phenotype, (Fig. 4B, bottom left). In contrast, macrophages stimulated with recombinant IL-4 (20 ng mL^{-1}) showed a fibroblast-like appearance, the characteristic morphology of M2 phenotype (Fig. 4B, top right). Strikingly, macrophages stimulated with *Tv*DNA (50 μg mL^{-1}) largely displayed a 'fried egg shape' after 24 h of treatment (Fig. 4B, bottom right). The aspect ratio of macrophages reflecting the morphological changes induced by *Tv*DNA and the other treatments was quantified and it is shown in Fig. 4C. These data indicate that the majority of *Tv*DNA-treated cells displayed the M1 morphology profile, which prevailed over the M2 profile. Taken together, these findings show that *Tv*DNA induces a burst of ROS release due to NADPH-oxidase activity, which may lead to a morphological and functional M1 phenotype in RAW264.7 macrophages. As previously shown (Sica *et al.*, 2015), this kind of macrophage activation can contribute to infection resolution.

Tv*DNA pretreatment promotes inflammation in female mice challenged with live parasites*

The CpG motifs contained in the microbial genome have an immunomodulatory effect, and it has been shown that these sequences are able to counteract an infection using *in vivo* models (Shivahare *et al.*, 2014). Shivahare *et al.* (2014) observed in both hamster and murine models of visceral leishmaniasis an increase in mRNA expression for iNOS and pro-inflammatory cytokines when the infected animals had been pretreated with CpGODN enveloped in liposomes. On the other hand, IL-10 and TGF-beta mRNA levels decreased. This characteristic Th1 immune response was associated with a decrease in the parasitic burden for both infectious models.

Our results suggest that *Tv*DNA modulates macrophages' response through NO, cytokines and ROS production *in vitro*, presumably due to the CpG motifs contained in *T. vaginalis* genome. To determine how *Tv*DNA impacts both infectivity and pathogenicity of this parasite, we investigated the *Tv*DNA immunomodulatory effect in female BALB/c mice infected with *T. vaginalis*, using the *in vivo* infection model that we previously reported (Olmos-Ortiz *et al.*, 2017) (Fig. 5A). *Tv*DNA was vaginally administered two days before infecting female mice with live trichomonas. Four days post-infection, both untreated and *Tv*DNA-treated female mice, showed signs of trichomoniasis,

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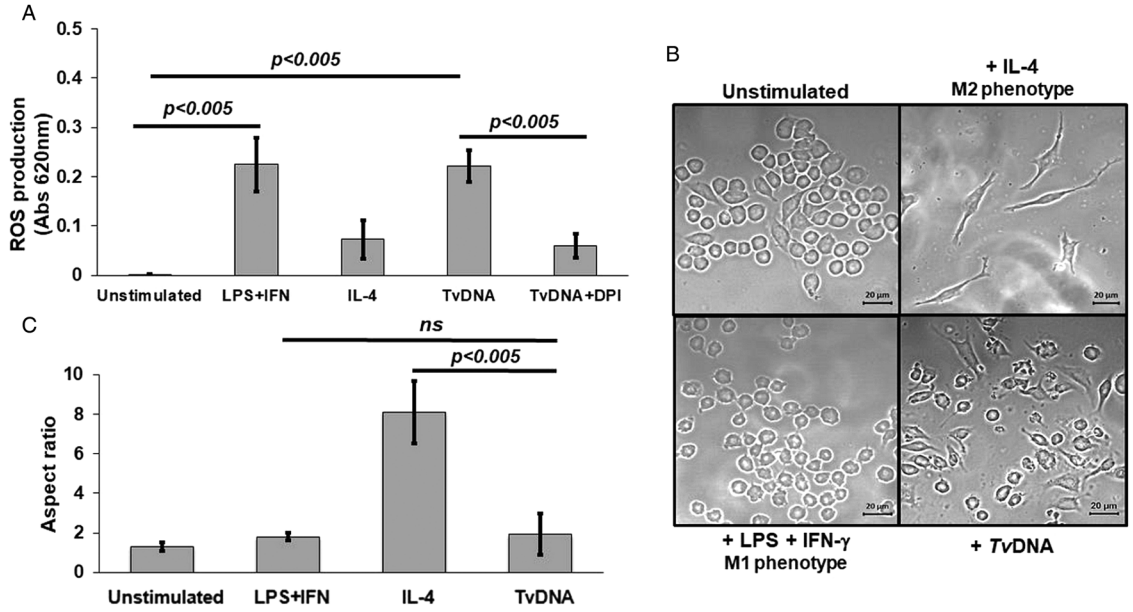

Fig. 4. ROS production and morphology changes induced by *Tv*DNA in RAW264.7 cells. (A) ROS production in RAW264.7 was determined using the NBT assay after 40 min of treatment with different stimuli. Cells were incubated with LPS (500 ng mL^{-1}) plus IFNγ (10 ng mL^{-1}) or recombinant IL-4 (20 ng mL^{-1}) as M1 or M2 phenotype induction controls, respectively. Macrophages were incubated with *Tv*DNA (50 μg mL^{-1}) in the presence or absence of DPI (30 μM), a Nox2 inhibitor. Unstimulated macrophages were used as a negative control. Data are representative of three independent experiments expressed as the mean ± S.D. Significance was determined by Kruskal–Wallis analysis and Dunn's test ($n = 18$, $^{*}P < 0.025$). ns = non-significant. (B) Morphology changes were observed in RAW264.7 cells incubated for 24 h with LPS (10 ng mL^{-1}) plus IFNγ (10 ng mL^{-1}) as M1 phenotype control, while recombinant IL-4 (20 ng mL^{-1}) was used as M2 phenotype control. Macrophages were pretreated with *Tv*DNA (50 μg mL^{-1}) to determine its effect or left untreated to use as a negative control. Images were taken by confocal microscopy, Scale bar 20 μm. Data are representative of three independent experiments. (C) Quantitative analysis of the morphological changes in macrophages induced by *Tv*DNA. The aspect ratio for cells under different stimuli was measured and calculated using ImageJ software. Significance was determined by Kruskal–Wallis analysis and Dunn's test ($^{***}P < 0.005$). One hundred cells per condition were analysed. ns = non-significant.

characterized by soft vulvar swelling, oedema formation, and whitish discharges. The vulvar oedema persisted up to 10 days post-infection in both groups of trophozoite-inoculated female mice. However, the infection signs were less evident in the infected group pre-treated with *Tv*DNA (Fig. 5B). In parallel, we also measured the extent of the vulvar oedema by quantifying the number of pixels of vulvar diameter over the rear width for each mouse, at each post-infection day. Comparing the two mice groups, untreated and pretreated with *Tv*DNA, there was no significant difference in vulvar oedema throughout the infection period, up to 10 days post-infection (Fig. 5C). For vulvar oedema measurements, we considered as zero time of infection, the mice prior to being subjected to infection. These findings suggest that pre-treatment of *Tv*DNA induces and maintains an inflammatory reaction in the host, which could be important for infection resolution.

Tv*DNA pretreatment of female mice affects the viability of* T. vaginalis *during parasite infection*

It has been reported that the immune stimulation triggered by CpG DNA induces a strong Th1 response, which is necessary to decrease parasite burden and to resolve the infection in a murine model of leishmaniasis (Ehrlich *et al.*, 2017). Our data suggest that *Tv*DNA pretreatment induces a sustained inflammatory response in infected female mice (Fig. 5B and C), thus we hypothesized that this sustained inflammatory response could affect parasite viability. To demonstrate our hypothesis, we evaluated the viability of *T. vaginalis* trophozoites in vaginal washes obtained from infected female mice that were pretreated or not with *Tv*DNA using a fluorescence microscopy assay that discriminates live *vs* dead trophozoites. As shown in Fig. 6B, we observed an increasing number of nonviable parasites from 4 to 10 days post-infection in *Tv*DNA pretreated mice compared to the *Tv*DNA untreated and infected mice (Fig. 6A). Next, we quantified the viability of *T. vaginalis* recovered from vaginal washes in untreated and *Tv*DNA pretreated mice. *Tv*DNA pretreatment significantly decreased the viability of *T. vaginalis* at four days post-infection, with the most pronounced decrease at ten days post-infection. In contrast, the viability of trophozoites in untreated mice was not changed throughout the 10 days of infection (Fig. 6C). These findings suggest that *Tv*DNA modulates the host response to favour the parasite elimination.

Tv*DNA pretreatment induces a higher production of pro-inflammatory cytokine IL-6 during parasite infection in mice*

As shown in Fig. 5 we observed a sustained vulvar inflammation in mice pretreated with *Tv*DNA and later infected with live trophozoites. Furthermore, we observed that *T. vaginalis* viability was significantly decreased by *Tv*DNA pretreatment (Fig. 6). Thus, we hypothesized that these *Tv*DNA-mediated events may be caused by a pro-inflammatory environment. To test this hypothesis we quantified IL-6, IL-10 and IL-17 cytokines in vaginal washes from mice untreated and pretreated with *Tv*DNA and later infected with live trophozoites (Fig. 7A). Results from these measurements revealed significantly higher IL-6 levels from 4 to 14 days post-infection in *Tv*DNA-pretreated mice compared to untreated ones (Fig. 7B). In striking contrast, IL-10 levels fell to undetectable levels at 4 to 10 days post-infection and only slightly rising after 14 days in *Tv*DNA-pretreated mice (Fig. 7C). Interestingly, *Tv*DNA-pretreated mice displayed significantly higher IL-17 cytokine production throughout the 14 days of infection compared to untreated but infected mice (Fig. 7D). Increased IL-17 levels are likely responsible for maintaining the inflammatory process in this condition. Taken together, these data indicate that *Tv*DNA pretreatment promotes a pro-inflammatory environment that may contribute to decreasing the parasite viability to favour infection resolution.

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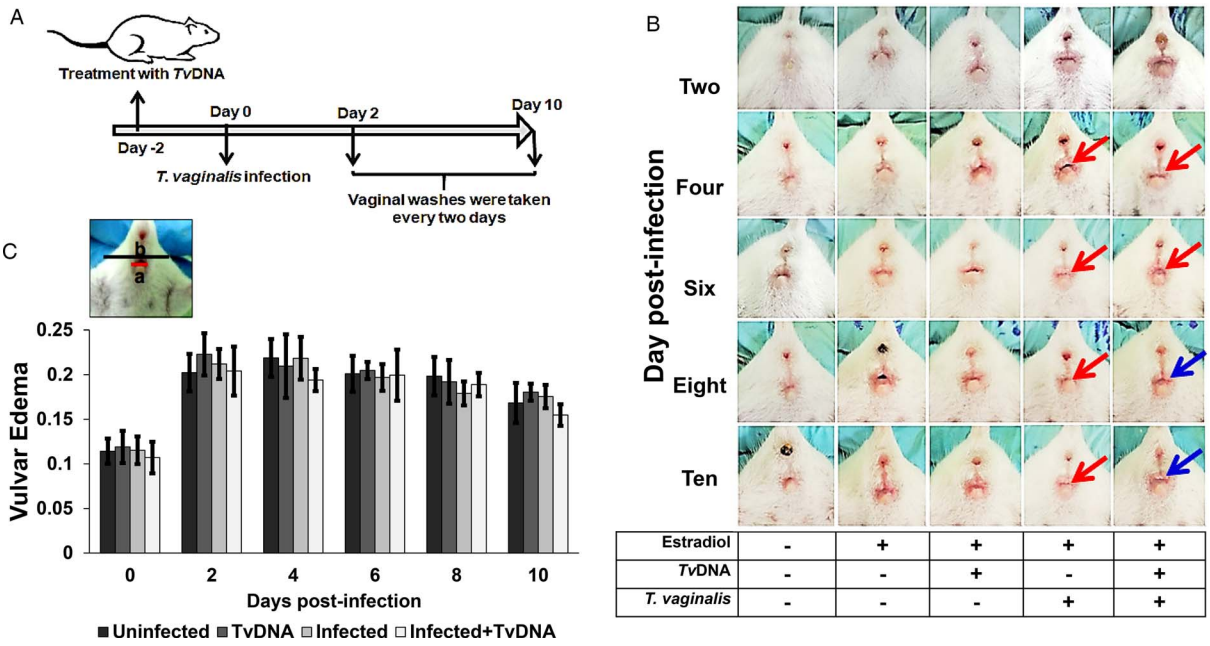

Fig. 5. *Tv*DNA promotes vulvar inflammation in infected mice. (A) Female mice were *Tv*DNA-pretreated or not, then infected with live trophozoites and vaginal washes were collected at 0, 4, 6 and 10 days post-infection. (B) Development of vulvar oedema in untreated and *Tv*DNA-pretreated (50 μg mL^{-1}) mice during ten days of infection with 5×10^6 trophozoites. Red arrows show the vulvar oedema and blue arrows show the persistence of vulvar oedema. (C) Evaluation of vulvar oedema at different days post-infection in untreated (black bars) and *Tv*DNA-pretreated (grey bars) mice. Vulvar oedema at different days post-infection was calculated as the ratio of vulvar diameter (a = red line) over the mouse rear width (b = black line). Data are representative of two independent experiments expressed as the mean ± s.d. Significance was determined by Kruskal–Wallis analysis and Dunn's test ($n = 5$ per group, $P > 0.1$).

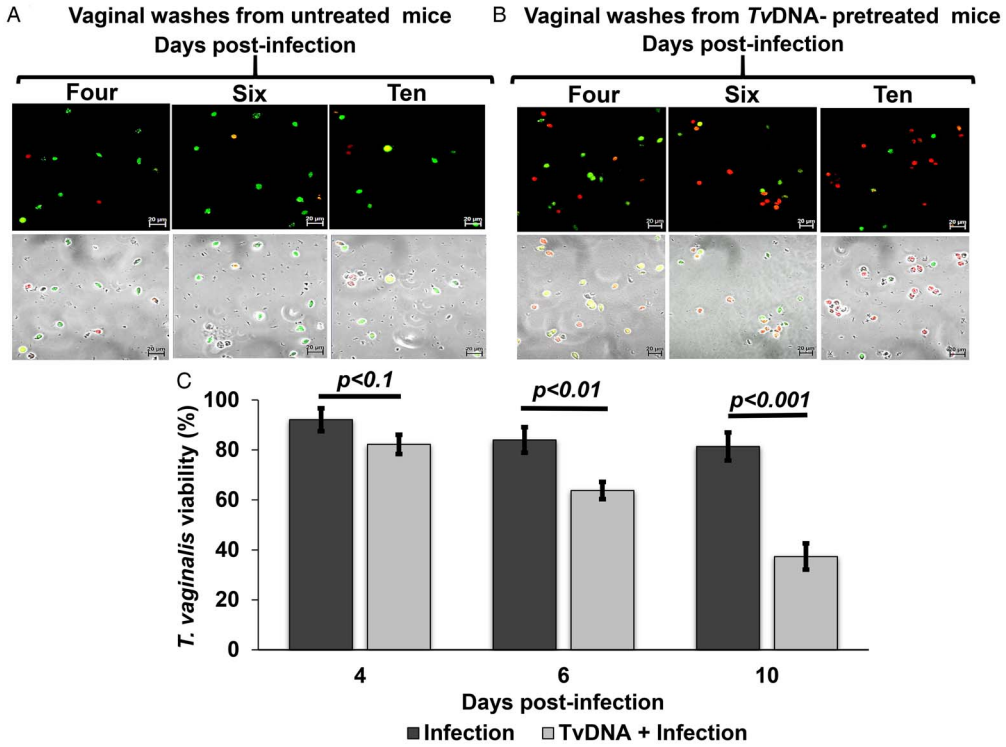

Fig. 6. Effect of *Tv*DNA pretreatment on parasite viability in infected mice. (A) Vaginal washes from untreated and infected mice collected at 4, 6 and 10 days post-infection. Scale bar, 20 μm. (B) Vaginal washes collected from infected mice pretreated with *Tv*DNA at 4, 6 and 10 days post-infection. Scale bar 20 μm. Live trophozoites are stained in green, while dead trophozoites are stained in red. (C) Viability rate of parasites in vaginal washes from untreated (black bars) and *Tv*DNA-pretreated (grey bars) mice. Data are representative of two independent experiments expressed as the mean ± s.d. Significance was determined by Kruskal–Wallis analysis and Dunn's test ($n = 5$ per group, $^*P < 0.1$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Discussion

The mechanisms by which *T. vaginalis* establishes the infection and persist chronically in humans are still not completely elucidated. This is in spite of its ranking as first among STDs and its alarming association with an elevated risk of cervical and prostate cancers (Shui *et al.*, 2016; Ghosh *et al.*, 2017). Trophozoites' adhesion to host cells is one of the first steps whereby *T. vaginalis* initiates the infection. This action triggers a cascade of events

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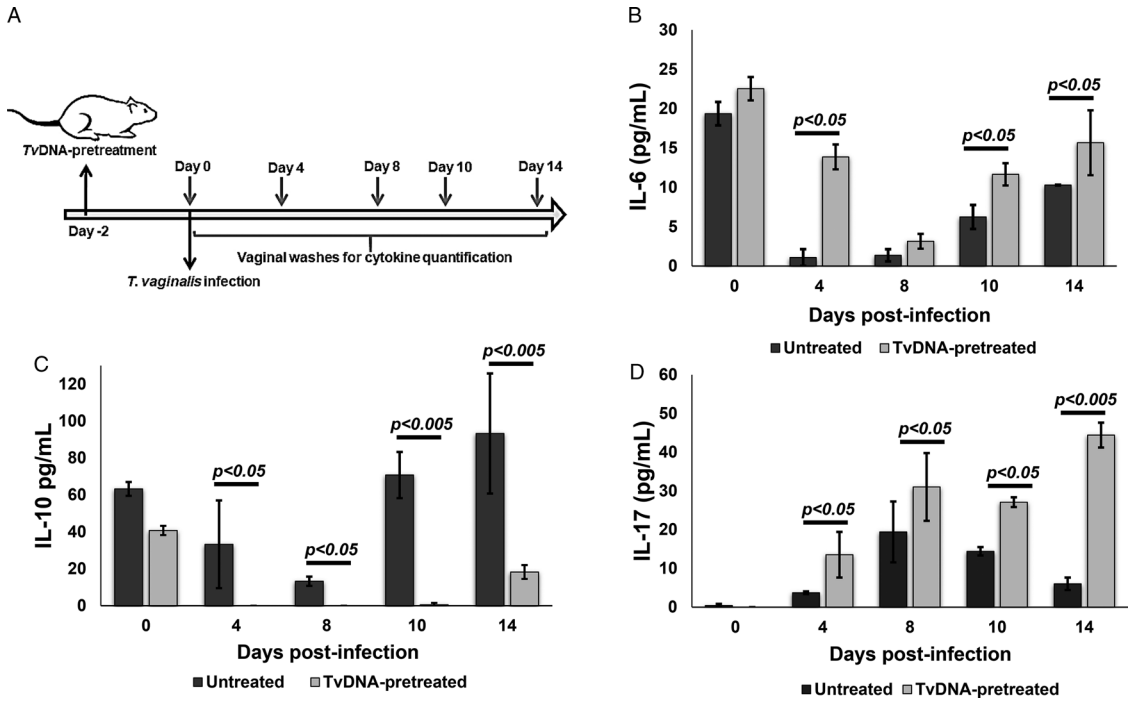

Fig. 7. Proinflammatory cytokine profile induced by *Tv*DNA pretreatment on infected mice. (A) Female mice were *Tv*DNA-pretreated or not, 2 days prior to being infected with live trophozoites. Vaginal washes were collected at 0, 4, 8, 10 and 14 days post-infection to quantify different cytokines. (B) IL-6, (C) IL-10 and (D) IL-17 levels in *Tv*DNA-pretreated (grey bars) and untreated (black bars) mice infected with *Tv* were measured by ELISA at different days post-infection. Data were obtained from two independent experiments, with ten mice in total per evaluated condition. Graphs show the mean with standard error. Significance was determined by Kruskal–Wallis analysis and Dunn's test ($n = 5$ per group, $^{**}P < 0.05$, $^{***}P < 0.005$).

mediated by Pathogen-Associated Molecular Patterns (PAMPs) and the expression of compounds at the plasma membrane, including lipophosphoglycan (LPG) (Ryan *et al.*, 2011; Pasare, 2017) that contribute to the establishment of the parasite within the host. There is also a large body of evidence about PAMPs' ability to activate immune cells through nitric oxide, cytokine production and changes in the expression of some receptors on the surface of immune cells (Takeuchi and Akira, 2010; Kumar *et al.*, 2011; Broz and Monack, 2013).

Previous studies have demonstrated an important immunostimulatory effect of CpG DNA motifs from microbes and parasites that induce both the maturation and activation of professional phagocytes (Shoda *et al.*, 2001; Ahmad-Nejad et al., 2002; Das *et al.*, 2015) as well as the promotion of a Th1 immune response (Krieg, 2000; Sabatel *et al.*, 2017) and T lymphocyte differentiation (Moseman *et al.*, 2004). Our study reveals *Tv*DNA (a CpG DNA specific type) as a robust promoter of macrophages' proinflammatory profile (Figs 2, 3 and 4) that enables them to fight against the parasite (Fig. 6). That is, our data strongly suggest that *Tv*DNA pretreatment can directly support the host in mounting an effective immune response. First, *Tv*DNA induces a cytotoxic response by enhancing NO and ROS production in macrophages. Second, *Tv*DNA promotes a substantial alteration on macrophages towards an M1 profile, inducing the expression of pro-inflammatory cytokines in addition to drastic morphological changes. Third, and most significant, *Tv*DNA pretreatment has a prominent negative impact on parasite survival and an enhanced proinflammatory cytokine profile in our *in vivo* model of trichomoniasis. A potent adjuvant effect of CpG DNA has been previously reported on leishmaniasis, toxoplasmosis and trypanosomiasis (Zimmermann *et al.*, 2008; Rodríguez-Morales *et al.*, 2015; Gong *et al.*, 2016) showing an important protective Th1 immune response in parasitic disease (Chu *et al.*, 1997; Zimmermann *et al.*, 1998; Ramirez *et al.*, 2014). The transition of the immune response to a Th1 profile against a parasite like *T. vaginalis*, possibly induced by polarization of macrophages to an M1 profile, led our interest on CpG DNA to understand the specific mechanisms of an immune response that would favour the host (Abou Fakher *et al.*, 2009). Thus, our data suggest that *Tv*DNA could exert an adjuvant effect on at least one type of immune cells, improving macrophage function during *T. vaginalis* infection. The early effect of *Tv*DNA on macrophages could trigger a strong response potentially able to change the outcome of the host–parasite interaction. Apart from the general adjuvant effect of CpG DNA, it has been shown that specific sequences present in each pathogen genome could promote the specificity of the host immune response (Gupta *et al.*, 2015). Thus, there is a need to better understand the mechanisms regulating the response to CpG DNA from parasites such as trichomonas.

The transition of macrophages to M1 profile includes morphological changes that lead to the modulation of cytokines production by mechanisms still not well understood. McWhorter *et al.* (2015) have suggested that the lamellipodia formation could induce a new actin distribution pattern and showed that the macrophage plasticity is linked to its activation and function. It will be interesting to establish how cytoskeleton reorganization promotes macrophage polarization to achieve specific functional phenotypes and how these changes contribute to enhance the host response by decreasing trichomonas' viability.

In the present study, the lack of effect of *Tv*DNA on vulvar oedema but the dramatic effect on trophozoite viability are opposite effects to those observed with *T. vaginalis* exosomal fractions (*Tv*ELV) in the pretreatment of infected mice which increased the IL-10 production, decreased the vulvar oedema and favoured the trophozoite viability in our murine infection model (Olmos-Ortiz *et al.*, 2017). Thus, it is possible that *Tv*DNA induces macrophages to extend the proinflammatory response to prevent parasite population expansion in the host. The specific role of innate immune response in the host–parasite relationship during trichomoniasis is limited and requires further study.

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Overall, our work suggests that *Tv*DNA is an immunomodulator that could redirect the result of the host–parasite interaction. Finally, *Tv*DNA could be considered as a potential adjuvant for vaccine development against trichomoniasis.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182019001094.

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Conflict of interest. The authors declare that they have no conflict of interest.

Ethical standards. Permission for this study was granted by the Ethics Committee of the University of Guanajuato. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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