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Increased intracellular Cl^- concentration mediates *Trichomonas vaginalis*-induced inflammation in the human vaginal epithelium

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ABSTRACT

Trichomonas vaginalis is a primary urogenital parasite that causes trichomoniasis, a common sexually transmitted disease. As the first line of host defense, vaginal epithelial cells play critical roles in orchestrating vaginal innate immunity and modulate intracellular Cl^- homeostasis via the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel that plays positive roles in regulating nuclear factor-κB (NF-κB) signalling. However, the association between *T. vaginalis* infection and intracellular Cl^- disequilibrium remains elusive. This study showed that after *T. vaginalis* infection, CFTR was markedly down-regulated by cysteine proteases in vaginal epithelial cells. The intracellular Cl^- concentration ($[Cl^-]_i$) was consequently elevated, leading to NF-κB signalling activation via serum- and glucocorticoid-inducible kinase-1. Moreover, heightened $[Cl^-]_i$ and activated NF-κB signalling could be sustained in a positive feedback regulatory manner resulting from decreased intracellular cAMP through NF-κB-mediated up-regulation of phosphodiesterase 4. The results conclusively revealed that the intracellular Cl^- of the human vaginal epithelium could be dynamically modulated by *T. vaginalis*, which contributed to mediation of epithelial inflammation in the human vagina.

1. Introduction

Trichomoniasis, caused by the flagellated parasitic protozoan *Trichomonas vaginalis*, is the most common non-viral genitourinary sexually transmitted infection in humans (World Health Organization, 2011). *Trichomonas vaginalis* infection has been implicated as a cause of serious adverse pregnancy outcomes (Schwebke and Burgess, 2004; Bouchemal et al., 2017), higher risk of HIV acquisition (Sorvillo and Kerndt, 1998; Schwebke and Burgess, 2004), cervical neoplasia and cervical cancer (Bouchemal et al., 2017). Considering the high morbidity and the various complications, trichomoniasis has been chosen by the Centers for Disease Control and Prevention (USA) as a priority for public health action (Meites, 2013). Oral treatment with 5-nitroimidazoles (eg. metronidazole) reveals a powerful therapeutic effect on trichomoniasis (Schwebke and Burgess, 2004). However, novel therapeutic strategies are still required in cases of allergy or resistance with regard to the use of conventional drugs (Pearlman et al., 1996; Land et al., 2004), and the risks of side-effects in pregnant women (Schwebke and Burgess, 2004). Thus, elucidating the mechanism underlying the host-parasite interaction is a necessity for the prevention and treatment of *T. vaginalis* infection.

As sentinels of vaginal immune protection, vaginal epithelial cells provide the first line of host defense against invading pathogens in a variety of ways. These include formation of tight junctions, secretion of anti-microbial peptides, antigen presentation, transport of IgA and IgG, and secretion of cytokines and chemokines (Wira et al., 2005; Ochiel et al., 2008; Patel et al., 2013). In addition, epithelial cells participate in regulating the microenvironment of the vaginal lumen through ion transportation to maintain homeostasis within the vaginal mucosa (Chan et al., 2013; Li et al., 2014). Cl^- is the predominant anion in biological systems,

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both in the extra- and intracellular environments of animal cells (Duran et al., 2010; Stauber and Jentsch, 2013). Various Cl^- transporters and channels participate in maintaining intracellular Cl^- homeostasis. The cystic fibrosis transmembrane conductance regulator (CFTR), which is widely expressed throughout the body, is a cAMP-regulated anion channel that mainly conducts Cl^- transport in epithelial cells (Anderson et al., 1991; Pilewski and Frizzell, 1999). Recent investigations revealed decreased Cl^- secretion after infection by various pathogens, including influenza virus M2 (Londino et al., 2015), *Toxoplasma gondii* (Guo et al., 2015) and *Campylobacter jejuni* (Negoro et al., 2014) in epithelial cells. The decreased expression of CFTR in H5N1 virus-infected alveolar epithelial cells has also been observed (Chan et al., 2016). These observations suggest that an alteration in CFTR expression or function may be involved in the pathogenic process and in the unbalanced innate immune response induced by the invasion of pathogens. It has been demonstrated that changes in intracellular Cl^- concentrations ($[Cl^-]_i$) regulate the expression of specific genes and the activity of kinases (Piala et al., 2014; Valdivieso et al., 2016). Furthermore, our previous research revealed that intracellular Cl^- may serve as an intracellular signal following lipopolysaccharide (LPS) stimulation, resulting in activation of the serum- and glucocorticoid-inducible kinase-1 (SGK1), a Cl^- sensitive serine/threonine protein kinase, to promote the activation of nuclear factor κB (NF-κB) in the airway epithelium (Zhang et al., 2018). These discoveries prompted us to speculate that aberrant CFTR-Cl^--SGK1 signalling may be implicated in mediating vaginal epithelial inflammation following infection.

Thus, this study aimed to explore the regulatory role of intracellular Cl^- in the inflammatory process provoked by *T. vaginalis* infection in the vaginal epithelium. Additionally, the possible mediatory role of CFTR and SGK1 in this process was explored. The outcomes might complement and expand our knowledge of pathogenic mechanisms during trichomoniasis and lead to new theoretical bases for drug development.

2. Materials and methods

2.1. Culture of parasites and host cells

Trichomonas vaginalis strain CPO 02 was cultured in Diamond's Trypticase Yeast Extract Maltose (TYM) medium composed of (in $g\ L^{-1}$): 20 tryptone (Oxoid, UK), 10 yeast extract (Oxoid), 5 D-maltose (Sigma Aldrich, USA), 1 L-cysteine hydrochloride (Whiga, China) and 0.2 L-ascorbic acid (Sigma Aldrich) (pH 6.8), supplemented with 10% heat-inactivated foetal bovine serum (FBS, Tianhang Biotechnology, China), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Hyclone, USA) in 5% CO_2 at 37 °C, and used for the next assays during log phase (Diamond, 1957). The human vaginal epithelial cell line VK2/E6E7 (ATCC® CRL-2616) was purchased from Beijing ZhongYuan Ltd (China), and cells were cultured as previously described (Sun et al., 2014, 2016). In brief, the cells were cultured in keratinocyte serum-free medium (KSFM, Gibco, USA) supplemented with 0.1 ng ml^{-1} recombinant epidermal growth factor (Gibco), 50 μg ml^{-1} of bovine pituitary extract (Gibco), 100 units ml^{-1} penicillin (Hyclone), 100 μg ml^{-1} streptomycin (Hyclone), and an additional 0.4 mM $CaCl_2$ (Guangzhou Chemical Pharmaceutical Factory, China). Cells were cultured in 5% CO_2 at 37 °C, and subcultured at nearly 80% of confluence.

2.2. Infection of VK2/E6E7 cells with T. vaginalis

The human vaginal epithelial VK2/E6E7 cell line was established from normal human vaginal epithelia immortalised by human papillomavirus 16/E6E7 (Fichorova et al., 1997). The VK2/E6E7 cells were seeded into a 12-well plate, and when the plate was near 80% of confluence, the cells were incubated with different numbers of live or inactivated *T. vaginalis* parasites for different time periods at 37 °C in 5% CO_2. *T. vaginalis* were harvested by centrifugation at 2000*g* for 5 min. The parasites used in experiments with or without inhibitors were viable (>99%), as determined by a trypan blue exclusion assay (beyotime, China, Supplementary Fig. S1). Inactivated *T. vaginalis* were prepared at 60 °C for 30 min, or fixed in 10% formalin (Guangzhou Chemical Pharmaceutical Factory, China) at room temperature for 30 min. Trypan blue staining showed that no viability was found in the inactivated parasites, whereas the cell structure and integrity remained undestroyed (Supplementary Fig. S2A).

2.3. Western blotting

Total protein was extracted from cultured cells. Briefly, samples were lysed by boiling for 5 min and electrophoresed by 6–10% SDS/PAGE. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA) and blocked at room temperature for 1 h. Blots were then incubated at 4 °C overnight with primary antibodies. The following antibodies were purchased from Cell Signaling Technology (USA): anti-phospho-IκB-α (Ser32, #2859), anti-CFTR (#2269), anti-β-actin (#4970). Antibody against phospho-SGK1 (Ser255/Thr256, 36–002) was purchased from Upstate (USA). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat antibody against rabbit (EarthOx, USA), with 1 h incubation at room temperature. Western HRP Substrate was used for protein band detection (Millipore, USA).

2.4. Quantitative real-time PCR analysis

Total RNA was extracted using a RNAprep Pure Cell/Bacteria Kit (TIANGEN, China). The mRNA was reverse transcribed using HiScript II Q RT SuperMix for real-time quantitative PCR (qPCR) (+genomic DNA (gDNA) wiper) (Vazyme, China). qPCR was performed with ChamQ SYBR qPCR Master Mix (Vazyme, China). The levels of target genes were normalised to the levels of GAPDH mRNA, and relative expression was determined using the $2^{-\Delta\Delta Ct}$ method. The primers used in qPCR were as follows: (forward) 5′-AAACAGATGAAGTGCTCCTTCCAGG-3′ and (reverse) 5′-TGGAGAACACCACTTGTTGCTCCA-3′ for *IL1B*; (forward) 5′-CAGCCCTGAGAAAGGAGACAT-3′ and (reverse) 5′-GCTCTGGCTTGTTCCTCACTA-3′ for *IL6*; (forward) 5′-AACATGACTTCCAAGCTGGCC-3′ and (reverse) 5′-TTATGAATTCTCAGCCCTCTTC-3′ for *CXCL8*; (forward) 5′-TGCCCTTCGGCGATGTTT-3′ and (reverse) 5′-GCGATAGAGCGTTCCTCCTTG-3′ for *CFTR*; (forward) 5′-TGCACCACCAACTGCTTAGC-3′ and (reverse) 5′-GGATGCAGGGATGATGTTCT-3′ for *GAPDH*. The primers for human four subfamilies of type 4 phosphodiesterase (PDE4), *PDE4A*, *PDE4B*, *PDE4C* and *PDE4D* were described previously (Zhang et al., 2018).

2.5. Intracellular Cl^- measurement

The VK2/E6E7 cells were subcultured on glass cover slips and treated under different conditions when the confluence reached nearly 20%. The fluorescence was recorded after the incubation of the Cl^- sensitive dye, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE, Invitrogen, USA), as described previously (Zhang et al., 2018). The calibration curve and its construction were shown in Supplementary Fig. S3.

2.6. ELISA analyses

Intracellular cAMP content under different treatment conditions was determined by ELISA from R&D Systems (USA) as

described in the manufacturer's protocol, followed by the measurement of protein concentrations of the lysates, as described previously (Zhang et al., 2018).

2.7. Statistical analysis

Data were presented as the mean ± S.D. The pairwise comparisons of the results were performed by the Student's two-tailed *t* test, and one-way ANOVA followed by Bonferroni was used for multiple comparisons, using Origin Pro software (OriginLab Corporation, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Trichomonas vaginalis triggered inflammation, accompanied by an increase in $[Cl^-]_i$ resulting from down-regulation of CFTR expression via cysteine proteases in vaginal epithelial cells

Trichomonas vaginalis infections are usually associated with inflammatory processes (Bouchemal et al., 2017). To investigate the pro-inflammatory effect of *T. vaginalis* on vaginal epithelial cells, we initially evaluated *T. vaginalis*-induced activation of the NF-κB signalling pathway in human vaginal epithelial VK2/E6E7 cells. After incubation with *T. vaginalis* for 3 h, the inhibitor of NF-κB (IκB) phosphorylation was positively enhanced during infection in a dose-dependent manner in VK2/E6E7 cells (Fig. 1A). Meanwhile, pro-inflammatory cytokines/chemokines including interleukin-1β (IL-1β), IL-6 and IL-8, which were considered as the cytokine markers of vaginal inflammation (Fichorova, 2004), were significantly up-regulated after *T. vaginalis* infection (Fig. 1B). These findings were consistent with previous findings in vaginal epithelial cells (Fichorova et al., 2013, 2016), indicating the activation of NF-κB signalling triggered by *T. vaginalis* in the vaginal epithelium.

In light of the positive role of CFTR in regulating inflammation (Anderson et al., 1991; Pilewski and Frizzell, 1999; Stauber and Jentsch, 2013), we interrogated whether *T. vaginalis* dynamically modulated the expression of CFTR in vaginal epithelial cells. Interestingly, the protein expression of CFTR was dose-dependent but decreased after incubation with *T. vaginalis* for 3 h (Fig. 1C), whereas no significant difference was observed at the transcriptional level in VK2/E6E7 cells (Supplementary Fig. S4). Considering the critical roles of CFTR in modulating $[Cl^-]_i$ in epithelial cells, we then investigated whether $[Cl^-]_i$ was dynamically changed after *T. vaginalis* infection. Using a Cl^- sensitive dye, MQAE, we found that after incubation with *T. vaginalis* for 3 h, $[Cl^-]_i$ was significantly increased from 44.06 ± 1.21 mM at basal conditions to 61.29 ± 1.91 mM in VK2/E6E7 cells (Fig. 1D). These findings suggested the possibility that the CFTR protein might be degraded by *T. vaginalis*, which led to the increase in $[Cl^-]_i$ in vaginal epithelial cells. Previous evidence has shown that the *T. vaginalis* secreted cysteine proteases (CPs) play crucial roles in the pathogenesis of trichomoniasis, and CFTR has been proven to be the substrate for CPs (Arroyo and Alderete, 1989; Hasegawa et al., 1992; Stratford et al., 2003). Thus, we next sought to verify the involvement of

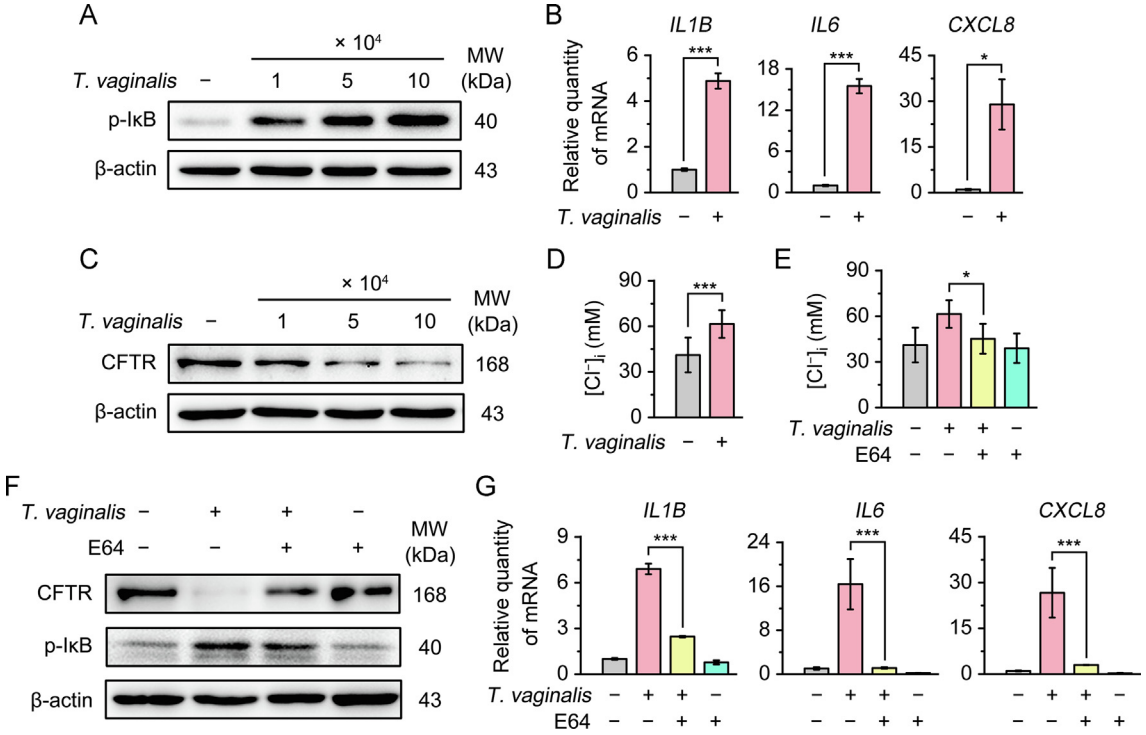

Fig. 1. *Trichomonas vaginalis* infection induced down-regulation of cystic fibrosis transmembrane conductance regulator (CFTR), increased $[Cl^-]_i$ and inflammation in the human vaginal epithelial VK2/E6E7 cells. (A) Whole-cell lysates from VK2/E6E7 cells, incubated with indicated numbers of *T. vaginalis* for 3 h, were subjected to immunoblotting with the antibody against phospho-IκB (p-IκB), using β-actin as a loading control. Data shown are representative blots of three independent experiments. (B) Quantitative real-time PCR analyses of IL-1β, IL-6 and IL-8 mRNA were measured in VK2/E6E7 cells incubated with 1×10^5 *T. vaginalis* for 3 h. Data are means ± S.D., $n = 3$. $^{*}P < 0.05$ and $^{***}P < 0.001$. (C) Whole-cell lysates from VK2/E6E7 cells incubated with indicated numbers of *T. vaginalis* for 3 h were subjected to immunoblotting with the antibody against CFTR, using β-actin as a loading control. Data shown are representative blots of three independent experiments. (D) Alteration in $[Cl^-]_i$ after 1×10^5 *T. vaginalis* infection for 3 h in VK2/E6E7 cells. Data are means ± S.D., $n = 9$ cells for each group. $^{***}P < 0.001$. (E) Alteration in $[Cl^-]_i$ in VK2/E6E7 cells treated with E64 (150 μM), the selective inhibitor of cysteine proteases, for 1 h, followed by infection with 1×10^5 *T. vaginalis* for 3 h in VK2/E6E7 cells. Data are means ± S.D., $n = 9$ cells for each group. $^{*}P < 0.05$. (F) Whole-cell lysates from VK2/E6E7 cells treated with E64 (150 μM) together with live 1×10^5 *T. vaginalis* for 3 h were subjected to immunoblotting with the antibody against CFTR and p-IκB, using β-actin as a loading control. Data shown are representative blots of three independent experiments. (G) Quantitative real-time PCR analyses of IL-1β, IL-6 and IL-8 mRNA were measured in VK2/E6E7 cells treated with E64 (150 μM) together with live 1×10^5 *T. vaginalis* for 3 h. Data are means ± S.D., $n = 3$. $^{***}P < 0.001$.

CPs in *T. vaginalis*-elicited down-regulation of CFTR, elevated $[Cl^-]_i$ and inflammation in vaginal epithelial cells. After treating VK2/E6E7 cells with inactivated *T. vaginalis* (either heat-killed or formalin-treated), the phosphorylation of IκB was weakly heightened, without the observation of down-regulation of CFTR expression (Supplementary Fig. S2B), revealing that down-regulation of CFTR was live parasite-dependent. Pretreatment with E64 (150 μM), the selective inhibitor of CPs (Stratford et al., 2003), abrogated the effect of *T. vaginalis* on CFTR expression and $[Cl^-]_i$ in VK2/E6E7 cells (Fig. 1E, F). Notably, *T. vaginalis* triggered IκB phosphorylation and pro-inflammatory cytokines/chemokines were also inhibited by E64 (Fig. 1F, G). The above evidences indicated a possible link between CFTR impairment, heightened $[Cl^-]_i$ and inflammation in the pathogenesis of *T. vaginalis*-induced vaginal epithelial inflammation.

3.2. Increased $[Cl^-]_i$ mediated Trichomonas vaginalis-induced vaginal epithelial inflammation via SGK1 activation

We have previously observed that the activity of an inflammation-associated serine–threonine kinase, serum- and glucocorticoid-inducible kinase-1 (SGK1) (Tai et al., 2009), could be augmented by higher Cl^- concentrations (Zhang et al., 2018). These prompted us to investigate whether heightened $[Cl^-]_i$ induced by *T. vaginalis* infection led to SGK1 activation and inflammatory responses in vaginal epithelial cells. Firstly, we validated the activation of SGK1 in higher $[Cl^-]_i$-elicited inflammation in VK2/E6E7 cells. To establish a vaginal epithelial cell model with different $[Cl^-]_i$, we used $CFTR_{inh-172}$ (10 μM), a selective blocker of CFTR (Melis et al., 2014), to increase $[Cl^-]_i$ by functionally damaging CFTR (Fig. 2A). Analogous to infection with *T. vaginalis*, treatment with $CFTR_{inh-172}$ (10 μM) led to more pronounced IκB phosphorylation (Fig. 2B). Moreover, the phosphorylation of SGK1 was also strengthened by $CFTR_{inh-172}$ (10 μM, Fig. 2C), which indicated that the inflammation elicited by increased $[Cl^-]_i$ might be associated with SGK1 activation. To exclude a possible non-specific effect of $CFTR_{inh-172}$, we established a cell model by adding NaCl (40 mM) into the cell culture medium. Correspondingly, Na-gluconate (40 mM) was used to exclude the effect of Na^+ and mannitol was used as the osmotic control. Similarly, NaCl treatment significantly resulted in intracellular Cl^- accumulation (Supplementary Fig. S5A), higher levels of IκB phosphorylation and SGK1 activation in VK2/E6E7 cells, whereas no significant difference was observed in the Na-gluconate group or the mannitol control group (Supplementary Fig. S5B). These results strongly confirmed the correlation between higher $[Cl^-]_i$-elicited inflammation and SGK1 activation. Given that $[Cl^-]_i$ was increased after *T. vaginalis* infection in vaginal epithelial cells, we then evaluated the possible regulatory role of SGK1 in *T. vaginalis*-triggered inflammation. The results showed that SGK1 phosphorylation was enhanced after *T. vaginalis* infection, accompanying a pronounced enhanced IκB phosphorylation (Fig. 2D). Furthermore, pharmacological inhibition of SGK1 using the selective inhibitor, EMD638683 (Ackermann et al., 2011), significantly inhibited the up-regulation of pro-inflammatory cytokines/chemokines induced by *T. vaginalis* in a concentration-dependent manner (Fig. 2E), revealing the vital role of SGK1 in regulating *T. vaginalis*-triggered inflammatory responses in vaginal epithelium.

3.3. Trichomonas vaginalis infection reduced intracellular cAMP via NF-κB–phosphodiesterase 4 signalling pathways, resulting in ongoing inflammation in vaginal epithelial cells

A positive feedback loop in NF-κB activation via the NF-κB–PDE4–cAMP–$[Cl^-]_i$–NF-κB pathway has been described in LPS-induced airway epithelial inflammation (Zhang et al., 2018). We thus investigated the effect of *T. vaginalis* on PDE4 in vaginal epithelial cells. Using qPCR technique, the mRNA levels of PDE4A–D were detected in VK2/E6E7 cells. The results showed that *T. vaginalis* elicited up-regulation of PDE4B and PDE4C (Fig. 3A), but not the other two variants (Supplementary Fig. S6), after incubation for 3 h. This was consistent with the findings that intracellular cAMP significantly decreased after *T. vaginalis* infection (Fig. 3C). Furthermore, JSH-23 (20 μM), a translocational inhibitor of the NF-κB p65 subunit (Shin et al., 2004), suppressed both the up-regulation of PDE4B/C and the decrease in intracellular cAMP triggered by *T. vaginalis* (Fig. 3B, C). This suggested that *T. vaginalis* induced the initial NF-κB activation, which subsequently resulted in PDE4B/C up-regulation and the decrease in intracellular cAMP levels. In light of the cAMP-regulated property of CFTR activation and the cAMP-hydrolyzing role of PDE4 (Hasegawa et al., 1992; Banner and Trevethick, 2004; Spina, 2008), we then investigated

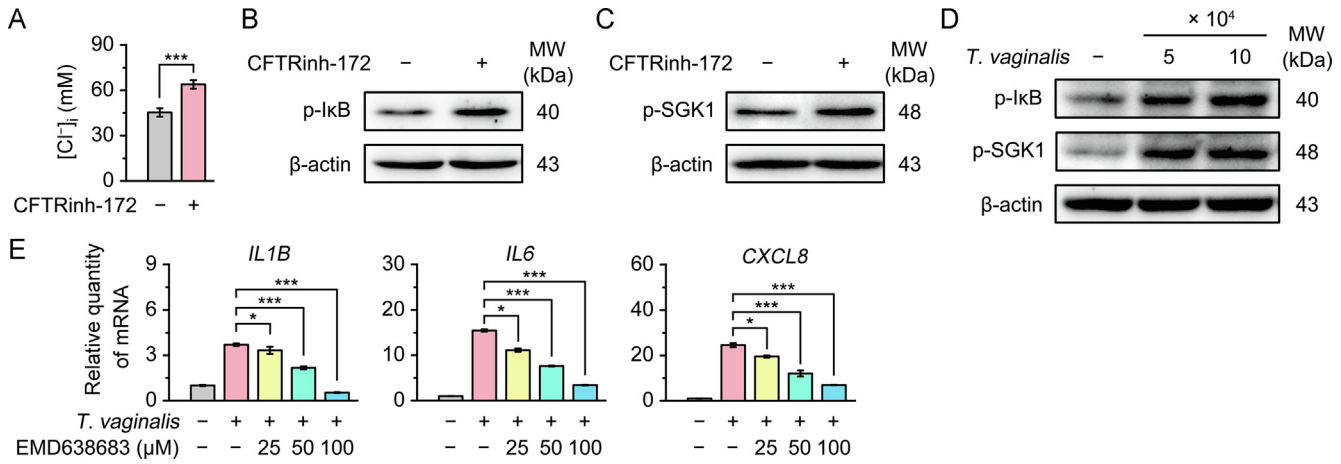

Fig. 2. Elevated $[Cl^-]_i$ elicited by *Trichomonas vaginalis* mediated inflammatory responses through activating serum- and glucocorticoid-inducible kinase-1 (SGK1) in the human vaginal epithelial VK2/E6E7cells. (A) $[Cl^-]_i$ was measured in VK2/E6E7 cells pretreated with $CFTR_{inh-172}$ (10 μM), the selective blocker of cystic fibrosis transmembrane conductance regulator for 12 h. Data are means ± S.D., $n = 9$ cells for each group. $^{***}P < 0.001$. Whole-cell lysates were subjected to immunoblotting with antibody against (B) phospho-IκB (p-IκB), or (C) phospho-SGK1 (p-SGK1), using β-actin as a loading control in VK2/E6E7 cells pretreated with $CFTR_{inh-172}$ (10 μM) for 12 h. Data shown are representative blots of three independent experiments. (D) Whole-cell lysates were subjected to immunoblotting with antibody against p-IκB or p-SGK1, using β-actin as a loading control in VK2/E6E7 cells incubated with indicated numbers of *T. vaginalis* for 3 h. Data shown are representative blots of three independent experiments. (E) The effect of different concentrations of EMD638683 on the mRNA expression of IL-1β, IL-6 and IL-8 in VK2/E6E7 cells incubated with 1×10^5 *T. vaginalis* for 3 h. Data are means ± S.D., $n = 3$. $^{*}P < 0.05$ and $^{***}P < 0.001$.

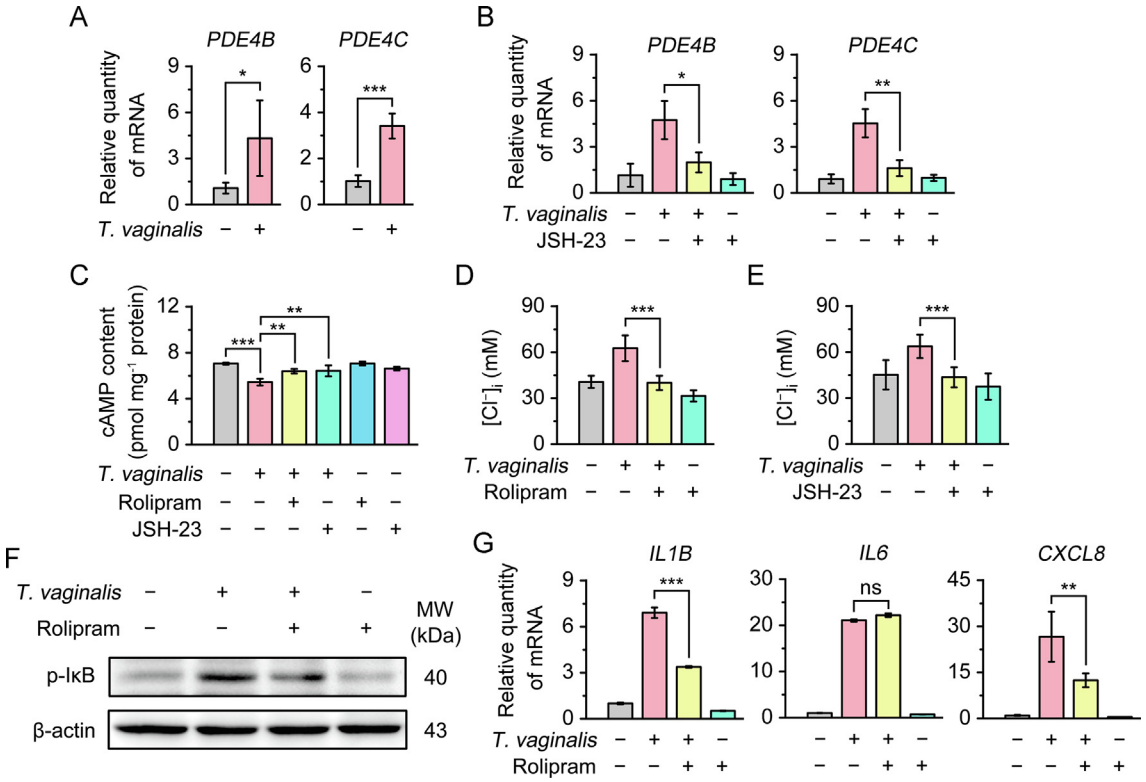

Fig. 3. *Trichomonas vaginalis* decreased intracellular cAMP and induced inflammation via up-regulation of phosphodiesterase 4 (PDE4) in a nuclear factor-κB (NF-κB) - dependent manner. (A) The human vaginal epithelial VK2/E6E7cells were infected by 1×10^5 *T. vaginalis* for 3 h and the mRNA levels of PDE4B and PDE4C were measured by quantitative PCR. Data are means ± S.D., $n = 3$. $^{*}P < 0.05$ and $^{***}P < 0.001$. (B) Effect of JSH-23, an inhibitor of NF-κB p65 nuclear translocation (20 μM), on the mRNA expression of PDE4B and PDE4C after 1×10^5 *T. vaginalis* infection for 3 h in VK2/E6E7 cells. Data are means ± S.D., $n = 3$. $^{*}P < 0.05$ and $^{**}P < 0.01$. (C) Effect of JSH-23 (20 μM) and rolipram (50 μM) on the intracellular concentration of cAMP using ELISA in VK2/E6E7 cells incubated with 1×10^5 *T. vaginalis* for 3 h. Data are means ± S.D., $n = 3$–4. $^{**}P < 0.01$ and $^{***}P < 0.001$. Alteration in $[Cl^-]_i$ in VK2/E6E7 cells treated with (D) rolipram (50 μM) or (E) JSH-23 (20 μM) for 1 h, followed by 1×10^5 *T. vaginalis* infection for 3 h in VK2/E6E7 cells. Data are means ± S.D., $n = 9$ cells for each group. $^{*}P < 0.05$ and $^{***}P < 0.001$. Effects of rolipram (50 μM) on (F) p-IκB and (G) mRNA expression of three pro-inflammatory cytokines in VK2/E6E7 cells incubated with 1×10^5 *T. vaginalis* for 3 h. The western blot images are representative blots of three independent experiments. Data from real-time quantitative PCR experiments are means ± S.D., $n = 3$. $^{**}P < 0.01$ and $^{***}P < 0.001$.

the involvement of PDE4 in maintaining *T. vaginalis*-induced aberrant $[Cl^-]_i$ in vaginal epithelial cells. Pre-treatment with a selective PDE4 inhibitor (Torphy, 1998), rolipram (50 μM), inhibited the decreased intracellular cAMP (Fig. 3C) and increased $[Cl^-]_i$ triggered by *T. vaginalis* in VK2/E6E7 cells (Fig. 3D). Moreover, JSH-23 (20 μM) also showed an inhibitory effect on the *T. vaginalis*-induced increase in $[Cl^-]_i$ (Fig. 3E), which indicated an anti-inflammatory approach by interrupting NF-κB–PDE4–cAMP signalling pathways in *T. vaginalis*-infected vaginal epithelial cells. Notably, pre-treatment with rolipram (50 μM) suppressed the *T. vaginalis*-elicited IκB phosphorylation (Fig. 3F) and up-regulation of the cytokines/chemokines, with the exception of IL-6 (Fig. 3G) in VK2/E6E7 cells.

4. Discussion

Trichomoniasis, an extremely common global infection, has received more attention recently due to a better understanding of the associated severe complications (Meites, 2013). Some virulence factors including lipophosphoglycan and CPs that participate in trichomonal cytoadherence and cytotoxicity, have been shown to be associated with the pathogenesis of *T. vaginalis* (Arroyo and Alderete, 1989; Schwebke and Burgess, 2004; Malla et al., 2014; Bouchemal et al., 2017). However, little is known about the molecular mechanisms occurring during lower genital tract inflammation caused by infection with *T. vaginalis*. Here, we show the link between *T. vaginalis*-triggered inflammation and aberrant higher $[Cl^-]_i$ resulting from CFTR impairment, thereby confirming that intracellular Cl^- might be a positive regulator for NF-κB.

The mediating role of CFTR in transcellular Cl^- transport has been elucidated in past decades. As an anion channel distributed extensively throughout the body, CFTR plays a crucial role in fluid homeostasis at the surface of the epithelium, dysfunction of which may cause mucous abnormalities, infection and inflammation (Guo et al., 2015; Elborn, 2016). Besides, CFTR reportedly regulates MAPK/NF-κB signalling (Dong et al., 2015; Chen et al., 2016), presenting a positive role in inflammatory regulation. Previous studies have shown significant down-regulation of CFTR in cells infected by various pathogens including the H5N1 virus (Chan et al., 2016), *Helicobacter pylori* (Cao et al., 2015) and *Pseudomonas aeruginosa* (Rubino et al., 2014). Here, we have demonstrated that the protein level, but not the transcriptional level, of CFTR was down-regulated after *T. vaginalis* infection in a CP-dependent manner. Considering that CFTR has been proven to be the substrate for CPs (Stratford et al., 2003), we speculated that the degradation of CFTR via CPs secreted by *T. vaginalis* was probably the main cause, although further investigation is required to verify this hypothesis. *T. vaginalis* is estimated to secrete up to 156 CPs (Hirt et al., 2011). These hydrolases have trypsin-like activity and participate in nearly all the pathogenic processes of *T. vaginalis* infection such as the invasion of the mucous layer, cytoadherence, cytotoxicity, degradation of immunoglobulins, apoptosis and type 2 immune responses (Arroyo and Alderete, 1989; Hernandez et al., 2014; Mielczarek and Blaszkowska, 2016; Oh et al., 2017). Several CPs contributing to the virulence properties of *T. vaginalis*, such as TvCP39 and TvCP65, have been characterised (Arroyo et al., 2015). The identification of the CPs involved in *T. vaginalis*-elicited CFTR impairment would be helpful in better understanding

the pathogenesis of trichomoniasis, although the difficulty of CP protein purification and characterisation remains a limitation (Hernandez et al., 2014).

In most cells, $[Cl^-]_i$ is dynamically modulated by multiple related channels and transporters. Although our mainstream knowledge of Cl^- comprises relevant physiological functions including fluid secretion, regulation of excitability and volume regulation (Nilius and Droogmans, 2003; Duran et al., 2010), the pathological roles of intracellular Cl^-, such as apoptosis and inflammation, have only been sporadically reported in recent years (Willumsen et al., 1989; Heimlich and Cidlowski, 2006; Yang et al., 2012). Additionally, some Cl^--sensing kinases and genes such as with-no-lysine kinase 1 (WNK1) (Piala et al., 2014), the ribosomal protein RPS27 and glutaredoxin-related protein 5 (Valdivieso et al., 2016) have been identified. We have previously demonstrated that SGK1 was a Cl^--sensitive kinase, which had potentiated activity in higher levels of Cl^-. In this study, we found the phosphorylation of SGK1 was significantly enhanced in VK2/E6E7 cell models with higher $[Cl^-]_i$, or after *T. vaginalis* infection, which further illustrated the close relationship between $[Cl^-]_i$ and SGK1. Moreover, the inhibitor of SGK1 abrogated the increased expression of pro-inflammatory cytokines/chemokines caused by this parasite. SGK1 reportedly regulates multiple cellular and physiological functions, including the activity of NF-κB. SGK1 phosphorylates IκB kinase (IKK) at Thr-23 directly and Ser-180 indirectly (Tai et al., 2009), and the SGK1 inhibitor harbours anti-inflammatory properties by blocking Nod-like receptor protein 3 (NLRP3) inflammasome activation (Gan et al., 2018). Our findings confirmed the regulatory role of SGK1 in mediating inflammatory responses, which is also consistent with our previous work in the respiratory system (Zhang et al., 2018).

Inflammation is beneficial in appropriate amounts. However, when the response is excessive or persistent, it may become detrimental. Here, we found that after *T. vaginalis* infection, the content of intracellular cAMP decreased in an NF-κB dependent manner because of higher expression of PDE4B/C, a phosphodiesterase that can degrade intracellular cAMP through enzymatic hydrolysis (Francis et al., 2011). These results suggested that in vaginal epithelial cells, *T. vaginalis* may impair the activity of CFTR in a positive feedback regulatory manner via NF-κB–PDE4–cAMP signalling. This is because CFTR is a phosphorylation-activated anion channel regulated by elevation of cAMP and activation of protein kinase A (PKA) (Gadsby and Nairn, 1999). PKA mediated the phosphorylation of serine residues leading to the opening of the Cl^- ion permeation pathway (Cheng et al., 1991), which means that the content of intracellular cAMP is closely related to the activity of CFTR. Persistent dysfunction of CFTR maintains a higher $[Cl^-]_i$ in *T. vaginalis*-infected cells, which then contributes to the ongoing inflammation. The anti-inflammatory action of PDE4 inhibitors through increasing cAMP levels has been reported extensively (Fabbri et al., 2009; Crilly et al., 2011; Komatsu et al., 2013). Here, we have demonstrated that the inhibition of PDE4 ameliorated *T. vaginalis*-induced vaginal epithelial inflammation. However, it should be pointed out that rolipram failed to suppress *T. vaginalis*-induced up-regulation of IL-6. One possible reason might lie in the involvement of other immune regulatory pathways activated by *T. vaginalis* which could modulate the transcription of IL-6 in a cAMP-independent way.

In conclusion, *T. vaginalis* infection induced CFTR degradation via CPs secreted from the parasites in vaginal epithelial cells. $[Cl^-]_i$ was then heightened and this activated NF-κB by enhancing the activity of SGK1. Furthermore, PDE4B and PDE4C were up-regulated in an NF-κB-dependent way, resulting in a decreased content of cAMP, elevated $[Cl^-]_i$ and ongoing inflammation (Fig. 4). This study confirmed that intracellular Cl^- may act as a pivotal mediator in the *T. vaginalis*-induced epithelial inflammation response, indicating a positive feedback loop in NF-κB activation. Therefore, medicines targeting CPs or PDE4 that may alleviate intracellular Cl^- accumulation, or the downstream kinase SGK1, may have therapeutic potential for the treatment of *T. vaginalis*-induced epithelial inflammation in the vagina.

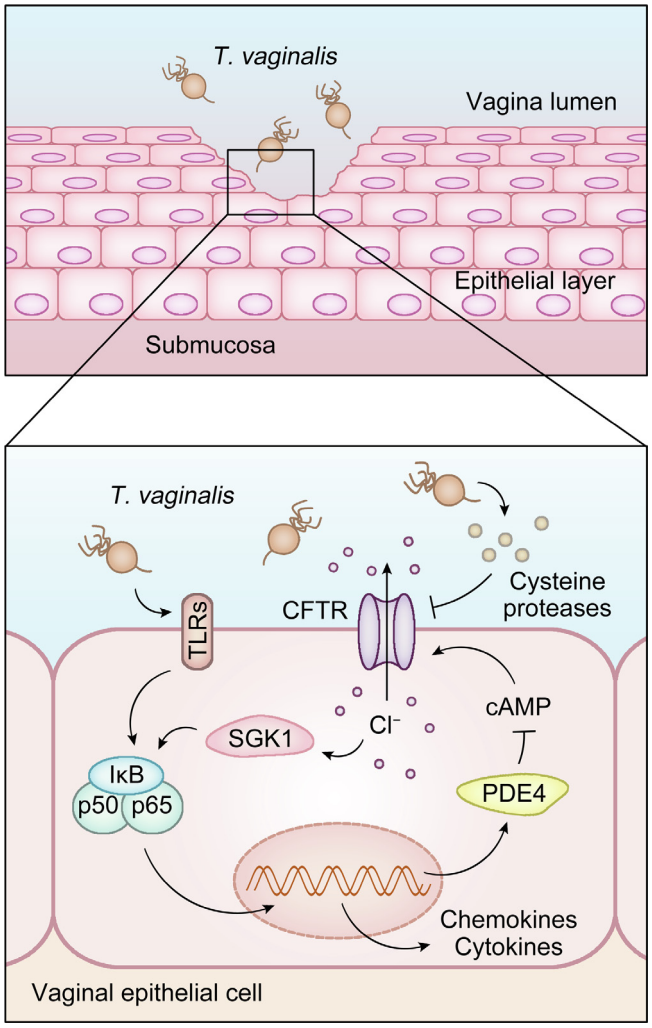

Fig. 4. Schematic model of increased $[Cl^-]_i$-mediated vaginal epithelial inflammation triggered by *Trichomonas vaginalis* infection. *Trichomonas vaginalis* can give rise to an increase in $[Cl^-]_i$ as a result of the impairment of cystic fibrosis transmembrane conductance regulator (CFTR). The increased $[Cl^-]_i$ may initiate inflammatory responses through activation of serum- and glucocorticoid-inducible kinase-1 (SGK1), thereby activating nuclear factor-κB (NF-κB) signalling pathways. The inflammatory response may persist due to NF-κB-mediated up-regulation of phosphodiesterase 4 (PDE4), which promotes cAMP hydrolysis and persistent elevated $[Cl^-]_i$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.04.005.

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