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▣ ORIGINAL ARTICLE

Adenosine A_3 Receptor Mediates ERK1/2- and JNK-Dependent TNF-α Production in *Toxoplasma gondii*-Infected HTR8/SVneo Human Extravillous Trophoblast Cells

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Abstract: *Toxoplasma gondii* is an intracellular parasite that causes severe disease when the infection occurs during pregnancy. Adenosine is a purine nucleoside involved in numerous physiological processes; however, the role of adenosine receptors in *T. gondii*-induced trophoblast cell function has not been investigated until now. The goal of the present study was to evaluate the intracellular signaling pathways regulated by adenosine receptors using a HTR-8/SVneo trophoblast cell model of *T. gondii* infection. HTR8/SVneo human extravillous trophoblast cells were infected with or without *T. gondii* and then evaluated for cell morphology, intracellular proliferation of the parasite, adenosine receptor expression, TNF-α production and mitogen-activated protein (MAP) kinase signaling pathways triggered by adenosine A_3 receptor (A_3AR). HTR8/SVneo cells infected with *T. gondii* exhibited an altered cytoskeletal changes, an increased infection rate and reduced viability in an infection time-dependent manner. *T. gondii* significantly promoted increased TNF-α production, A_3AR protein levels and p38, ERK1/2 and JNK phosphorylation compared to those observed in uninfected control cells. Moreover, the inhibition of A_3AR by A_3AR siRNA transfection apparently suppressed the *T. gondii* infection-mediated upregulation of TNF-α, A_3AR production and MAPK activation. In addition, *T. gondii*-promoted TNF-α secretion was dramatically attenuated by pretreatment with PD098059 or SP600125. These results indicate that A_3AR-mediated activation of ERK1/2 and JNK positively regulates TNF-α secretion in *T. gondii*-infected HTR8/SVneo cells.

Key words: *Toxoplasma gondii*, adenosine A_3 receptor, TNF-α, HTR8/SVneo trophoblast cell, MAPK

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect many vertebrate animals, including humans, and is highly prevalent worldwide [1]. Although *T. gondii* infection in humans is frequently asymptomatic, it can lead to severe disease in immunocompromised patients and congenitally infected children, leading to several manifestations, such as retinochoroiditis and miscarriage during the first trimester of pregnancy [2]. Host protection against *T. gondii* infection results from a complex cell-mediated immune response involving inflammatory cells, lymphocytes and macrophages, which is characterized as a T helper type 1 (Th1)-immune response with prominent production of interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-1β [3].

Disorders due to congenital *T. gondii* infection likely involve both cellular and molecular changes in the placenta. *T. gondii* is known to infect all nucleated host-cells and can trigger host-cell apoptosis [4]. In pregnant mice infected by *T. gondii*, increased trophoblast apoptosis was observed and was associated with increased FAS expression in trophoblast cells and IFN-γ and TNF-α in decidual cells [5]. *T. gondii* infection-induced apoptosis was also observed in femur bone marrow cells of mice and was associated with increased TNF-α expression [6]. TNF-α, a multifunctional cytokine, has been detected in many tissues including ovary, oviduct, uterus, and placenta and is expressed in embryonic tissues. For many years, TNF-α was primarily considered to be a cytokine involved in triggering immunological pregnancy loss and as a mediator of various embryopathic stresses [7].

Adenosine is a potent immunomodulatory biomolecule

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that is produced by the ectoenzymes nucleoside triphosphate dephosphorylase (CD39) and ecto-5'-nucleotidase (CD73), which are highly expressed by several cell types, including leukocytes, during stress, injury, and infection [8]. Extracellular adenosine levels increase in response to hypoxia, ischemia and inflammation, preventing tissue damage during instances of cellular stress or injury [9]. The effects of adenosine are mediated via 4 adenosine receptor (AR) subtypes: A_1AR, A_{2A}AR, A_{2B}AR, A_3AR [10]. Of these, A_{2A}AR is recognized as mediating major adenosine anti-inflammatory activity. Iriyama et al.[11] revealed that a local increase of adenosine in the placenta is sufficient to trigger key features of preeclampsia using mouse models, and adenosine was identified as one of pathogenic factors for preeclampsia. A2B receptor activation has been shown to blunt trophoblast migration, possibly as a result of reduced activation of the ERK1/2 and SAPK/JNK signaling pathway and lower proMMP-2 and VEGF levels, which are crucial for trophoblast function [9]. These observations suggest the possible involvement of adenosine receptors in placental developmental processes.

Although adenosine receptor activity is important in the immune response against *T. gondii* during gestation, the role of adenosine receptors in *T. gondii*-infected human extravillous trophoblast cells remains unclear. HTR8/SVneo cells are widely used as a model of human extravillous trophoblast cells to evaluate the migration and proliferation processes of extravillous trophoblast cells [12] and better understand the factors involved during preeclampsia [13]. For this reason, HTR8/SVneo cells were used as to study *T. gondii* infection in the present study.

The goal of the present study was to evaluate the functional role of adenosine receptors using a HTR-8/SVneo trophoblast cell model of *T. gondii* infection, we evaluated *T. gondii*-mediated alterations in HTR8/SVneo cell morphology, intracellular proliferation of the parasite, adenosine receptor expression, TNF-α production and mitogen-activated protein kinase (MAPK) signaling pathways triggered by A_3AR in HTR8/SVneo cells.

MATERIALS AND METHODS

Cell culture and parasite infection

The human extravillous trophoblast cell line HTR8/SVneo was purchased from the American Tissue Culture Collection (Manassas, Virginia, USA). Briefly, these cells were cultured in RPMI 1640 medium (Hyclone, Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic-antimycotic (all from Gibco, Grand Island, New York, USA) in a humidified incubator at 37˚C under an atmosphere with 5% CO_2. HTR8/SVneo cells were infected with *T. gondii* at a multiplicity of infection (MOI) of 10 for 4, 8, and 24 hr. Then the cell morphology, viability, adenosine receptor family expression, TNF-α production and activation of MAPK signaling pathways were evaluated. To evaluate the role of A_3AR in *T. gondii*-induced MAPK activation and TNF-α production, HTR8/SVneo cells that had been incubated for 48 hr after transfection with control siRNA or A_3AR-specific siRNAs were infected with *T. gondii* at an MOI of 10 for 24 hr and then MAPK activation and TNF-α secretion levels were assessed.

In vitro cultivation of *Toxoplasma gondii*

Tachyzoites of RH and GFP-RH (constitutively express green fluorescent protein) strains of *T. gondii* were maintained in ARPE-19 cells under an atmosphere with 5% CO_2 and 37˚C. Infected cells were scraped, forcibly passed through a 27-gauge needle, and centrifuged at 1,350×g for 10 min using Percoll (Sigma, St. Louis, Missouri, USA) to pellet the parasites.

The human RPE cell line ARPE-19 was purchased from the American Tissue Culture Collection (Manassas, Virginia, USA). The cells were routinely grown in Dulbecco's modified Eagle's medium/F12 (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum and antibiotic–antimycotics (all from Gibco). The cells were cultured at 37˚C under an atmosphere with 5% CO_2 and passaged every 3-4 days. ARPE-19 cells were used between passages 4 and 8 in the present study.

Immunofluorescence microscopy

HTR8/SVneo cells were seeded onto coverslips in 12-well plates at a density of 2×10^4 cells/well and incubated for 24 hr. The cells were then mock-infected or infected with the GFP-RH strain of *T. gondii* at a multiplicity of infection (MOI) of 10 for 4, 8, and 24 hr. Subsequently, the cells were washed with Hank's balanced salt solution (HBSS) and fixed with freshly prepared 4% paraformaldehyde for 1 hr at room temperature. After being washed 5 times with PBS containing 0.3% Triton X-100 (PBS-T) for 10 min, the cells were incubated with a primary antibody against α-tubulin for 2 hr at room temperature. The cells were washed to remove excess primary antibody and then incubated with an anti-mouse Alexa Fluor 647 secondary antibody for 2 hr. After mounting the samples with VECTA-

SHIELD HardSet antifade mounting medium with DAPI (Vector Laboratories, Burlingame, California, USA), fluorescence images were acquired using a laser scanning confocal microscope (Leica TCS SP5 II).

Cell viability assay

HTR8/SVneo cells were either mock-infected or infected with *T. gondii* at an MOI of 10 for 4, 8 or 24 hr, after which cell viability was estimated using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, Wisconsin, USA) following the manufacturer's instructions.

Western blot analysis

After washing HTR8/SVneo cells with phosphate-buffered saline (PBS), proteins were isolated using ice-cold RIPA buffer (Thermo Scientific, Waltham, Massachusetts, USA) with complete protease inhibitor cocktail (Roche, Basel, Switzerland) for 30 min on ice. After centrifugation at 14,000×g for 15 min at 4˚C, the supernatants were collected, and equal amounts of protein from each sample were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline (20 mM Tris and 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBST) and 5% skim milk. After being washed once with TBST, the membranes were incubated overnight at 4˚C with the primary antibodies diluted in TBST supplemented with 5% bovine serum albumin. Primary antibodies against the following proteins were used: adenosine A_1-R (A_1AR), adenosine A_{2A}-R (A_{2A}AR), adenosine A_{2B}-R (A_{2B}AR), adenosine A_3-R (A_3AR), and α-tubulin (all from Santa Cruz Biotechnology); and phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2, ERK1/2, phospho-JNK, and JNK (all from Cell Signaling Technology Inc., Danvers, Massachusetts, USA). Following 3 consecutive washes in TBST, the membranes were incubated for 90 min with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories), diluted 1:5,000 with incubation buffer, as described above. Subsequently, the membranes were soaked with Immobilon Western Chemiluminescent HRP Substrate (Jackson ImmunoResearch Laboratories), and chemiluminescence was detected with a Fusion Solo System (Vilber Lourmat, Collegien, France). Band intensity was quantified using ImageJ (NIH, Bethesda, Maryland, USA), and the results were normalized to α-tubulin protein levels and expressed as fold-changes compared to the control group.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total cellular RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, California, USA). RNA (2 μg) was reverse transcribed in a final volume of 20 μl using Superscript II reverse transcriptase (Invitrogen Life Technologies), as specified by the manufacturer, and then used in RT-qPCR analyses with specific primer sets (Table 1). RT-qPCR was conducted using an ABI 7500 FAST System (Applied Biosystems, Carlsbad, California, USA) in 20-μl reactions containing cDNA (100 ng) with SYBR-Premix Ex Taq II (Takara Bio Inc., Otsu, Japan). The hypoxanthine phosphoribosyltransferase 1 (HPRT1) gene was amplified for normalization of the cDNA amount used in RT-qPCR. Reactions were performed in triplicate, and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

ELISA for TNF-α

HTR8/SVneo cells were mock-infected or infected with the RH strain of *T. gondii* at an MOI of 10 for 4, 8, and 24 hr. The supernatants from the mock-or *T. gondii*-infected HTR8/SVneo cells were collected in triplicate, and TNF-α levels were mea-

Table 1. Adenosine receptor family primer sequences used for quantitative reverse transcription PCR (RT-qPCR) in the present study

Gene name	GenBank Accession No.	Primer sequence (5'-3')	Product size (bp)
ADORA1 (A_1AR)	NM_000674.3	F-ATTGCTGTGGACCGCTACCTCC R-CGCACTCAGATTGTTCCAGCCA	153
ADORA2 (A_{2A}AR)	NM_000675.6	F-ACCGCTACATTGCCATCCGCAT R-TCCTTTGGCTGACCGCAGTTGT	151
ADORA2B (A_{2B}AR)	NM_000676.2	F-GCTCCATCTTCAGCCTTCTGGC R-AAGGACCCAGAGGACAGCAATG	125
ADORA3 (A_3AR)	BC029831.1	F-ATACAAGAGGGTCACCACTCA R-CAGGTGAGGAAGCTGAAGTATAC	204
HPRT1	NM_000194.2	F-GACCAGTCAACAGGGGACAT R-CTGCATTGTTTTGCCAGTGT	111

sured using commercially available TNF-α ELISA kits following the manufacturer's instructions (R&D System, Minneapolis, Minnesota, USA). The cytokine concentrations in the samples were calculated from standard curves obtained using recombinant cytokines.

siRNA transfection

Cells were transfected with siRNA duplexes specific for human A_3AR (Santa Cruz Biotechnology) using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, California, USA) following the manufacturer's protocol. Briefly, the cells were seeded into 6-well plates, grown for 24 hr (70% confluence), and then transfected with 20 nM adenosine A_3-R siRNA or negative control siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) for 48 hr. Subsequently, the cells were pre-infected with the RH strain of *T. gondii* at an MOI of 10 for 24 hr, and knockdown efficiency was determined by western blot analysis.

Inhibition of MAPK activation

To confirm the roles of p38, ERK1/2, and JNK in the *T. gondii*-induced production of TNF-α by HTR8/SVneo cells, we performed experiments using inhibitors of p38 MAPK (SB203580), ERK1/2 (PD098059), and JNK (SP600125). HTR8/SVneo cells were pretreated with 30 μM SB203580, PD098059 or SP600125 for 1 hr followed by infection with *T. gondii* at an MOI of 10 for a further 24 hr. Then, the cell culture supernatants were collected, and TNF-α secretion was assessed as described above.

Statistical analyses

All assays were performed at least 3 times in triplicate. The data are presented as means ± standard deviation (SD). Statistical analysis of the data was performed using unpaired, 2-tailed Student's t-tests with Bonferroni adjustment or ANOVA for multiple comparisons. A *P*-value less than 0.05 was considered to indicate statistical significance.

RESULTS

T. gondii infection induced cytoskeletal changes in HTR8/SVneo cells and reduces cell viability

For detecting morphologic changes, the integrity of the microtubule network was assessed by immunofluorescence microscopy using an α-Tubulin antibody and DAPI to stain cellular microtubules and DNA, respectively. Immunofluorescence staining analysis of HTR8/SVneo cells by confocal microscopy showed a well-developed array of hair-like microtubule networks of slim fibrous microtubules (red) wrapped around the cell nucleus (blue) in control cells. In contrast, cells infected with *T. gondii* showed a α-tubulin staining pattern that was diffuse and disorganized. In addition, *T. gondii* infection induced nuclear fragmentation and cellular shrinkage, whereas untreated cells grew well with a clear complete cytoskeleton (Fig. 1A). The numbers of *T. gondii*-infected cells and the total number of cells were counted under a fluorescence microscope. As shown in Fig. 1B, the *T. gondii* infection rate significantly increased in an infection time-dependent manner. Furthermore, to evaluate the effects of *T. gondii* infection on HTR8/SVneo cell viability, the cells were incubated with *T. gondii* at an MOI of 10 for various times and then subjected to cell viability assay. Compared to mock-infected control cells, *T. gondii* infection significantly reduced cell viability, with viabilities of 82.83 ± 4.78%, 70.37 ± 7.77%, and 48.50 ± 3.49% observed for cells infected with *T. gondii* for 4, 8, and 24 hr, respectively (Fig. 1C). These results indicate that *T. gondii* infection alters HTR8/SVneo cell cytoskeletal and reduces cell viability.

T. gondii infection upregulated adenosine A_3 receptor expression in HTR8/SVneo cells

In the present study, to explore the mechanism associated with the *T. gondii*-induced alterations in HTR8/SVneo cell morphology and the potential involvement of adenosine receptors, HTR8/SVneo cells were infected with *T. gondii* at an MOI of 10 for 4, 8, and 24 hr. Subsequently, the gene expression of adenosine receptor family members was assessed by RT-qPCR, and the results indicated that adenosine A_3 receptor (A_3AR) mRNA levels were significantly increased after 4, 8, and 24 hr of *T. gondii* infection in a time-dependent manner. Unexpectedly, *T. gondii* infection did not alter A_1AR, A_{2A}AR, and A_{2B}AR mRNA expression (Fig. 2A). We also evaluated protein levels of adenosine receptor family members by western blot analysis. Consistent with RT-qPCR results, *T. gondii* infection dramatically increased A_3AR protein levels in a time-dependent manner, while those of A_1AR and A_{2B}AR were unaffected (Fig. 2B). In addition, A_{2A}AR protein was not detected in either the control or *T. gondii*-infected HTR8/SVneo cells (data not shown). These results support the hypothesis that adenosine receptors are associated with the *T. gondii*-induced HTR8/SVneo cell cytoskeletal changes, which may specifically involve the time-dependent upregulation of A_3AR levels.

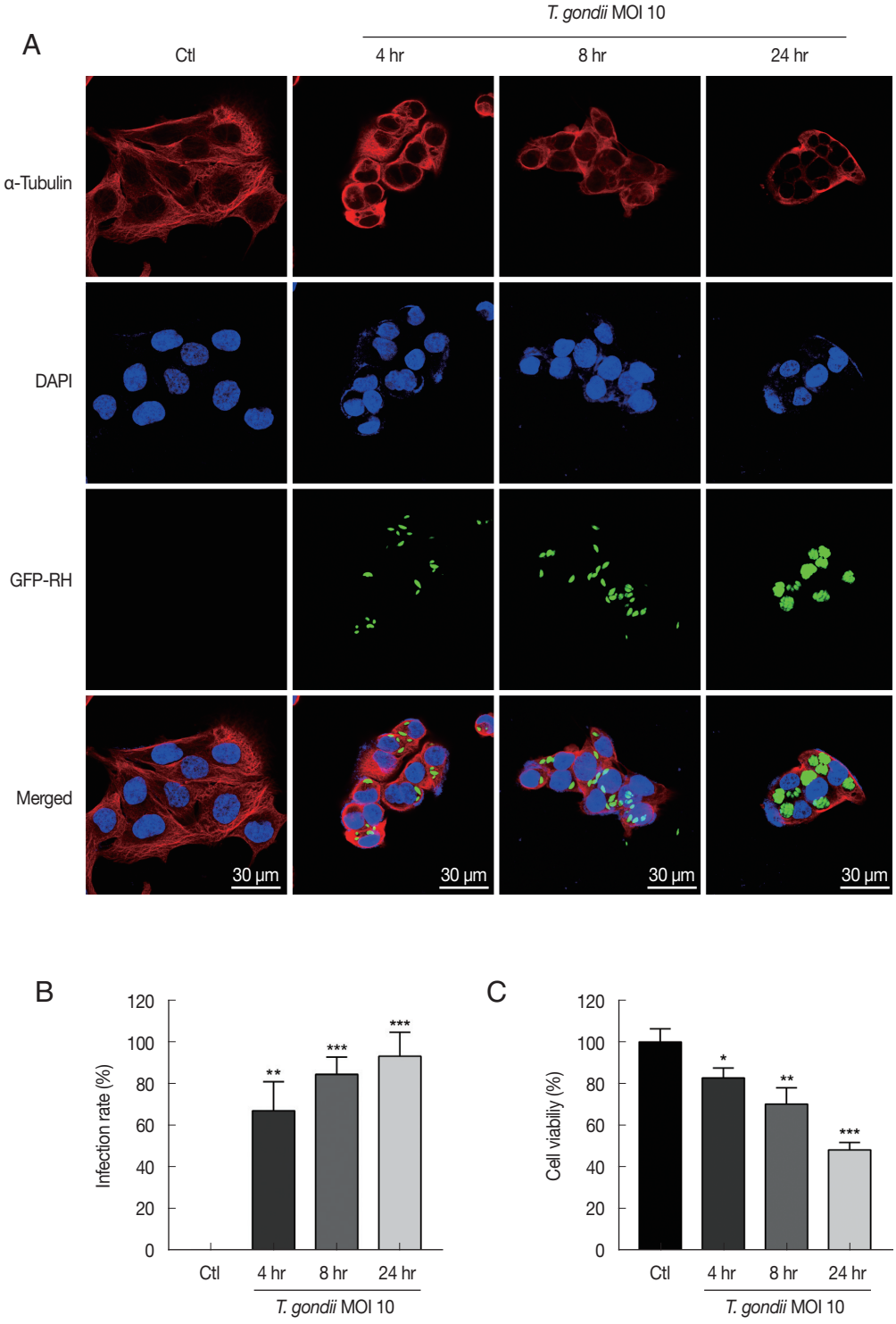

Fig. 1. *Toxoplasma gondii* infection induced morphological changes in HTR8/SVneo cells and reduces cell viability. (A) HTR8/SVneo cells were infected with GFP-expressing *T. gondii* at an MOI of 10 for the indicated time durations. Cells were fixed and probed against α-tubulin (red), after which they were counterstained with DAPI (blue) and visualized by confocal microscopy. Scale bar = 30 μm. (B) Number of *T. gondii*-infected cells and total number of cells were counted to calculate infection rate. (C) Cell viability was measured by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (mean ± SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with mock-infected control cells.

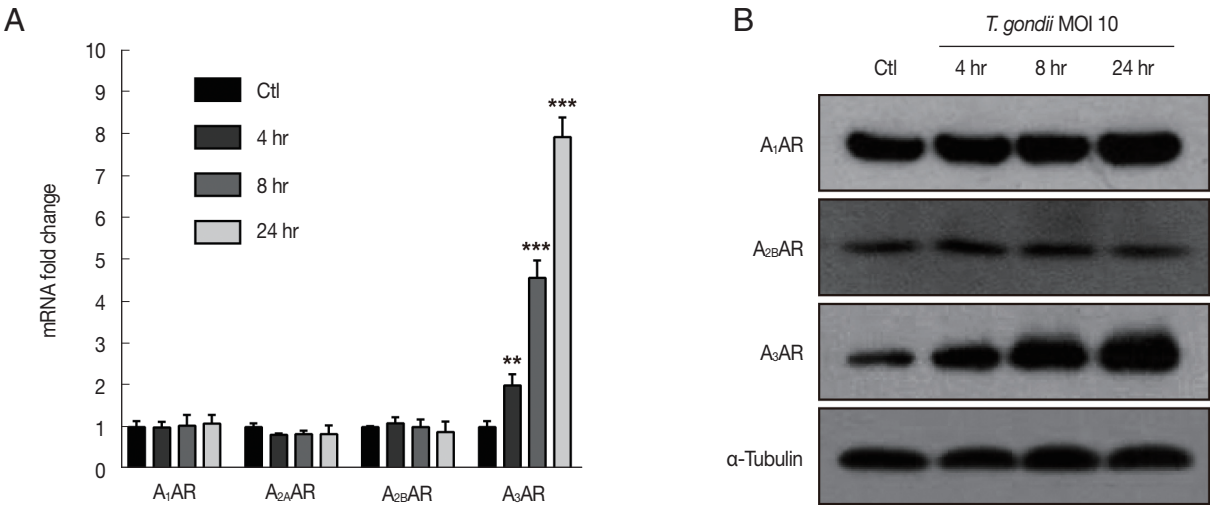

Fig. 2. *Toxoplasma gondii* infection upregulated adenosine A_3 receptor expression in HTR8/SVneo cells. HTR8/SVneo cells were infected with the RH strain of *T. gondii* at an MOI of 10 for the indicated time durations. (A) Adenosine A_1, A_{2A}, A_{2B} and A_3 receptor expression assessed by RT-qPCR. HPRT1 was used as an internal control. $^{**}P<0.01$, $^{***}P<0.001$ compared with mock-infected control cells. (B) Expression level of adenosine receptors assessed by western blot analysis. α-Tubulin used as the loading control.

T. gondii infection increased TNF-α secretion and MAPK activation in HTR8/SVneo cells

HTR8/SVneo cells were mock-infected or infected with the RH strain of *T. gondii* at an MOI of 10 for 4, 8, and 24 hr and then evaluated for TNF-α secretion and MAPK activation. Significantly increased TNF-α secretion by *T. gondii*-infected HTR8/SVneo cells was detected by ELISA with a time-dependent manner (Fig. 3A). A previous study convincingly demonstrated that intracellular *T. gondii* induces MAPK pathways and the production of proinflammatory cytokines in macrophages [15]. In turn, MAPKs promote the activation of transcription factors, ultimately resulting in the production of IL-12 and TNF-α [16], leading us to ask whether the *T. gondii*-induced secretion of TNF-α by HTR8/SVneo cells was associated with the activation of MAPK. To evaluate this possibility, HTR8/SVneo cells were incubated with *T. gondii* at an MOI of 10 for 4, 8, and 24 hr and then assessed for MAPK kinase pathway activation. As shown in Fig. 3B, the phosphorylation levels of p38 and ERK1/2 protein levels were markedly increased and peaked at 4 hr postinfection and then gradually decreased. More importantly, *T. gondii* infection induced the sustained phosphorylation of JNK in a time dependent manner. Based on these findings, we conclude that phosphorylation of MAPK components may be involved in the *T. gondii*-induced production of TNF-α by HTR8/SVneo cells.

Effect of A_3AR siRNA treatment on MAPK activation and subsequent TNF-α cytokine secretion

To evaluate the role of A_3AR in *T. gondii*-induced MAPK activation and TNF-α production, HTR8/SVneo cells that had been incubated for 48 hr after transfection with control siRNA or A_3AR-specific siRNAs were infected with *T. gondii* at an MOI of 10 for 24 hr and then MAPK activation and TNF-α secretion levels were assessed. As shown in Fig. 4A, western blot results revealed significantly increased levels of A_3AR and phosphorylated p38, ERK1/2 and JNK in the control siRNA-transfected *T. gondii*-infected cells. In contrast, the levels of these proteins were dramatically downregulated in A_3AR knockdown cells. Subsequently, to further assess whether A_3AR is involved in the regulation of TNF-α secretion, we examined TNF-α production in A_3AR siRNA-transfected *T. gondii*-infected cells. Notably, *T. gondii* infection markedly increased TNF-α production in control siRNA transfected cells, while A_3AR siRNA transfection significantly inhibited the *T. gondii*-induced production of TNF-α (Fig. 4B). These results strongly suggest that A_3AR is involved in the *T. gondii*-induced activation of MAPK and TNF-α secretion. Subsequently, to obtain direct evidence for the association between MAPK activation and TNF-α secretion, HTR8/SVneo cells were preincubated with or without inhibitors of p38 MAPK (SB203580), ERK1/2 (PD098059), and JNK (SP600125) for 2 hr and then were infected with *T. gondii* for 24 hr. ELISA results revealed that TNF-α levels were reduced by PD098059

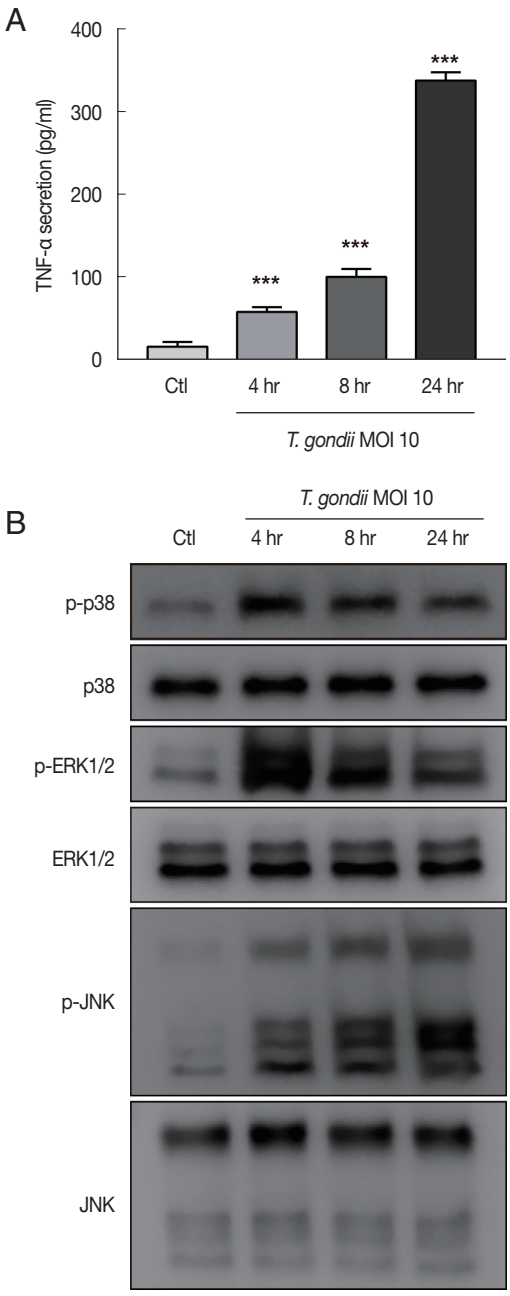

Fig. 3. *Toxoplasma gondii* infection increased TNF-α secretion and MAPK activation in HTR8/SVneo cells. HTR8/SVneo cells were infected with *T. gondii* at an MOI of 10 for the indicated time durations. (A) TNF-α secretion levels evaluated by ELISA. ***$P<0.001$ compared with mock-infected control cells. (B) Expression level of MAPK pathway molecules assessed by western blots analysis. α-Tubulin was used as a loading control.

and SP600125 (Fig. 4C). These data indicate that A_3AR-mediated ERK1/2 and JNK signaling activation may be responsible for the increased TNF-α secretion observed in *T. gondii*-infected HTR8/SVneo cells.

DISCUSSION

In the present study, we observed that *T. gondii* infection upregulated A_3AR expression and TNF-α production in HTR8/SVneo cells in a time dependent manner. *T. gondii* also caused morphological changes and reduced the viability of HTR8/SVneo cells in an infection time-dependent manner. We further evaluated the effect of A_3AR function by assessing the activation of MAPK signaling pathway components and the expression of TNF-α in *T. gondii*-infected HTR8/SVneo cells. We demonstrated that A_3AR siRNA transfection downregulated TNF-α release from HTR8/SVneo cells in response to *T. gondii* infection and dramatically attenuated the *T. gondii* infection-mediated increase in p38, ERK1/2 and JNK phosphorylation levels compared to that observed in control siRNA-transfected cells. Notably, PD098059 and SP600125 pretreatment reduced the *T. gondii* infection mediated increase in TNF-α production. These data suggest that the effect of *T. gondii* infection on TNF-α release was in part regulated by A_3AR-mediated ERK1/2 and JNK signaling activation.

First, we investigated whether *T. gondii* infection affects HTR8/SVneo human extravillous trophoblast cell morphology and viability. We evaluated morphological changes in *T. gondii*-infected HTR8/SVneo cells by assessing the integrity of the microtubule network. After infection with *T. gondii* at an MOI of 10 for 4, 8, and 24 hr, we observed cells with condensed chromatin, nuclear fragmentation, and cellular shrinkage, which are associated with cell cytotoxicity [17]. Subsequently, we further evaluated *T. gondii*-induced HTR8/SVneo cell viability, and the results showed that *T. gondii* infection significantly reduced cell viability, with viabilities of 82.83±4.78%, 70.37±7.77%, and 48.50±3.49% observed for cells infected with *T. gondii* for 4, 8, and 48 hr, respectively. These findings are consistent with those of our previous study showing that GFP-RH (MOI 5) *T. gondii* infection of human umbilical cord mesenchymal stem cells significantly reduced viability by 21 and 30% in cells infected with *T. gondii* for 24 and 48 hr, respectively [18].

Second, we investigated the mechanisms associated with the *T. gondii* infection-mediated reduction in cell viability in HTR8/SVneo cells. Adenosine is a purine nucleoside involved in numerous physiological processes [19]. Recently, Pinheiro et al. reported that adenosine A_{2A} receptor mediates dexamethasone induces morphological alterations in primary rat hippo-

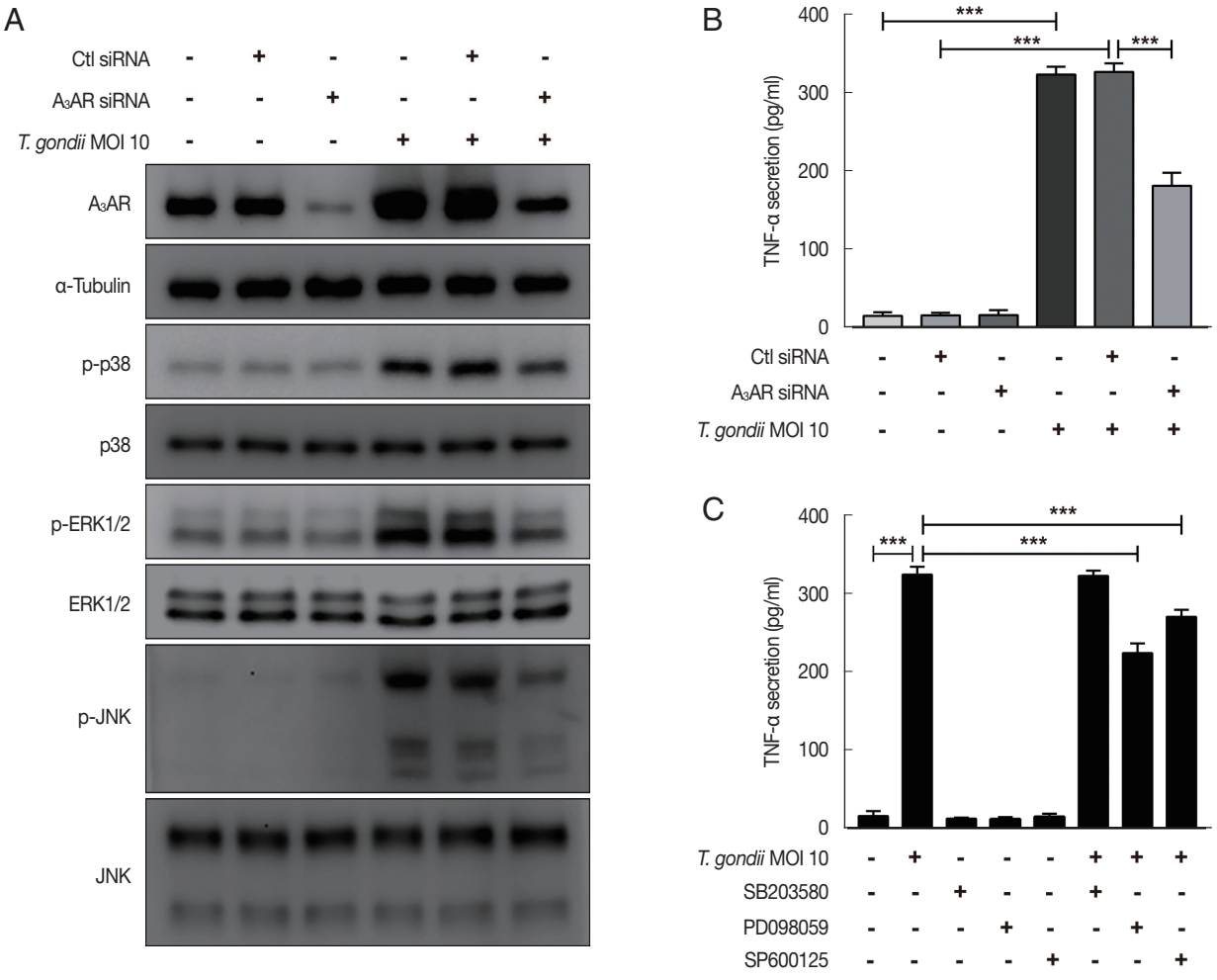

Fig. 4. Roles of A_3AR in *T. gondii*-induced MAPK activation and TNF-α secretion in HTR8/SVneo cells. HTR8/SVneo cells were transfected with control siRNA or A_3AR-specific siRNAs and infected with *T. gondii* at an MOI of 10 for 24 hr. (A) Expression level of A_3AR and MAPK pathway molecules assessed by western cell culture supernatants blot α-Tubulin was used as a loading control. (B) Concentration of TNF-α in was evaluated by ELISA. (C) Inhibitory effect of p38 MAPK (SB203580), ERK1/2 (PD098059), and JNK (SP600125) on TNF-α secretion levels in *T. gondii*-infected cells. HTR8/SVneo cells were pretreated with or without 30 μM SB203580, PD098059 or SP600125 for 2 hr and subsequently infected with *T. gondii* at an MOI 10 of for 24 hr. TNF-α secretion levels was evaluated by ELISA. ***$P<0.001$ compared with respective control.

campal neurons [14]. Thus, in the present study, we evaluated whether this adenosine receptor is involved in the regulation of *T. gondii*-induced HTR8/SVneo cell morphological changes. *T. gondii* infection dramatically increased A_3AR mRNA and protein levels in a time-dependent manner but not those of A_1R, $A_{2A}AR$, and $A_{2B}AR$. Lima et al. previously reported that Leishmania infantum parasites subvert the host inflammatory response through adenosine A_{2A} receptor to promote the establishment of infection [20]. The greatest differences between these 2 studies are the infection source of different species. In the present study, the number of *T. gondii*-infected HTR8/SVneo cells and the intracellular parasite content were significantly increased in a time-dependent manner. These findings suggested that *T. gondii* infection of HTR8/SVneo cells may alter morphological changes and accelerate the infection rate and intracellular proliferation through regulation of the adenosine A_3A receptor.

Third, we evaluated the functional role of A_3AR in the *T. gondii*-induced production of TNF-α. Adenosine binding to A_1R and A_3R also modulates TNF-α release from adult monocytes, whereas $A_{2b}R$ appears to have little effect [21]. In the present study, *T. gondii* significantly enhanced TNF-α release from HTR8/SVneo cells in a time-dependent manner, which is similar to the results of other studies [22-24]. However, *T. gon-*

dii exploits STAT3 to downregulate IL-12 and TNF-α expression in infected macrophages [25]. Differences in the type of host cell, *T. gondii* virulence and experimental conditions resulted in different results between these studies. A previous study convincingly demonstrated that intracellular *T. gondii* induces MAPK pathway activation and the production of proinflammatory cytokines in macrophages [15]. Regarding *T. gondii*-induced intracellular signaling in HTR8/SVneo cells, Milian et al. recently showed that increased *T. gondii* intracellular proliferation in human extravillous trophoblast cells (HTR8/SVneo line) is sequentially triggered by MIF, ERK1/2, and COX-2 [2]. Thus, we further evaluated *T. gondii*-induced MAPK intracellular signaling pathway activation in HTR8/SVneo cells. *T. gondii* dramatically increased the phosphorylation levels of p38 and ERK1/2, which peaked at 4 hr postinfection and then gradually decreased. In addition, *T. gondii* induced sustained phosphorylation of JNK in time-dependent manner. Subsequently, the role of A_3AR in *T. gondii*-induced MAPK activation and TNF-α production were examined. *T. gondii* significantly increased TNF-α production, A_{3A}R protein levels and the phosphorylation levels of p38, ERK1/2 and JNK in control siRNA-transfected *T. gondii*-infected cells. Moreover, the inhibition of A_3AR by A_3AR siRNA transfection apparently suppressed the *T. gondii*-mediated upregulation of TNF-α and A_3AR levels and the activation of MAPK. These results are supported by those of many other studies showing that adenosine receptors are involved in MAPK activation and TNF-α secretion [9,21]. Finally, the role of MAPK activation in the *T. gondii*-mediated regulation of TNF-α secretion was evaluated by TNF-α ELISA. The results showed that the *T. gondii*-mediated increase in TNF-α secretion was dramatically attenuated by pretreatment of cells with PD098059 or SP600125. These results indicate that the A_3AR-mediated activation of ERK1/2 and JNK positively regulate TNF-α secretion in *T. gondii*-infected HTR8/SVneo cells.

In summary, in the present study, we elucidated a mechanism of adenosine A_3 receptor-mediated ERK1/2- and JNK-dependent TNF-α production in *T. gondii*-infected HTR8/SVneo human extravillous trophoblast cells that ultimately may induce abnormal pregnancy. This is the first study to systemically evaluate the effect of *T. gondii* infection on the adenosine receptor family proteins and the regulatory mechanism of TNF-α production mediated by adenosine A_3 receptor. Further studies are need to assess the role of *T. gondii* infection in placental development and possibly in the pathophysiology of preeclampsia.

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CONFLICT OF INTREST

The authors declare that they have no conflicts of interest.

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