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# Infection by *Toxoplasma gondii*, a severe parasite in neonates and AIDS patients, causes impaired anion secretion in airway epithelia

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Contributed by Francisco J. Ayala, February 20, 2015 (sent for review September 5, 2014; reviewed by Paul Denny)

The airway epithelia initiate and modulate the inflammatory responses to various pathogens. The cystic fibrosis transmembrane conductance regulator-mediated Cl<sup>-</sup> secretion system plays a key role in mucociliary clearance of inhaled pathogens. We have explored the effects of *Toxoplasma gondii*, an opportunistic intracellular protozoan parasite, on Cl<sup>-</sup> secretion of the mouse tracheal epithelia. In this study, ATP-induced Cl<sup>-</sup> secretion indicated the presence of a biphasic short-circuit current (*I<sub>sc</sub>*) response, which was mediated by a Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel (CaCC) and the cystic fibrosis transmembrane conductance regulator. However, the ATP-evoked Cl<sup>-</sup> secretion in *T. gondii*-infected mouse tracheal epithelia and the elevation of [Ca<sup>2+</sup>]<sub>i</sub> in *T. gondii*-infected human airway epithelial cells were suppressed. Quantitative reverse transcription-PCR revealed that the mRNA expression level of the P2Y2 receptor (P2Y2-R) increased significantly in *T. gondii*-infected mouse tracheal cells. This revealed the influence that pathological changes in P2Y2-R had on the downstream signal, suggesting that P2Y2-R was involved in the mechanism underlying *T. gondii* infection in airways. These results link *T. gondii* infection as well as other pathogen infections to Cl<sup>-</sup> secretion, via P2Y2-R, which may provide new insights for the treatment of pneumonia caused by pathogens including *T. gondii*.

ATP | Cl<sup>-</sup> secretion | P2Y2 receptor | pneumonia | cystic fibrosis

The airway epithelium is the initial cell type exposed to both inhaled environmental factors and medications for airway diseases (1). Therefore, epithelial cells are recognized as being very important in host defense. In the lungs of mammals, the first line of defense against pathogen infection is the thin layer of airway surface liquid (ASL) lining the airway surface. Maintenance of the proper height and ion composition of ASL is crucial for proper lung defense. The airway epithelium exhibits several complex regulatory pathways to adjust ASL volume to maintain proper mucociliary clearance (2). These pathways blend ion transport by regulating epithelial sodium channel (ENaC)-mediated Na<sup>+</sup> absorption (3) and Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel (CaCC) and cystic fibrosis transmembrane conductance regulator (CFTR)-mediated Cl<sup>-</sup> secretion (4).

It has become increasingly clear that extracellular nucleotides are important regulators of mucus clearance in the airways as a result of their ability to stimulate fluid secretion (5), mucus hydration (6), and cilia beat frequency (7, 8). ATP released by epithelial cells plays a key role as an important autocrine and paracrine signaling molecule (9). In normal airway epithelia, ATP is released in response to local stress in ASL and is sufficient to induce purinoceptor-mediated increases in ASL height and maintain proper mucociliary clearance (10). This unusual ASL level will result in abnormal airway functioning and suggests

that ATP has a vital role in airway function. It has been shown that the exogenous application of ATP produces a regulatory effect on airway epithelia ion transport, including the inhibition of Na<sup>+</sup> absorption (11, 12) and activation of Cl<sup>-</sup> secretion (13, 14).

Extracellular ATP and its analogous nucleotides exert their effects through a class of cell surface receptors known as P2 receptors, which are divided into two subfamilies—P2Y and P2X (15). Seven P2Y receptors have been identified in mammals; they contain seven-transmembrane segments, are associated with G-protein activation, and are involved in regulating various biological processes such as secretion, proliferation, differentiation, wound repair, and anti-inflammatory and other processes (16, 17). A knockout study confirmed that the P2Y2 receptor (P2Y2-R) is the dominant P2Y purinoceptor that regulates airway epithelial ion transport, whereas other P2Y receptor subtypes are relatively more important in other nonrespiratory epithelia (18). P2Y2-R is linked to phospholipase C (PLC)-generated inositol (1,4,5) trisphosphate (IP3)-mediated release of [Ca<sup>2+</sup>]<sub>i</sub> in human airway epithelia (19).

*Toxoplasma gondii* is an obligate intracellular parasitic protozoan, and its definitive host is the cat and other feline species. Beside felines, it can also infect many warm-blooded animals including humans, resulting in toxoplasmosis (20). Reactivation

## Significance

The airway epithelia modulate the inflammatory responses to various pathogens. Pulmonary disease caused by *Toxoplasma gondii* infection affects human neonates, children, and immunocompromised individuals. However, it is not clear how *T. gondii* infection impacts airway epithelia. We report the use of a short-circuit current (*I<sub>sc</sub>*) technique to determine the Cl<sup>-</sup> secretion induced by ATP in tracheal epithelia infected by *T. gondii*. We surprisingly found that the ATP-evoked Cl<sup>-</sup> secretion in *T. gondii*-infected mouse tracheal epithelia was significantly suppressed. We also found that the mRNA expression level of the P2Y2 receptor increased significantly in *T. gondii*-infected mouse trachea, via real-time quantitative PCR. Our study provides previously unidentified insights into the mechanism underlying host impairment caused by *T. gondii* infection.

Author contributions: H.-M.G., W.-L.Z., and Z.-R.L. designed research; H.-M.G., J.-M.G., Y.-L.L., and Y.-Z.W. performed research; H.-M.G., Y.-L.Z., G.H., F.J.A., and Z.-R.L. analyzed data; and H.-M.G., G.H., W.-L.Z., F.J.A., and Z.-R.L. wrote the paper.

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of a latent infection in immune-deficient patients such as AIDS and organ transplantation subjects can become life-threatening (21). Besides the mechanical destruction of the host cell, little is known about the mechanisms underlying *T. gondii* infection resulting in multiorgan injury. Pulmonary disease caused by *T. gondii* infection is seen in neonates, children, and immunocompromised hosts. Pneumonitis caused by *T. gondii* in immunocompromised hosts results in dyspnoea, minimal sputum production, and other clinical and radiologic effects. It evokes diffuse disease in the lung, such as interstitial infiltrates, bronchopneumonia, and pulmonary infection (22). Although the key players in host defense against pathogenic microorganisms are airway epithelia, it is not clear how *T. gondii* infection affects the functions of airway epithelia.

In view of the significant roles of ATP and P2Y2-R in airway epithelia function, we set out to investigate ion transport induction by ATP in tracheal epithelia infected by *T. gondii* and to elucidate the intracellular signaling pathways involved in the pathogenesis of *T. gondii* infection. The results uncover a previously unidentified mechanism that enables us to understand the pathogenesis of the infection and could offer potential therapeutic applications for pulmonary toxoplasmosis.

## Results

**Characteristics of ATP-Induced Short-Circuit Current Response.** Previous studies on extracellular ATP-induced transepithelial  $\text{Cl}^-$  transport in different epithelial cells have shown that the time course for the ATP response is biphasic. Different regulatory pathways are involved in mediating the ATP-induced short-circuit current ( $I_{sc}$ ), such as the cAMP-dependent and the traditional P2-purinoreceptor-linked  $\text{Ca}^{2+}$ -dependent or P1-like receptor-dependent pathways, which activate different  $\text{Cl}^-$  channels (23–25). In this study, we investigate the  $I_{sc}$  response to extracellular ATP at 10  $\mu\text{M}$  in mice trachea epithelia. The normal trachea tissues used in the present study had a transepithelial electrical resistance of  $74.93 \pm 2.06 \Omega/\text{cm}^2$  ( $n = 4$ ), with a basal  $I_{sc}$  ( $I_b$ ) of  $13.38 \pm 0.37 \mu\text{A}/\text{cm}^2$  ( $n = 4$ ) in the unstimulated state when bathed in Krebs–Henseleit (K–H) solution. The transepithelial resistance did not change significantly after ATP stimulation.

ATP added to the apical side induced a biphasic response. This response consisted of a transient upstroke followed by a sustained phase (first peak,  $111.85 \pm 26.85 \mu\text{A}/\text{cm}^2$ ; second peak,  $69.55 \pm 13.80 \mu\text{A}/\text{cm}^2$ ; Fig. S1A). Removing ambient  $\text{Cl}^-$  (gluconate substitution) made a very highly significant reduction in the whole  $I_{sc}$  response (first peak,  $32.48 \pm 11.42 \mu\text{A}/\text{cm}^2$ ; second peak,  $18.15 \pm 5.54 \mu\text{A}/\text{cm}^2$ ; Fig. S1B) ( $P < 0.001$ ). A summary of these effects is shown in Fig. S1C. This suggests that the ATP-induced  $I_{sc}$  in trachea tissues is primarily due to  $\text{Cl}^-$  currents.

Compared with the control data, the ATP induced  $\text{Cl}^-$  secretion was not changed by 100  $\mu\text{M}$  amiloride (an ENaC blocker) (first peak,  $107.64 \pm 23.55 \mu\text{A}/\text{cm}^2$ ; second peak,  $56.05 \pm 8.95 \mu\text{A}/\text{cm}^2$ ; Fig. S2A), was partially blocked by 100  $\mu\text{M}$  disulfonic acid stilbene (DIDS, a CaCC blocker) (first peak,  $37.26 \pm 3.18 \mu\text{A}/\text{cm}^2$ ; second peak,  $68.47 \pm 7.01 \mu\text{A}/\text{cm}^2$ ; Fig. S2B), and was significantly blocked by 1 mM diphenylamine-2-carboxylate (DPC, a nonselective  $\text{Cl}^-$  channel blocker) (first peak,  $41.72 \pm 8.85 \mu\text{A}/\text{cm}^2$ ; second peak,  $15.61 \pm 2.18 \mu\text{A}/\text{cm}^2$ ; Fig. S2C). These results show that DIDS can inhibit a large proportion of the initial  $I_{sc}$  response but had no effect on the second phase. A summary of the above data are shown in Fig. S2D. This suggests that the ATP-induced initial  $I_{sc}$  response ( $\text{Cl}^-$  secretion) involves activation of the CaCC, whereas the sustained phase may involve other  $\text{Cl}^-$  channels (such as the CFTR-like channel). To further confirm that the ATP-induced  $I_{sc}$ -sustained phase is indeed due to the CFTR opening, we used the most specific commercial CFTR blocker (CFTRinh-172) to test the ATP-induced  $I_{sc}$ . When the ATP-induced  $I_{sc}$  response reached a plateau, 10  $\mu\text{M}$  CFTRinh-172 was applied to the apical side of the trachea tissue.

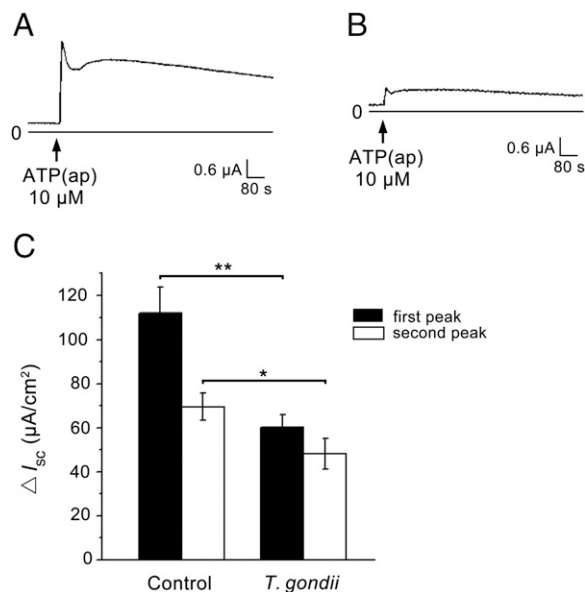
The results showed that 10  $\mu\text{M}$  CFTRinh-172 can inhibit the ATP-induced sustained phase from  $69.55 \pm 13.80 \mu\text{A}/\text{cm}^2$  to  $37.90 \pm 9.15 \mu\text{A}/\text{cm}^2$  (Fig. S2B and E). These results indicate that CaCC and CFTR channels located on the trachea epithelium mediate the ATP-induced  $\text{Cl}^-$  secretion.

ATP can activate PLC through the P2Y receptor, to elevate the intracellular  $\text{Ca}^{2+}$  in different cells (26). To determine whether the pathway mentioned above was used in ATP-induced  $\text{Cl}^-$  secretion in mice trachea epithelia, the effects of the P2-R agonist and antagonist were examined. The administration of ATP (10  $\mu\text{M}$ , apical), a P2-R agonist, led to an increase in  $I_{sc}$  (Fig. S3A). However, preincubation of the tracheal epithelia with the P2-R antagonist, suramin (100  $\mu\text{M}$ , apical), greatly suppressed the  $I_{sc}$  response to ATP (Fig. S3B), and the change in  $I_{sc}$  was reduced by 50% (Fig. S3F).

Further experiments were conducted to investigate the role of PLC and intracellular  $\text{Ca}^{2+}$  in ATP-induced  $\text{Cl}^-$  secretion response. The  $I_{sc}$  response to ATP was eliminated by pretreatment with U73122 (100  $\mu\text{M}$ , apical), which inhibits PLC activation; with BAPTA-AM (100  $\mu\text{M}$ , apical), which chelates intracellular  $\text{Ca}^{2+}$ ; and with 2-aminoethoxydiphenyl borate (2-APB) (100  $\mu\text{M}$ , apical), which blocks IP3 receptors (Fig. S3C–F). These data demonstrate that PLC activation and intracellular  $\text{Ca}^{2+}$  mobilization play significant roles in the ATP-induced  $I_{sc}$  response.

The evidence has shown that P2Y2-R is linked to PLC-generated IP3-mediated release of  $[\text{Ca}^{2+}]_i$  in human airway epithelia (19). Together with the above study, it is thus likely that ATP can induce  $\text{Cl}^-$  secretion response via the P2Y2-R.

**Inhibitory Effect of *T. gondii* on ATP-Induced  $\text{Cl}^-$  Secretion.** Compared with the normal control group data, the dual peaks of ATP-induced  $\text{Cl}^-$  secretion were reduced significantly by *T. gondii* infection for 3 d [first peak,  $60.19 \pm 11.46 \mu\text{A}/\text{cm}^2$  ( $P < 0.01$ ); second peak,  $48.09 \pm 14.16 \mu\text{A}/\text{cm}^2$  ( $P < 0.05$ ); Fig. 1A and B]. A summary of these effects is shown in Fig. 1C. This suggests that *T. gondii* can depress ATP-induced  $\text{Cl}^-$  secretion.



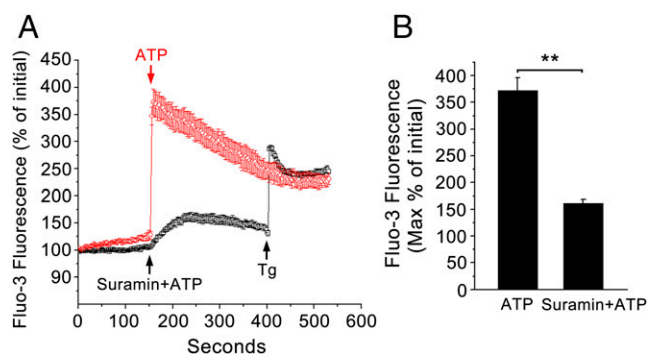
**Fig. 1.** Effects of *T. gondii* infection on ATP-induced  $I_{sc}$  currents. Addition of 10  $\mu\text{M}$  ATP to the apical side (ap) of the proximal trachea tissues caused a rise in  $I_{sc}$  response (A). This response was found to be partially inhibited by *T. gondii* pretreatment (B). (C) A summary of the effects of *T. gondii* on the dual phases of ATP-induced  $I_{sc}$  response. Data are presented as mean change in  $I_{sc} \pm \text{SEM}$  ( $n = 4$ ) and are representative of four independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Inhibitory Effect of *T. gondii* on ATP-Induced  $[Ca^{2+}]_i$  Response.** To further investigate intracellular  $Ca^{2+}$  involvement in *T. gondii*-reduced  $Cl^-$  secretion in response to ATP, the change of intracellular  $Ca^{2+}$  level was measured by confocal imaging using Fluo3 dye in the presence of extracellular  $Ca^{2+}$ . Fig. 2 shows the effect of ATP (10  $\mu$ M) on the  $[Ca^{2+}]_i$  in single 16 human bronchial epithelial (16HBE) cells. It can be seen that 10  $\mu$ M ATP stimulation gave rise to a  $Ca^{2+}$  peak and that in the presence of the P2-R antagonist, suramin, ATP did not lead to a corresponding  $[Ca^{2+}]_i$  increase, reflecting the effect of P2-R expression on the cells. However, in *T. gondii*-pretreated cells, the elevation of  $[Ca^{2+}]_i$  showed a very highly significant attenuation ( $P < 0.001$ , Fig. 3). The results show that  $Ca^{2+}$  responses in *T. gondii*-pretreated cells are functionally reduced compared with those in normal cells.

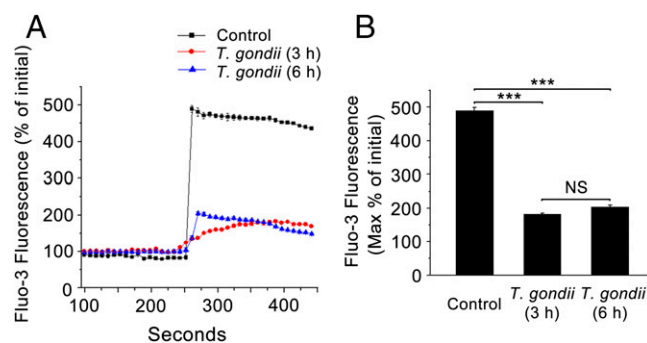
**The Effect of *T. gondii* on Expression of P2Y2-Rs.** Several studies have demonstrated that P2Y2-R is expressed in epithelial cells, smooth muscle cells, endothelial cells, leukocytes, and cardiomyocytes (27–31). Particularly, mice deficient in P2Y2-R lost 85–95% of the nucleotide-stimulated  $Cl^-$  secretion in mouse tracheal epithelium (18). To investigate the change in the mRNA expression level of the P2Y2-R in *T. gondii*-infected cells, quantitative real-time PCR analysis was carried out in mouse tracheal tissue that had been infected with *T. gondii* for 3 d. The mean value of the P2Y2-R mRNA expression level in normal tissue was normalized to 1. After infecting the mouse with *T. gondii*, the relative levels of the P2Y2-R mRNA increased to 1.90 (normalized by GAPDH; Fig. 4) and 1.55 (normalized by beta-actin; Fig. S4), which was significantly higher than that in the normal tissue ( $P < 0.05$ ). These results indicate that the mRNA expression of the P2Y2-R increased in *T. gondii*-infected tracheal tissue.

## Discussion

Acute infection with *T. gondii* during pregnancy and its potentially tragic outcome for the fetus and newborn continue to occur worldwide (21), with a significant proportion of pregnancies being at risk (32, 33). Toxoplasmosis is being recognized with increased frequency, especially in patients with AIDS (34). The mechanism of pathogenesis for this disease is complex, although some results from ours and other laboratories indicate that it might be linked with NO production by the host (35–37) and



**Fig. 2.** Effect of P2 receptor blocker suramin on intracellular  $Ca^{2+}$  induced by ATP in 16HBE cells. Intracellular  $Ca^{2+}$  measurement in single 16HBE cells using Fluo 3/AM as a probe. (A) The addition of ATP (10  $\mu$ M) significantly increased the intracellular  $Ca^{2+}$ , which was inhibited in the presence of the P2-R antagonist, suramin (100  $\mu$ M); the addition of 1  $\mu$ M Thapsigargin (Tg, an inducer of stored  $Ca^{2+}$  release) was done to check the activity of cells. (B) A summary of the effects of suramin on ATP-induced calcium elevation. Data are presented as mean of fluorescence intensity  $\pm$  SEM ( $n = 4$ ) and are based on four independent experiments.  $**P < 0.01$ .

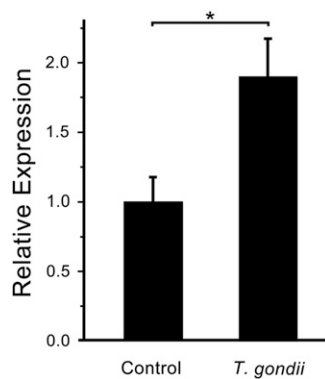


**Fig. 3.** Effect of *T. gondii* on intracellular  $Ca^{2+}$  induced by ATP in 16HBE cells. Intracellular  $Ca^{2+}$  measurement in single 16HBE cells using Fluo 3/AM as a probe. (A) Addition of ATP (10  $\mu$ M) significantly increased the intracellular  $Ca^{2+}$  inhibited after *T. gondii* pretreatment for 3 h or 6 h. (B) A summary of the effects of *T. gondii* on ATP-induced calcium elevation. Data are presented as mean fluorescence intensity  $\pm$  SEM ( $n = 4$ ) and are based on four independent experiments.  $***P < 0.001$ ; NS,  $P > 0.05$ .

transepithelial migration of *T. gondii* (38). Little is known about the pathogenesis of host lung infection with *T. gondii*. In this study, we have examined the role of the predominant subtype P2Y2-R purinoceptors that are expressed in the lungs.

Airway epithelial cells are traditionally known as barrier cells that line the surface of the airways. The apical membrane faces the lumen of the airways, which in turn are in direct contact with the external environment. The fluid-covered microenvironment allows autocrine or paracrine mediators to diffuse easily and rapidly. This provides an ideal setting for autocrine and paracrine purinergic signaling, and this has been implicated in the regulation of airway epithelial cell functions including transepithelial ion transport. In general, extracellular nucleotides and nucleosides, through P2Y, P2X, and P1 receptors, stimulate secretory  $Cl^-$  and  $H_2O$  transport (39). Our results demonstrate that ATP could stimulate  $Cl^-$  secretion via a P2Y2-R-mediated PLC- $Ca^{2+}$  pathway across mice tracheal epithelia, but that the secretion was attenuated in the epithelia of *T. gondii*-infected mice. The  $[Ca^{2+}]_i$  elevation was suppressed in *T. gondii*-pretreated 16HBE cells. Moreover, the mRNA expression level of P2Y2-R was abnormal in the *T. gondii*-infected mice trachea.

Abnormal ion transport and mucociliary clearance in airway epithelia is one of the general features of respiratory infection. Extracellular ATP is considered a key signaling molecule to maintain the normal homeostasis of these processes in airway epithelia (40, 41). Our studies in mouse trachea epithelia show that the response of the ATP-stimulated  $Cl^-$  secretion consisted of an initial spike and a long-term component, which is consistent with previous studies in various organs (23–25). P2Y2-R is a Gq-coupled protein that is activated by extracellular ATP to promote Gq-dependent activation of PLC, which in turn regulates calcium flux and protein kinase C (PKC) activity, by an IP3 and diacylglycerol (DAG)-dependent mechanism, in Schwann cells and eye suprachoroid (42, 43). Based on this fact, in our study, the suppression of the ATP-induced  $I_{sc}$  increase by a P2-R inhibitor, suramin, revealed that transepithelial  $Cl^-$  transport induced by ATP was mediated through the P2-R. Similarly, suppression by a PLC inhibitor, U73122, indicated that PLC activation participated in the response and triggered downstream signaling, in which  $[Ca^{2+}]_i$  played a major role in stimulation of  $Cl^-$  secretion. This can be concluded because pretreatment of tracheal epithelia with apical addition of the specific inhibitors BAPTA-AM, a  $[Ca^{2+}]_i$  chelator, and 2-APB, an inhibitor of  $[Ca^{2+}]_i$  release, prevented the ATP-induced  $I_{sc}$  response. These results demonstrated the role of ATP on the P2-R to stimulate the transfer of signals through the PLC- $Ca^{2+}$  pathway.



**Fig. 4.** *T. gondii* infection results in increased P2Y2-R expression. Quantitative real-time PCR analysis of mRNA expression of P2Y2-R in mice trachea treated without or with *T. gondii* for 3 d. Absolute values were corrected using GAPDH as a reference gene. Gene expression was reported as the relative variation to unstimulated control tissue mRNA levels. Bars represent means  $\pm$  SEM ( $n = 3$ ) and are based on three independent experiments. \* $P < 0.05$ .

However, the physical  $\text{Cl}^-$  secretion in mouse tracheal epithelia evoked by the apical administration of ATP was impaired in the mice infected with *T. gondii* for 3 d. In a  $\text{Ca}^{2+}$  measurement experiment, it was shown that ATP could raise cytosolic  $\text{Ca}^{2+}$  in control cells. Pretreatment of the cells with the P2-R antagonist could block this  $[\text{Ca}^{2+}]_i$  elevation response. However, this ATP-induced intracellular  $\text{Ca}^{2+}$  increase was attenuated in *T. gondii*-infected cells, showing that the interference by  $\text{Ca}^{2+}$ -dependent signals may account for the inhibited  $\text{Cl}^-$  current in the *T. gondii*-infected epithelial cells. The experiments traced the effects back to the function of the P2Y receptor, which acts upstream of the  $\text{Ca}^{2+}$  signal. Moreover, the P2Y2-R mediates the majority of epithelial  $\text{Cl}^-$  secretory responses (18). From this, we deduce that *T. gondii* can inhibit the activation of purinergic receptors to reduce  $\text{Cl}^-$  secretion. We hypothesize that *T. gondii* infection can result in abnormal airway function by controlling the function and expression of purinergic receptors. In *T. gondii*-infected mice trachea, the mRNA expression levels of P2Y2-R were compared with the control using real-time PCR. However, the mRNA expression in *T. gondii*-infected mice trachea was higher than that in normal trachea. The abnormal expression level may be caused by the dysfunction of P2Y2-R. The cells need higher receptor mRNA expression to counteract the functional decline in the P2Y2-R. Despite the receptor mRNA expression being increased, it could not rescue the activity of P2Y2-R to exploit the downstream pathways. These results show that *T. gondii* infection has had a negative potential impact on the cell membrane P2Y2-R, which plays an important role in regulation of airway water homeostasis. These results were in line with the suppressed  $[\text{Ca}^{2+}]_i$  and  $I_{sc}$  increase when *T. gondii*-infected epithelial cells were stimulated by the addition of ATP and the P2-R antagonist. Because the extracellular ATP (and its analog), secreted from airway tissue, can modulate  $\text{Cl}^-$  secretion in airway epithelial cells, the deficiency in the P2Y2-R can result in the decrease of  $\text{Cl}^-$  secretion. This, in turn, can lead to an unusual ASL height as well as poor mucociliary clearance and then to a weakening of the airway innate immune system in fighting against *T. gondii* infection.

A series of studies have demonstrated that P2Y2-R has important functions in different tissues. The activation of P2Y2-R can promote neuroprotective responses in glial cells (44), mediate cytoprotection in alveolar epithelial cells (45), inhibit inducible NO synthase in cultured rat mesangial cells (46), regulate expression of cyclooxygenase-2 and release of prostaglandin E2 in airways (47), and exert a protective role against infection of

the lungs by *Pseudomonas aeruginosa* (48). Moreover, our studies reveal that *T. gondii* could result in the abnormal expression and function of P2Y2-R in airways of the infected host. This evidence suggests that *T. gondii*, by means of the effect on P2Y2-R, could lead to various abnormal changes in the physiological and immune functions involved in the pathological mechanism underlying *T. gondii*-determined pulmonary infection. This could provide new insights for better understanding the pathogenesis, the prevention, and the therapy of pulmonary toxoplasmosis.

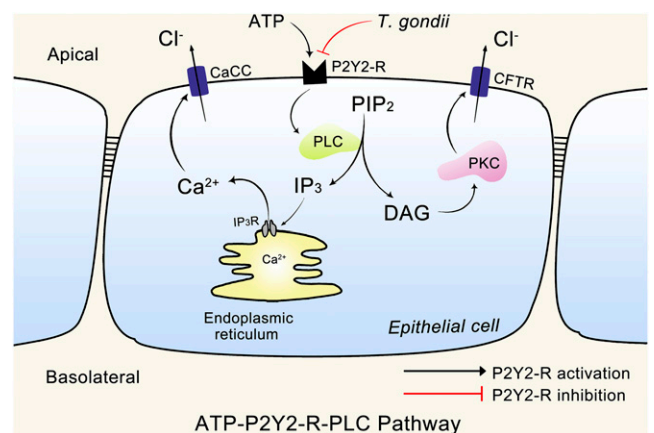
This investigation addressed the questions of whether the ion transport induced by ATP is shifted in *T. gondii*-infected mammalian hosts, including human tracheal epithelia, and whether purinergic signaling is potentially involved in the mechanisms underlying the disease caused by *T. gondii* infection in airway epithelial cells. This research may widen our view not only on the physiological functions of purinergic signaling in the airway epithelia but also on the pathophysiology and the pathogenesis of *T. gondii* infection.

In summary, our results demonstrate that *T. gondii* can reduce ATP-induced  $\text{Cl}^-$  secretion in airway epithelium by inhibiting the function of the P2Y2-R. The impairment action of *T. gondii* on chloride secretion was related to its ability to inhibit P2Y2-Rs that are activated by extracellular ATP to promote the Gq-dependent activation of the PLC/IP<sub>3</sub>/Ca<sup>2+</sup>- and PLC/DAG/PKC-sensitive  $\text{Cl}^-$  channel pathway (summarized in Fig. 5). The finding that the P2Y2-R can be an essential target blocked by *T. gondii* provides previously unidentified insights into the mechanism underlying host impairment caused by the infection with *T. gondii*.

## Materials and Methods

**Chemicals.** Dulbecco's modified Eagle medium/nutrient mixture F12 (DMEM/F12), FBS, penicillin/streptomycin, Hank's Balance Salt Solution, and trypsin were purchased from Gibco Laboratories. ATP disodium salt, amiloride, DPC, DIDS, CFTRinh-172, suramin sodium salt, U73122, BAPTA-AM, and 2-APB were from Sigma Chemical Co. Fluo 3/AM was obtained from Molecular Probes, Inc. ATP and suramin were dissolved in water. The other chemical stocks were dissolved in dimethyl sulfoxide (DMSO).

**Animals and *T. gondii* Infection.** Swiss mice (local name, Kunming mice) were purchased from the Animal Center of Sun Yat-Sen University. Animals were housed and fed according to the guidelines of the Sun Yat-Sen University



**Fig. 5.** Mechanisms underlying the impairment action of *T. gondii* on chloride secretion in airway epithelia. The schematic model shows that the impairment action of *T. gondii* on chloride secretion was related to its ability to inhibit P2Y2-Rs. Black arrows represent the P2Y2-R activation signaling pathways, or cellular events identified in the present and previous studies (42, 43), and a red block symbol denotes the P2Y2-R inhibition signaling pathways and cellular events described in airway epithelia [PIP<sub>2</sub>, phosphatidylinositol (4,5) biphosphate].

Animal Use Committee, and all procedures were approved before each experiment. Animals were housed in a constant-temperature room (25 °C) with a 12 h light/dark photoperiod and were allowed food and water ad libitum. Mice were infected for 3 d with tachyzoites from the *T. gondii* RH strain by peritoneal injection ( $5 \times 10^5$  per mouse).

**Cell Culture and Stimulation of Cells with *T. gondii*.** The HBE cell line, 16HBE (a gift from J. Xu, Guangzhou Institute of Respiratory Disease, Guangzhou Medical University, Guangzhou, China) was cultured in DMEM/F12 with 10% (vol/vol) FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C, 5% CO<sub>2</sub>, in a humidified atmosphere.

16HBE cells were seeded at a density of  $1 \times 10^5$  cells per well in six-well tissue culture plates with medium. Cells were cultured for 48–72 h with a medium change after 24 h, and then incubated with *T. gondii* at a ratio of 5:1 (parasites:cells, 5:1) in medium without serum and antibiotics for the duration of the experiment.

**Tissue Preparation and Short-Circuit Current ( $I_{sc}$ ) Measurement.** Mice were anesthetized using CO<sub>2</sub>, and the proximal tracheas were dissected out and put in K-H solution. After carefully removing the blood vessels and connective tissue, the trachea was longitudinally cut open with fine scissors under a stereo microscope and clamped between the two halves of an Ussing Chamber as previously described (49).

**Measurement of Intracellular Calcium.** The cells were grown in culture medium on six-well tissue plates with a piece of cover glass at 37 °C. After 2 d of culture and *T. gondii* infection for 3 h and 6 h, the cells were prepared and loaded with the fluorescence dye Fluo 3/AM, as previously described (50). Data analysis was processed with Origin 8.0. The change of fluorescence intensity after the drug treatments was normalized with the initial intensity.

**Total RNA Extraction, Reverse Transcription (RT), and Quantitative Real-Time PCR.** The total RNA was extracted by TRIzol reagents (Invitrogen). The RT reaction of RNA was performed using PrimeScrip First Strand cDNA Synthesis Kit (Takara Bio Inc.) according to the manufacturer's protocol. The P2Y<sub>2</sub>-R (forward primer, 5'-TGCCGCTGGTCTATT-3', and reverse primer, 5'-GG-AGCGCAGAGGTCTGAA-3') and GAPDH (forward primer, 5'-ACATCATCCTT-GCATCCACTG-3', and reverse primer, 5'-TCATTGAGAGCAATGCCAGC-3') primer sequences were designed using Primer 5.0 software and synthesized by Sangon. The reaction was performed in an iQ5 Gradient Real Time PCR Amplification System (Bio-Rad) using the SYBR Premix Ex Taq (Takara Bio Inc.). After the optimization of RT-PCR conditions, the reaction was conducted with SYBR Green master mix, 10 µM forward/reverse primers, and 100 ng of each cDNA sample. The P2Y<sub>2</sub>-R primers' amplification curve was carried out using the following parameters: a hot start at 95 °C for 30 s and then 40 cycles of the 95 °C denaturing step for 5 s and 30 s annealing at 60 °C. The products generated were confirmed by melting curves. The results were analyzed by the comparative threshold cycle (C<sub>t</sub>) method, and the mRNA expression of P2Y<sub>2</sub>-R was normalized by GAPDH. Each experiment was carried out in triplicate.

**Statistical Analysis.** For  $I_{sc}$  measurements, the changes in ion transport (Delta  $I_{sc}$ ) were given as peak values. All of the results were expressed as mean ± SEM. Statistical comparisons were carried out using the Student's two-tailed *t* test or ANOVA where appropriate. Differences were considered statistically significant at  $P < 0.05$ .

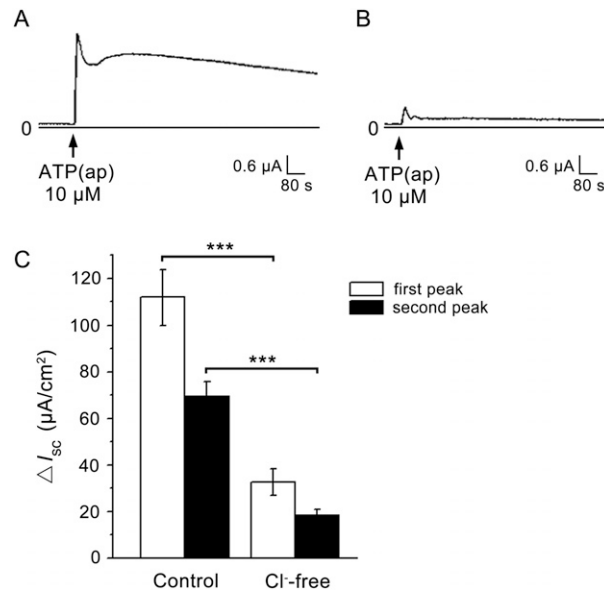
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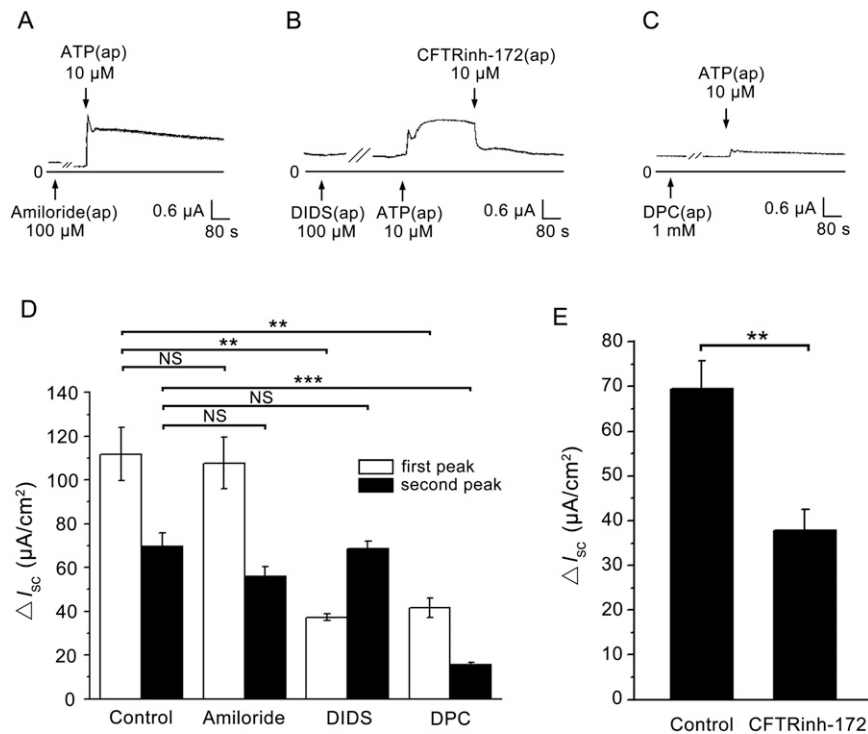
# Supporting Information

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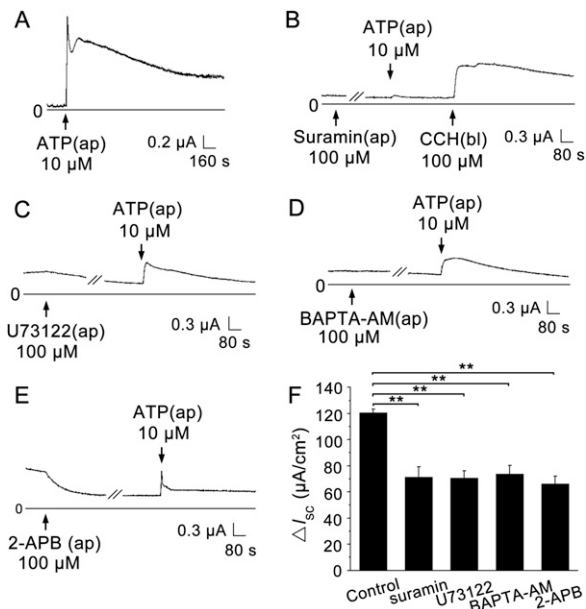


**Fig. 51.** Cl<sup>-</sup>-dependence of the  $I_{sc}$  response to ATP.  $I_{sc}$  recordings obtained from proximal trachea tissues bathed in normal K-H solution (A) and in Cl<sup>-</sup>-free solution (B). (C) A summary of the effects in the different conditions. Data are presented as mean change in  $I_{sc} \pm$  SEM ( $n = 4$ ) and are based on four independent experiments (A–C). \*\*\* $P < 0.001$ .

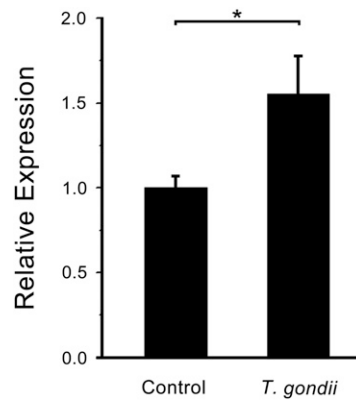




**Fig. S2.** Effects of ENaC and Cl<sup>-</sup> channel blockers on ATP-induced  $I_{sc}$  currents. Addition of 10  $\mu$ M ATP to the apical side (ap) of the proximal trachea tissues caused a rise in  $I_{sc}$  response. This response was not changed by adding 100  $\mu$ M Amiloride (an ENaC blocker) (ap) (A) but was partially inhibited by adding 100  $\mu$ M DIDS (a CaCC blocker) (ap) and 10  $\mu$ M CFTRi-172 (a CFTR blocker) applied to the cells (ap) when the ATP-induced  $I_{sc}$  response stays at the plateau (B) and was almost inhibited by 1 mM DPC (a nonselective Cl<sup>-</sup> channel blocker) (ap) (C). (D) A summary of the effects of Amiloride, DIDS, and DPC on the ATP-induced  $I_{sc}$  response. (E) A summary of the effects of CFTRi-172 on the second phase of ATP-induced  $I_{sc}$  response. Data are presented as mean change in  $I_{sc} \pm$  SEM ( $n = 4$ ) and are based on four independent experiments (A–E). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; NS,  $P > 0.05$ .



**Fig. S3.** ATP-induced  $I_{sc}$  responses were mediated by the P2Y2-R. Addition of 10  $\mu$ M ATP to the apical side (ap) of the proximal trachea tissues caused a rise in  $I_{sc}$  response (A). The response was found to be partially inhibited after 100  $\mu$ M suramin (a P2-R antagonist) (ap) pretreatment for 15 min; the addition of 100  $\mu$ M Carbachol (CCH, an analog of acetylcholine) to the basolateral side (bl) of cells was done to check the activity of cells (B). The response was also found to be partially inhibited after 100  $\mu$ M U73122 (a PLC activation inhibitor) (ap) (C) or BAPTA-AM (a [Ca<sup>2+</sup>]<sub>i</sub> chelator) (ap) (D) and/or 2-APB (a IP3 receptor blocker) (ap) (E) pretreatment for 15 min. (F) A summary of the effects of suramin, U73122, BAPTA-AM, and 2-APB. Data are presented as mean change in  $I_{sc} \pm$  SEM ( $n = 4$ ) and are based on four independent experiments. \*\* $P < 0.01$ .



**Fig. S4.** *T. gondii* infection results in increased P2Y2-R expression. Quantitative real-time PCR analysis of mRNA expression of P2Y2-R in mice trachea treated without or with *T. gondii* for 3 d. Absolute values were corrected using beta-actin as a reference gene. Gene expression was reported as the relative variation to unstimulated control tissue mRNA levels. Bars represent means  $\pm$  SEM ( $n = 3$ ) and are based on three independent experiments.  $*P < 0.05$ .