Early Response of Mucosal Epithelial Cells during *Toxoplasma gondii* Infection

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The innate immune response of mucosal epithelial cells during pathogen invasion plays a central role in immune regulation in the gut. Toxoplasma gondii is a protozoan intracellular parasite that is usually transmitted through oral infection. Although much of the information on immunity to T. gondii has come from i.p. infection models, more recent studies have revealed the importance of studying immunity following infection through the natural peroral route. Oral infection studies have identified many of the key players in the intestinal response; however, they have relied on responses detected days to weeks following infection. Much less is known about how the gut epithelial layer senses and reacts during initial contact with the pathogen. Given the importance of epithelial cells during pathogen invasion, this study uses an in vitro approach to isolate the key players and examine the early response of intestinal epithelial cells during infection by T. gondii. We show that human intestinal epithelial cells infected with T. gondii elicit rapid MAPK phosphorylation, NF-κB nuclear translocation, and secretion of IL-8. Both ERK1/2 activation and IL-8 secretion responses were shown to be MyD88 dependent and TLR2 was identified to be involved in the recognition of the parasite regardless of the parasite genotype. Furthermore, we were able to identify additional T. gondii-regulated genes in the infected cells using a pathway-focused array. Together, our findings suggest that intestinal epithelial cells were able to recognize T. gondii during infection, and the outcome is important for modulating intestinal immune responses. The Journal of Immunology, 2009, 183:7420–7427.

A single layer of intestinal epithelial cells that line the mucosal surface must prevent the entry of exogenous Ags, allow absorption of essential nutrients, and yet initiate effective and appropriate immune responses when pathogens are present (1). Host defenses at mucosal surfaces include the secretion of IgA, defensins, and cytokines and chemokines. IgA and defensins prevent bacterial adherence and contribute to pathogen elimination, whereas cytokines and chemokines participate in gut homeostasis as well as the recruitment of immune cells during infection. Epithelial cells express several innate immune receptors, including nucleotide oligomerization domain proteins and TLRs that participate in initiating the immune response (2). However, activation is tightly controlled to prevent pathology due to mucosal inflammation (2–6). Upon encounter with pathogenic bacteria, epithelial cells elicit a potent response that shapes the ensuing immune response (7, 8).

Toxoplasma gondii is an orally acquired apicomplexan protozoan parasite (9). Human infections are usually asymptomatic, but reactivation of chronic infection in immunosuppressed individuals results in toxoplastic encephalitis (10, 11). Serological surveys have estimated that one-third of the world’s population has been exposed to this parasite (12). However, there is no vaccine and therapeutic treatment regimens have significant side effects. T. gondii infections are controlled primarily by T lymphocytes. IL-12 and TNF-α are critical cytokines for stimulating Th1 CD4+ T cell-induced protection (13, 14), whereas IFN-γ plays a major role in protection through CD8+ T cells (14, 15).

TLRs are innate immune receptors that directly recognize microbial structures and initiate an inflammatory response. All TLRs, except TLR3, use the adapter molecule MyD88 to initiate the signaling cascade. MyD88-deficient mice are highly susceptible to T. gondii infection due to a failure to produce IL-12 (16). Multiple TLRs have been linked to protective immunity against T. gondii infection. In mice, TLR11 expressed by dendritic cells (DCs)2 is required for the secretion of IL-12 in response to stimulation with the T. gondii protein profilin (17, 18). TLR2-deficient mice show increased susceptibility with high-dose i.p. infection (19). Oral infection of mice results in intestinal inflammation, ileitis, in wild-type mice but not in mice deficient in TLR9 or TLR4 (20, 21). This suggests that TLR9 and TLR4 may play much more important roles in initiating immunity to T. gondii at the mucosal surface. The role of TLRs in human cell recognition of T. gondii infection is much less well studied. Human TLR2 can respond to GPs from T. gondii, but the role of TLRs during live infection of human cells, especially in the gut, has not been studied.

T. gondii can infect the gut mucosa by direct invasion of epithelial cells in the small intestine (22). Therefore, epithelial cells may respond directly to T. gondii infection and initiate early local mucosal immune responses. This is supported by in vitro RNase protection studies using an immortalized mouse small intestinal enterocyte cell line, which demonstrated that the chemokines MCP-1, MIP-1, and eotaxin were induced upon infection (23). In the present study, we have investigated whether human intestinal epithelial cells respond directly to infection with T. gondii and by what mechanism this recognition occurs.
Materials and Methods

Cell culture and parasites

Henle 407 (human embryonic intestinal epithelial cells), HEK293 (human embryonic kidney cells), and HS27 (human foreskin fibroblasts) cells were maintained in DMEM supplemented with 2 mM t-glutamine, 50 μM penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 10% low endotoxin FBS. T. gondii tachyzoite strains were maintained by serial 2-day passages on HS27 monolayers in DMEM. All cell cultures and parasites were routinely checked for mycoplasma by a high sensitivity PCR-ELISA based Mycoplasma detection kit (Roche).

In vitro infections

T. gondii tachyzoites were added to Henle 407 cells and briefly centrifuged (400 × g for 1 min) to initiate parasite and cell contact. At time points indicated in each figure legend, supernatants or cell lysates were collected for further analysis. In some experiments, 50 ng/ml wortmannin (WM) was added 2 h prior to infection.

In vivo passage of T. gondii tachyzoites

To pass the parasite through mice, 5 × 10⁶ RH tachyzoites were i.p. injected into C57BL/6 mice. After 3 days of infection, the parasites were collected from the peritoneal fluid and washed once with HBSS. The parasites were selected with neomycin (1.0 mg/ml) for 14 days, and antibiotic-resistant individual colonies were isolated for further analysis and maintained in DMEM supplemented with 2 mM t-glutamine, 50 μM penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 10% low endotoxin FBS. T. gondii tachyzoite strains were maintained by serial 2-day passages on HS27 monolayers in DMEM. All cell cultures and parasites were routinely checked for mycoplasma by a high sensitivity PCR-ELISA based Mycoplasma detection kit (Roche).

Antibodies

Abs specific to total and phosphorylated forms of ERK1/2, p38, and PKB (Akt) were from Cell Signaling Technology. The anti-MyD88 polyclonal Ab was from Alexis Biotechnology. The NF-κB p65 Ab was from Santa Cruz Biotechnology, and Alexa Fluor 594 was from Molecular Probes (Invitrogen).

NF-κB translocation assay

For immunofluorescence analyses, Henle 407 cells were plated at a density of 2–3 × 10⁵ cells per well on sterile coverslips placed in a 24-well plate. Cell monolayers were infected with T. gondii RH-yellow fluorescent protein (YFP) tachyzoites and then fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 in TBS (TBS-TX) for 15 min and blocked in 1% BSA in TBS-TX for 20 min. The cells were then stained with primary rabbit anti-NF-κB p65 (1/1,000) followed by conjugated secondary Ab goat anti-rabbit IgG Alexa Fluor 594. Nuclei were stained using DAPI (1/10,000). Confocal images were taken with a Leica laser scanning confocal microscope using a 63× lens. Contrast and brightness of individual channels were adjusted linearly in Photoshop (Adobe). For Western blot analyses, Henle 407 cells were plated at a density of 1.5 × 10⁶ cells per well in 6-well plate and infected with T. gondii RH for the indicated times. Cytoplasmic and nuclear proteins were isolated according to previous protocols (24) and blotted for p65 (1/500).

RNA interference

SureSilencing human MyD88 short hairpin RNA (shRNA), TLR2 and TLR9 shRNA, and control plasmids were purchased from SuperArray Bioscience. Henle 407 cells were transfected using TransIT transfection reagent (Mirus Bio) according to manufacturer’s protocols. Transfected cells were selected with neomycin (1.0 mg/ml) for 14 days, and antibiotic-resistant individual colonies were isolated for further analysis and maintained in the presence of neomycin. For transient transfections, Henle 407 cells were transfected with shRNA plasmids by electroporation. Cells were used 48–96 h post-transfection.

RNA extraction and PCR analysis

Total RNA of Henle 407 cells infected with T. gondii was extracted using the RNaseasy mini kit (Qiagen). Reverse transcription of the RNA (1 μg) was performed using ImProw-II reverse transcription system (Promega). PCR was performed in 25 μl of a reaction mixture containing 1 μl of the reverse-transcribed RNA. The final PCR products were electrophoresed on 2% agarose gels and visualized using UV light illumination after ethidium bromide staining. Real-time PCR was performed in the Applied Biosystems 7500 real-time PCR system according to the manufacturer’s instructions (Applied Biosystems). The reaction was performed using the Power SYBR Green PCR master mix. GAPDH was used as an internal control for each sample. The primers used were as follows: IL-8 forward, 5'-AGCTTGGTCTTGGCCTGCTTGGATG-3'; CCL20 reverse, 5'-CTGGCGTGGAAACGGCCCCCA ATAAA-3'; CCL15 forward, 5'-TTGATCCTCACGCGGGACGG-3'; CCL24 forward, 5'-ATGCTCCAAGGCGAGTGATCTT-3'; CCL24 reverse, 5'-TCTCTATGACCTGGGACCCACT-3'; MyD88 forward, 5'-AGATGATCCGGCAACGTGGGACACA-3'; and MyD88 reverse, AGTCCATTTGCTTCGTCAGGT. TLR and GAPDH primers were used as previously described (25, 26).

Luciferase reporter assays

HEK293 cells were plated at 1 × 10⁶ cells per well in 96-well plates. Cells were transfected using TransIT transfection reagent (Mirus Bio) and a total of 200 ng of DNA per well consisting of human TLR2 plasmids and NF-κB or IL-8 luciferase reporters. Cells were stimulated with TLR2 ligand or infected with T. gondii tachyzoites, lysed in reporter lysis buffer (Promega), and assayed for luciferase (Promega) activity. NF-κB activity was calculated and processed by Microsoft Excel.

Gene array analysis

The commercial pathway-focused oligonucleotide microarrays (OHS-011; Oligo GEArray human inflammatory cytokines and receptors microarray) were purchased from SuperArray Bioscience. The array analyses were performed using a chemiluminescence-based detection system according to the manufacturer’s instructions. Images of the array were developed on x-ray films. Image data sets were scanned and analyzed using ScanAlyze (Eisen Lab), and Microsoft Excel software. Background adjustment was performed by subtracting the lowest measured value on the membrane from the values of all genes. The signals from the expression of each gene on the array were normalized against the signal from the internal housekeeping gene GAPDH to obtain the processed data sets. Fold changes were calculated as the normalized ratio of average experimental processed data sets divided by the average medium control processed data sets. Thresholds were set to select for genes up-regulated 2-fold or more. The original array data were deposited in National Center for Biotechnology Information Gene Expression Omnibus database under accession no. GSE18085.

Cytokine ELISA

Production of IL-8 was measured with the human CXCL8/IL-8 DuoSet ELISA development kits (R&D Systems) according to the manufacturer’s instructions.

Statistical analysis

Minitab 15 statistical software (Minitab) was used for Student’s t tests. A value of p < 0.05 was considered significant.

Results

Intestinal epithelial cells respond to T. gondii infection in vitro

Oral infection with T. gondii results in ileitis in C57BL/6 mice mediated by a robust Th1 type of response (23). CD4⁺ T cells synergize with intestinal epithelial cells to drive the secretion of Th1 type cytokines and various chemokines. The response of intestinal epithelial cells directly to the parasite during the early stage of infection in mice, and especially in humans, remains unclear. To determine whether human intestinal epithelial cells respond directly to T. gondii, we incubated T. gondii tachyzoites (RH strain) with human intestinal epithelial cell lines. Consistent with other studies on the large intestine, colon cell lines did not activate MAPKs in response to T. gondii infection even though they were permissive for infection and parasite replication (data not shown). T. gondii also infected the human small intestinal epithelial cell line Henle 407 (supplemental Fig. 1). The kinetics of infected Henle 407 cells showed increased parasite invasion during the first few hours, followed by parasite replication after 8 h as indicated by the appearance of rosettes (supplemental Fig. 1, arrows). Unlike colon cell lines, T. gondii infection of Henle 407 cells induced phosphorylation of both ERK1/2 and p38 MAPKs by 15 min.

3 The online version of this article contains supplemental material.
Henle 407 cells were infected with RH tachyzoites (parasite to cell ratio, 6:1) for the indicated times. Whole cell lysates were collected at the indicated time points (p.i., postinfection) and assayed by ELISA (triplicate assays; error bar, SD). Experiments were performed three times with similar results; *, p < 0.05, compared with IL-8 levels of uninfected cells. C and D, Henle 407 cells were infected with RH (C) or RH-YFP (D) parasites for the indicated times. NF-κB p65 localization was visualized by immunoblotting of cytosolic (Cy) and nuclear (Nu) extracts (C) or by immunofluorescent staining (D) for anti-NF-κB p65 (red).

FIGURE 1. Intestinal epithelial cells respond to T. gondii infection. A, Henle 407 cells were infected with RH tachyzoites (parasite to cell ratio, 6:1) for the indicated times. Whole cell lysates were collected at the indicated time points (p.i., postinfection) and assayed by ELISA (triplicate assays; error bar, SD). Experiments were performed three times with similar results; *, p < 0.05, compared with IL-8 levels of uninfected cells. C and D, Henle 407 cells were infected with RH (C) or RH-YFP (D) parasites for the indicated times. NF-κB p65 localization was visualized by immunoblotting of cytosolic (Cy) and nuclear (Nu) extracts (C) or by immunofluorescent staining (D) for anti-NF-κB p65 (red).

NF-κB regulates proinflammatory and antiapoptotic genes in response to pathogens. To determine whether T. gondii infection of Henle 407 cells results in activation of NF-κB, we infected the cells for various times with RH tachyzoites and immunoblotted cytosolic and nuclear extracts for p65 NF-κB. Translocation of NF-κB to the nucleus was observed as early as 15 min, peaked at 60 min, and was still detected 2 h postinfection (Fig. 1C). Infection with T. gondii-infected intestinal epithelial cells express several inflammatory genes

Oral infection studies have demonstrated an increase in several cytokines and chemokines in response to T. gondii infection; however, the mixed population of the intestinal mucosa did not allow for the determination of the relative role that specific cell populations play in the production of these mediators. To address the role of intestinal epithelial cells to modulate the cytokine environment early following infection, we used specific pathway arrays to identify cytokines and chemokines induced 4 h after exposure to T. gondii. At this time point most cells have become infected, but the parasites have not replicated. Consistent with previous studies on late responses, infected epithelial cells expressed higher levels of proinflammatory chemokines, including MIP-1α/CCL3, MIP-2/CXCL2, RANTES/CCL5, MCP-2/CCL8, MCP-3/CCL7, and IFN-γ-inducible protein-10 (Table I). The induced cytokines and transgenic parasites expressing YFP allowed the visualization of infected cells. Staining for p65 NF-κB demonstrated that infected, but not uninfected, cells translocated NF-κB to the nucleus (Fig. 1D).

Table I. Pathway-specific microarrays revealed up-regulation of several T. gondii-induced inflammatory cytokines, chemokines, and receptors

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*Henle 407 cells were infected with RH parasites and total RNA was collected at 4 h postinfection. RNA from infected and uninfected cells was compared using a pathway-focused oligonucleotide microarray containing 113 genes involved in the inflammatory response. Genes that were up-regulated 2-fold or more after infection were shown.

*Non-cytokine gene.
chemokines included ones that induce neutrophil chemotaxis (IL-8 and MIP-2/CXCL2), homing of mucosal DCs (CCL20), and migration of DCs to sites of infection (MIP-1a/CCL3 and RANTES/ CCL5) (27, 32). Of note, IL-18, which enhances IL-12 mediated immune responses to *T. gondii*, was highly induced in infected epithelial cells (33–35). By PCR analysis, there was little or no IL-8 mRNA but a low level of IL-18 mRNA in uninfected cells. Four hours after infection both IL-8 and IL-18 mRNA were up-regulated, supporting our array data (supplemental Fig. 2). Real-time PCR analysis confirmed the up-regulation of several neutrophil and monocyte chemoattractants, including IL-8, CCL15, CCL20, and CCL24 upon *T. gondii* infection (Fig. 2). Together, these data demonstrate that human intestinal epithelial cells induce chemotactic and inflammatory mediators capable of modulating the local immune response early (minutes to hours) after infection.

Epithelial cell response to *T. gondii* is PI3K independent

*T. gondii* infection of mouse macrophages induces MAPK and protein kinase B (also known as Akt) activation through a Gαi-dependent PI3K signaling pathway (36). Similarly as in macrophages, phosphorylation of Akt occurred in Henle 407 cells 60 min after *T. gondii* infection (Fig. 3A). The PI3K inhibitor WM completely blocked *T. gondii*-induced phosphorylation of Akt but only slightly reduced the phosphorylation of ERK1/2 and p38 at 15 min and had no detectable effect on ERK1/2 at 60 min (Fig. 3A). Constitutive production of IL-8 was dramatically inhibited by treatment with WM (Fig. 3B; compared with no infection controls). However, *T. gondii* infection of WM-treated cells still resulted in an induction of IL-8 secretion with similar fold induction as that of untreated cells (Fig. 3B). Real-time PCR analysis demonstrated that IL-8 mRNA up-regulation was not affected by WM in infected or uninfected cells (Fig. 3C). This suggests that although the steady-state production of IL-8, but not mRNA production, is dependent on PI3K, *T. gondii*-induced up-regulation is independent of PI3K.

Epithelial cell response to *T. gondii* infection is MyD88 dependent

TLR9 has been implicated in the host response to oral infection with *T. gondii* because TLR9-deficient mice fail to develop ileitis that is observed in wild-type mice (21). Both hematopoietic and nonhematopoietic cells express TLR9, and experiments using bone marrow chimeras suggest that both compartments are critical for the host response. However, it is unclear whether nonhematopoietic cells, such as epithelial cells, respond directly to *T. gondii* through TLR9 or whether the response is secondary to commensal bacterial leak into the lamina propria following damage to the epithelium. To determine whether TLR9 or other TLRs play a role in the early response of epithelial cells to *T. gondii*, we depleted MyD88 from Henle 407 cells by stable expression of a shRNA against MyD88. All TLRs, except TLR3, are dependent on MyD88 for signal transduction. Control shRNA-transfected Henle 407 cells expressed similar levels of MyD88 protein as nontransfected cells. However, cells transfected with the MyD88 shRNA expressed significantly less MyD88 protein, confirming the effect of the RNA interference-mediated knockdown (Fig. 4A). Upon *T. gondii* infection, cells deficient in MyD88 had a significantly reduced level of ERK1/2 phosphorylation and a slightly reduced level of p38 phosphorylation, compared with control cells (Fig. 4B and data not shown). Furthermore, MyD88-deficient cells failed to induce IL-8 upon exposure to *T. gondii* (Fig. 4C). MyD88 is also an adapter protein for IL-1 and IL-18. Because IL-18 is induced upon *T. gondii* infection and contributes to small intestinal pathology in C57BL/6 mice (33, 34), it remains possible that the MyD88 dependence is via IL-18 signaling. However, in Henle 407 cells the bioactive forms of both IL-18 and caspase-1 were not detected until 6 h post-*T. gondii* infection (C. Ju, unpublished observation).
This suggested that although the IL-18 mRNA level is regulated after infection, the post-translational cleavage to generate the bioactive form of IL-18 did not occur until later. Therefore, MyD88 plays a critical role in the response of Henle 407 small intestine epithelial cells to T. gondii infection, most likely through a TLR.

T. gondii activates TLR2 on intestinal epithelial cells

Similarly as in other studies on primary human small intestine cells, Henle 407 cells expressing most of the TLRs except TLR8 (37) (Fig. 5A). Stimulation with TLR ligands or PMA as a control induced phosphorylation of ERK1/2 and p38 within 15 min (TLR1/2, Pam3Cys; TLR2/6, Malp-2; TLR3, poly(I:C); TLR4, LPS; TLR5, flagellin; TLR7, loxoribine; and TLR9, CpG DNA) (Fig. 5B and data not shown). To specifically identify which human TLR was involved in T. gondii recognition, we reconstituted HEK293 cells with each human TLR independently and measured the activation of NF-κB following stimulation with positive control ligands, soluble Toxoplasma Ag (STAg) or live T. gondii infection using a luciferase reporter. Although each positive control ligand stimulated NF-κB activation in the respective TLR expressing cells, only TLR2 expression was permissive for NF-κB response to live T. gondii infection (Fig. 6A). STAg failed to activate NF-κB, suggesting that live infection was required.

To confirm the role of TLR2 in Henle 407 cell response to T. gondii infection, we knocked down TLR2 expression using shRNA. Transient transfection with TLR2 shRNA depleted the mRNA relative expression levels to 6% of wild-type levels (Fig. 6B). Knockdown of TLR2 reduced the activation of ERK1/2, p38, and IL-8 induction in response to T. gondii infection (Fig. 6, C and D). Transient transfection of MyD88 shRNA gave similar results compared with those of stably transfected cells (Fig. 6, C and D, compared with Fig. 4). TLR2 knockdown in Henle 407 cells does not completely block the response of MAPK activation and IL-8 induction. Therefore, the response is almost entirely MyD88 dependent but only partially dependent on TLR2. Attempts to combine TLR2 with other TLRs, including TLR4 and TLR9, in our HEK293-based stimulation assay did not result in further increases in NF-κB activation (data not shown). TLR2 was required for IL-8 production because its expression in HEK293 cells was sufficient to permit induction of an IL-8 regulated luciferase reporter (Fig. 6E). Pretreatment with the ERK1/2 inhibitor U0126 inhibited IL-8 luciferase reporter activity. These data demonstrate that TLR2-induced IL-8 production was through the ERK1/2 pathway (Fig. 6E). In our system, TLR9 was neither necessary nor sufficient for epithelial cell response to T. gondii infection (supplemental Fig. 3 and Fig. 6A). Taken together, T. gondii induced IL-8 is dependent on ERK1/2, MyD88, and TLR2.

Genotype of T. gondii does not influence epithelial cell response

T. gondii strains have been classified into three clonal lineages that differ in their pathogenicity in mouse models (38). Several strains of each clonal lineage were tested for their ability to induce epithelial cell response and activate TLR2. Henle 407 cells phosphorylated ERK1/2 in response to all strains from each of the three lineages (Fig. 7A and data not shown; type I: RH and GT; type II: PT-G, CC, and DEG; and type III: VEG). The various strains differed dramatically in their capacity to induce phosphorylation of p38, but there was no correlation with genotype (Fig. 7A). All strains induced NF-κB activation through TLR2 (Fig. 7B and data not shown). The type I RH strain had a lower capacity to induce NF-κB when compared with another type I strain, GT-1 (data not shown). RH tachyzoites grown in cell cultures have reduced virulence when compared with those passed in mice (39). Similarly, we observed that RH tachyzoites passed in mice induced significantly more TLR2-dependent NF-κB activation than those passed through fibroblasts in vitro (data not shown). The ability of live T. gondii to activate cellular responses through TLR2 was not unique to human cells, because cells transfected with mouse TLR2 responded similarly (data not shown). Together, we conclude that TLR2-dependent activation of signaling cascades by T. gondii is not genotype dependent.

Discussion

In the present study, we evaluated the initial cellular responses of human intestinal epithelial cells to T. gondii infection. This type of

FIGURE 4. T. gondii-induced MAPK activation and IL-8 secretion is MyD88 dependent. A, Henle 407 cells were either untransfected (lane 1), transfected with control shRNA plasmids (lane 2), or transfected with MyD88 shRNA plasmids carrying the MyD88 RNA interference sequence (lane 3). Cell lysates from the stably transfected cell lines were collected for immunoblotting with Abs against MyD88 or β-tubulin. B, Activation of ERK1/2 in T. gondii-infected MyD88 knockout Henle 407 cells. Control Henle 407 cells (lanes 1), control shRNA cells (lanes 2), and MyD88 shRNA cells (lanes 3) were infected with RH tachyzoites (parasite to cell ratio, 6:1) at the indicated time points. Cell lysates were collected for immunoblotting with Abs against total and phosphorylated (p-) forms of ERK1/2. C, Control Henle 407 (lane 1), control shRNA (lane 2), and MyD88 shRNA (lane 3) cells were infected with T. gondii (parasite to cell 6:1). Supernatants from each sample were collected for IL-8 ELISA analysis (triplicate assays; error bar, SD; *, p < 0.05, compared with IL-8 levels of uninfected cells).

FIGURE 5. TLR genes are expressed in Henle 407 cells. A, Total RNA was collected from Henle 407 cells, reverse transcribed to cDNA, and then amplified for human TLR or GAPDH by PCR. Genomic DNA (gDNA) was used as a positive control. B, Henle 407 cells were treated with 10 ng/ml PMA as a positive control or TLR1/2, TLR4, TLR2/6, and TLR9 ligands (1 μg/ml Pam3Cys, 100 ng/ml LPS, 1 μg/ml Malp-2, and 5 μg/ml CpG, respectively). Total cell lysates were collected and immunoblotted for total and phosphorylated (p-) forms of ERK1/2 and p38.
study is critical for identifying the very early innate immune responses to parasitic infection of the intestinal mucosa. Using an in vitro model in which an isolated cell type is directly exposed to the infectious agent offers the advantage over mixed cell populations in identifying the response of a specific cell type. Most studies on the immune response to *T. gondii* infection have used a peritoneal challenge model for this orally acquired pathogen. More recently, the importance of studying the natural route of infection has revealed the importance of epithelial cell response in influencing the outcome of the local and systemic immune response (40, 41). By examining the response of the cells most likely to first encounter the pathogen, we can begin to uncover the early responses that may limit or induce the spread of *T. gondii* to other tissues such as muscle and brain, where a persistent infection results. Therefore, it is highly relevant to study the response of these cells during *T. gondii* infection, which occurs both locally in the intestine and systemically.

Very little is known about the human intestinal response to *T. gondii* infection; therefore, a major finding of this study is that human small intestinal epithelial cells respond directly to *T. gondii* within minutes by activating signaling cascades. The neutrophil chemoattractant IL-8 is up-regulated both at the protein and mRNA level within hours. Several additional cytokines and chemokines are also up-regulated at the mRNA level within 4 h. During mouse infections with *T. gondii*, neutrophils are critical for host defense and are one of the first cells recruited to the site of infection. They play a key role in the recruitment and activation of macrophages and DCs (27, 31, 42). Therefore, our findings that the human small intestine epithelial cell line Henle 407 responds directly to *T. gondii* infection suggests that an in vivo epithelial response would modulate the local inflammatory environment to initiate host defense against infection.

This series of studies also elucidates the molecular mechanism for epithelial response to *T. gondii* infection. We show that, similarly as macrophages and DCs, epithelial cells activate the MAPK pathway. The inability of MyD88-deficient epithelial cells to secrete IL-8 and activate ERK1/2 during infection suggests that TLRs play a critical role in initiation of the mucosal inflammatory process. Human TLR2 responds to live *T. gondii* in our heterologous reconstitution assay. However, knocking down TLR2 with shRNAs in intestinal epithelial cells only partially reduced the activation of ERK1/2. Thus, although TLR2 contributes to the epithelial response to *T. gondii* infection, there is likely an interaction with additional TLRs or other receptors that we could not detect in our assay. In fact, preliminary examination of the dependence of several cytokines and chemokines on MyD88 and TLR2 by using shRNA knockdown revealed a complex pattern. Although several genes were TLR2 dependent (IL-8, CCL10, CCL15), a few were TLR2 independent and MyD88 dependent (CCL5 (RANTES) and CCL11). IL-18 and CCL20 did not depend on either MyD88 or...
Cell lysates were collected at 18 h and assayed for luciferase activity (triplicates). The transfected HEK293 cells were stimulated with Abs against total and phosphorylated (p-) forms of ERK1/2 and p38. Total cell lysates were collected for immunoblotting at indicated time points. Total cell lysates were collected at 18 h and assayed for luciferase activity (triplicate assays; error bar, SD; *, p < 0.05, compared with NF-κB activity of uninfected controls).

FIGURE 7. MAPK activation and TLR2-dependent response to T. gondii is not strain type specific. A, Henle 407 cells were infected with, GT-1 (type I), CC, DEG (type II), and VEG (type III). T. gondii strains for the indicated time points. Total cell lysates were collected for immunoblotting with Abs against total and phosphorylated (p-) forms of ERK1/2 and p38. B, HEK293 cells were transfected with human TLR2 and an NF-κB luciferase reporter plasmid. The transfected HEK293 cells were stimulated with the TLR2 ligand Pam3Cys or different T. gondii tachyzoite strains. Cell lysates were collected at 18 h and assayed for luciferase activity (triplicate assays; error bar, SD; *, p < 0.05, compared with NF-κB activity of uninfected controls).

TLR2 (data not shown). TLR4 and TLR9 are candidates for working in concert with TLR2 for the production of cytokines and chemokines, because mice deficient in these TLRs have reduced intestinal pathogenicity during oral T. gondii infection. Furthermore, TLR9 in either hematopoietic or nonhematopoietic compartments is important for efficient T cell responses to oral infection (21). However, in our in vitro system knocking down TLR9 in Henle 407 cells or reconstituting TLR9 in HEK293 cells does not affect the MAPK or elicit a NF-κB response to T. gondii. Commensal bacteria are present in the intestine and are capable of activating TLR9. Therefore, it is likely that the TLR9-dependent pathology induced during oral infection with T. gondii is secondary to epithelial damage and recognition by TLR9 of bacteria that penetrate the epithelial barrier.

Ligands for TLR2 include lipopeptides, lipoproteins, and GPIs. GPI-anchored proteins are abundant on the surface of T. gondii tachyzoites, and GPIs from T. gondii and Trypanosoma cruzi also stimulate epithelial cells during infection. These other receptors are unlikely to be TLRs, because attempts to coexpress other TLRs with TLR2 did not enhance the response in our HEK293 based stimulation assay.

There are three clonal lineages of T. gondii that differ in their ability to induce cytokines and virulence. Low virulence type II parasites show lower induction levels of IL-12p40, IL-10, IL-1β, and IL-6, whereas high virulence type I parasites attract more neutrophils during infection (51, 52). We predicted that the ability to activate TLR2 might correlate with lower virulence due to an increased activation of the immune response, especially because NF-κB activation and cytokine secretion in immune cells correlate with genotype (53). However, all types elicited ERK1/2 and p38 MAPK activation through TLR2. Although the strains varied in the level of activation, there was no correlation with genotype. Virulence and cytokine induction differences among strains are not due to an ability to activate epithelial cells via TLR2.

In summary, this study demonstrates that human intestinal epithelial cells respond directly to T. gondii infection via MyD88- and TLR2-driven ERK1/2 kinase and NF-κB signaling pathways. An interesting question for future studies is how epithelial cells crosstalk and influence immune cells during infection. Our preliminary data point to a complex pattern of cytokine regulation in which TLR2 is important but not the whole story. Understanding the local immune response against pathogens in the intestine will provide insight into the development of intestinal disorders, mechanisms for enhancing immune response to infection, or targets for vaccine development.

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Disclosures

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References


