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Regulation of toll like receptors in intestinal epithelial cells by stress and *Toxoplasma gondii* infection

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SUMMARY

Intestinal epithelial cells (IECs) form a barrier between invading microorganisms and the underlying host tissues. IECs express Toll-like receptors (TLRs) that recognize specific molecular signatures on microbes which activate intracellular signaling pathways leading to production of proinflammatory cytokines and chemokines. Stress hormones play an important role in modulation of proinflammatory cytokines and downregulation of immune responses. Here we demonstrated that expression levels of TLR-2, TLR-4, TLR-9 and TLR-11 were significantly increased in mouse IECs following infection with *Toxoplasma gondii* on day 8 post infection. In contrast, expression of TLRs was significantly decreased in infected mice subjected to cold water stress (CWS+INF). Expression of TLR-9 and TLR-11 in the mouse MODE-K cell line was significantly increased after infection. Expression of TLR-9 and TLR-11 in cells exposed to norepinephrine (NE) and parasites was significantly decreased when compared to cells exposed to parasites only. A significant increase was observed in SIGIRR, a negative regulator of TLRs in the CWS+INF group when compared to the INF group. Stress components were able to decrease expression levels of TLRs in IECs, decrease parasite load, and increase expression of a negative regulator thereby ameliorating intestinal inflammatory responses commonly observed during per oral *T. gondii* infection in *C57BL/6* mice.

Keywords

Toxoplasma gondii; Intestinal epithelial cells; Toll like receptors; stress hormones; innate immunity

INTRODUCTION

In addition to functioning as a barrier and absorptive organ, intestinal epithelial cells (IECs) are involved in defense and inflammatory diseases by releasing multiple cytokines and inflammatory mediators (1,2). The intestinal mucosa is exposed to a variety of bacteria and IECs sense microbial molecular patterns leading to activation inflammatory and immunomodulatory molecules (3,4). IECs express a variety of innate immune receptors called toll-like receptors (TLRs) and intra cytoplasmic NOD proteins which are critical for innate immune responses (5,6). They sense invading pathogens through pathogen associated molecular patterns (PAMPs) present in microorganisms such as bacteria, virus, fungi and protozoa. For Example, TLR-2 recognizes peptidoglycan, a main cell wall component of Gram positive bacteria, lipoprotein, lipotechoic acid and lipopeptides (7,8), TLR-4 recognizes lipopolysaccharide (LPS) of G (-) bacteria (9), TLR-9 binds to unmethylated CpG DNA of bacteria (10) and TLR-11 recognizes *T. gondii* profilin (11,12). After recognition of

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their ligand, TLRs activate intracellular signaling pathways through Toll/IL-1 receptor (TIR) (13) and recruitment of adaptor molecules such as MyD88, TICAM-1, TIRAP and TRAM (14). These adaptor molecules act independently or in combination based on the TLRs and activate MAPK (ERK, JNK and p38) and NF- κ B resulting in the transcription of inflammatory and immunomodulatory genes include chemokines, cytokines and co-stimulatory molecules (15,16). Negative regulation of TLR signaling is critical for downregulation of gene activation thereby controlling proinflammatory cytokine production and overwhelming inflammation. There are several proteins (Tollip, SIGIRR, IRAK-M and A20) which act directly on the TLRs or adaptors or different downstream molecules thereby reducing TLR expression (17-19).

Infection with the obligate intracellular parasite *T. gondii* is naturally acquired by oral ingestion of soil or raw meat contaminated with oocysts or parasite tissue cysts (20,21). Following per oral infection, bradyzoites in tissue cysts convert to tachyzoites and actively invade IECs and disseminate to different tissues until host immune responses finally stops parasite multiplication resulting in parasite tissue cysts in lungs, liver, kidney, pancreas, brain and skeletal muscles (21,22). Oral infection with *T. gondii* in susceptible C57BL/6, but not in the resistant BALB/c mice, leads to a Th1-type acute and lethal ileitis (23). Intestinal pathology is mediated by CD4⁺ T cells through among other the release of IFN- γ , TNF- α , and inducible NO synthase (iNOS) (24), as well as the regulatory effects of IL-10 (25) and TGF- β (26). After per oral infection, neutralization of either IFN- γ or CD4⁺ T cells prevents intestinal pathology and increases survival in C57BL/6 mice (23,27). The ensuing intestinal disease after per oral *T. gondii* infection is similar to that observed in human ileitis in respect to disease location, histological finding, and immunological imbalance (28). Although the involvement of Th-2 cytokines in intestinal pathology following per oral *T. gondii* infection is less clear, IL-4 and IL-5 have been shown to participate in the ensuing inflammation as IL-4 and IL-5 deficient mice had increased survival and presented decreased intestinal pathology (29-31).

In mammals, stress activates the hypothalamic pituitary adrenal (HPA) and sympathetic adrenal medullary (SAM) axes. Activation of these two major pathways releases stress hormones such as glucocorticoids and catecholamines. These stress hormones have been shown to alter immune function by downregulating inflammatory cytokines and upregulating anti-inflammatory cytokines (32-34). Resistance to West Nile virus was markedly reduced in mice stressed with cold water (CWS) or by isolation stress (35). Immobilization stress has been shown to enhance mortality in mice during acute infection with a virulent strain of *T. gondii* (36). Cold water stress decreased activation, cytokine production, and microbicidal properties of macrophages (37), splenocytes (38), and delayed parasite control in the brain after *T. gondii* infection (39). In the C57BL/6-*T. gondii* mouse model of per oral infection, modulation of intestinal immune responses by CWS would advantageous by limiting inflammatory responses due to over-production of Th1 cytokines (23,24). Stress components have been shown to down regulate proinflammatory cytokines and up regulate anti-inflammatory cytokines (32-34). Furthermore, the timing and intensity of stress seems to play an important role in the outcome when applied to a host-pathogen interaction. Tanaka *et al.* (40) demonstrated that mild but not severe stress protected mice against cold-restraint stress-induced gastric ulceration. The protective effect by mild stress was mediated through prostaglandins released through activation of COX-1 and COX-2. Our previous results employing the CWS paradigm have demonstrated the immunomodulatory effect of CWS during *T. gondii* infection in mice. This effect seems to be correlated with the route of infection as stressed mice were more susceptible following intraperitoneal infection (41).

As IECs are the first cells to encounter invading parasites after per oral infection and dictate early innate immune responses; we wanted to determine the effect of CWS on TLR-mediated immune response in IECs. In the present study, we have examined the expression levels of different TLRs, and SIGIRR, a negative regulator of TLRs in IECs during CWS and infection. Our data demonstrated CWS down regulated TLR-2, TLR-4, TLR-9 and TLR-11 expression in IECs isolated from infected mice subjected to CWS. The mouse intestinal cell line, MODE-K is an important model exhibiting many characteristics of mouse IECs and allows for in vitro studies. We mimicked the effects of stress by addition of NE to MODE-K cells *in vitro*. Significantly decreased expression of TLR-9 and TLR-11 was observed in MODE-K cells exposed to NE and *T. gondii* infection when compared to MODE-K cells exposed to infection alone. We have also demonstrated that *T. gondii* infection both *in vivo* and *in vitro* caused a significant reduction in SIGIRR expression, while increased expression was found in the stress groups. These results demonstrated that CWS as a mild stressor was beneficial after per oral infection by modulating TLR expression in IECs; the outcome of this modulation may be decreased parasite load, inflammation, and resulting intestinal pathology.

MATERIALS AND METHODS

Mice

Female C57BL/6J mice 7-8 wks of age (Jackson Laboratory, Bar Harbor, ME) were housed and allowed to adjust under approved conditions at Northern Arizona University. Temperature and light (12 light/ 12 dark) were controlled while intake of food and water were provided *ad libitum*. The Institutional Animal Care and Use Committee approved all experimental protocols involved in this study.

Cold water stress (CWS)

Cold water stress was applied by placing mice in shallow clear glass chambers filled with 3 cm of cold water ($1 \pm 0.5^\circ \text{C}$), deep enough to cover their backs, for 5 min a day for eight days (37-39,41). The animals were allowed to move freely within the chamber and not subjected to any physical restraint. Control animals were handled in the same way as the other group, but they will not be subjected to any CWS. After CWS animals were dried with a soft towel and place back in their cages.

Parasites and infection

For *in vitro* infection, *T. gondii* ME49 tachyzoites were maintained in human foreskin fibroblasts. The ME49 strain of *T. gondii* was obtained from brains of Swiss Webster mice infected 3 months earlier. Mouse brains containing cysts were homogenized with a glass pestle in 2 ml of 0.1M phosphate-buffered saline (PBS) pH 7.2. To disrupt tissues, the homogenate was passed several times (5-6) through a 20 gauge needle. Five μl aliquots of the suspension was placed in a glass slide and cysts enumerated under light microscopy and adjusted with PBS to contain 35 cysts/0.2 ml. On the 8th day of CWS treatment, mice were orally infected with 35 cysts in 0.2 ml by intragastric gavage using a 1ml syringe and a 1.5 inch intubation needle. Control animals were given equal PBS volume. Mice from all groups were sacrificed by CO₂ asphyxiation on days 3 and 8 post infection (PI) as this two time points provide information on the early and late host intestinal immune responses and its regulation by stress.

Preparation of *Toxoplasma* lysate antigen (TLA)

Cultures of HFF cells were infected with ME-49 parasite strain. Tachyzoites were purified after disruption of cells and filtration as described below. The cells were lysed, forced

through 21, 25 and 27 gauge needles, to release tachyzoites from the cells. Excess debris was removed by filtration through a 3µm filter, and parasites collected by centrifugation at 3000 rpm for 10 min. The supernatant was discarded and parasites resuspended in PBS. The parasites were sonicated (setting at 5 sec, 2 min once, and 6X total). Finally the lysate was filtered through a 0.45µm filter and protein concentration of the TLA measured using the RC DC protein assay (Bio-Rad laboratories, Hercules, CA).

Isolation of IECs

Intestinal epithelial cells were isolated as described by Evans *et al.*, (42). Small intestines were flushed with Ca²⁺, Mg²⁺ -free PBS, longitudinally spliced and cut into 0.5 cm pieces. The intestinal pieces were incubated at room temperature with 5mM EDTA for 20 min at pH 7.4 with agitation using a magnetic stirrer. Supernatants containing IECs were removed, centrifuged at 1,200 x g, and cells resuspended with PBS to remove excess EDTA. This process was repeated at least three times. The cell purity was confirmed by Indirect Fluorescent antibody technique using a cytokeratin-specific antibody (TROMA (I).

Indirect florescent antibody technique

Purified IECs were smeared on the slide and cells fixed with 4% formaldehyde in PBS for 20 minutes at 20° C. Cells were blocked by incubation in PBS containing 5% normal goat serum and 0.2% Tween-20 for 20 min at room temperature (Jackson Immuno Research Laboratories, West Grove, PA). Cells were then incubated with a 1:10 dilution of TROMA-I antibody (Developmental Studies Hybridoma Bank, University of IOWA, Iowa City, IO) for 30 min at room temperature. After rinsing the slides with PBS with 0.2% Tween-20 the slides were incubated with FITC-conjugated goat anti rat IgG (1:100 dilution) for another 30 min (BD Biosciences, San Jose, CA). Slides were washed three times and mounted using Fluoromount (Southern Biotech Associates Inc, Birmingham, AL). The slides were viewed under Leica DMIRE2 fluorescent microscope (20X, 40X) and fluorescence images captured with the Image Pro software (Leica Microsystems Inc., Mannheim, Germany).

MODE-K Cell Line

The mouse epithelial cell line, MODE-K was derived from small intestines of C3H/He mice created by Vidal and Colleagues (Institut Pasteur de Lyon, France) (43) and kindly provided by Dr. K. Croitoru (McMaster University, Canada). Cells were maintained in growth medium consisting of Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum, 10 mM HEPES, 0.4 g/liter L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 100 µg/ml gentamycin, and 50 µM 2-mercaptoethanol (Sigma, St. Louis, MO). MODE-K cells were split weekly and seeded at 1:10 dilution.

Isolation of RNA and conversion of cDNA by reverse transcription

Total RNA was isolated from IECs using the TRI reagent following the manufacturer's instructions (Molecular Research Center INC, Cincinnati, Ohio). Samples were treated with DNase I at 1µl/µg of RNA (Fermentas Inc, Glen Burnie, MD) for 30 min to remove contaminating genomic DNA. RNA was reverse-transcribed to cDNA following standard methods using the avian myeloblastosis virus reverse transcriptase (20U/µl), random hexamers (0.2µg/µl), RNase inhibitor (20U/µl), 10mM dNTP mix (First Strand cDNA Synthesis Kit, Fermentas) for 20µl reaction. RNA, 2-5 µg was used to obtain cDNA. Successful cDNA conversions were confirmed by amplification of β-actin by conventional PCR. The PCR product was viewed in a 1.5% agarose gel electrophoresis using ethidium bromide staining method.

Quantitative real time PCR

The primer sequences were either designed using the Roche diagnostics software (Roche Diagnostics corp. Indianapolis, IN), or from published sequences. The length of PCR products ranged from 50 to 300 bp. The primer concentrations used for our reactions were 250 nM in a 20 µl using the SYBR green master mix (SuperArray Bioscience Corporation, Frederick, MD) and run in 96-well PCR plates using the BioRad MyiQ real time PCR (BioRad Laboratories, Hercules, CA). All reactions were run in triplicate with 1 µl of cDNA per reaction. To check for amplicon contamination, every run contained at least two “no template” controls in which DNase/RNase free water instead of samples or standards were included. A standard curve was generated using 10-fold serial dilutions of purified DNA product (QIA Quick Gel Extraction kit, QIAGEN Sciences, Maryland) of gene of interest (44). The relative standards varying from 10^9 to 10^1 copies of DNA and this curve was considered acceptable if a difference of 3.3 ± 0.3 cycles was demonstrated between each of the 10-fold dilutions and if the correlation coefficient was at least 0.99. Samples were heated at 95°C for 10 min, and then subjected to 40 cycles of amplification by melting at 95°C, 15 sec, then annealing at 58°C, 1 min. Experimental samples and standards were run in duplicate every time. Threshold cycle numbers (Ct) were used to calculate as per the method outlined in “Relative Standard Curve Method” in user Bulletin provided by Applied Biosystems using GAPDH as the reference gene as indicated. Real-time data were collected and analyzed using the Excel program. Based on the standards the copy numbers were calculated by relative quantification

Measurement of TLRs

Expression levels of TLR- 2, TLR-4, TLR-9 and TLR-11 were measured by real time PCR as described above. For each TLR gene the primer sequences were obtained from Johnson *et al.* (45). Primers for TLR-2 were forward 5'-TGGAATGTCACCAGGCTGC-3' and reverse 5'-GTCCGTGGAATGGTGGC-3'; TLR-4 forward 5'-GCCCCGCTTTCACCTCTG-3' and reverse 5'-TGCCGTTTCTTGTTCTTCTCT-3'; TLR-9 forward 5'-TTCTCAAGACGGTGGATCGC-3' and reverse 5'-GCAGAGGGTTGCTTCTCACG-3'; and TLR-11 forward 5'-TCTTTGACGCTCGGAAGTGTAGCA-3' and reverse 5'-TAGGTCGATGCACAACTGGGTGAA-3'. Standards were made from gel-purified gene products for each TLR. PCR conditions were as follows; for TLR-2 and TLR-9 denaturing at 95° C for 30 sec, annealing at 60° C for 1 min, and extension for 40 sec. For TLR-4; denaturing at 95° C for 30 sec, annealing at 58° C for 40 sec, and extension 72° C for 40 sec. For TLR-11; denaturing 95° C for 30 sec, annealing at 58° C for 30 sec and extension at 72° C for 30 sec.

Measurement of negative regulator of TLR expression SIGIRR

The single immunoglobulin IL-1 receptor-related molecule (SIGIRR) is a negative regulator for Toll-IL-1 receptor (TIR) domain (18). We determined its expression by real time PCR by using specific primers (forward 5'-GTGGCTGAAAGATGGTCTGGCATTG-3', reverse 5'-CAGGTGAAGGTTCCATAGTCCTCTGC-3' (19). The PCR conditions were as follows: denaturing 95° C for 30 sec, annealing 60° C for 1 min, and extension at 72° C for 40 sec.

Measurement of parasitic burden

Amplification of *T. gondii* by PCR has been performed using primers against the 529 bp fragment that is repeated 200- to 300-fold in the *T. gondii* genome and it has shown to be more sensitive than the 35-copy B1 genomic region (46-48). We determined parasite burden by measuring expression levels of *T. gondii* by real time PCR using targeting the repeated 529 bp fragment with specific primers: forward 5'-CACAGAAGGGACAGA AGT-3'; reverse 5'-TCGCCTTCATCTACAGTC-3' (48). The PCR conditions were as follows:

denaturing 95° C for 15 sec, annealing 60° C for 1 min, and extension at 72° C for 15 sec for 40 amplification cycles.

Statistics

Data are presented as means $\pm$ standard error. Statistical differences were determined by one-way ANOVA followed by Student's *t*-test or Dennett's multiple comparison tests of all groups against the control group. All tests were performed using JMP5 statistical software.

RESULTS

Oral infection in cold water stressed C57BL/6 mice infected with *T. gondii* cysts down regulates TLR expression

Stress hormones such as adrenocorticotrophic hormone (ACTH) and catecholamines (nor epinephrine and epinephrine) act as an immunomodulators and suppress secretion of inflammatory cytokines preventing the overwhelming infection (32,33). We have previously reported that CWS induced immunomodulation in mice during *T. gondii* infection (37-39,41). CWS altered intracerebral immune responses against *T. gondii* by decreasing expression of IFN- γ , IL-2, TNF- α , iNOS and IL-12; and delayed encystation in BALB/c mice (39). Following per oral infection, IECs are the first cells to interact with *T. gondii*, and indication that TLR-9 is critical in Th1 mediated immune response during *T. gondii* infection (49). In this study we determined the role of stress hormones on TLR expression in IECs and how CWS modulated TLR-mediated immune responses to *T. gondii* infection. Our results showed that stress modulated expression of TLR-2, TLR-4, TLR-9 and TLR-11 in IECs in response to *T. gondii* infection. Animals were sacrificed 3 and 8 days PI, IECs were collected from small intestines in control (CON), infected (INF), cold water stress (CWS), and cold water stress and infection (CWS+INF) groups. The purity of IECs was checked by IFAT with TROMA-I antibody (Figure 1). Our isolation protocol consistently yielded >90% purity. RNA isolated from IECs was converted to cDNA and expression levels of TLR-2, TLR-4, TLR-9 and TLR-11 determined by real time PCR. On day 3 PI, expression levels of all TLRs was significantly higher in IECs from the CWS group when compared to day 8 PI. In particular, expression of TLR-2 was significantly higher in the CWS group when compared to the other groups. Expression of TLR-4 and TLR-11 was significantly higher in the CWS and CWS+INF groups than in the CON and INF groups (Figure 2). On day 8 PI, expression of TLR-2, TLR-4, TLR-9 and TLR-11 was significantly higher in the INF group when compared to other groups. In contrast, IECs from the CWS+INF group showed a significant decrease in TLR-2, TLR-4 and TLR-9 and TLR-11 when compared to INF group. On day 8 PI, the CWS+INF group had significantly elevated expression of TLR-2, TLR-4 and TLR-11 when compared to CON group and significantly higher expression of TLR-2 and TLR-4 when compared to the CWS group (Figure 2).

TLR expression is altered in MODE-K cells exposed to *T. gondii* tachyzoites or *Toxoplasma* lysate antigen (TLA)

In IECs purified from mice subjected to CWS we found that stress down regulated the expression of TLR-2, TLR-4, TLR-9 and TLR-11 on day 8 PI. Intestines are highly innervated by sympathetic ganglia and it is expected that local released from these nerve endings modulates immune responses. Our *in vitro* study was designed to establish the effect of locally produced on NE in IECs by using the mouse intestinal epithelial cell line MODE-K. First we determined the effect of active infection (live parasites) or TLA alone on TLR expression in MODE-K cells. In our experiment we measured the expression of TLRs in MODE-K cells infected with ME-49 *T. gondii* tachyzoites (3:1 ratio) (INF) or exposed to TLA (5 μ g/ml). After 15 h incubation, excess supernatants containing parasites or TLA were removed and cells washed with PBS. RNA was extracted from MODE-K cells using the TRI

Reagent and expression of TLR-2, TLR-4, TLR-9 and TLR-11 was measured by real time PCR. Among the TLR tested, TLR-9 and TLR-11 had significantly higher mRNA expression levels in response to live parasites or TLA when compared to untreated CON. This effect was more pronounced and significant in cells infected with parasites than exposed to TLA. No significant differences were observed between the groups in TLR-2 and TLR-4 expression (Figure 3).

TLR expression is altered in MODE-K cells exposed to NE

In vitro studies were designed to mimic stress conditions by exposing a mouse IEC line, MODE-K cells to NE and determine its effect on TLR expression. Our previous studies have shown that CWS modulated immune response and offered a protective effect in mice infected with *T. gondii* (37-39,41). In addition, our previous experiment showed significant increase in TLR-9 and TLR-11 expression in MODE-K cells in response live parasites or TLA exposure. In this section, we wanted to determine the effect of the catecholamine, NE on TLR expression in MODE-K cells. For that we used three groups MODE-K cells without any treatment (CON), MODE-K cells exposed to NE (10^{-6} M) for 4 h, washed to remove excess NE and incubated for 15 h (CON+PreNE). In another group, MODE-K cells were exposed to NE for 15 h (CON+DurNE). After the 15 h incubation, excess media was removed and cells washed with PBS. RNA was extracted from MODE-K cells using the TRI Reagent and expression of TLR-2, TLR-4, TLR-9 and TLR-11 was measured by real time PCR. Expression of TLR-2 and TLR-11 was significantly higher in the CON+DurNE group when compared to CON and CON+PreNE groups; but no significant differences were observed when CON+PreNE group was compared with the CON group. There were no significant differences among the groups in expression of TLR-4 and TLR-9 (Figure 4).

TLR expression is altered in MODE-K cells exposed to *T. gondii* tachyzoites and exposed to NE

Catecholamines modulate immune functions such as cell proliferation, cell migration and balance of pro- and anti-inflammatory cytokines (50). Norepinephrine suppresses inflammatory cytokines such as IL-23 and IL-12p40 but had no effect on anti-inflammatory cytokine IL-10 (51). From the previous section, we showed alteration in TLR expressions in MODE-K cells in response to NE exposure. Here we determined the effect of active infection and NE on TLR expression in MODE-K cells. Cells were exposed to NE (10^{-6} M) for 4 h before infection with live ME49 tachyzoites at a 3:1 ration (INF+PreNE); or NE was added to the cultures at the time of infection and incubated for 15 h (INF+DurNE). Another group consisted of MODE-K cells exposed to tachyzoites without having any treatment (INF). After 15 h incubation, supernatants and excess media were removed, and cells washed with PBS. RNA was extracted from MODE-K cells using the TRI Reagent and expression of TLR-2, TLR-4, TLR-9 and TLR-11 was measured by real time PCR. Our results showed a significant decrease in TLR-2 and TLR-9 expression in the INF+DurNE group when compared to the INF and INF+PreNE groups. No significant differences were observed between the INF and the INF+PreNE groups. TLR-11 expression was significantly decreased in the INF+PreNE when compared to the INF group but no significant differences were observed when compared to the INF+DurNE group. There were no significant differences among the groups in TLR-4 expression in response to parasite or NE exposure (Figure 5).

TLR expression in MODE-K cells was not altered by exposure to *Toxoplasma* lysates and NE

We have shown that NE can modulate TLR expression on MODE-K cells (Figure 4) and when present during infection (CON+DurNE group) (Figure 5). In this section, we wanted to determine whether expression of TLRs was altered in MODE-K cells in response to TLA

and NE at different time periods. MODE-K cells were exposed to NE (10^{-6} M) for 4 h and TLA (5 µg/ml) added (TLA+PreNE), or NE was added to the cultures at the time of exposure to TLA (TLA+DurNE). Another group consisted of MODE-K cells exposed to TLA only (TLA). After 15 h incubation, supernatants and excess media were removed, and cells washed with PBS. RNA was extracted from MODE-K cells using the TRI Reagent and expression of TLRs was determined by real time PCR. Our results showed no significant differences among the groups (TLA, TLA+PreNE and TLA+DurNE) in TLR-2, TLR-4, TLR-9 and TLR-11 expression levels (Figure 6).

Increased expression of the negative regulator SIGIRR in IECs from infected and stressed mice

Some proteins act to negatively regulate TLR activation thereby down regulating inflammatory cytokines and chemokines. They act directly on TLRs or genes involved in downstream signaling. Negative regulators such as SIGIRR and Triad3 act directly on TLRs or downstream TLR signaling pathways (16). SIGIRR (single immunoglobulin interleukin-1 receptor-related protein) acts as a negative regulator of IL-1R and TLR-4 signaling (18,19). In this study we tested the effect of stress and *T. gondii* infection on SIGIRR expression in IECs. SIGIRR expression levels were measured in mouse IECs on days 3 and 8 PI. Expression of the housekeeping gene, GAPDH was used for normalization and fold differences between the groups calculated. On day 3 PI SIGIRR expression in IECs from CON and CWS groups was significantly higher than in the INF and CWS+INF groups. No significant differences were observed in IECs between CON and CWS as well as INF and INF+CWS. On day 8 PI SIGIRR expression levels in CON and CWS groups were significantly increased in the INF and CWS+INF groups. Expression of SIGIRR in the INF group was significantly decreased when compared to the CWS+INF group (Figure7).

SIGIRR expression is altered in mouse MODE-K cells exposed to *T. gondii* or TLA

In this section, we wanted to demonstrate in vitro the effect of on the negative regulator, SIGIRR by NE, parasite antigen, or active infection in MODE-K cells. We first measured SIGIRR expression in MODE-K cells alone (CON), exposed to NE for 4 h (CON+PreNE), or in the presence of NE (CON+DurNE). A similar approach was used to expose MODE-K cells to live parasites or TLA. After 15 h incubation, supernatants and excess media were removed, and RNA extracted using the TRI Reagent and expression of TLRs was determined by real time PCR.

Expression of SIGIRR was decreased by NE and this effect was more evident when MODE-K cells were exposed to NE for 15 h (CON+DurNE) when compared to the CON group, but no significant differences were observed when compared to the CON+DurNE group (Figure 8A). Exposure of MODE-K cells to parasites or TLA significantly decreased SIGIRR expression and this effect was more pronounced with live infection. MODE-K cells exposed to parasites had significantly lower expression levels of SIGIRR, when compared to CON and TLA but no significant differences were observed between the CON and the TLA group (Figure 8B). When MODE-K cells were infected or exposed to TLA in the presence of NE, SIGIRR expression was not significantly altered (Figure 8C,D). This effect was more pronounced when MODE-K cells were exposed to TLA than to live infection (Figure 8D).

Oral infection in cold water stressed C57BL/6 mice infected with *T. gondii* cysts down regulates Toxo-529 expression

Amplification of *T. gondii* specific genes is an indirect method to demonstrate parasite load in the infected host. Here we measured the expression levels of 529-bp repeat element in IECs isolated from small intestines of CON, INF, CWS, CWS+INF groups. Amplification was not observed in any of the control samples. Amplification of the 529bp segment was

low and no significant differences were observed between the INF and the INF+CWS groups at day 3 PI (data not shown). On day 8 PI, expression of Toxo-529 in IECs was significantly lower in INF+CWS group when compared to INF group further suggesting that CWS modulated parasite load following to *T. gondii* infection (Figure 9).

In this section, we determined the expression of *T. gondii* repeated fragment 529 in MODE-K as an indicator of parasite load in MODE-K cells. We measured *T. gondii* repeated fragment 529 expression in MODE-K cells exposed to live parasites (INF), exposed to NE for 4 h (INF+PreNE), or in the presence of NE (INF+DurNE). A similar experimental approach was designed but instead of live infection, MODE-K cells were exposed to TLA. After 15 h incubation, supernatants and excess media were removed, and RNA extracted using the TRI Reagent. Amplification was not observed in samples collected from control groups. Exposure of MODE-K cells to parasites or TLA modulated Toxo-529 expression. There were no significant differences in expression levels of Toxo-529 in the INF and in INF+PreNE groups when compared to INF+DurNE (Figure 9B). When MODE-K cells were exposed to TLA, expression levels in the TLA and TLA+DurNE groups was significantly higher when compared to TLA+PreNE (Figure 9C).

DISCUSSION

In the *T. gondii*-mouse model of intestinal pathology, following ingestion of cysts C57BL/6 mice succumb to infection 7-10 days PI due to the uncontrolled inflammatory responses mediated by lamina propria CD4⁺ T cells (24,27). Intestinal epithelial cells (IECs) first encounter the parasite and this initial interaction is critical in determining the outcome of infection. Central among these responses is the involvement of TLRs. Although *T. gondii* antigens have been described as agonists of TLRs (52,53), a clear involvement for only TLR-4 and TLR-9 in innate intestinal immune responses has been established in this model (49,54,55). Several studies have shown activation of TLRs during *T. gondii* infection but only a few have focused on intestinal immune responses following per oral infection. Mun et al. (56) reported the involvement of TLR-2 on survival of *T. gondii*-infected TLR-2 deficient mice. Survival was dependent on the dose of the parasite, at low dose infection (50-100 cysts) no protection was observed, but TLR-2 was essential for protective immunity when 300 cysts were used. Innate immunity mediated by TLR-9 has been reported to be essential by Minns et al. (49). Mice deficient of TLR-9 developed significantly less gut pathology, suffered less weight loss, and live longer than wild type mice (49). The role of TLR-4 in intestinal innate responses has been a bit more controversial. Furuta et al. (54) have shown TLR-4-mediated innate immune recognition mediated protective mechanisms against oral *T. gondii* ME49 infection. Both C3H/HeJ and TLR-4 deficient mice were significantly more susceptible to *T. gondii* infection and this susceptibility was in part attributed to reduced production of IFN- γ and IL-12 at 5 days PI. Conversely, Heimesaat et al. (55) found that a switch towards increased population of Gram negative bacteria in particular *Escherichia coli* in TLR-4 deficient mice during infection resulted in decreased intestinal pathology and reduced mortality. Protection in TLR-4 deficient mice was associated with decreased production of IFN- γ and nitric oxide (NO) levels in the inflamed terminal ileum. Furthermore, they concluded LPS primarily from *E. coli* played a central role in the induction and aggravation of small intestinal inflammation during peroral *T. gondii* infection. Although the role of TLR-11 has not been described in this model, it does not seem to participate in innate immune mechanisms during peroral infection as TLR-11 deficient mice exhibited moderate susceptibility to infection. Using *in vivo* bioluminescence imaging Hitziger *et al.* (57) studied the role of TLRs after intraperitoneal or intra venous infection with a type II strain. No differences were found among the TLR-deficient mice in total parasite load, and mortality suggesting a non essential role for TLR-1, TLR-2, TLR-4, TLR-6 and TLR-9 for control of *Toxoplasma* infection.

Our results clearly demonstrated that *T. gondii* infection increased expression of TLRs in IECs. This increased expression primarily occurs at day 8 PI as no significant differences in TLR expression were observed in IECs among the groups at day 3 PI. Furthermore, this increased TLR expression was abrogated by stress as IECs recovered from stressed and infected mice (CWS+INF) showed at least a two-fold decrease in all TLRs tested. Using the mouse IEC line, MODE-K we were able to determine that active *T. gondii* infection significantly increased expression of TLR-9 and TLR-11. Both of these TLRs have been described during *T. gondii* infection. For examples, TLR-9 seems to be critical during *T. gondii* infection and TLR-9 deficient animals showed less pathology and lower cytokine responses following oral infection with *T. gondii* (49). Although no description of TLR-11 in IECs has been reported during *T. gondii* infection, a *T. gondii* profilin has been shown to activate dendritic cells through TLR-11 and mediate IL-12 production (11).

The lack of significant increase in TLR-2 and TLR-4 expression from our *in vitro* experiments using the MODE-K cell line can be explained by the fact that our reagents lacked any ligands for these receptors (TLR-2, bacterial lipoarabinomannan, lipoprotein, and peptidoglycans; TLR-4, bacterial lipopolysaccharide and lipotechoic acid). However, parasite antigens (SAG1) have been shown to mediate production of pro-inflammatory cytokines and chemokines, and development of the lethal ileitis in C57BL/6 mice after oral infection (52). Parasite glycosylphosphatidylinositol (GPI) which anchors proteins to the surface of tachyzoites have been shown to activate both TLR-2 and TLR-4 in transfected Chinese hamster ovary (CHO) cells (53). In addition, increased expression of TLR-9 and TLR-11 occurred when MODE-K cells infected with live tachyzoites but not soluble tachyzoites lysate antigen (TLA). Similar requirement for active infection have been reported by Butcher and Denkers (58) in LPS-activated macrophages. They observed that only infection with tachyzoites blocked production of IL-12p40 and TNF- α in activated macrophages.

TLR signaling can be negatively regulated by various proteins acting at different stages in the signaling pathways; among these, single immunoglobulin IL-1R-related molecule (SIGIRR) function as inhibitory receptor for TLR and IL-1R, sequestering proteins from signaling complexes and preventing primarily TLR-4 signaling (17). It has been shown that SIGIRR increased protection against bacterial infection by down-regulating TLR-4 signaling (19). Furthermore, IECs lacking SIGIRR exhibited elevated expression of pro-inflammatory genes, and SIGIRR deficient mice were more susceptible to commensal bacteria-dependent intestinal inflammation (59). SIGIRR was consistently up-regulated by CWS but its expression was decreased by *T. gondii* infection; however SIGIRR expression was not significantly different in infected MODE-K cells exposed to NE. It is unclear whether decreased mRNA expression in IECs recovered infected mice was directly associated with invasion, parasite replication or exposure to the increased number of Gram negative bacteria known to occur during infection (55).

Catecholamines act through adrenergic receptors and suppress inflammation by inhibiting production of proinflammatory cytokines such as IFN- γ , TNF- α , IL-12 and activate the release of anti-inflammatory cytokines such as IL-10 (32). Relevant to these findings is our identification of adrenergic receptors α 1a, α 1b, α 2a, β 1 and β 2 in mouse IECs but only α 1b, α 2a and β 1 expression in MODE-K cells (unpublished data). In our study MODE-K cells exposed to NE showed significant increased expression in TLR-2 and TLR-11 in the CON +PreNE group when compared to the CON group. In contrast, we observed a significant decrease in expression of TLR-2, TLR-9 and TLR-11 in MODE-K cells exposed to NE and infected with tachyzoites (INF+DurNE). NE suppresses the production of IL-23, IL-12 p40, TNF- α and IL-6 in LPS-stimulated DC with no significant difference in IL-10 expressions (51). It is clear that IECs express adrenergic receptors and their activation by stress or NE

exposure was capable to modulate TLR expression in response to *T. gondii* infection. We have previously shown several immunomodulatory effects of CWS on murine immune responses during *T. gondii* infection (37-39,41). We have also observed a protective role for CWS in C57BL/6 mice during peroral infection (41, unpublished results). These results provided some evidence that the protective role of CWS may be in part due to modulation of TLR expression in IECs by stress hormones. We have postulated that after per oral infection activation of TLRs in IECs by parasite antigens and through subsequent exposure to luminal bacterial antigens are critical in mediating the initial production of inflammatory cytokines and chemokines. Furthermore, our CWS paradigm seems to modulate TLR expression and activation. At this time we are unable to assign a particular target or signaling pathway modulated by NE or stress hormones, but this is an area that we are actively pursuing.

We have shown that CWS modulated parasitic burden and down regulated TLR expression in IECs. Significant reduction in Toxo-529 expression levels in the CWS+INF group when compared to INF suggests a protective role for CWS by preventing invasion or multiplication of parasites in IECs. CWS has also been shown to decrease intestinal pathology after infection. Mild villous blunting with mild epithelial necrosis in small intestine were observed in sections from CWS+INF mice, whereas sections from INF mice showed marked multifocal villous necrosis, crypt inflammation, and blunting with edema (unpublished data).

The interactions of stress with the immune system are complex and likely made even more complex by the immune suppressive properties of the parasite itself (60). Nevertheless, it is clear that CWS is capable of decreasing inflammatory responses and the ensuing inflammation in the C57BL/6-*T. gondii* model of per oral infection. At this time, we want to stress that the model of ileitis developed by Liesenfeld (23) utilizes 100 cysts; while our model of stress only 35 cysts are used. The outcomes of stress are dependent among other factors on its timing and its intensity. Tanaka *et al* (40) demonstrated the protective effects of mild stress compared to severe cold-restraint stress. It was found that mild stress was protective while severe stress was deleterious causing gastric ulceration mediated by activation of COX-1 and COX-2 and the release of prostaglandins. Although CWS is a combination of hypothermia, confinement and exhaustion, it is considered a mild stressor and beneficial in our C57BL/6-*T. gondii* model. In addition, we have found that CWS is detrimental to the host when infection is given by the intraperitoneal route (37-39,41). Taken together, we can conclude that CWS is beneficial in this model of per oral infection, in part by suppressing pro-inflammatory responses and decreasing parasitic load.

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Abbreviations

TLRs	Toll-like receptors
IECs	intestinal epithelial cells
PI	post-infection
NE	norepinephrine
SIGIRR	single immunoglobulin IL-1 receptor-related molecule

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Figure 1.

Indirect Fluorescent antibody technique of mouse IECs stained with a cytokeratin-specific antibody (TROMA-I). The IECs were incubated with 1:10 dilution of TROMA-I antibody for 30 min at room temperature, and detected with FITC-conjugated goat anti rat IgG (1:100 dilution) for 30 min (BD Biosciences, San Jose, CA). Slides were mounted using Fluoromount and viewed under a Leica DMIRE2 fluorescent microscope (20X, 40X) and images were captured with the Image Pro software (Leica Microsystems Inc., Mannheim, Germany).

Figure 2.

Relative expression of TLR-2, TLR-4, TLR-9 and TLR-11 in IECs recovered from mice infected orally with *T. gondii* (INF), infected mice subjected to cold water stress (CWS +INF), mice subjected to cold water stress (CWS), and control mice with no infection or stress (CON). Female C57BL/6 mice were stressed for 5 min each day for 8 days and infected orally with 35 cysts of the ME49 strain on the last day of stress. Animals were sacrificed on day-3 and day-8 PI and IECs collected from their small intestines. Total RNA was isolated from these samples and converted to cDNA, and real time PCR was carried out for GAPDH, TLR-2, TLR-4, TLR-9 and TLR-11. Relative expression was calculated for all TLRs using GAPDH as the internal control gene. Data are presented as mean $\pm$ SD and are

representative of at least two experiments performed with five mice per group. Asterisks denote a statistically significant differences (* $p \leq 0.05$ or ** $p \leq 0.001$) between CON, INF, CWS and CWS+INF groups on day 3 and day 8 PI.

Figure 3.

Relative expression of TLRs in the mouse IEC cell line, MODE-K exposed to either live parasites or TLA for 15 hours. We measured expression of TLRs in response to ME49 tachyzoite infection or TLA (5 μ g/ml) using MODE-K cells grown at 80% confluent in 60 mm cell culture plates. MODE-K cells were divided into the following groups: cells without any treatment (CON), cells infected with live parasites at 1:3 ratio (INF), and cells exposed to *Toxoplasma* lysate antigen (TLA, 5 μ g/ml) for 15 hours. Total RNA was isolated from these groups and converted to cDNA. Expression of GAPDH, TLR-2, TLR-4, TLR-9 and TLR-11 was measured and relative expression was calculated using GAPDH as the internal control. Data are presented as mean $\pm$ SD for and are representative of at least two

experiments performed with six plates per group. Asterisks denote a statistically significant differences (* $p \leq 0.05$ or ** $p \leq 0.001$) between CON, INF and TLA groups.

Figure 4.

Relative expression of TLR-2, TLR-4, TLR-9 and TLR-11 in the mouse IEC cell line, MODE-K exposed to NE at different periods of time. MODE-K cells were grown at 80% confluent in 60 mm cell culture plates and exposed to NE. MODE-K cells were divided into the following groups: cells without any treatment (CON), cells exposed to NE (10^{-6} M) for 4 hours and excess NE washed and kept for 15 hours (CON+PreNE), and cells exposed to NE for 15 hours (CON+DurNE). Total RNA was isolated from these samples and converted to cDNA. Expression of GAPDH, TLR-2, TLR-4, TLR-9 and TLR-11 was measured and relative expression was calculated for the above TLRs using GAPDH as the internal control. Data are presented as mean $\pm$ SD for and are representative of at least two experiments

performed with six plates per group. Asterisks denote a statistically significant differences (* $p \leq 0.05$ or ** $p \leq 0.001$) between CON, CON+PreNE, and CON+DurNE groups.

Figure 5.

Relative expression of TLRs in the mouse IEC cell line, MODE-K infected with tachyzoites and exposed to NE over a period of time. Cells were grown at 80% confluency in 60 mm cell culture plates and infected with tachyzoites (INF) at 1:3 ratio, cells exposed to NE for 4 hours and infected with tachyzoites (INF+PreNE), and MODE-K cells exposed to NE during infection for 15 hours (INF+DurNE). Total RNA was isolated from these samples, converted to cDNA, which was used in real time PCR to measure GAPDH, TLR-2, TLR-4, TLR-9 and TLR-11 expression. Relative expression was calculated for the above TLRs using GAPDH as the internal control. Data are presented as mean $\pm$ SD for and are representative of at least two experiments performed with six plates per group. Asterisks

denote a statistically significant differences (* $p \leq 0.05$) between INF, INF+PreNE and INF+DurNE groups.

Figure 6.

Relative expression of TLRs in MODE-K cells exposed to *Toxoplasma* lysate antigen (TLA, 5 μ g/ml) and MODE-K cells exposed to NE at different time periods. The effect of NE on TLR expression was determined in response to TLA. MODE-K cells were grown at 80% confluent in 60 mm cell culture plates and treated with TLA for 15 hours (TLA), exposed to NE for 4 hours before addition of TLA (TLA+PreNE), or MODE-K cells exposed to NE for 15 hours in the presence of TLA (TLA+DurNE). Total RNA was isolated from these samples, converted to cDNA, which was used in real time PCR to measure TLR-2, TLR-4, TLR-9 and TLR-11 expression. Relative expression was calculated for the above TLRs using GAPDH as the internal control. Data are presented as mean $\pm$ SD for and are

representative of at least two experiments performed with six plates per group. Asterisks denote a statistically significant differences (* $p \leq 0.05$) between TLA, TLA+PreNE and TLA+DurNE groups.

Figure7.

Relative expression of SIGIRR in IECs. IECs were collected from mice infected with *T. gondii* (INF), infected mice subjected to cold water stress (CWS+INF), cold water stress mice (CWS), and from control animals without infection or exposure to CWS (CON). Female C57BL/6 mice were stressed for 5 min each day for 8 days and infected orally on the last day of stress with 35 cysts of the ME49 strain. Animals were sacrificed at days 3 and 8 PI and IECs collected from all groups of animals. Total RNA was isolated from these samples and converted to cDNA. Real time PCR was carried out for GAPDH and SIGIRR and relative expression calculated. Data are presented as mean $\pm$ SD for and are representative of at least two experiments performed with five mice per group. Panels A and

B show SIGIRR mRNA expression of IECs collected from CON, INF, CWS, CWS+INF on days 3 and 8 respectively. Asterisks denote statistically significant differences (* $p \leq 0.05$ or ** $p \leq 0.001$) between CON, INF, CWS and CWS+INF groups on days 3 and 8 PI.

Figure 8.

Relative expression of SIGIRR in the mouse intestinal epithelial cell line MODE-K. In this *in vitro* experiment, MODE-K cells were grown at 80% confluency in 60 mm cell culture plates and cells were treated as follows. Panel (A) shows untreated MODE-K cells exposed to NE for 4 h (CON+PreNE), cells exposed to NE for 15 h (CON+DurNE), and MODE-K cells alone (CON). In panel (B) MODE K cells were exposed to live parasites at 1:3 ratio (INF), exposed to NE for 4 h and infected (INF+PreNE), or cells were exposed to NE during infection for 15 h (INF+DurNE). In panel (C) uninfected MODE-K cells were infected with live tachyzoites at 1:3 ratio (INF), or exposed to TLA (5 µg/ml) for 15 h (INF), or exposed to NE during infection for 15 h (INF+DurNE). In panel (D) shows SIGIRR expression in MODE-K cells treated with TLA for 15 h (TLA), exposed to NE for 4

h before addition of TLA (TLA+PreNE), and MODE-K cells exposed to NE for 15 h in addition to TLA (TLA+DurNE). Total RNA isolated from these samples, converted to cDNA and used in real time PCR to measure SIGIRR expression. Data are presented as mean $\pm$ SD for and are representative of at least two experiments performed with six plates per group. Asterisks denote a statistically significant differences (* $p \leq 0.05$ or ** $p \leq 0.001$) between groups.

Figure 9.

Relative expression of Toxo-529 in IECs as indicator of parasitic load. IECs were collected from mice infected with *T. gondii* (INF), infected mice subjected to cold water stress (CWS +INF), cold water stress mice (CWS), and from control animals without infection or exposure to CWS (CON). On the last day of CWS, female C57BL/6 mice were infected orally with 35 cysts of the ME49 strain. Animals were sacrificed at days 3 and 8 PI and IECs collected from all groups. Total RNA was isolated from these samples and converted to cDNA. Real time PCR was carried out for GAPDH and Toxo-529 and relative expression calculated. Data are presented as mean $\pm$ SD for and are representative of at least two experiments performed with five mice per group. No amplification was observed in mRNA

from IECs from CON, CWS group of animals. Panel A) Expression of Toxo-529 mRNA in IECs collected from INF and CWS+INF mice on day 8. In addition, we also determined relative expression of Toxo-529 in the mouse intestinal epithelial cell line MODE-K. In this *in vitro* experiment, MODE-K cells were treated as follows. Panel B) Untreated MODE-K cells exposed to live parasites at 1:3 ratio (INF), cells exposed to NE for 4 h and infection (INF+PreNE), or cells exposed to NE during infection for 15 h (INF+DurNE). Panel C) Untreated MODE-K cells exposed to TLA for 15 h (TLA), cells exposed to NE for 4 h before addition of TLA (TLA+PreNE), and MODE-K cells exposed to NE for 15 h in the presence of TLA (TLA+DurNE). Total RNA isolated from these samples was used in real time PCR as described above. Data are presented as mean $\pm$ SD for and are representative of at least two experiments performed with six plates per group. Asterisks denote a statistically significant differences (* $p \leq 0.05$ or ** $p \leq 0.001$) between groups.