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ROLE OF GLUCOCORTICOIDS AND *Toxoplasma gondii* INFECTION ON MURINE INTESTINAL EPITHELIAL CELLS

Stacy L. Johnson¹, Radha Gopal², Amber Enriquez¹, and Fernando P. Monroy¹

¹Department of Biological Sciences, Northern Arizona University, P.O. Box 5640, Flagstaff, Arizona 86011, USA

²Children's Hospital of Pittsburgh, University of Pittsburgh Medical Center (UPMC), 4401 Penn Avenue, Pittsburgh, PA 15224. USA

Abstract

Glucocorticoids (GCs) are stress hormones secreted in response to perceived psychological and or physiological stress. GCs have been shown to reduce tissue inflammation by down-regulating the production of inflammatory chemokines produced by epithelial cells. The protozoan parasite *Toxoplasma gondii* is known to increase cytokine, chemokine, and Toll-like receptors (TLRs) expression in parasite infected mouse intestinal epithelial cells (IECs). We sought to analyze the role of an anti-inflammatory protein, glucocorticoid-induced leucine zipper (GILZ) in MODE-K cells during infection with *T. gondii*. GILZ expression in MODE-K cells was assessed by PCR and immunoblotting after stimulation with GCs (corticosterone, CORT) or *T. gondii* infection. GILZ mRNA was constitutively expressed in MODE-K cells but not its protein product. While infection and pre-exposure to CORT decreased GILZ isoforms of 28 and 17 kD, the presence of CORT during infection increased levels of 17 kD isoform. Infected cells treated with CORT had decreased expression of chemokines (IP-10/CXCL10, MCP-1/CCL2, MIP-2/CXCL8) while their expression was increased when endogenous GILZ was removed by siRNA treatment. GILZ up-regulation during infection may serve as a mechanism to decrease epithelial cell responses and facilitate parasite replication.

Keywords

GILZ; immunity; glucocorticoids; apicomplexa; *Toxoplasma*; parasite; siRNA; immunosuppression

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Address for Correspondence: Fernando P. Monroy, Department Biological Sciences, Northern Arizona University, P.O. Box 5640, Flagstaff, AZ 86011. USA. Tel. 928-523-0042. Fax: 928 523 7500. Fernando.Monroy@nau.edu.

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1. Introduction

During acute and chronic stress (such as with severe injury and prolonged fear/anxiety), the hypothalamic-pituitary-adrenal (HPA) axis is activated elevating blood glucocorticoids (GCs) levels, which induces an immunosuppressive effect such as inhibition of the inflammatory response as well as increased antibody production [1,2]. GCs (cortisol in humans and corticosterone in mice) regulate immune cell function, such as differentiation, trafficking, cytokine production, proliferation, chemokine expression, adhesion, molecule-ligand expression, and leukocyte effector function [3]. The role of GCs on the mammalian immune system is generally known to exert an anti-inflammatory effect by down regulating the production and secretion of inflammatory cytokines such as tumor necrosis factor (TNF)- α , and interleukin (IL)-1, IL-2, IL-6, and the chemokines macrophage inhibitory protein (MIP)-1, and macrophage chemotactic protein/s (MCP)-1 and 2 from epithelial cells and macrophages, dendritic cells (DCs), T cells, and through apoptosis of T cells [4,5].

The effects of GCs are mediated through the glucocorticoid receptor, which regulates the expression of GC-responsive genes [6,7,]. One of the prominent GC responsive genes is the glucocorticoid-induced leucine zipper (GILZ) [8,9]. GILZ has been found to be expressed in immune tissues, T-cells, macrophages, dendritic cells, mast cells, airway epithelial cells, endothelial cells, and ovarian cancer/tumor cells, and is known to have anti-inflammatory functions [9–11]. GILZ was up-regulated by the synthetic GC dexamethasone (DEX), and exerted anti-inflammatory effects in airway cells presumably through inactivation of NF- κ B and AP-1 by direct binding of GILZ to NF- κ B's p65 subunit, and AP-1 respectively [12–16]. GILZ has been shown to bind cytosolic Raf-1/Ras and suppression of ERK MAPK pathway activation [15,17].

Toxoplasma gondii is an intracellular parasite found worldwide and infects most mammalian hosts [18]. Humans may acquire *T. gondii* infection orally from ingestion of oocysts in infected water, cat boxes, and from tissue cysts after consuming raw or undercooked meat or unwashed vegetables. After ingestion and release in the small intestine, parasites may quickly invade epithelial cells forming a parasitophorous vacuole [19]. Intestinal epithelial cells (IECs) are the first cells to come in contact with the invading parasite and respond to infection by secreting cytokines and chemokines that drive the ensuing inflammatory immune response [20–23]. Stress activates two major neuroendocrine systems, the hypothalamic-pituitary adrenal (HPA) axis and the sympathetic nervous system (SNS). Their activation results in the production of glucocorticoids (GC) hormones and catecholamines respectively [24]. Although both systems mediate adaptive metabolic, cardiovascular, and anti-inflammatory effects; in the context of this infection model, the role of GCs is largely unknown. We have shown that cold water stress (CWS) and nor-epinephrine (NE) in particular modulated IECs immune responses during *T. gondii* infection both in vivo and in vitro. IECs recovered from stressed mice had decreased expression of Toll-like receptors (TLRs) and chemokine secretion [22,23]. The aim of this study was to determine the effect of corticosterone (CORT) on GILZ in the mouse MODE-K cell line during in *T. gondii* infection. We determined gene expression levels of chemokines (MCP-1, MIP-2, IP-10), and provided an insight on the role-played by the endogenous GC responsive

gene, GILZ that has been shown to be an important anti-inflammatory protein in animal models of inflammatory diseases [25].

2. Materials and methods

2.1. Cell culture

The murine intestinal epithelial cell line MODE-K was established from the duodenum of C3H/HeJ mice by K. Vidal [26], and provided to us by Dr. K. Croitoru, McMaster University, Canada. Cells were grown in T-75 flasks in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum, 10 mM HEPES, 0.4 g/L L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 0.1 mM non-essential amino acids, 50 μ M 2-mercaptoethanol, and incubated at 37° C.

2.2. *Toxoplasma gondii* maintenance

Human foreskin fibroblasts and MODE-K cells were infected with tachyzoites of *T. gondii* RH strain and allowed to multiply until host cells were nearing lysis. The flasks were scraped and the intracellular parasites released by forced passage through a 21, 25, and 27 gauge hypodermic needles. Remaining cell debris was removed by filtration through a 3 μ m filter. Parasites were counted under 40X magnification and diluted to 1×10^6 *T. gondii* tachyzoites per milliliter.

2.3. RNA Isolation and conversion to cDNA by reverse transcription

Total RNA was isolated from MODE-K cells using the RNA-easy kit following the manufacturer's instructions (Quiagen, Valencia, CA). Briefly, samples were treated with DNase I at 1 μ L/ μ g of RNA (Fermentas Inc, Glen Burnie, MD) for 30 min, and to remove contaminating genomic DNA. RNA was reverse-transcribed to cDNA following standard methods using the avian myeloblastosis virus reverse transcriptase (20 U/ μ L) in 20 μ L reaction containing random hexamers (0.2 μ g/ μ L), RNase inhibitor (20 U/ μ L), 10 mM dNTPs mix for 20 μ L reaction (First Strand cDNA Synthesis Kit, Fermentas). RNA, 2–5 μ g was used to obtain cDNA. Successful cDNA conversions were confirmed by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by conventional PCR. The primer sequence for GAPDH are shown below and the conditions were: 30 cycles of 95° C for 30 sec, 55° C for 30 sec, and 72° C for 30 sec. PCR reactions were performed in a MJ minicycler. The PCR product was viewed in a 1.5% agarose gel electrophoresis staining with ethidium bromide.

2.4. Quantitative Real Time PCR

The PCR primers were obtained from published sequences or designed with the Roche diagnostics software (Roche Diagnostics Corp. Indianapolis, IN). The PCR products ranged in length from 90 to 300 base pairs, and used in concentrations of 250 nM in 20 μ L reactions using SYBR green master mix by (SuperArray Bioscience Corp, Frederick MD). Samples were ran in 96 well PCR plates using the BioRad MyiQ real time PCR machine (BioRad Labs, Hercules, CA). Each gene of interest, (MCP-1, IP-10, MIP-2, *Toxoplasma* 529, and GILZ) was run in separate plates/runs relative to their respective prepared standards (see below). The primers for GAPDH were: forward 5'-CGTCGTGGACATGACGTG-3' reverse

5'-CCTGCTTCACCACTTCTTG-3'; MCP- 1/CCL2 forward 5'-CTTCTGGGCTGCTGTTCA-3' and reverse 5'-CCAGCCTACTCATTGGGATCA-3'; MIP-2/CXCL2 forward 5'-TCCAGAGCTTGAGTGTGACG-3' and reverse 5'-TTCAGGGTCAAGGCAAACCTT-3'; IP- 10/CXCL10 forward 5'-GCCGTCATTTTCTGCCTCAT-3' reverse 5'-GCTTCCCTATGGCCCTCATT-3'; *Toxoplasma* p529 forward 5'-CACAGAAGGGACAGAAGT-3' reverse 5'-TCGCCCTTCATCTACAGTC-3'; TLR-9 forward 5'-TTCTCAAGACGGTGGATCGC-3' reverse 5'-CGAGAGGGTGCTTCTCA-3'; and GILZ forward 5'-GTGGCTCTGTCTTAGGGTGG-3' reverse 5'-CCAGATGGGCATGTGCTTG-3'. All reactions were run in triplicate and appropriate controls in which DNase/RNase free water instead of samples or standards were included in each run.

A standard curve was generated using 10-fold serial dilutions of purified DNA product (QIA Quick Gel Extraction kit, QIAGEN, Valencia, CA) of gene of interest [23]. The relative standards varied from 10^9 to 10^1 copies of DNA and this curve was considered acceptable if a difference of 3.3 + or - 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 s, annealing at 58° C, 1 min, and extension at 72° C for 30 sec. Experimental samples and standards were run in triplicate 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 Microsoft Excel program. Based on the standards the copy numbers were calculated by relative quantification.

2.5. SDS-PAGE and Western Blotting

Cells were collected directly from 6-well culture plates by addition of Laemmli buffer. Samples were boiled for 3 min and resolved in a 15% separating gel. SDS-PAGE was carried out in a Bio-Rad mini Protein unit (Bio Rad Labs, Hercules, CA) for 1.5 hr at 200 volts. After electrophoresis, samples were transferred onto 0.2 µm PVDF membranes using a Bio-Rad transfer unit at 10 volts overnight. The membranes were blocked overnight with 5% powdered milk in PBS (blocking buffer), followed by incubation in primary antibody (rabbit anti-GILZ, Santa Cruz Biotechnology) diluted 1:500 with 5% milk + PBS containing 0.1% Tween 20 (PBST) for 2 h. The secondary antibody was horse radish peroxidase-conjugated goat anti-rabbit (ZYMED, San Francisco, CA) diluted at 1:30,000 in 5% fat free milk +PBST solution. After extensive washing with PBST, membranes were incubated with chemiluminescence substrate, FEMTO ECL reagent (Pierce Inc. Glen Burnie, Maryland) for 5 min, and exposed for 30 sec to X-ray film. After exposure, the PVDF membranes were stripped of all previous antibodies by immersing in 0.2 M NaOH solution for 20 min under gentle rocking. The membranes were neutralized by washing 3 times for 10 min with PBST, and subsequently placed in 5% PBST-5% fat free milk over night. Membranes were reacted with anti-β-actin antibody (Santa Cruz Biotechnology Inc, Santa Cruz CA) at a 1:500 dilution. Secondary antibody, horse-radish peroxidase donkey anti-mouse antibody (Santa Cruz Biotechnology Inc.) was used at 1:10,000 dilution. Membranes were incubated with FEMTO ECL reagent as described above.

2.6. GILZ knockdown by small interfering RNA (siRNA)

Knockdown of GILZ by siRNA was undertaken using siRNA reagents from Santa Cruz Biotechnology. MODE-K cells were grown in 6-well plates in DMEM media without antibiotics. Groups were as follows: Control (CON); Dexamethasone (DEX, 10^{-7} M); *Toxoplasma* infection (TOXO); Corticosterone (CORT, 10^{-5} M); DEX plus positive siRNA (DEX-siRNA⁺); *Toxoplasma*-CORT; *Toxoplasma* plus negative siRNA (TOXO-siRNA⁻; *Toxoplasma*-siRNA⁺; CORT-siRNA⁺; *Toxoplasma*-siRNA⁻, *Toxoplasma*-siRNA⁻, and CORT-siRNA⁻. Positive siRNA and negative wells were first washed with siRNA transfection medium, and overlaid with 10 μ M positive and negative siRNA duplexes. At this time, *Toxoplasma* tachyzoites were added to the infected groups along with the addition of DEX and CORT. Positive and negative siRNA treated cells were allowed to incubate for 5 hr, after which 1 ml normal DMEM (with antibiotics) was added to all groups, incubated for 10 hr, and cells collected for mRNA analysis as indicated above. All samples treated with CORT were exposed for 15 hr, which is similar to the DUR treatment.

2.7. Experimental Designs

Experiments were undertaken to determine: 1) expression of GILZ mRNA by MODE-K cells, 2) induction and optimal concentration of CORT for expression of GILZ in MODE-K cells, 3) GILZ expression in MODE-K cells in response to two CORT treatments, and 4) expression of GILZ mRNA in MODE-K cells during CORT exposure and infection with *T. gondii*. Two exposure protocols were used: pre-exposure of cells to CORT for 4 hr (Pre-CORT) or continuous exposure to CORT for 15 hr (Dur-CORT). MODE-K cells were grown in six well plates using DMEM media until cells reached 80% confluency. Briefly, after 4 hr incubation, the groups designated Pre-CORT were washed with warmed sterile PBS to remove CORT and fresh DMEM media added and kept for 11 more hours while in the Dur-CORT group cells were exposed continuously for 15 hr. When using *T. gondii*, infection in the PRE-INF group cells were infected with 1×10^6 *T. gondii* tachyzoites when CORT was removed and incubated for the remaining 11 hr. Cells in the Dur-INF group were infected at the time of CORT addition and incubated for 15 hr. MODE-K RNA was collected using the RNeasy kit (Qiagen) and processed immediately or stored at -20° C.

2.8. Statistical Analysis

All data are demonstrated as means plus or minus standard errors. All experiments were repeated at least twice. Statistical differences were calculated by one-way ANOVA followed by non-parametric one tailed Mann Whitney *t*-test using GraphPad Prism 5 (GraphPad Software, Inc. La Jolla).

3. Results

3.1. MODE-K cells express GILZ

GILZ is expressed in human airway epithelial cells [16] but there have been no reports of its expression in mouse small intestinal epithelium. Using real time PCR we determined GILZ mRNA expression in mouse small intestines (data not shown), RAW 264. 7 macrophages, and the mouse IEC line MODE-K (Fig. 1A). GILZ mRNA expression was found in all

samples. There were no difference in basal GILZ mRNA expression levels between macrophages and MODE-K cells. These results showed that MODE-K cells and RAW 264.7 macrophages constitutively expressed GILZ mRNA.

3.2. Corticosterone (CORT) induces expression of GILZ in MODE-K cells

Dexamethasone was shown to induce GILZ mRNA expression in airway epithelial cells, monocytes, and macrophages [15]. We then determined the optimal concentration of CORT by performing PCR on MODE-K cells treated with different concentrations of CORT (10^{-3} through 10^{-7} M) for 4 hr and 15 hr. GILZ expression was determined after agarose gel electrophoresis and semi-quantitation using the Alpha Innotech 2000 imager system (Alpha Innotech, Indianapolis, IN) for GAPDH and the chemokines MCP-1, MIP-2, and IP-10. Although not a single concentration was optimal for expression of all analyzed genes, we found no significant differences when CORT was used at 10^{-4} to 10^{-5} M. We selected 10^{-5} M CORT for these studies (Fig. 2). To determine if GILZ mRNA was specifically induced by CORT, we incubated MODE-K cells for 15 hr in the presence of CORT (10^{-5} M) or in combination with the glucocorticoid receptor antagonist RU486 (10^{-4} M). We found that GILZ mRNA expression was induced by CORT and its expression was significantly ($P < 0.0141$, unpaired t test) inhibited (47%) by RU486 (Fig. 1B).

3.3. CORT induction of gene expression in uninfected MODE-K cells

We next determined the effects of CORT on MODE-K cells by analyzing genes expressed in activated cells [21,22]. MODE-K cells in culture were pre-exposed (4 hr, CON+Pre) or cultured in the continuous presence of CORT (15 hr, CON+Dur). Our results showed that in MODE-K cells exposed to CORT, IP-10 was significantly up regulated 5-fold and 2-fold in the CON+Pre cells and the CON+Dur cells respectively when compared to controls. MCP-1 was up regulated 13-fold when cells were pre-exposed to CORT (CON+Pre) but no significant differences were seen in the CON+Dur samples. MIP-2 expression was significantly increased in the CON+Pre, but no significant differences were seen in the CON+Dur cells compared to control samples. GILZ expression was significantly decreased nearly 3-fold in the CON+Pre cells, but significantly increased in the CON+Dur samples compared to controls (Fig. 3). With the exception of GILZ, MODE-K cells pre-exposed to CORT increased mRNA gene expression in uninfected cells over the short incubation period.

3.4. CORT induction of gene expression in *T. gondii* infected MODE-K cells

In a similar experiment as the one described above, MODE-K cells were pre-exposed to CORT for 4 hr or 15 hr after infection (INF). IP-10 expression was down regulated in both groups and more significant differences were observed in the INF+DUR group when compared to the INF group (Fig. 4). MCP-1 mRNA expression followed a similar trend to that of IP-10, and was significantly decreased in both groups. MIP-2 showed decreased mRNA expression but it was only significant in the INF+Pre group. *T. gondii* mRNA levels were not significant among the groups. However, increased mRNA was found in the INF+Dur which indicate increased parasite replication. Expression of *Toxoplasma*-p529 region was used as a positive control to show that samples exposed to parasites were indeed

infected. GILZ mRNA expression was significantly increased in both groups but higher expression levels were found in the INF+Dur group compared to the INF group. These results showed that infection increased chemokine mRNA expression while infection in the presence of CORT led to decreased mRNA gene expression; GILZ mRNA expression was increased regardless of CORT treatment.

3.5. GILZ protein expression in MODE-K cells exposed to CORT and *T. gondii*

After GILZ mRNA analysis, we determined whether GILZ protein was expressed in mouse MODE-K cells and if its expression was affected by CORT and/or *T. gondii* infection. SDS-PAGE and western blot were carried out on samples from MODE-K cells treated with 10^{-5} M CORT and infected with *T. gondii* as described above. Dexamethasone (DEX, 10^{-7} M) was used as a positive control. We detected the presence of 3 distinct bands at ~ 17, 22, and 28 kD. Figure 4 shows that GILZ protein expression was not detected in CON samples while faint expression was observed in the INF group at 28 and 17 kD. Uninfected MODE-K cells pre-(Pre 4 hr) or continuously exposed (Dur 15 hr) to CORT showed three distinct bands at 17, 22, and 28 kD. In the groups exposed to both parasites and CORT there were some observable differences. In the INF+Pre group two very distinct bands at 17 and 28 kD were observed; while three bands of more intensity were present in the INF+Dur group. Our positive control group (10^{-7} M DEX) showed three intense products (~ 17, 22, and 28 kD). To ensure that loading was consistent among the different groups, the gel was stripped of anti-GILZ antibody and samples exposed to anti- β -actin. Equal intensity among the different lanes was observed (Fig. 5). Our results showed that CORT treatment in uninfected and infected MODE-K cells induced GILZ; while infection induced very low levels of GILZ protein.

3.6. GILZ mRNA expression after siRNA knockdown in CORT- and *T. gondii*-exposed MODE-K cells

The role of GILZ in MODE-K cells during CORT exposure and infection was confirmed by siRNA gene knockdown (Fig. 6A). The control (CON) group showed very low GILZ mRNA expression in relation to our house-keeping gene, GAPDH. Both CORT and DEX induced significant GILZ mRNA expression, and their expression was decreased in the presence of siRNA⁺ treatment, leading to a 6-fold and 3.6-fold decreased expression suggestive of a successful gene knockdown.

We next investigated if decreased GILZ expression would affect chemokine gene expression. Both chemokines analyzed have been shown to increase in MODE-K cells infected with *T. gondii* (Fig. 4; [23]). Figure 6B and 6C show that infection (TOXO) induced significantly higher mRNA expression of IP-10 and MCP-1 while significantly reducing their expression in the presence of CORT. A different pattern was observed in infected cells treated with GILZ siRNA⁺ where significantly higher expression levels for both chemokines were observed when compared to TOXO alone. We also included in our approach control groups consisting of Con+siRNA⁺ and Con+siRNA⁻ for GILZ, IP-10 and MCP-1 analysis. Values for these groups were never higher than CON group alone (data not shown). These results suggest that GILZ is mediating some of the immunosuppressive effects of CORT.

4. Discussion

IECs are integral players in the ensuing inflammatory response observed in C57BL/6 mice following oral *T. gondii* infection [21–23]. Our model of oral infection in C57BL/6 mice combined with cold water stress (CWS+INF) we have shown the involvement of the sympathetic nervous system and its neurotransmitter, NE in down-regulating inflammatory chemokines/cytokines and TLRs from IECs [21,22]. In contrast, the involvement of the hypothalamic-pituitary-adrenal (HPA) axis and its main mediator, GCs has not been investigated.

MODE-K cells were exposed to CORT to mimic the effects of CWS in mice. We focused our efforts on the role played by the GC-responsive gene, GILZ, which is produced by both innate and acquired immune cells [7]. It has been proposed that GILZ up-regulation negates the unwanted expression of pro-inflammatory molecules in many kinds of cells by interfering with the activity of biologically relevant molecules such as AP-1, NF- κ B, NFAT, p38 MAPK, and Raf-MEK/ERK [2,5,12–17,27].

Our results showed that MODE-K cells constitutively expressed GILZ mRNA and its expression was specifically induced by CORT and DEX. This effect was more evident when cells were incubated with CORT for 15 hr. Furthermore, MODE-K cells pre-exposed to CORT increased chemokine mRNA expression but longer incubations abrogated this effect. We have shown that infection of MODE-K cells with *T. gondii* increases chemokine gene expression [22] but in the presence of CORT their expression was significantly decreased. It is likely that GILZ up-regulation is necessary to regulate increased inflammatory responses. GILZ is known to mediate the anti-inflammatory effects of GCs in epithelial cells [15,28] and endothelial cells [11]. Our results support the fact that MODE-K cells exposed to CORT and infection with *T. gondii* had decreased chemokine mRNA gene expression while increased GILZ protein expression. Our protein data in Figure 5 showed that CORT alone or in the presence of the parasite induced translation of GILZ while our mRNA data showed that GILZ was only induced by the continuous presence of CORT (CON-Dur, Fig 3). Infection with *T. gondii* is known to interfere with several cellular mechanisms to increase cell survival and parasite replication [29–31]. Although it is unclear why infection resulted in enhanced GILZ mRNA transcription but limited protein product, this observation is worth pursuing.

Here, we present the first report of constitutive GILZ expression in a murine in murine IECs. Treatment of MODE-K cells with CORT resulted in the expression of three GILZ variants; while infection mostly induced the 28 kD isoform. Four GILZ isoforms (GILZ 1–4) have been previously described and unique functions have been associated with particular isoforms [32]. GILZ2 has been reported as the predominant transcript variant in human alveolar macrophages [33]. The human GILZ2 protein (~ 17 kDa) corresponds to the dexamethasone-inducible isoform exhibiting the NF- κ B-inhibitory action described for this anti-inflammatory mediator [15,34], whereas GILZ1 was shown to inhibit NF- κ B transcription and epithelial sodium transport, GILZ4 was a potent inhibitor of cell proliferation [32]. Whether or not infection interferes with post-transcriptional events of some GILZ isoforms is unclear at the moment. We wanted to determine the role of GILZ

during *T. gondii* infection. We addressed this question, by knocking out GILZ from MODE-K cells and expected that removal of endogenous GILZ would exacerbate MODE-K cell responses to infection. We found indeed that absence of GILZ enhanced IP-10 and MCP-1 mRNA induction upon *T. gondii* infection. Our findings are in line with previous reports using siRNA GILZ knockdown that have resulted in increased cytokine production upon IL-1 β -mediated IL-8 production in lung A549 cells [28], and enhanced NF- κ B activity and TNF- α mRNA induction upon LPS-treatment in *in vitro* differentiated macrophages [33]. These findings support the notion that decreased levels of GILZ within the cell promote an inflammatory response. It is unclear if *T. gondii* interferes directly with GILZ expression to promote its growth and survival by interfering with transcription factors involved in the expression of inflammatory cytokines. The recent creation of a GILZ^{-/-} mouse would be helpful in addressing the role of endogenous GILZ during *T. gondii* infection [11].

Oral infection of C57BL/6 mice with *T. gondii* cysts results in intestinal pathology and mortality driven by IFN- γ and TNF- α produced by CD4⁺ lamina propria T cells [35]. Recruitment of CD4⁺ T cells is in part due to activated IECs [21,22]. Our previous findings led us to propose that stress increased mouse survival by targeting recruitment of CD4⁺ T cells by diminishing chemokine secretion from IECs. We investigated the role of nor-epinephrine in mediating this effect but the role of glucocorticoids remains unknown. Of great interest would be to determine if inflammatory cytokines or chemokines regulate GILZ expression in MODE-K cells. Pro-inflammatory cytokines in airway epithelial cells have been shown to decrease both GILZ mRNA and protein expression in bronchial epithelial (BEAS-2B) cells [15], but these effects were observed in alveolar epithelial cells (A549) stimulated with IL-1 β [28]. Eddleston et al. [15] also showed that inflammatory cytokines (IL-1 β , TNF- α , or IFN- γ) decreased GILZ mRNA and protein expression. This finding is interesting because TNF- α and IFN- γ are crucial cytokines involved in determining intestinal pathology or immune control of *T. gondii* infection. It is known that oral *T. gondii* infection induces intestinal pathology that is dependent on the host genotype. We can hypothesize that levels of GILZ would be increased in both macrophages and intestinal epithelial cells from resistant BALB/c mice while GILZ levels would be decreased in susceptible C57BL/6 mice similar to findings observed in patients with inflammatory bowel disease [10]. Future work will investigate the role of GILZ in purified IECs from stressed and infected mice and their modulation by inflammatory cytokines. This is necessary because of the potential for new drug therapies designed to specifically up-regulate GILZ in human intestinal cells and targeting the repression of pro-inflammatory transcription factors in patients with irritable bowel disease. Indeed, in a mouse model of colitis, GILZ over expression decreased Th1-derived cytokines, inhibited colonic epithelial cell apoptosis, and prevented mucosal damage induced by DNBS administration [36].

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Highlights

- GILZ detection in murine small intestine epithelial cells
- GILZ expression is induced by glucocorticoids and decreased by *T. gondii* infection
- Silencing GILZ increases inflammatory responses during infection
- Manipulation of GILZ could be useful in treating chronic inflammatory diseases

Fig. 1. Detection of GILZ mRNA expression in the IEC line MODE-K and RAW 264.7 macrophages by real-time PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene (Panel A). MODE-K cells were treated with CORT (10^{-5} M) alone or in combination with RU486 (10^{-4} M) for 4 hr in triplicate, and GILZ mRNA levels were assessed by real time PCR (Panel B). Relative expression was calculated as the quantity of GILZ mRNA over the quantity of GAPDH mRNA detected in each sample. Asterisks indicate statistically significant differences (* $P < 0.05$, Mann-Whitney t test).

Fig. 2.

Optimization of CORT concentrations in MODE-K cells ranging from 10^{-3} to 10^{-7} M and analyzed by PCR for induction of MCP-1, MIP-2, and IP-10 mRNA expression. PCR products were separated in 1.5% agarose gels and product density analyzed using the Alpha Innotech 2000 imager system. Only data for the 15 hr incubation is shown but a similar trend was observed in the 4 hr incubation.

Fig. 3.

MODE-K cells grown in 6-well plates were exposed to CORT (10^{-5} M) for 4 hr (CON +PRE) after which CORT was removed and incubated for 11 more hr. A second group was exposed to CORT for 15 hr (CON+DUR). Total RNA was isolated from these groups and used to determine relative expression of MIP-2, MCP-1, IP-10, TLR-9, and GILZ at the end of the 15 hr incubation. Data are representative of two different experiments and samples were run in triplicate in real time PCR using GAPDH as the internal control. Asterisks indicate statistically significant differences (* $P < 0.05$, Mann-Whitney t test).

Fig. 4.

Exposure of MODE-K cells to *T. gondii* in the presence of CORT. Cells were grown in 6-well plates and exposed to CORT (10^{-5} M) for 4 hr after which CORT was removed, and 1×10^6 parasites were added to the cultures (INF+PRE) and incubated for 11 hr. A second group was exposed to CORT and parasites at the same time and incubated for 15 hr (CORT +DUR). Total RNA was isolated from these groups and used to determine relative expression of MIP-2, MCP-1, IP-10, TLR-9, and GILZ at the end of the 15 hr incubation. Data are representative a two different experiments and samples were run in triplicate in real

time PCR using GAPDH as the internal control. Asterisks indicate statistically significant differences ($*P < 0.05$, Mann-Whitney t test).

Fig. 5.

Detection of GILZ protein in MODE-K cells by Western blotting. Cells were unexposed (CON), exposed to CORT (10^{-5} M) for 4 hr (CON+PRE), or for 15 hr (CORT+DUR). Similarly, cells were infected with 1×10^6 parasites (INF) or exposed to CORT (10^{-5} M) for 4 hr. After CORT removal, cells were infected and incubated for 11 more hr (INF+DUR). A second group was exposed to CORT and parasites at the same time and incubated for 15 hr (CORT+DUR). A positive control group consisted of cells exposed to 10^{-7} M dexamethasone (DEX) for 15 hr. Cell lysates were immunoblotted and reacted with rabbit anti-GILZ sera. Blots were stripped and rehybridized with an antibody to β -actin. Results are representative of 3 separate experiments.

Fig. 6.

Small interfering RNA (siRNA) knockdown of GILZ in MODE-K cells. In Panel A, uninfected groups were exposed to GILZ siRNA⁺ after treatment with CORT. Infected groups were exposed to parasites after incubation for 4 hr in CORT. At this time, groups were transfected with positive (siRNA⁺) or negative siRNA (siRNA⁻) peptides and incubated for 5 hr. Panels B and C show the gene expression for inflammatory cytokines. Groups included are controls (CON), cells exposed to 10^{-5} M CORT (CORT), CORT + siRNA⁻, cells exposed to 10^{-7} M dexamethasone (DEX), and DEX+siRNA⁺. The infected

groups are, cells infected with 1×10^{-6} parasites (TOXO), TOXO+siRNA⁻, TOXO+siRNA⁺, TOXO+CORT, TOXO+siRNA⁻, TOXO+siRNA⁺. RNA was isolated and used to determine GILZ, IP-10, and MCP-1 relative expression using GAPDH as the internal control. Asterisks indicate statistically significant differences (* $P < 0.05$, Mann-Whitney t test). Data shown are representative of 2 separate experiments, each performed in triplicate.