Entamoeba histolytica Cyclooxygenase-Like Protein Regulates Cysteine Protease Expression and Virulence

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The intestinal protozoan parasite Entamoeba histolytica (Eh) causes amebiasis associated with severe diarrhea and/or liver abscess. Eh pathogenesis is multifactorial requiring both parasite virulent molecules and host-induced innate immune responses. Eh-induced host pro-inflammatory responses plays a critical role in disease pathogenesis by causing damage to tissues allowing parasites access to systemic sites. Eh cyclooxygenase (EhCox) derived prostaglandin E$_2$ stimulates the chemokine IL-8 from mucosal epithelial cells that recruits neutrophils to the site of infection to exacerbate disease. At present, it is not known how EhCox is regulated or whether it affects the expression of other proteins in Eh. In this study, we found that gene silencing of EhCox (EhCoxgs) markedly increased endogenous cysteine protease (CP) protein expression and virulence without altering CP gene transcripts. Live virulent Eh pretreated with arachidonic acid substrate to enhance PGE$_2$ production or aspirin to inhibit EhCox enzyme activity or addition of exogenous PGE$_2$ to Eh had no effect on EhCP activity. Increased CP enzyme activity in EhCoxgs was stable and significantly enhanced erythrophagocytosis, cytopathic effects on colonic epithelial cells and elicited pro-inflammatory cytokines in mice colonic loops. Acute infection with EhCoxgs in colonic loops increased inflammation associated with high levels of myeloperoxidase activity. This study has identified EhCox protein as one of the important endogenous regulators of cysteine protease activity. Alterations of CP activity in response to Cox gene silencing may be a negative feedback mechanism in Eh to limit proteolytic activity during colonization that can inadvertently trigger inflammation in the gut.

Keywords: Entamoeba histolytica, parasites, cox like protein, cysteine protease, actinin like protein, virulence, pro-inflammatory cytokines

INTRODUCTION

Entamoeba histolytica (Eh) is an invasive extracellular protozoan parasite responsible for amebic colitis and liver abscess (World Health Organization, 1998). It is one of the major cause of severe diarrhea in areas of poor sanitation and nutrition particularly in tropical and developing countries. While the majority of Eh infection remains asymptomatic, about 10% of infection converts to an invasive phenotype where Eh invades the mucosal epithelium resulting in 100,000 death/year (World Health Organization, 1997; Stanley, 2003).
The host innate immune status and parasite virulence factors play major roles in disease pathogenesis (Faust and Guillen, 2012; Verkerke et al., 2012; Marie and Petri, 2014; Nozaki and Bhattacharya, 2015). The host immune response during *Eh* invasion of the colonic mucosa is characterized by increased levels of pro-inflammatory cytokines that recruits inflammatory cells including macrophages and neutrophils (Seydel et al., 1997; Mortimer and Chadee, 2010; Nakada-Tsukui and Nozaki, 2016) to the site of infection. The major *Eh* virulent factors identified to date are the galactose/N-acetyl-D-galactosamine (Gal/GalNAc) lectin (Gal-lectin), cysteine proteases, amoeboapone, and prostaglandin E2 (Moonah et al., 2013; Marie and Petri, 2014).

Prostaglandins are lipid mediators synthesized from arachidonic acid through cyclooxygenase and are associated with various diarrheal disease including bacterial and inflammatory bowel diseases (Ahrenstedt et al., 1994; Alcantara et al., 2001; Resta-Lenert and Barrett, 2002). We have shown that *Eh* synthesizes PGE2 through a cyclooxygenase like enzyme as confirmed by gas chromatography/mass spectrometry analysis (Belley and Chadee, 2000). Surprisingly, *Eh*PGE2 was not immunosuppressive to reduce host defenses but rather, PGE2 bound EP4 receptors on colonic epithelial cells and stimulated the potent neutrophil chemoattractant, IL-8 mRNA expression and protein production. In addition, EhPGE2 also altered tight junction proteins and increased ion permeability that led to diarrhea in intestinal amebiasis (Lejeune et al., 2011). Even though *Eh* produces high levels of PGE2 in the presence of arachidonic acid the parasite can also stimulate host cells such as macrophages and colonic epithelial cell to produce PGE2 as part of the pro-inflammatory response elicited by the parasite (Stenson et al., 2001; Sanchez-Ramirez et al., 2004). *EhCox* encodes a functional cyclooxygenase enzyme as evidenced by Cox enzymatic assays. An unusual aspect is that EhCox is primitive and has little homology to other Cox proteins from mammalian cells and some eukaryotes. At present, the biological function of EhCox other than being an enzyme that catalyzes the production of PGE2 in *Eh* is not known.

In this study, we made the seminal observation that silencing EhCox enhanced cysteine protease protein expression and enzyme activity independent of EhCox-induced PGE2 production. These results suggest that increased cysteine protease activity in EhCoxgs is linked to increase parasite-induced inflammation and pathogenicity. These findings increase our understanding on the molecular basis of pathogenicity in *Eh* and how dissimilar enzymes can regulate their activity in the parasite.

### MATERIALS AND METHODS

#### Reagent

E64, leupeptin, aprotinin, and Nonidet P-40 detergent were obtained from Sigma-Aldrich. Z-VVR-AMC substrate was purchased from Enzo Life Sciences. The Z-Arg-Arg-pNA.2 HCl substrate was purchased from Bachem. Mouse monoclonal anti-acid clone C4 antibody was purchased from MP Biomedical, LLC. Antibodies to EhCP4 and the CP inhibitors WRR483 and WRR605 were a kind gift from Dr. Sharon Reed, University of California, San Diego. EhCP5 and EhCox1 like antibodies were generated in rabbits using recombinant proteins expressed in *E. coli* (Belley and Chadee, 2000). Ubiquitin antibody (P4D1) was from Cell Signaling Technology and cycloheximide from Sigma-Aldrich.

### Cultivation and Harvesting of *E. histolytica*

G3 *Eh* were grown axenically in TYI-S-33 medium at 37°C. After 72 h, logarithmic-growth-phase Eh cultures were harvested by chilling on ice for 9 min, pelleted at 200 g, and washed two times with PBS. For the detection of proteins and enzymatic activity, *Eh* lysis was prepared by using three cycles of freeze-thawlysis and proteins quantified by the bicinchoninic acid protein assay, using bovine serum albumin as protein standard (#23225, Thermo Scientific). *Eh* secretory protein (SP) were prepared as described previously (Lidell et al., 2006). Briefly, secreted components were collected from mid-log phase *Eh* incubated in Hanks’ balanced salt solution (Invitrogen) for 2 h at 37°C at a final concentration of 2 × 10⁵ *Eh* per ml. Following incubation, *Eh* was removed by centrifugation at 10,000 × g for 10 min. Secretory proteins were quantified by the bicinchoninic acid protein assay. To quantify the growth of control and *EhCoxgs, 2.5 × 10⁵ log phase *Eh* were inoculated in 14 ml TYI-S-33 medium and the number of parasites counted every 24 h using a hemocytometer.

### Cloning of the EhCox-Like Gene

*EhCox*-like gene (Acc No. AF208390) 500 bp long 5’e end of protein-coding region was amplified by PCR from cDNA using specific primer containing stu1 and sac1 restriction sites (Table 1). The PCR product was sub cloned using the pGEM-T Easy vector system (Promega) and then digested with the restriction enzymes, stu1 and sac1. The digested DNA insert was cloned into Stu1- and Sac1-digested pSAP2-gumma (kind gift from Dr. Tomoyoshi Nozaki, Tokyo, Japan). This construct was verified by sequencing. G3 *Eh* were harvested during mid-log growth and transfected with a silencing plasmid (pSAP2-gumma) using the Lipofectamine (Life technologies) and OPTIMEMI medium (Life Technologies) supplemented with 5 mg/ml L-cysteine and 1 mg/ml ascorbic acid (transfection medium) and pH 6.8 as previously described (Fisher et al., 2006) Transfected *Eh* were selected with G418 (Sigma) over a 3-week period, starting with 6 µg/ml and ending with 24 µg/ml. Silencing was assessed in these strains using quantitative reverse transcription-PCR (qRT-PCR) and western blot analysis. Once gene silencing was confirmed, the G418 selection was removed, and then silencing was again confirmed and quantified.

### Preparation of *E. histolytica* Nuclear Proteins (EhNP)

*Eh* were washed twice in PBS and suspended in lysis buffer (100 mM Tris (pH 7.4), 1 µg/ml E-64 (Sigma), 2 µg/ml leupeptin, 7.4 µg/ml aprotinin and 0.5% Nonidet P-40 detergent) on ice for 15 min. Nuclei were pelleted by centrifugation at 2,000 × g for 15 min, washed with lysis buffer, and suspended in 0.1 M sodium phosphate buffer (pH 7.0). The protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as protein standard (Thermo Scientific).
Quantitative Real-Time (qRT) PCR-Based Analysis of Gene Expression

Total RNA was extracted from logarithmic-growth-phase *Eh* using a Trizol reagent method (Invitrogen; Life Technologies, Burlington, ON) and the yield and purity determined by the ratio of absorbance at 260/280 nm (NanoDrop, Thermo Scientific). DNase I-treated total RNA was used in the RT reaction using qScript cDNA Synthesis kit and PerfeCTa SYBR Green Supermix (Quantabio). Real-time qPCR was performed using a Rotor Gene 3000 real-time PCR system (Corbett Research). The PCR reaction mix (20 µl) comprised 1x SYBR Green, 25 ng cDNA and 1 µM of primers. A complete list of the primer sequences and conditions used are listed in *Table 1*. Results were analyzed using the 2−ΔΔCT methods and expressed as fold changes.

Sample Preparation for Proteomics

*Eh* lysates were prepared as described before. After proteins precipitation with TCA, the supernatant was discarded and proteins were air dried and suspended in 200 µL of urea/HCl. The sample was then treated with 10 mM of dithiothreitol and incubated at 37°C for 30 min and transferred into Microcon YM-30 (Millipore) and centrifuged at 14,000 × g for 15 min. The eluates were discarded, 200 µL of UA was pipetted into the filtration unit, and the units were centrifuged again. Then 100 µL of 0.05 M iodoacetamide in Urea/Tris was added to the filters, and samples were incubated in darkness for 20 min.

Filters were washed twice with 100 µL of Urea/Tris and, with 100 µL of 0.05 M ammonium bicarbonate at 14,000 × g for 15 min. Proteins were digested in 40 µL of trypsin in 0.05 M ammonium bicarbonate at 37°C overnight. The released peptides were collected by centrifugation at 14,000 × g for 10 min followed by two washes with 0.05 M, 40 µL ammonium bicarbonate and with 0.05 M NaCl. After isolation of the peptides, samples were transferred into new Eppendorf tube.

LC-MS/MS Analysis

Total protein and peptides content were analyzed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific) operated with Xcalibur (version 4.0.21.10) and coupled to a Thermo Scientific Easy-nLC (nanoflow Liquid Chromatography) 1,200 system. Isolated trypsin treated peptides (2 µL) were loaded onto an Easy Spray Column (ES803) at a maximum of 700 bars (2 µm particle column). Further, peptides were eluted using a 120 min gradient from 5 to 40% (5 to 28% in 105 min followed by an increase to 40% B in 15 min) of 0.1% formic acid in 80% LC-MS grade acetonitrile at a flow rate of 0.3 µL/min and separated on a C18 analytical column (ES803). Then, peptides were electrospayed using 2.0 kV voltages into the ion transfer tube (300°C) of the Orbitrap Lumos operating in positive mode. A full MS scan was performed by Orbitrap at a resolution of 12,000 FWHM to detect the precursor ion having a *m/z* between 375 and 1575 and a +2 to +7 charge. The Orbitrap AGC (Auto Gain control) and the maximum injection time set were at 4e5

### Table 1 | Primers used in this study.

| Name         | Sequence 5′-3′                  | Annealing temp (°C) |
|--------------|--------------------------------|---------------------|
| *E. histolytica* |                              |                     |
| EhCox        | Fwd:5′ stu1: AGGCCTATGACTGAAATAAAGAAT | 55                  |
|              | Fwd: 3′ sac1: GAGCTGAAAATCTGCTGTTA |                     |
| EhCox RT     | Fwd: TGACTGAAAATACCATGCTGTA | 59                  |
|              | Rev: CCATAAGACTACATCAGTACTGTA |                     |
| CPS RT       | Fwd: AATTCTAGGGAAGCTATTTTG | 59                  |
|              | Rev: CATCAAGAATCCCAACTGGA |                     |
| CP4 RT       | Fwd: GTTAACCATGTTGTCATTGCTGTA | 59                  |
|              | Rev: GCTACATGGAACACAGTTGGAAGA |                     |
| CP1 RT       | Fwd: ATAAACACTTCCGACGATTTGTA | 59                  |
|              | Rev: TTCTTCATTTCGACTAAGCAGC |                     |
| CP2 RT       | Fwd: ATCCAAAGCCACAGATCTGTA | 59                  |
|              | Rev: TCTCCATCAGGACGATTTGTA |                     |
| rDNA RT      | Fwd: TCAAAAAGCAGCTGTTGACGTA | 59                  |
|              | Rev: AGCCGTTAAGTGTATTCTT |                     |
| Murine       |                              |                     |
| IL-1β        | Fwd: GCTGCTGCTGCTGCTGAGCCA | 58                  |
|              | Rev: GTGAGGCTGCTGCTGCTGACG |                     |
| TNF-α        | Fwd: ATGACACAGAAGACATGAC | 60                  |
|              | Rev: TACAGGCCTTGTCCTCTGGAATT |                     |
| IFN-γ        | Fwd: TCAAGTGCATAGTGCAGAAGAA | 58                  |
|              | Rev: TGGCCTGCGGAGATTTGATG |                     |
| Actin        | Fwd: CTCATCGAGGCGTCTGCTG | 58                  |
|              | Rev: TGGGTGTGAGCAGTTC |                     |
| KC           | Fwd: ACCCAACCCGAGATCTAAGC | 60                  |
|              | Rev: TCTCCGTACTTCTGGAGCAC |                     |

Frontiers in Cellular and Infection Microbiology | www.frontiersin.org 3 January 2019 | Volume 8 | Article 447
and 50 ms, respectively. The Orbitrap was working with a 3 s
cycle time for precursor selection and most intense precursor
ions presenting a peptic isotopic profile, having an intensity
threshold of at least 5,000 were isolated using the quadrupole and
fragmented with HCD (30% collision energy) in the ion routing
multipole. The fragment ions (MS²) were analyzed in the ion trap
at a rapid scan rate. The AGC and the maximum injection time
were set at 1e4 and 35 ms, respectively, for the ion trap. Dynamic
exclusion was enabled for 45 s to avoid the acquisition of same
precursor ion having a similar m/z (plus or minus 10 ppm).

**Database Search**

With the help Raw Converter (v1.1.0.18; The Scripps Research
Institute), raw data files (*.raw) were converted into Mascot
Generic Format (MGF). Monoisotopic precursors having a
charge state of +2 to +7 were selected for conversion. The
mgf file was used to search a database by using Mascot
algorithm (Matrix Sciences; version 2.4). Search parameters for
MS data included trypsin as enzyme, a maximum number of
missed cleavage of 1, a peptide charge equal to 2 or
higher, cysteine carbamidomethylation as fixed modification,
methionine oxidation as variable modification and a mass error
tolerance of 10 ppm. A mass error tolerance of 0.6 Da was
selected for the fragment ions. Only peptides identified with a
score having a confidence higher than 95% were kept for further
analysis. The Mascot dat files were imported into Scaffold (v4.3.4,
Proteome Software Inc.) for comparison of different samples
based on their mass spectral counting.

**Western Blots**

Proteins (30 µg) were loaded on 12% SDS-PAGE gel followed
by transfer onto polyvinylidene fluoride (PVDF) membrane
and blocking in 5% skim milk. Blots were then probed with
1:5,000 *Eh*Cox antibody or 1:1,000 *Eh*CP5 antibody/1:500 *Eh*CP4
antibody/1:1,000 Actin antibody for 16 h at 4°C. After incubation
with one of the previously described primary antibodies, the blots
were incubated with appropriate HRP-conjugated secondary
antibody for 1 h at RT, and then developed using ChemiLucent
ECL detection (EMD Millipore).

**Eh*Cox Activity Assay**

*Eh*Cox activity assay was performed on nuclear fractions of *Eh*
(*Eh*N). *Eh*N (100 µg) was incubated for 30 min with 1 mM
aspirin (ASA) followed by 100 µM arachidonic acid (AA) or
vehicle for 1 h at 37°C in sodium phosphate buffer containing
200 µM tryptophan and 2 µM hematin (Sigma) in 500 µl volume.
After centrifugation, PGE₂ in the supernatant was extracted with
Amprep C2 ethyl columns (Amersham Biosciences) following
manufacturer's protocol. PGE₂ was quantified by using an
enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

**Eh Cysteine Proteinase Activity**

*Eh*CP5 and *Eh*CP4 activity in lysate and secretory protein
was determined using known chromophoric substrate
benzoyloxycarbonyl-L-arginyl-L-arginine p-nitroanilide
(Z-Arg-Arg-pNA) and fluorogenic substrate benzoyloxycarbonyl-
L-val-L-val-7-amino-4-methylcoumarin (Z-VVR-AMC),
respectively, as previously described (Leippe et al., 1995; He et al.,
2010). Briefly, substrate was incubated for 0–20 min at 37°C with
either *Eh* lysate/secretory proteins (50 µg) alone or pretreated
with CP inhibitor-E64 [L-trans-epoxysuccinyl-leucyl-amido-
(4-guanidino) butane] and CP-1(WRR483) and CP-4 (WRR605)
inhibitors. Cleavage of the chromophoric (Z-Arg-Arg-pNA) and
fluorogenic (Z-VVR-AMC) substrate were detected at the 405
and 460 nm wavelength, respectively.

**Gelatinase Gel Substrate Gel Electrophoresis**

For analysis of protease activity by gelatinase substrate gel
electrophoresis, 12% SDS polyacrylamide was copolymerized
with 0.1% gelatin as described previously (Hellberg et al., 2000).
Briefly, *Eh* lysate were prepared for three freeze-thaw cycles in
HBSS and centrifuged for 10 min at 10,000 × g. Supernatant was
separated on 0.1% gelatin copolymerized 12% SDS PAGE. After
separation of proteins, SDS was removed by two washings in
2.5% Triton X-100 for 1 h at room temperature. Gel was then
incubated in developing buffer (20 mM DTT, 100 mM sodium
acetate, pH 4.2, and 1% Triton X-100) at 37°C for 3 h. The gel
was stained with Coomassie blue. Clear band represent cysteine
protease activity.

**Eh Erythropagocytosis Assay**

Fresh human erythrocytes were obtained in DPBS and stained
with Phicoerythin by using PKH26 Red Fluorescent cell linker
kit (PKH26, Sigma-Aldrich). The erythrocytes were counted
and used in 1:100 (Eh: erythrocyte) ratio. *Eh* and erythrocytes
were incubated for 20 min at 37°C in DPBS. After interaction,
cells were washed twice with DPBS and centrifuged at 3,000
× g for 5 min at 4°C. Lysis buffer (RBC lysis buffer, Sigma-
Aldrich) was added for 1 min at RT followed by 0.5 ml addition
of PBS and washed again with DPBS. The cells were fixed with
4% p-formaldehyde for 20 min at RT and washed with DPBS.
To each sample a drop of fluorescein reagent was used for the
slide preparation. The slides were observed under confocal
microscope and fluorescent intensity was quantified by selecting
region of interest (ROI) containing phagocytes erythrocyte and
measuring the fluorescent intensity by using Image J software.
The data was plotted as mean fluorescent intensity.

**Eh Cytopathic Assay**

Caco-2 human colonic adenocarcinoma cells (ATCC, Manassas,
VA) were grown to confluent monolayers in DMEM
medium (Invitrogen-Gibco) supplemented with 5% fetal bovine
serum and 5 mg/ml penicillin-streptomycin under 5% CO₂ at
37°C (Sigma-Aldrich). *Eh* disruption of a Caco-2 cell monolayer
was determined using a previously described protocol (Belley
et al., 1996). Briefly, *Eh* (10⁵/well) were incubated with Caco-2
cell monolayers in 24-wells tissue culture plates at 37°C. The
incubation was stopped by placing the plates on ice and *Eh
were removed by washing with cold PBS. The Caco-2 cells that
remained attached to the plates were stained with methylene blue
(0.1% in 0.1 M borate buffer, pH 8.7). The dye was extracted from
stained cells with 0.1 M HCl and color intensity of the extracted
dye was measured spectrophotometrically at OD 660.
Stability of EhCP-A4 and EhCP-A5 Protein
Eh and EhCoxgs were inoculated at 2.5 × 10⁵ and grown in TYI-S-33 media for 48 h before treatment with the protein synthesis inhibitor, cycloheximide at 100 µg/ml for 6, 12, and 24 h. After treatment Eh were harvested, washed in PBS and cell lysates were prepared in RIPA buffer 100 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1% sodium deoxycholate pH 7.4 containing a protease inhibitor cocktail and 2 nM PMSF (Cruz-Vera et al., 2003). EhCP protein remaining after treatment with cycloheximide was calculated as percentage protein remaining of levels at time zero (0 h). Protein half-life (t₁/₂) was calculated by linear regression analysis.

Mice Colonic Loop Studies
Math1GFP mice were purchased from Jackson laboratory and bred in-house. Colonic loops studies were done by inoculating live Eh suspended in 100 µL PBS (1 × 10⁶) into closed colonic loops as described previously (Belley and Chadee, 1999). This is a short-term infection model (3 h after infection). After 3 h, the colons were excised and tissue pro-inflammatory gene expression and myeloperoxidase activity was analyzed.

Colonic Myeloperoxidase Activity Assay (MPO)
MPO activity was assayed in mouse colon samples as described previously (Kumar et al., 2017). Briefly, fresh frozen tissues were homogenized in 0.5% hexadecytri-methylammonium bromide. Homogenized tissue was freeze-thawed three times, sonicated, and centrifuged at 10,000 g for 10 min at 4°C. Clear supernatant was collected and the reaction was initiated by addition of 1 mg/ml dianisidine dihydrochloride (Sigma, St. Louis, MO) and 1% H₂O₂, and change in optical density was measured at 450 nm.

Math1 Expression via Non-invasive Whole-Body Imaging ex vivo
After 3 h of Eh infection, colons of Math1GFP mice were surgically removed, imaged ex vivo using an in vivo Xtreme 4MP-imaging platform (Bruker, Billerica, MA, USA) to detect GFP expression. The imaging was performed in two steps. The first one is reflectance imaging (2 s exposure time) and the second one was with excitation at 470 nm and emission at 535 nm (5 s exposure time) i.e., fluorescent imaging. Images were acquired and analyzed from the in vivo Xtreme using Bruker molecular imaging software MI SE (version 7.1.3.20550). GFP expression in the colon was quantified by measuring the mean fluorescence (after background subtraction) in a constant ROI.

Ethics Statements
All studies were carried out with the approval of the University of Calgary Animal Care Committee. Animal care committee have approved experimental procedure proposed and certifies that animal care was in accordance with recent policies by the Canadian council on Animal care.

Statistical Analysis
Data was analyzed using Graphpad Prism 7 (GraphPad Software, San Diego, CA) for all statistical analysis. Student’s t-test was used when two groups were compared. Statistical significance was assumed at P < 0.05.

RESULTS
Silencing of the Eh Cyclooxygenase Like Gene Responsible for PGE₂ Biosynthesis
Eh-derived PGE₂ not only induces pro-inflammatory IL-8 production but also disrupts colonic epithelial cell tight junction by coupling through EP4 receptors (Dey et al., 2003; Dey and Chadee, 2008; Lejeune et al., 2011). To analyze the biological functions of endogenous EhCox and EhPGE₂ mediated pro-inflammatory responses, we silenced the expression of the gene by small RNA-mediated transcriptional gene silencing in the G3 strain (Bracha et al., 2006). The specific gene repression was confirmed by reverse transcription PCR of corresponding cDNA and immunoblot analysis of proteins by using EhCox specific antibody. Complete silencing of EhCox was achieved in comparison to the Eh control (Figures 1A,B, Supplementary Figure 1). Immunoblot analysis detected the 72 and 66 Kda protein band in the lysate of control Eh as previously described (Dey et al., 2003). As predicted, EhCox enzymatic assay using nuclear fractions isolated from log-phase Eh showed almost no aspirin (ASA) inhibited PGE₂ release (Dey et al., 2003) by EhCoxgs in comparison to control Eh incubated with arachidonic acid (AA) substrate (Figure 1C). We used ASA inhibited PGE₂ release to accurately quantify PGE₂ levels as EhCoxgs showed modest non-specific binding by enzyme immunoassay (EIA) that was not inhibited with ASA.

Growth Kinetics of EhCoxgs
To determine whether EhCoxgs had an effect on cell proliferation, the growth kinetics of EhCoxgs and control Eh were compared. We analyzed the growth of Eh over time and showed that during the first 24 h growth kinetics were similar, however at 48 and 72 h EhCoxgs proliferation was significantly slower than control Eh (Figure 1D). These results suggest that EhCoxgs might be essential for cell growth and proliferation.

EhCox Gene Silencing Caused Limited Proteome Change
To determine if EhCoxgs also affected the expression of other proteins, we took a proteomic approach and analyzed the proteome from EhCoxgs and control Eh. Only a limited number of proteins showed three-fold or higher expression (Supplementary Table 1). In EhCoxgs, 23 proteins were up regulated and 19 proteins were down regulated compared to control Eh (Table 2). EhCox protein (Q9U328_ENTHII) was not detected in EhCoxgs proteome that confirms complete absence of the EhCox protein and gene specific silencing. Among the proteins that were up regulated included those encoding for several uncharacterized proteins (M7X297_ENTHII, A0A175JH8X_ENTHII, A0A175JQ8_ENTHII, A0a175JU71_ENTHII, A0A175JQL9_ENTHII, A0A175LQ0_ENTHII, A0A175JJP8_ENTHII) and Rab family GTPase proteins (A0A175JL18_ENTHII, A0A175JT01_ENTHII, A0A175JSR0_ENTHII), UDP-glucose-glycoprotein glucosyltransferase (C4M0W6_ENTHII), WD domain containing protein...
Gene Silencing of EhCox Increases Cysteine Protease Expression and Activity

Cysteine proteases play major roles in the pathogenesis of Eh. Specifically, EhCP-A5 has been shown to be involved in tissue invasion by disrupting the protective mucus layer and stimulating pro-inflammatory response in colonic cells (Moncada et al., 2003; Hou et al., 2010). To determine the functional significance of EhCox derived PGE2 in Eh virulence, we analyzed the expression of EhCPs protein and mRNA in EhCoxgs. Cell lysates from EhCoxgs and control Eh were assayed for CP expression by western blotting using EhCP-A5 and EhCP-A4 specific antibodies. These proteases are highly expressed and released extracellular basally and during infection (He et al., 2010; Kissoon-Singh et al., 2011). An unexpected finding was that EhCoxgs showed a slight increased in EhCP-A5 and significantly up regulated EhCP-A4 protein expression compared to control Eh (Figure 2A, Supplementary Figure 2). Surprisingly, we did not detect a corresponding increase in CPs transcripts by qPCR (Figure 2B) using primer specific for CP1, CP2, CP4, and CP5 (Table 1). These results suggest that EhCox was regulating CPs at the translational level independent of transcription. To assess whether the increase in EhCPs protein expression resulted in a corresponding increase in EhCPs enzymatic activity, gelatin substrate gel electrophoresis was performed with Eh lysates that showed prominent bands of CPs activity in EhCoxgs as compared to control Eh lysate which was not present in E64 (CP inhibitor) treated Eh lysate (Figure 2C). To verify increased enzymatic activity, EhCoxgs and control Eh lysates were incubated with known substrates and proteinase activity was quantified by the liberation of chromogenic leaving group p-nitroanilide.
TABLE 2 | List of genes that are up or down regulated ≥3-fold upon EhCox gene silencing.

| Protein name                                                                 | Accession no.  | Regulation |
|------------------------------------------------------------------------------|----------------|------------|
| Uncharacterized protein                                                      | M7X297_ENTHII  | Up         |
| Uncharacterized protein                                                      | A0A175JH08_ENTHII | Up         |
| UDP-glucose-glycoprotein glucosyltransferase                                  | C4M0W6_ENTHII  | Up         |
| Uncharacterized protein                                                      | A0A175JQ08_ENTHII | Up         |
| tRNA cytosine 5 methyltransferase putative                                   | A0A175JG04_ENTHII | Up         |
| Leucine-rich repeat containing protein                                       | A0A175J648_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JU71_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JQ09_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JU00_ENTHII | Up         |
| Alpha-soluble rnf attachment protein putative                                | A0A175JU90_ENTHII | Up         |
| Serine-rich protein                                                          | A0A060N047_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JF08_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JF02_ENTHII | Up         |
| Rab family GTPase                                                           | A0A175JL08_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JH08_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JG04_ENTHII | Up         |
| LIM zinc finger domain containing protein                                     | A0A060N66K9_ENTHII | Up         |
| WD domain containing protein                                                 | A0A175JG04_ENTHII | Up         |
| Galactose-specific adhesin light subunit                                     | A0A175JF02_ENTHII | Up         |
| Rab family GTPase                                                           | A0A175JL01_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JG04_ENTHII | Up         |
| tRNA (guanine-N(7)-)-methyltransferase non-catalytic subunit                | A0A175J648_ENTHII | Up         |
| Rab family GTPase                                                           | A0A175JF08_ENTHII | Up         |
| Cysteine protease                                                           | A0A175JG04_ENTHII | Up         |
| Uncharacterized protein                                                      | A0A175JU73_ENTHII | Down       |
| Uncharacterized protein                                                      | A0A175JU90_ENTHII | Down       |
| Uncharacterized protein                                                      | A0A175JU90_ENTHII | Down       |
| DNAJ family protein                                                          | A0A175JG06_ENTHII | Down       |
| Ras guanine nucleotide exchange factor putative                              | A0A175JY44_ENTHII | Down       |
| Uncharacterized protein                                                      | A0A175JL03_ENTHII | Down       |
| Uncharacterized protein                                                      | A0A175JL28_ENTHII | Down       |
| Ph domain containing protein                                                 | A0A175JQ04_ENTHII | Down       |
| V-type proton ATPase subunit                                                 | A0A175JF04_ENTHII | Down       |
| 70kDa heat shock protein                                                     | A0A060N091_ENTHII | Down       |
| Spry domain protein                                                          | A0A175JL04_ENTHII | Down       |
| Mov34 mnp pd1 1 family protein                                               | A0A175JY43_ENTHII | Down       |
| Uncharacterized protein                                                      | A0A175JL08_ENTHII | Down       |
| Rho guanine nucleotide exchange factor putative                              | A0A175JX09_ENTHII | Down       |
| Casein kinase putative                                                       | A0A175JL04_ENTHII | Down       |
| Uncharacterized protein                                                      | A0A175JN08_ENTHII | Down       |
| Type a flavoprotein putative                                                 | A0A175JL06_ENTHII | Down       |

and the fluorescent leaving group 7-amino-4-methylcoumarin (AMC) from EhCP5 and EhCP4 peptide substrates, Z-Arg-Arg-pNA and Z-VVR-AMC, respectively. Degradation of EhCPs substrate occurred in linear mode over time (Figures 2D,E) with significantly higher enzymatic activity in EhCoxgs as compared to control Eh lysates. Specificity for CPs enzymatic activity was confirmed using specific EhCP-A5 and EhCP-A4 inhibitors, WRR483 and WRR605, respectively (Figures 2D,E). EhCP-A5 and EhCP-A1 are unique as they both cleave the common substrate Z-Arg-Arg-pNA and here we used the EhCP-A1 specific inhibitor WRR483 to show that EhCP-A5 enzyme activity was specifically increased (St-Pierre et al., 2017). E64 inhibits all CPs in Eh. As EhCPs are secreted extracellular we also found significantly increased EhCP5/4 enzyme activity in EhCoxgs
compared to control *Eh* (Figures 2F,G). These results clearly show that *EhCoxgs* expressed higher levels and activity of CPs, which was regulated by *EhCox* protein.

**Effect of Arachidonic Acid (AA), Aspirin and Prostaglandin E₂ on Cysteine Protease Activity**

From the studies above it was unclear if inhibition of *Eh* PGE₂ biosynthesis and/or Cox enzyme activity was regulating CP expression and enzyme activity in *EhCoxgs*. In live *Eh*, PGE₂ is produced in a time-dependent manner in the presence of AA substrate (Dey et al., 2003). AA is the most important rate-limiting step in *EhCox* driven biosynthesis of PGE₂ and aspirin is the only inhibitor known to inhibit *EhCox* enzymatic activity (Dey et al., 2003). To evaluate if PGE₂ played a role in regulating EhCP5 activity, control *Eh* was incubated with exogenous AA or aspirin and they had no effect on enzyme activity (Figures 3A,B). Similarly, the addition of exogenously prostaglandin (PGE₂) to *EhCoxgs* showed no difference in EhCP5 activity (Figure 3C). These results strongly suggest that the increase in *EhCoxgs* activity was not dependent on Cox enzymatic activity but rather appear to be a direct effect of the Cox protein on EhCP expression and activity.

**EhCoxgs Stabilizes EhCP-A5 Protein Degradation**

To address the mechanism whereby *EhCoxgs* increased protein expression and enzymatic activity, we hypothesize that the CPs in *EhCoxgs* were not degraded based on increased protein expression (Figure 2A). As several proteins were up/down regulated in *EhCoxgs* (Table 2), we were surprised that more proteins were ubiquitinated and destine for 26S proteasome degradation in *EhCoxgs* as compared to *Eh* (Figure 4A). Based on these findings we theorize that proteins critical in regulating the stability of EhCPs maybe degraded and quantified the half-lives of both EhCP-A4/5. To do this, *Eh* and *EhCoxgs* were treated with cycloheximide and the percentage protein remaining over 24 h determined. Surprisingly, EhCP-A4 protein was not degraded whereas the half-life for EhCP-A5 was 19.3 h in *EhCoxgs* as compared to 12.2 h for control *Eh* (Figures 4B,C). The increase in EhCP-A5 protein stability in *EhCoxgs* may account for increase protein accumulation and increase enzyme activity. In contrast, EhCP-A4 protein was very stable and turnover rate low. We did not treat *Eh* longer than 24 h with cyclohexamid (95% viable by trypan blue exclusion) as cells became rounded and detached from the glass tubes and we were concern about cell death.

**Increased Cysteine Protease Expression Results in Increased Erythrophagocytosis and Cytopathic Activity**

Experimental evidence suggests a direct correlation between *Eh* virulence and the rate of phagocytosis and protease activity (Ankri et al., 1998, 1999; Okada et al., 2005; Hirata et al., 2007). Erythrophagocytosis is considered as one of the prominent marker of *Eh* virulence (Trissel et al., 1978; Orozco et al., 1983; Bhattacharya et al., 2002). Accordingly, we assayed the ability of *EhCoxgs* to phagocyte fluorescently labeled RBCs and measured the fluorescence intensity by confocal microscopy. *EhCoxgs* showed significantly higher RBCs uptake in comparison to control *Eh* (Figure 5A). *EhCoxgs* also significantly destroyed 27% of a Caco-2 monolayer after 2 h incubation compared to control *Eh* that destroyed 11% (Figure 5B). These results show that *EhCoxgs* is highly phagocytic with increased cytopathic activity.

**Differential Math1 Transcriptional Activity in Math1<sup>GFP</sup> Mice Exposed to EhCoxgs**

Based on the results above, we then determined if there was a similar increase in *EhCoxgs* virulence using closed colonic loops in mice (Kissoon-Singh et al., 2013). We have previously shown that *Eh* induces hyper secretion of mucus by goblet cells and elicits an acute pro-inflammatory response in colonic loops (Darmanli et al., 2009). In particular, EhCP5 RGD motif has been shown to bind αvβ3 integrin on goblet cells to elicit mucus hyper secretion (Corknici et al., 2016). We hypothesized that increase CPs activity would result in a differential response toward mucin biosynthesis and secretion to *EhCoxgs* as compared to control *Eh*. To interrogate this, we used Math1<sup>GFP</sup> mice containing the green fluorescent protein (GFP) reporter for Math1-expressing goblet cells. In the colon, Math1 is expressed in epithelial cells to differentiate into muc2-producing goblet cells lineage. Basally, Math1<sup>GFP</sup> activity was higher in the proximal colon in control mice. However, following *Eh* infection, Math1 activity was significantly decreased in the proximal colon, which was decreased further when mice were infected with *EhCoxgs* (Figure 6). We have previously shown that decrease in Math1<sup>GFP</sup> activity correlates with increase pro-inflammatory activity in response to DSS-induced colitis (Tawiah et al., 2018).

**Pro-inflammatory Responses Are Exacerbated in Math1 Mice Exposed to EhCoxgs Compared Control Eh**

It is well-known that *Eh* infection in the gut elicits an acute pro-inflammatory response with the production of pro-inflammatory cytokines IL-1β, IL-8, IFN-γ, and TNF-α (Bansal et al., 2009; Galván-Moroyoqui et al., 2011). Based on the results above showing a marked decrease in Math1<sup>GFP</sup> activity in the proximal
Gene silencing of EhCox increases cysteine protease expression and activity. (A) Immunoblot blot analysis was performed on lysate prepared from control Eh and EhCoxgs. The proteins were separated on 12% SDS PAGE gels and analyzed with CP5, CP4 or actin antibody. Quantifications of CP5/4 were performed by densitometric analysis from three independent experiments shown in right panel. (B) qPCR was used to monitor CPs expression using cDNA from Eh and EhCoxgs. Data indicate changes in mRNA expression compared with controls. (C) Gelatin substrate gel electrophoresis of lysate from control Eh and EhCoxgs treated/untreated.
FIGURE 2 | with E64. (D,E) CPs enzymatic activity was evaluated by incubating inhibitor-treated/non-treated Eh with EhCP-A5 or EhCP-A4 substrates for 10 and 20 min, respectively and calculated in µM/min/mg, shown in the bottom panel. (D) EhCP-A5 enzymatic activity in lysate with known substrates (Z-RR) and 20 µM of inhibitor WRR483. (E) EhCP-A4 enzymatic activity in lysate with known substrates (Z-VVR) and 20 µM of inhibitor WRR605 and 100 µM E64. (F) EhCP-A5 and (G) EhCP-A4 enzymatic activity in secreted protein. The bars indicate the means and the error bars indicate the standard errors of the means for three different experiments. The asterisks indicate the results of comparisons with the controls. *P < 0.05, **P < 0.01, ***P < 0.002, ****P < 0.001.

FIGURE 3 | Effect of arachidonic acid, aspirin and prostaglandin on EhCP-A5 cysteine protease activity. Live control Eh were pretreated with drugs for 16 h at 37°C. (A) Arachidonic acid (100 µM), (B) aspirin (1 mM) and (C) PGE₂ (1 µM) for 16 h at 37°C. Lysates were prepared from drug treated/non-treated Eh using three cycles of freeze-thaw lysis. The bars indicate the means and the error bars indicate the standard errors of the means for three different experiments.

FIGURE 4 | EhCoxgs stabilizes EhCP-A5 protein degradation. (A) Log phase Eh and EhCoxgs proteins (30 µg) were loaded on a 12% SDS-PAGE gel and immunoblotted with an ubiquitin antibody (P4D1). Actin was used as a loading control. (B,C) The half-life of EhCP-A4 and EhCP-A5 protein was determined by treating Eh with cyclohexamide (see details in Materials and Methods) and the remaining CPs quantified by Western blot over 24 h. Protein half-life ($t_{1/2}$) was calculated using regression analysis.
colon, we hypothesize that enhanced pro-inflammatory cytokines were suppressing Math1 activity. Indeed, pro-inflammatory cytokines expression in colonic tissues after 3 h exposure with EhCoxgs showed increased expression of IL-1β and KC (human IL-8 homolog) but not TNF-α and IFN-γ as compared to Eh-inoculated loops (Figure 7). Both IL-1β and KC are released by epithelial and immune cells recruited to the site of infection that can exacerbate tissue injury (Mortimer and Chadee, 2010). Specifically, KC is a potent neutrophil chemo attractant that recruits neutrophils to the site of infection but is ineffective in clearing the parasite. Pro-inflammatory cytokine expression correlated with increased myeloperoxidase (MPO) activity in response to EhCoxgs as compared to control Eh (Figure 7). These results reveal that EhCoxgs with increased CP enzyme expression and activity enhanced virulence in the mouse colon to elicit increased pro-inflammatory responses.

**DISCUSSION**

In this study, we describe how Eh cysteine protease expression and activity is regulated by another enzyme, cyclooxygenase (Cox). Cox is the critical rate limiting step in the biosynthesis of PGE2 (Smith and Marnett, 1991; Vane et al., 1998). Cox was identified in Eh that showed little homology to known Cox1/2 enzyme across different species with absence of conserved arachidonic acid binding domain and catalytic site that are present in other species. However, endogenous and recombinant EhCox showed Cox like enzymatic activity by using arachidonic acid as substrate that was inhibited only by aspirin but not by another Cox-1/2 inhibitors (Dey et al., 2003). In a later study, Cox like protein (Acc No. AF208390) was characterized as an actin like protein in Eh as it has actin- and calcium-binding domains. The actin binding domain of Eh actin like protein share only 30% identity with other actin binding proteins however, the protein showed 28% sequence identity to D. discoideum actinin protein. Based on some of the unusual domain architecture feature of Eh actinin like proteins it was proposed that this unusual protein might differ in function from known actinin proteins (Heike et al., 2005). Beside actin bundling, multiple cellular functions of the actinin protein have been proposed with putative interacting partners (Otey and Carpen, 2004; Cabello et al., 2007). For example, mammalian actinin regulates several receptor activity by linking the cytoskeleton to a variety of trans membrane proteins (Cabello et al., 2007). From these studies and our findings, we proposed that besides Cox like activity and actin polymerization function, this protein might have multifunctional roles in the regulation of several other proteins in Eh. Based on its molecular structure and proposed multiple functions we developed EhCoxgs to determine its biological function in the pathogenesis of amebiasis.

Cysteine proteases (CPs) are ubiquitous and are differentially regulated in virulent and non-virulent strains of Eh. Previous studies have showed that CPs are key virulent factors in the pathogenesis of Eh that are released during tissue invasion and
FIGURE 6 | Differential Math1 transcriptional activity in Math1 GFP mice exposed to EhCoxgs due to increased cysteine proteases activity. (A) Math1 expression heatmap in Math1 GFP mice colons, dotted line indicates the colonic loop ligation. (B) GFP signal quantification in the proximal colon. GFP, green fluorescent protein; AU, arbitrary units. *P < 0.05.

FIGURE 7 | Pro-inflammatory responses are exacerbated in Math1 mice exposed to EhCoxgs compared control Eh. Math1 GFP mice were infected with control Eh or EhCoxgs for 3 h. Gene expression of IL-1β, KC, TNF-α, and IFN-γ was examined from excised tissues from the proximal colon. Myeloperoxidase activity in the proximal colons of mice inoculated with control Eh or EhCoxgs. The bars indicate the means and the error bars indicate the standard errors of the means for different experiments. *P < 0.05.
one of the most important protein involved in phagocytosis (Okada et al., 2005; Hirata et al., 2007). In the present study, we have shown that silencing the Cox gene increased cysteine protease transfection and activity endogenously and extracellularly withpout affecting CPs transcript. Increased cysteine protease activity led us to hypothesize that Cox derived PGE2 was negatively regulating cysteine protease activity. However, the addition of exogenous arachidonic acid to live Eh to increase PGE2 production or aspirin to inhibit PGE2 or exogenous PGE2 did not affect CP activity. These observations suggest that increase in CPs expression and activity was not the effect of Cox enzymatic activity/end product of biosynthesis but rather was regulated by the Cox protein itself. The EhCox protein acted as a negative regulator of CPs. Since expression of CPs was not regulated at the transcriptional level, there is the possibility of post-translational modification of CPs up regulating its expression and enzymatic activity. In support of this we have shown increased EhCP-A5 protein stability in EhCoxgs as compared to control Eh. While the mechanism of this post-translational regulation is not known, our results suggest that proteins critically involved in regulating EhCP-A5 turnover were degraded in EhCoxgs by the appearance of more ubiquitinated proteins. In contrast, EhCP-A4 protein was stable and turnover rate low over 24 h that suggest different regulatory mechanisms between the CPs. No doubt uncovering the post-translational regulation of CPs enzyme will provide the basis to understand the mechanism of Cox mediated regulation and promote the development of more efficient therapeutic strategies for indirectly targeting CPs enzyme.

Proteomic analysis of the EhCoxgs revealed high expression of Rab family GTAPase and WD domain containing proteins as compared to control Eh. These proteins are involved in various cellular process including membrane trafficking, cytoskeletal assembly and cell proliferation (Saito-Nakano et al., 2005; Nakada-Tsukui and Nozaki, 2015). Based on this observation, we proposed that these proteins might be involved in up regulating CPs when EhCox is silenced. However, it needs to be further determined what functional advantages or constraints drive CPs up regulation. We also found iron-sulfur falvoprotein (A0A175JR31, M7W6A3; Supplementary Table 1) among the down regulated proteins in EhCoxgs which supports reduced growth of EhCoxgs as these proteins have been shown to be essential for the growth and survival of Eh under different condition (Nozaki et al., 1998; Shahi et al., 2016). However, the question that remains to be answered is the mechanism of how EhCox regulates Eh growth.

Phagocytosis is an active process in Eh and prominent marker of parasite pathogenicity. Phagocytosis involves several steps and activation of signaling pathways (Orozco et al., 1983; Hirata et al., 2007). A proteomic study of phagosome showed the involvement of several proteins in this process including cysteine protease and vesicular transport proteins (Okada et al., 2005). Since more cysteine protease (activity assay and expression) and several vesicular trafficking proteins (proteomics analysis) were observed in EhCoxgs, we analyzed the phagocytosis capacity of EhCoxgs as compared to control Eh. Unexpectedly, we found increased erythrophagocytosis and cytopathic activity in EhCoxgs as compared to control Eh. While it is difficult to explain how increased in Eh CPs can enhance phagocytosis, these data are consistent with previous studies that showed CPs are directly involved in destruction of colonic epithelial cells, tissue invasion, phagocytosis and degrade host antibodies and complement in immune evasion and disease pathogenesis (Hirata et al., 2007; Mortimer and Chadee, 2010; Nakada-Tsukui and Nozaki, 2016).

Several in vivo and ex vivo assays reported the recruitment of inflammatory cell in Eh-induced infection/lesion as a consequence of localized expression of chemokines and cytokines at the site of infection, leading to an inflammatory response (Bansal et al., 2009; Kissoon-Singh et al., 2013; Nakada-Tsukui and Nozaki, 2016). To address whether EhCoxgs were more virulent, we used closed colonic loop as short infection model to quantify acute inflammatory responses. Our study revealed that the Cox protein played a major role in Eh-induced fluid secretions and pro-inflammatory cytokine responses by regulating CPs activity. In particular, EhCoxgs elicited high levels of IL-1β and KC expression demonstrating cysteine protease dependent induction of IL-1β and IL-8 expression as observed in Eh infected SCID mouse-human intestinal xenograft (Seydel et al., 1997). Previous studies have shown CPs to be important in enhancing mucosal inflammation by cleaving the released pro-IL-1β into its active form and inducing IL-8 expression in mast cell (Zhang et al., 2000; Lee et al., 2014). Both TNF-α and IFN-γ were up regulated in response to Eh and was not enhanced further by EhCoxgs. In EhCoxgs inoculated colonic loops we also found elevated MPO activity, a marker for increased flux of neutrophils that are responsible for exacerbating tissue injury.

Increased CP expression and virulence in EhCoxgs is intriguing which led us to propose the following hypotheses. First, EhCox is an endogenous inhibitor of cysteine proteases. While examples of identified cysteine proteinase inhibitors produced by parasites are rare they can be targeted to treat disease related to increased protease activity. For example, papain inhibitors were detected in parasitic protozoa including Leishmania, Trichomonas, and Trypanosoma, suggesting existence of these inhibitors are widespread (Irvin et al., 1992). In Schistosoma mansoni, a gene was identified which encode papain inhibitors (Cao et al., 1993). In Eh, few CP inhibitors have been characterized that negatively regulates CP secretion and thus, virulence of the parasite (Sato et al., 2006). Second, CP regulation by Cox is a negative feedback mechanism to reduce host inflammation. This negative feedback regulation may counteract excessive cysteine protease function at site of inflammation and thus, decrease the likelihood of tissue damage that led to amebic lesion/colitis. This phenomenon may furthered explain why most Eh infections are asymptomatic. Consistent with this hypothesis, a study in mice showed that ribosomal protein S19 interact with macrophage migration inhibitory factor and function as an extracellular inhibitory factor by attenuating its pro-inflammatory function (Filip et al., 2009). Another study in Arabidopsis thaliana proposed the formation of a complex network of cysteine protease-Serpin1 interaction controlling innate immunity during plant development (Rustgi et al., 2017). Based on these findings it would be interesting to determine whether these proteins can...
interact together; it is likely these interactions could regulate CPs activity either as a result of conformational changes of the enzyme or by impacting subcellular localization of CPs and thus affecting its interactions with Cox, to further modulate its activity. Furthermore, the effect of this interaction on activity and expression of cysteine protease or vice versa can be analyzed. This information can be used to develop chemotherapeutic target against *Eh* infection. Clearly further studies are needed to understand the underlying molecular mechanisms by which *Eh*Coxs increases CPs expression and activity.

**AUTHOR CONTRIBUTIONS**

PS and KC conceived and design the experiments and wrote the manuscript. PS and FM performed the experiments. PS analyzed the data.

**FUNDING**

This work was supported by an operating grant from the Canadian Institutes of Health Research (KC; MOP-142776).

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**ACKNOWLEDGMENTS**

We thank the staff of the SAMS Center at the University of Calgary for help with proteomic analysis of the data. We also thank Dr. Björn Petri from the Snyder Mouse Phenomics Resources Laboratory for acquiring the non-invasive whole-body imaging *ex vivo* and the Snyder Live Cell Imaging facility for technical support. PS was supported by an Eyes High Postdoctoral Scholar award from the University of Calgary.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00447/full#supplementary-material

**Supplementary Table 1** | Entire proteomics profile of control *Eh* and *Eh*Coxs with mass spectrometry counts, accession number and protein ID.

**Supplementary Figure 1** | Full scan of blots for Cox protein shown in Figure 1B.

**Supplementary Figure 2** | Full scan blots of CPS/4/actin shown in Figure 2A.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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