Title: Activation of human potassium channels by the parasite *Entamoeba histolytica* causes cell death and inflammasome activation

Authors: Chelsea Marie 1#, Hans P. Verkerke 1, Dan Theodorescu 2-4, William A. Petri, Jr. 1

1 Division of Infectious Diseases and International Health. University of Virginia School of Medicine, Charlottesville, Virginia USA

2 Department of Surgery, University of Colorado, Denver, CO, USA

3 Department of Pharmacology, University of Colorado, Denver, CO, USA

4 University of Colorado Comprehensive Cancer Center, Denver, CO, USA

**ABSTRACT**

Pathogens manipulate and exploit host factors to survive and cause disease. *Entamoeba histolytica* invades the intestinal epithelium and kills host cells, resulting in tissue destruction, inflammation, and diarrhea. We used the cytotoxic activity of *E. histolytica* to select a genome-wide RNAi library to identify novel human genes required for amebic cytotoxicity. shRNA silencing constructs that caused increased resistance to *E. histolytica* cytotoxicity in human cells were mapped to 281 candidate susceptibility genes, and assigned evidence scores based on incorporated bioinformatics analyses, in vivo expression data and sequencing data. Ion transport was a significantly overrepresented function of candidate susceptibility genes, with potassium (K+) being the major ion substrate. Five K+ transporters were hits in the RNAi screen (*KCNA3, KCNB2, KCNIP4, KCNJ3,* and *SLC24A3)*. Blocking K+ efflux from human cells with pharmacologic inhibitors and with excess extracellular K+ protected multiple human cell types from *E. histolytica* induced death*.* Additionally, *E. histolytica* parasites triggered K+ channel activation and K+ efflux by intestinal epithelial cells, which preceded cell lysis. Inhibitor studies indicated that Ca2+-dependent K+ channels in intestinal epithelial cells and in macrophages are the main mediators of cell death. In addition to preventing cell death, blocking K+ efflux also inhibited caspase-1 and inflammasome activation in human macrophages, suggesting that K+ efflux mediates pyroptosis during amebic killing. There was no evidence of inflammasome activation by *E. histolytica* in intestinal epithelial cells, however K+ efflux may be the common trigger of cell-type specific death induced by *E. histolytica*. This work demonstrates that host K+ efflux is required for amebic cytotoxicity in multiple cells types, and for parasite-mediated inflammasome activation in macrophages.

**INTRODUCTION**

*E. histolytica* is a major cause of severe diarrhea globally 1–3. Amebiasis has a global distribution of more than 50 million cases worldwide, with an estimated 40,000-110,000 deaths and there are limited effective therapeutic options. For invasive amebiasis the nitroimidazoles are the only approved drug class, for which toxicity and the emergence of resistance are clinical concerns. Forty five percent of infants in Dhaka were infected with *E. histolytica* by one year of age and 10.9% suffered from *E. histolytica* diarrhea 4. *E. histolytica* was the leading cause of unadjusted mortality from 12 to 24 months of age in a 7-site study of moderate to severe diarrhea in low income countries 5, and is associated with growth shortfall and impaired cognitive development 6–8.Amebiasis causes significant global morbidity, and unacceptably remain a major cause of mortality in children in the developing world.

The parasite’s name is derived from its potent cytotoxic activity toward host cells—*histolytica-* is a composite of Greek roots meaning tissue - loosening. Detailed analysis of *E. histolytica* killing of host cells has uncovered distinctive features of amebic cytotoxicity. *E. histolytica* parasitesbind to target cells and internalize pieces of host cell membrane, leading to Ca2+ elevations and death of target cells, termed trogocytosis (nibbling)9. Parasites invade intestinal crypts and induce a highly inflammatory immune response leading to macrophage and neutrophil infiltrates10.

Clinical studies indicate that inflammatory mediators including leptin11, tumor necrosis factor-α (TNF-α)12, interferon-γ (IFN-γ)13, and nutritional status8 greatly influence amebic pathogenesis in humans. In vitroresearch has shown that amebic invasion depends on the cytotoxic activity of the parasite and that cytoxicity of *E. histolytica* is contact-dependent. Parasites bind to target cells and cause rapid alterations include dephosphorylation of host proteins, a spike in intracellular Ca2+, activation of caspase-3, DNA fragmentation and phosphatidylserine exposure on the cell surface 14–18. Parasites also trigger host inflammatory signaling cascades at the molecular level by activation of extracellular regulated kinases 1 and 2 (ERK1/2) and NADPH-oxidase-derived reactive oxygen species (ROS) 19–22. Other host molecules implicated in amebic pathogenesis at the cellular level include B-cell lymphoma-2 (BCL-2), nuclear factor ’kappa-light-chain-enhancer’ (NF-κB) 23,24, and signal transducer and activator 3 (STAT3)25,26. Overall these studies demonstrate the importance of host factors in the outcome of amebic infection.

In order to identify novel and biologically relevant host factors required for amebic cytotoxicity, a whole genome pooled RNAi library of human cells was selected for resistance to amebic cytotoxicity. This approach has been used successfully to identify host factors that mediate susceptibility to viral and bacterial pathogens and recently for the parasite *Trypansoma cruzi* 27. We hypothesized that cells silenced for host factors that are exploited by *E. histolytica* would exhibit increased survival to killing by *E. histolytica* parasites.

The RNAi screen identified a novel and important role for ion transport in amebic killing of host cells. Host ion transport dysregulation is a common feature of enteric pathogens28–30. Diarrhea results from reduced ionic absorption and increased luminal secretion at the intestinal epithelium28–30. The role of host ion transport in the pathogenesis of *E. histolytica* at the intestinal epithelium is relatively unexplored. Prior work described the effect of amebic lysates inhibiting colonic Na+ and Cl- absorption and stimulatating Cl- secretion in rat colonic tissue31,32. Cl- secretion was mediated by a Ca2+-dependent response activated by amebic serotonin32 and by Ca2+-independent activation of CFTR through elevation of intracellular cAMP31. *E. histolytica* analogs of serotonin and prostaglandin E2 (EhPGE2) have been shown to induce increased intracellular cAMP and Ca2+ upstream of inflammatory and secretory responses33,34.

Based on novel identification in the RNAi screen, we further characterized the role of K+ channels in amebic cytotoxicity. Inhibition of K+ efflux during cell contact with *E. histolytica* blocked amebic killing in multiple cell types including intestinal epithelial cells and macrophages. *E. histolytica* activated host K+ channels in human cells upon contact and inhibitor studies indicated a primary role for Ca2+-dependent K+ channels. K+ efflux was necessary for *E. histolytica* activation of caspase-1 and inflammasome mediated secretion of IL-1β in human macrophages. These results demonstrate that *E. histolytica* parasites actively modify cellular ion transport resulting in ionic secretion, activation of the host inflammatory cascade23 in some cell types, and cell death. Here we report the methodology and results of the RNAi screen, analysis and validation of RNAi candidate genes and characterization of K+ transport as a critical mediator of amebic cytotoxicity.

**METHODS**

**Cell culture**. UMUC3 human bladder epithelial cells were maintained in Minimum Essential Medium (MEM) supplemented with 2 mM L-glutamine and 10% heat-inactivated FBS. HT-29 cells were maintained in McCoy's 5a Medium (Life technologies) supplemented with 10% heat-inactivated FBS. Human cell lines were maintained in a humidified incubator at 37°C with 5% CO2. *E. histolytica* strain HM1:IMSS trophozoites were grown at 37°Cin TYI-S-33 medium supplemented with penicillin (100 U/ml) andstreptomycin sulfate (100 µg/ml) (Gibco/BRL). All co-culture of human cells with *E. histolytica* trophozoites was done in M199 supplemented with 5.7 mM cysteine, 0.5% BSA, and 25 mM HEPES (pH 6.8) (M199S). The UMUC3 cells were infected by lentiviral transduction with the RNAi Consortium (TRC) 1.0 shRNA library or an empty vector control as described by Guinn et al.Transduced cells were maintained under puromycin (Sigma-Aldrich) selection at 2 µg/ml. shRNAs were identified by a clone number (TRCN) and comprised of hairpin sequences containing a 21 base stem and a 6 base loop. A minimum of 3-5 different shRNA constructs targeted different regions of the mRNA transcript for each gene.

**Library Screening.** The UMUC3 TRC 1.0 library was distributed into eight tissue culture treated T75 flasks (Corning) to a final concentration of 2.0 X 105 cells/ml. UMUC3 cells transduced with an empty vector shRNA were seeded equivalently into two T75 flasks. Cells were allowed to adhere for three hours after which the medium was removed and *E. histolytica* trophozoites were added at a concentration of 2.0 X 104 trophozoites/ml (a ratio of 1 parasite to 5 host cells) in M199S. Host cells were incubated with *E. histolytica* parasitesfor three hours. Flasks were mixed by gentle rocking every 15 minutes. After selection, the host cell monolayers were washed one time with 5 ml MEM. The wash was pooled with the M199S supernatants to recover detached host cells and centrifuged at 1000 X *g* for 5 minutes. The pellet was resuspended in complete MEM supplemented with puromycin and transferred back to the surviving monolayers to allow cells that had detached but were not dead to recover. The following day, surviving cells were added to the flask from which they were originally removed and cells were allowed to recover until they were approximately 90% confluent. Trophozoites did not survive culturing in MEM with puromycin. For re-screening, monolayers were trypsinized and plated at a density 2.0 X 105 cells/ml. The remainder of the cells were frozen and reserved for later analysis. Cells were allowed to adhere for 3 hours prior to replacement of MEM with M199S media containing parasites for re-selection.

**Next generation sequencing to identify candidate susceptibility genes.** Genomic DNA was isolated from the pools of selected cells using the GenElute mammalian DNA kit (Sigma Aldrich). Sequencing libraries were created from genomic DNA using primers designed to specifically amplify shRNA inserts. The flow cell was built on an Illumina cBot cluster generation station using GA Compatible (cBOT) cluster generation kits (Illumina). Sequencing was carried out on an Illumina GaIIx sequencer using a 36-cycle single-end run with a 36-Cycle Sequencing Kit v4 (Illumina). Raw sequencing reads were aligned to the expected computational target sequences from the TRCN clones. Reads containing a full-length perfect match alignment to a clone target sequence were flagged as hits. Hits with fewer than ten sequence reads were omitted from the analysis.

**Secondary validation.** 54 genes identified in the original screen were selected for secondary screening using endoribonuclease-prepared siRNAs (esiRNA) (Sigma-Aldrich). esiRNA transfection conditions were optimized with an esiRNA targeted to firefly luciferase (FLUC) in UMUC3 cells stably expressing FLUC. Knockdown efficiency was also confirmed by qRT-PCR for 5 knockdowns. esiRNA knockdown yielded >75% knockdown of the target relative to FLUC esiRNA control transfected cells. Transfection was carried out in triplicate in UMUC3 cells as follows: 500 cells in 100 μl of media were distributed into each well of a microtiter plate. esiRNA (24 ng/well) and oligofectamine RNAi max (0.2 μl/well) (Life Technologies) were mixed with Optimem media (Life technologies) and added to each well. The medium was changed after 24 hours and cytotoxicity assays were performed 48 hours post-transfection. Cytotoxicity assays were performed as described below. Cytotoxicity was expressed as a percentage of the control FLUC transfected controls.

**Amebic cytotoxicity assays.** A representative well from each plate was used to determine the average cell count. Cell culture medium was removed and *E. histolytica* trophozoitesat indicated concentrations were resuspended in M199S and added to each well. Plates were incubated for the indicated time at 37° C with 5% CO2. At the end of the incubation period, plates were centrifuged at 500 X *g* for 5 minutes and 50 μl of supernatant from each well was transferred to a black microtiter plate. Lactate dehydrogenase (LDH) levels in the supernatant were measured using the CytoTox-ONE Homogeneous Membrane Integrity assay (Promega, Madison, WI) as directed. Briefly, 50 μl reconstituted CyTox-ONE reagent was added to each well. Plates were incubated for 10 minutes at 22° C and 50 μl stop solution was added to each well. Fluorescence was quantified at 560 nm excitation/590 nm emission (560ex/590em) using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA). Percent cytotoxicity was calculated using the following equation: *[LDH release in presence of E. histolytica - LDH release in absence of E. histolytica] / [maximum LDH release].* Maximum LDH release was determined by the addition of 0.2% Triton-X to cells alone. Each experiment was repeated at least 3 times and representative experiments are shown.

**Bioinformatics.** The DAVID Bioinformatics resource 6.7 was used to generate functional annotation gene lists for the selected pool 35–38. The starting lentiplex shRNA input library of 16,058 gene knockdowns was used as the background list. 278 of the 281 candidate susceptibility genes mapped to a DAVID ID. Tissue expression was determined using Unigene EST quartile in DAVID 6.7. Functional annotation clustering was performed by gene ontology (GO) analysis of biological process(BP), molecular function (MF) and cellular component (CC). BP represents a recognized series of events or a collection of molecular events with a defined beginning and end. MF describes the cellular functions of a gene product. CC describes the locations of proteins, at the levels of subcellular structures and macromolecular complexes. Statistical significance was determined by the EASE Score, a modified Fisher Exact *P*-value, for gene-enrichment analysis. We used an EASE score cutoff of 0.05, which is generally considered strongly enriched in the annotation categories. The significantly enriched terms in the BP GO graph (Fig. 1D) were manually curated to remove parental and/or redundant terms when appropriate. The complete analysis is provided in Table S3. Additional functional annotation for enriched keywords was performed using the UniProt Knowledgebase (UniProtKB), which provides a comprehensive database of functional protein information.

**Gene expression analysis.** The methods are described in the original report of this analysis 40. Colonic biopsy samples were obtained from 8 subjects with acute *E.* *histolytica* colitis, and again 60 days later during convalescence. Gene expression in the human colon during acute and convalescent *E. histolytica* disease was evaluated by microarray (GEO accession GSE23750)40.

**Inhibition of ion channels.** *In vitro*: 5 x 104 cells/well were distributed into 96 well microtiter plates and incubated overnight. THP1 cells were treated with 1 ng/mL PMA to induce differentiation. The day of the experiment media was changed to M199S. Cells were treated with inhibitors at the indicated concentrations in M199S for 30 minutes prior to the addition of *E. histolytica* (5 x 103 trophozoites/well). *E. histolytica* was added in a volume that resulted in a 0.1 dilution of inhibitors. For washout experiments, medium containing the inhibitor was removed from the cells and monolayers were washed twice in warm M199S prior to the addition of *E. histolytica.* Cytotoxicity was measured as described above after 60 minutes.

**IC50 concentrations** were determined with GraphPad Prism software (version 6.0e; GraphPad Software, San Diego, CA). Cellular LDH values were normalized to 100% and 0% based on external controls where the mean of vehicle-treated cells in the presence of *E. histolytica* was set to 100% and the mean of vehicle-treated cells in the absence of *E. histolytica* was set to 0%*.* DMSO at the same concentration used for compound addition served as the negative vehicle control. IC50 inhibition curves were fitted using the normalized least squares (ordinary) fit. In cases where inhibition curves plateau above the control values (0%) the IC50 defines the middle of the curve and the concentration of which yielded the maximum inhibitory concentration (ICmax) is shown. Several inhibitors resulted in cellular toxicity at higher concentrations in the absence of *E. histolytica*. In these instances, the points were shown for reference, but values were excluded from IC50 determinations.

**Gene expression analysis.** The methods are described in the original report of this analysis 40. Colonic biopsy samples were obtained from 8 subjects with acute *E.* *histolytica* colitis, and again 60 days later during convalescence. Gene expression in the human colon during acute and convalescent *E. histolytica* disease was evaluated by microarray (GEO accession GSE23750)40.

**K+ channel activation assays.** The FluxOR™ potassium channel assay was performed as outlined in the product information sheet and previously described39. Briefly, FluxOR™ loading buffer stock was diluted and Powerload™ concentrate and water-soluble probenecid were used as directed to enhance the dye solubility and retention, respectively. Media were removed from the 96-well cell plates manually, and 40 µL of loading buffer containing the loading buffer and dye mix was added**.** Inside thecell, the nonfluorescent AM ester form of the FluxOR™ dye is cleaved by endogenous esterases into a thallium-sensitive indicator. The dye was loaded for 60 min at room temperature and then removed manually. The cell plates were subsequently washed once with dye-free assay buffer, before adding a final volume of 80 µL assay buffer containing water-soluble probenecid +/- inhibitors and incubated at room temperature (23-25 °C) for 30 min to allow equilibration of the test compounds. Stimulation buffer was prepared from the 5× chloride-free buffer and thallium and contained 10 mM free thallium (5 mM Tl2SO4). *E. histolytica* trophozoites were resuspended in stimulation buffer, immediately prior to addition to cells loaded with FluxOR dye. The final added concentrations were 2 mM free Tl+ and 1 trophozoite to 5 host cells after a 1:5 dilution by addition of 20 µL stimulus buffer/well. *E. histolytica* (+EH) or vehicle (-EH) was added and fluorescence was measured every 40 seconds for 12 minutes.

Data analysis for the FluxOR™ screen were analyzed as previously described39. The baseline fluorescence value for each well was determined from the average of 3 readings prior to addition of stimulus buffer. Each well F value was normalized to the mean initial baseline value (F0). The effect of inhibitors on K+ channel activation by *E. histolytica* was assessed by calculating the area under the curve (AUC) of each inhibitor and control in the presence (+EH) and absence of *E. histolytica* (-EH). Inhibitor concentrations tested were: (KCl - 25 mM, 293B-10 um CLO -10 um, PAX - 10 um, ChoCl – 25 mM). The AUC (% of control) for each inhibitor was normalized to the mean of the AUC media control (+EH was normalized to +EH control, -EH was normalized to –EH control). DMSO at the same concentration used for compound addition served as the negative vehicle control. Inhibition Values were graphed using GraphPad® Prism 6.0e (GraphPad Software, San Diego, CA).

*P*-values were calculated relative to untreated cells (\*, *P* < 0.001) by Fisher's LSD test.

**PBFI intracellular K+ measurements.** Intracellular K+ was determined in cells using the K+-sensitive fluorophore PBFI. HT-29 cells were seeded in 96-well plates at 5 × 105 cells/well and allowed to settle, after which the medium was changed to M199S. Cells were loaded with the cell permeant acetoxymethyl ester of PBFI (PBFI-AM) (Molecular probes) at 5 μM with Pluronic F-127, a non-ionic detergent polyol used to facilitate cell loading of large dye molecules (Invitrogen) for 60 min at room temperature, according to the manufacturer's instructions. Cells were washed twice in pre-warmed M199S following loading with PBFI. Control incubations using valinomycin to equilibrate intracellular and extracellular K+ were performed as described in the manufacturer's instructions and confirmed the expected decrease in PBFI 340/380 fluorescence ratio. Fluorescence emission at 500 nm was recorded with excitation alternating between 340 and 380 nm. PBFI shows K+-dependent emission at 500 nm when excited at 340 nm and K+-independent fluorescence when excited at 380 nm, near its isosbestic point. Hence, the ratio of fluorescence emission at 500 nm at excitations of 340 and 380 nm with provided a measure of intracellular K+ concentration that was independent of dye concentration and photobleaching. After a five-minute initial period of stabilization, cells were treated as indicated.

**Measurement of extracellular K+.** 1 x 105 -1 x 106 HT-29 cells/ml were plated in 6 well plates and grown overnight. The following day, 1 well was used to obtain a cell count. For the remaining wells, medium was replaced with M199S containing *E. histolytica*. 100 μl samples were taken at the time points indicated and centrifuged at 1000 X *g*. Supernatants were transferred to a new microfuge tube and immediately stored at -20°C. The experiment was performed in triplicate. K+ concentrations in sample supernatants were measured at the University of Virginia Health system clinical chemistry labs. The coefficient of variance of the assay at this level was 1%.

**Inflammasome activation.** HT-29 or THP-1 cells were seeded in 6 well plates the day before the experiment in cell culture media supplemented with PMA (5 ng/ml) the day before the experiment. The next day, medium was replaced with serum free RPMI 1640 prior to addition of relevant inhibitors. All inhibitors with the exception of KCl were washed out prior to addition of *E. histolytica* trophozoites in serum free RPMI1640. After a 3 hour incubation, the plates were centrifuged and supernatants were analyzed separately for IL-1β secretion and caspase-1 secretion by ELISA (R&D biosystems) and cell lysis by LDH release as described above. The caspase-1 ELISA is specific to the p20 subunit of Caspase-1. The processing of IL-1β in supernatants was verified by immunoblot.

**Statistical analysis.** Statistical significance was calculated using the two-tailed student t test, Fisher least significant difference (LSD) t test or by Analysis of Variance (*ANOVA)* with GraphPad Prism software (version 6.0e; GraphPad Software, San Diego, CA). A *P-*value of ≤0.05 was considered significant unless otherwise noted. The results were expressed as means and standard errors of the means (SEM) unless indicated otherwise. Statistical significance of bioinformatics data was calculated by fisher exact *P-*value using DAVID 6.7 tools and a *P-*value of ≤ 0.05 was considered significant unless otherwise noted.

**RESULTS**

**Design and implementation of a whole genome shRNA screen to identify novel host factors critical for *E. histolytica* cytotoxicity.** We directly select a pooled genome-wide RNAi library for clones with increased resistance to amebic killing with *E. histolytica* parasites. The library was constructed in UMUC3 cells, which were susceptible to killing, by *E. histolytica*. In addition UMUC3 killing was blocked by galactose, which blocks amebic adherence and contact-dependent killing (Fig. S1A). To define optimal screening conditions, we tested the effect of cell density and host cell: parasite ratio. A high plating density of 5 x 105 cells/ml and low plating density of 1 x 105 cells/ml. We also varied the ratio of parasites to host cells from 1:5 to 1:100). We selected a ratio of 1:5 parasites to host cells, which yielded ~22% killing of host cells after 3 hours of contact with lower density plated library cells (Fig.S1B).

The input pooled shRNA library was subjected to successive rounds of selection with *E. histolytica* trophozoites. After each round of selection, resistant cells were separated from trophozoites and cultured to obtain a sufficient cell number for rescreening. Samples were taken after every round of selection to track the loss of susceptible clones (Fig. 1A). The RNAi library had increased resistance to *E. histolytica* cytotoxicity relative to the empty vector control library screened in parallel after 6 rounds of selection (Fig. 1B). The screen was continued for three additional rounds of selection, with the final round of selection at the higher ratio of 1:2 parasites to host cells.

**Candidate susceptibility gene identification by next generation sequencing.** DNA from resistant clone pools was purified and sequenced by next generation sequencing. The number of sequence reads corresponding to a given shRNA construct was used to estimate the relative abundance of individual clones. The sequence reads displayed a normal distribution in all the sequenced pools. Clones with fewer than 10 sequencing reads were excluded from this analysis (Fig. S1C). Pool six contained 5320 unique TRCN clones targeting 4314 genes, representing ~27% of the genes in the input library. This was reduced to 410 TRCNs targeting 395 genes in pool 8 and further to 284 TRCNs targeting 281 genes in pool 9 (Table 1). The low number of clones lost between round 8 and round 9 was consistent with saturation of the selection with *E. histolytica*. A full list of TRCN clone IDs, sequence abundance, and corresponding gene targets is shown in Table S1.

The selection of knockdowns in genes in pathways with previously defined roles in *E. histolytica* cytotoxicity, including sugar modifying enzymes (*ALG1, ALG9, B3GNT7, OGT, GBE1, PIGV, PGK2, GP6)*, fibronectin genes (*FNDCB3, FLRT3)*, caspase-8 (*CASP8)*, the chloride channel cystic fibrosis transmembrane conductance regulator (*CFTR*), protein phosphatases (*ACP1, PPP1R13L, PPP1R14C, PPP2R1B, PPP3R1, PPP4R1L)* and Ca2+ binding proteins (*CIB3, CABP2, CACNG8, SCGN, SLC24A3)* added confidence to the biological significance of our approach14,15,18,31,41,42.

**Bioinformatics Analysis of Resistant Clones.** Enrichment analysis classified the susceptibility candidate genes into statistically significant over-represented functional categories (Table S3). Several overrepresented gene categories with previously documented roles in amebic cytotoxicity included cell death (25 genes, fold enrichment = 2.2*,* *P* = 0.0004) and calcium signaling (9 genes, KEGG, 3.5 fold enrichment*,* *P* = 0.0035) were identified (Table 3 and Table S3).

Ion transport was a novel significantly overrepresented molecular function (2.2 fold enriched, *P* = 0.00065). 25 of 277 genes were classified as ion transporters. 18 of which were classified as cation transporters (2.0 fold enriched, *P* = 0.01). The predominant substrate was K+ (6 genes), followed by Cl- (4 genes including *CFTR*), Na+ (3 genes) and Ca2+ (2 genes) (Fig. 1D). Transport was also the main biological process (BP) by gene ontology analysis, with 56 genes in this category (1.4 fold enrichment, *P* = 0.014) with related process and their kappa values shown in figure 1D. Cellular component (CC) analysis found that 8 genes were localized to ion channel complexes (2.6 fold enrichment, *P* = 0.033) as was expected based on MF and BP enrichment (Table 3). Ion transport was significantly overrepresented as a molecular function, biological process and cellular component in candidate susceptibility genes (Table 3, Fig. 1D). Furthermore, it indicated a novel and unexpected role specifically for K+ ion transport in ameba-induced cell death.

**Evidence score analysis of candidate susceptibility genes**. A combination of data sets was used to rank and prioritize the candidate susceptibility genes identified in pool 9. This technique generates an evidence score and has been used previously for candidate gene analysis in other whole genome screens 43. The evidence score incorporated the relative survival of individual clones (determined by sequence reads), genes independently selected by multiple shRNA constructs, defined intestinal expression, KEGG annotation in amebiasis and regulation in response to *E. histolytica* *in vitro* and *in vivo* (Fig. 4) (Table 2)40,44. The intestinal epithelium is the main tissue site of *E. histolytica* colonization andinfection and 64/277 candidate susceptibility genes were expressed in normal human colon (EST database)45. The KEGG amebiasis pathway (hsa05146) was used to identify genes and pathways with previously characterized roles in amebic infection. 19/277 susceptibility candidate genes are annotated in KEGG as associated with susceptibility to amebiasis. Transcriptional analyses of host genes regulated during amebic infection were incorporated in the evidence score: one compared colonic gene expression in patients with acute amebic colitis and one examined expression in response to *E. histolytica* in host cells *in vitro* 40,44. The candidate genes and evidence scores are given in Table S2. Fibronectin type III domain containing 3B (*FNDC3B*) had the highest evidence score of 8 (Table S2). The fibronectin leucine rich transmembrane protein 3 (*FLRT3*) also had an evidence score of 4. The prioritization of these genes added assurance to our approach as *E. histolytica* is known to recognize and bind host fibronectin42,46.

**Validation of selected candidate susceptibility genes in a secondary RNAi screen.** Due to the high probability of off target effects in pooled shRNA screens, a secondary screen was performed on 55 candidate susceptibility genes from the final pool. Silencing in the secondary screen was accomplished using endoribonuclease-digested siRNA (esiRNA). Each knockdown was tested individually in a well-based assay for amebic cytotoxicity in the same cell line used for the primary screen (UMUC3). Silencing of 35 out of 54 candidate susceptibility genes tested reduced amebic cytotoxicity relative to cells transfected with esiRNA targeting firefly luciferase (FLUC) as a control. Of the 55 genes tested, silencing of 15 significantly reduced amebic killing of transfected cells (\**P* < 0.05 by two-tailed students t-test). 9 knockdowns had marginal effects on amebic killing (< 5%) and 12 knockdowns increased susceptibility to amebic cytotoxicity, 8 of which reached significance (\**P* < 0.05) (Fig. 2). Overall, 65% of hits from the primary screen reduced amebic cytotoxicity in the secondary screen, including *FLRT3*,the gene with the highest evidence score. *FLRT3* knockdown reduced amebic killing by 20% relative to controls. It is also of note that the K+ ion channels that not validated (*KCNIP4* and *SLC24A3*) had evidence scores of <2, while the K+ channels with higher evidence scores (>2) were validated (*KCNB2, KCNA3, KCNJ3*). Knockdown of each channel reduced amebic killing by ~16% (\**P* < 0.05) (Fig. 2).

**Inhibition of ion transport blocked amebic cytotoxicity**. To further validate the role of ion transport in amebic cytotoxicity, we tested if pharmacological blocked amebic killing of UMUC3 cells. The broad-spectrum K+ channel inhibitors ibutilide and tetraethylammonium chloride (TEA) inhibited amebic cytotoxicity to undetectable levels (Fig. 3). Ibutilide inhibits both K+ and Ca2+ channels. Ibutilide blocked amebic killing with an IC50 of ~5 μM, nearly 50-fold higher than the reported IC50 of 0.01-2 μM 47–50. TEA blocks a range of K+ channels with varying efficacy: Ca2+-activated (IC50: 150 μM), delayed-rectifier (IC50 = 3 mM) and ATP-activated K+ channels (IC50: 15 mM) 51. The IC50 of TEA for inhibition of amebic cytotoxicity was ~50 μM, consistent with specific inhibition of Ca2+-activated K+ channels. Both inhibitors were toxic to cells at higher concentrations (> 1 mM ibutilide and > 2.34 mM TEA).

Quinine and 4-aminopyrimadine (4-AP) are also broad-spectrum K+ channel inhibitors. Both decreased amebic cytotoxicity marginally. 4-AP caused toxicity above 18.75 mM while quinine did not exhibit cause cytotoxicity at the concentrations tested. Quinine is an antimalarial compound with similar inhibitory effects on ion flux as TEA, but is less effective against Ca2+-activated K+ channels (IC50: 300 μM)51 and did not inhibit amebic killing beyond 46% of control (Fig.3).

Inhibition of other ion channels was also effective for blocking amebic killing of UMUC3 cells. The K+/Na+/Ca2+ channel inhibitor benzamil inhibited amebic cytotoxicity with an IC50 of ~8 μM consistent with other reported efficacy studies52,53*.* The anesthetic procaine blocked amebic cytotoxicity with an IC50 36 μM. Procaine has reported effects on Na+ and K+ channels (IC50 for Na+ channels: 110 μM, voltage-activated K+ channels: 6302 μM, inward-rectifying (hERG) K+ channels: 35 μM)54.The Ca2+ channel inhibitor diltiazem is active in the colon55 and has a range of biological activities depending on the concentration (0.5 - 2500 μM)56. Diltiazem blocked amebic cytotoxicity with an IC50 25-60 μM, consistent with inhibition of Ca2+ channels56. The Cl- channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) strongly inhibited amebic cytotoxicity with an IC50 5-15 μM. NPPB inhibited Cl- flux in intestinal T84 cells at 20 μM52 (Fig. 3). Overall, the finding that pharmacological ion channel inhibitors strongly blocked amebic killing supported a role for ion channels during amebic cell killing.

**Colonic gene regulation in human amebic colitis** The primary RNAi screen identified ion channels as mediators of cell killing in a bladder epithelial cell line (UMUC3) (Table 4). As the expression and function of ion channels is variable across cell types and tissues we asked if similar mechanisms are important during amebic infection of the human colon. To answer this question we analyzed the expression of K+ channel (*KCN*) genes in acute amebic colitis in humans. We determined the regulation of the 94 annotated human *KCN* genes. We found that *KCN* genes were globally down-regulated during acute amebiasis (Fig. 4). Furthermore, the level of down-regulation was correlated with the overall level of expression in the colon (day 60 expression levels, corresponding to samples from recovered patients) (R=-0.54, *P* < 0.0001). The Cl- channel *CFTR* was also highly expressed in the colon and highly down-regulated during acute amebiasis. *CFTR* is a known regulator of K+ channels. This correlation indicated that *KCN* genes with higher colonic expression were more transcriptionally repressed during acute amebic infection. Down-regulation of colonic *KCN* genes and *CFTR* may be a protective physiological response to prevent excessive luminal secretion and cell death during amebiasis. Additionally, cells with higher *KCN* gene expression may be more sensitive to *E. histolytica* killing leading to an overall depletion of *KCN* transcripts.

**Inhibition of ion channels blocked amebic killing of intestinal epithelial cells and macrophages.** We tested if chemical inhibition of ion efflux also blocked amebic cytotoxicity in biologically relevant target cells including HT-29 intestinal epithelial cells (IECs) and THP1 macrophages. Increased KCl, K2SO4, NaCl and ChoCl were tested for the ability to block amebic cytotoxicity and chemical inhibition of ion transport blocked amebic cytotoxicity in both IECs and macrophages (Fig. 5A). Potassium ions were most effective in blocking amebic cytotoxicity. The IC50 of KCl was 8 mM for blocking killing of IECs and 12 mM for macrophages (Fig. 5A). K2SO4 yielded similar inhibition as KCl. NaCl also blocked amebic cytotoxicity higher concentrations (IEC IC50=25 mM and macrophages IC50=59 mM). Conversely, choline chloride, which was included as an osmotic control, was more effective in blocking cytotoxicity in macrophages (IC50=29 mM) than IECs (IC50=57 mM). The differential inhibitory effect of NaCl and ChoCl may be due to ionic effects on tissue-specific K+ channels including KCNMA1, which may also be regulated by extracellular Na+ and Cl-58.

Several studies have demonstrated that *E. histolytica* raises intracellular Ca2+ prior to cell death9,15 but the subsequent cellular events that trigger cellular death are not understood. To characterize Ca2+-activated K+ efflux during amebic killing we tested if specific Ca2+-activated K+ channel inhibitors blocked amebic cytotoxicity. Paxilline inhibits big conductance (BK) Ca2+-activated K+ channels, mainly KCNMA1, localized in the apical membrane of goblet cells in the intestine59. Paxilline binds the closed conformation of KCNMA and the IC50 ranges from 10 nM for the closed conformation to near 10 μM for the open conformation60,61. Paxilline strongly blocked amebic killing of IECs (84± 3% inhibition, IC50=5 μm) and of macrophages (85± 7% inhibition of killing, IC50 = 6 μM) (Fig. 5B and Table 6). Clotrimazole, a potent inhibitor of intermediate-conductance (IK) Ca2+-activated K+ channels also blocked cell killing by *E. histolytica*. Clotrimazole reduced killing of IECs by 76± 4% in IECs (IC50=6 μM) and by 60 ± 6% in macrophages (IC50 = 14 μM) (Fig. 5B and Table 6). The major target of clotrimazole in the intestine is KCNN4 which can also be activated by cAMP in parallel with CFTR to drive intestinal CI− secretion62.

We also tested chromanol 293B, an inhibitor of KCNQ1. In the intestine, KCNQ1 forms a voltage-insensitive channel with the KCNE1 subunit that is activated by elevated intracellular cAMP and may be co-activated with CFTR 62–65. Chromanol 293B reduced killing of IECs by 57± 5% in IECs and by 62± 3% in macrophages with an IC50 of 7 μM for both cell lines (Fig. 5B and Table 6). Overall, inhibition of KCNMA1 by paxilline, KCNN4 by clotrimazole and KCNQ1 by chromanol 293B blocked amebic killing of intestinal epithelial cells. The effective concentrations of paxilline and chromanol 293B have been reported to block K+ current by these targets when studied by electrophysiological techniques66–68. These three channels were also highly expressed in colonic biopsies (Fig.4). Paxilline was the most effective inhibitor in both IECs and macrophages, and the paxilline target KCNMA1 was the third most down-regulated *KCN* gene in acute amebic colitis (Fig.4).

To further define the mechanism of *E. histolytica* activation of K+ channels in the intestine we tested specific K+ channel inhibitors of K+ channels identified in the RNAi screen (Table 2) in IECs and macrophages. AM 92016 and CP 339818 are potent blockers of voltage-gated K+ channels, mainly KCNA3 and KCNA269–71, while TEA is a broad inhibitor of K+ channel activity. AM 92016 and CP 339818 blocked killing of IECs and macrophages but were more effective in protecting macrophages from amebic killing (Fig. 5C and Table 6). It is of note that KCNA2 and 3 had lower expression relative to KCNMA1, KCNQ1 and KCNN4 in colonic biopsies (Fig. 3), suggesting a correlation with expression and cell-type specific efficacy.

***E. histolytica* trophozoites activated K+ channels in IECs and macrophages.** We monitored K+ channel activation using the FluxOR assay (Fig. 5D,E) (described in methods). We found that increased extracellular K+ blocked K+ channel activation in both IECs and macrophages. Similar to the effect on cell killing by *E. histolytica* choline choloride had no effect on K+ channel activation in HT-29 IECs but moderately reduced K+ channel activation in THP1 macrophages. To test if inhibitors specifically inhibited K+ channel activation by *E. histolytica* we compared channel activation in the presence and absence of trophozoites (fig. 5E). KCl, paxilline, clotrimazole and chromanol 293B reduced K+ channel activation in HT-29 cells in response to *E. histolytica*. KCl and clotrimazole also reduced K+ channel activation in the absence of *E. histolytica.* A similar response was observed in THP1 macrophages, however chromanol 293B did not block K+ channel activation by *E. histolytica* and choline chloride did moderately reduce K+ channel activation in response to parasites (Fig. 5E).

***E. histolytica* trophozoites caused K+ efflux in IECs.** We monitored extracellular levels of K+ levels and LDH when host cells exposed to *E. histolytica* for an hour (Fig. 6A). We found that increased extracellular K+ preceded elevated LDH, suggesting that K+ efflux occurs prior to cell death. To further determine if *E. histolytica* induced efflux of intracellular K+, cells were loaded with the K+-sensitive fluorescent intravital dye PBFI. Changes in intracellular K+ concentrations in HT-29 cells during contact with *E. histolytica* were monitored over 30 minutes in media M199 (KCl = 5.33 mM). Cells co-incubated with *E. histolytica* displayed a significant reduction in intracellular K+ after 30 minutes (Fig.6B). The extracellular ionicconcentrations of K+ in the supernatants of HT-29 cells were measured in parallel and an increase of 0.1 mM K+ in the presence of *E. histolytica* was detected after 30 minutes*.* No increase in extracellular K+ concentration was observed in the absence of *E. histolytica* or with *E. histolytica* in the absence of HT-29 cells(data not shown).

**Inflammasome activation.** *E. histolytica* has recently been reported to activate the host cell inflammasome72. K+ efflux is a well defined mechanism of inflammasome activation and pyroptotic cell death via caspase-1 activation in human cells73–75. We tested whether *E. histolytica* activated the host inflammasome by measuring IL-1β secretion and cell death in HT-29 IECs in response to *E. histolytica*. Intestinal epithelial cells have recently been shown to express a Caspase-4 dependent IL-18 secreting inflammasome (76,77). We tested if specific inhibitors of caspase 1, 3, 4 and the pan-caspase inhibitor zVAD-FMK blocked amebic cytotoxicity and cytokine secretion in HT-29 cells. HT-29 cells did not secrete detectable levels of IL-1β or IL-18 in response to *E. histolytica* (measured after 3 and 16 hours of co-incubation)regardless of pretreatment with LPS as a priming signal. Treatment of LPS-primed HT-29 cells with ATP, a positive control for NLRP3 inflammasome activation also failed to induce IL-1β secretion (data not shown). Overall, inhibition of host caspases protected HT-29 IECs from amebic killing but *E. histolytica* did not induce secretion of IL-18 or IL-1β in HT-29 cells (Fig. S2).

*E. histolytica* has recently been demonstrated to activate the NLRP3 inflammasome in THP-1 macrophages72. The inflammasome can be activated by multiple stimuli, however K+ efflux may be the common trigger74. *E. histolytica* inflammasome activation also required K+ efflux. Excess K+ and specific K+ channel inhibitors (AM 92016 and CP 339818 blocked IL-1β secretion and amebic cytotoxicity in THP1 cells (Fig. 7A). We tested the effect of caspase inhibitors on inflammasome activation and cell killing by *E. histolytica.* Caspase-1 inhibition blocked both cell killing and IL-1β secretion. Caspase-3 inhibition blocked cell killing but not IL-1β secretion, while caspase-4 inhibition had no effect. The pan-caspase inhibitor zVAD-FMK significantly inhibited IL-1β secretion and amebic cytotoxicity in THP-1 cells (Fig. S2). Overall, IL-1β secretion required both K+ efflux. There is also evidence that caspase-1 is required for IL-1β cleavage by the inflammasome in response to *E. histolytica* in THP-1 macrophages.

To further investigate the role of the inflammasome in cell killing by *E. histolytica* we tested the susceptibility of ASC-deficient THP1 cells to *E. histolytica.* ASC (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain) is a key adaptor molecule between pathogen-sensing NOD-leucine-rich repeat (NLR) proteins, and pro-caspase-1. ASC mediates oligomerization into the inflammasome complex, which leads to auto-activation of pro-caspase-1 and the secretion of IL-1β and IL-1878. We found that ASC-deficient THP1 cells were significantly more resistant to amebic killing (39.3 .7 ± 5.9% reduction in killing vs. wild type THP1 cells, *P=* 0.02). ASC-deficient cells also secreted significantly less of IL-1β, but displayed only a moderate defect in caspase-1 secretion (Fig. 7B). Secreted, processed caspase-1 in THP1 supernatants was measured by ELISA specific for the active p20 subunit. Inflammasome assembly occurs when NLRP3 oligomerization with ASC and pro-caspase-1 is triggered by K+ efflux, and We found that caspase-1 activation by *E. histolytica* required K+ efflux and could be inhibited by excess K+ and the K+ channel inhibitor, AM 92016. It is of note that excess K+ was more effective than the caspase-1 inhibitor, YVAD, for blocking caspase-1 activation, IL-1β secretion and cell killing by *E. histolytica* (Fig. 7B).

**DISCUSSION**

We set out to identify novel and biologically relevant host factors that are required for amebic killing of host cells by screening a whole-genome RNAi library of human cells. This approach successfully identified genes and pathways previously implicated in amebic cytotoxicity as well as numerous genes and gene families novel to the field of amebiasis. The identification of K+ channels was an important finding that we pursued and validated. Further investigation found that blockade of K+ efflux, genetically, pharmacologically, and electromotively, inhibited *E. histolytica* cytotoxicity. We also showed that *E. histolytica* activated human K+ channels (fig. 5d), decreased intracellular K+ concentrations in host cells while simultaneously raising extracellular K+ concentrations (fig.6), indicating direct activation of K+ efflux by *E. histolytica* parasites. Finally, we found that *E. histolytica-*induced K+ efflux was required for inflammasome activation and cell death in human THP-1 macrophages. The importance of K+ ion transport for *E. histolytica* cytotoxicity makes sense in light of the physiologic diarrheal symptoms associated with amebic colitis. Enterotoxic bacteria cause diarrhea by manipulating ion-transport in the intestinal epithelium. Toxins secreted during colonization by *Vibrio cholera*79and *Escherichia coli* 80 activate adenylate cyclase, raising intracellular cAMP, leading to Cl- ­ secretion via CFTR and severe diarrhea.

Analogously, previous work has defined a role for Na+, Cl- and Ca2+ ion transport in amebic cytotoxicity. Two studies described the ionic effects of amebic lysates on sections of rabbit and rat colon. The authors concluded that lysates of *E. histolytica* inhibited colonic Na+ and Cl- absorption while stimulating luminal Cl- secretion31,32. Cl- secretion occurred via a Ca2+-dependent response activated by amebic serotonin32 and by a Ca2+-independent response mediated by increased cellular cAMP activating host CFTR channels31. These studies clearly demonstrate the impact of *E. histolytica* on host ion transport. Though it is important to note the prior studies used amebic lysates as opposed to intact parasites, an approach that may limit the relevance of the findings since amebic lysates contain many toxic insults, which may indirectly activate ion channels or permeabilize cell membranes. Our work used intact parasites but relied mainly on cultured IECs as a model for host ion transport. In light of this earlier work using primary tissue, our work corroborates the observation that ion transport is critical for *in vivo* amebic infection. Others have also investigated the effect of Ca2+ and Na+ channel inhibitors on amebic cytotoxicity *in vitro*. The Na+ channel blocker tetrodotoxin had no effect on amebic cytotoxicity but the slow Na+-Ca2+ channel blockers verapamil and bepridil both decreased amebic cytotoxicity toward Chinese hamster ovary cells 81. Inhibitors of Ca2+ flux as well as Ca2+ chelators also blocked amebic cytotoxicity 15,82. In combination these studies support our conclusion that ion flux is required for amebic killing of target cells.

A model of ion transport and *E. histolytica* cytotoxicity is shown in Fig. 8. Previous work has found that *E. histolytica* increases intracellular Ca2+ and cAMP in host cells 15,31 while stimulating Na+ and Cl- secretion. In an intestinal epithelial cell, Cl- efflux is mediated by the apical CFTR (a susceptibility gene candidate), while K+ efflux occurs at both the luminal and basolateral surfaces28. Intestinal cells are extremely sensitive to intracellular Ca2+ and cAMP concentration which activate K+ channels in luminal and basolateral membranes 51,53,63,70,71. Alternatively, *E. histolytica* may directly activate luminal K+ and Cl- channels in the intestinal membrane by a novel mechanism. Cl- efflux may be activated directly in response to parasites or may be a secondary effect of K+ efflux to balance charge. In this model, K+ and Cl- efflux causes intracellular ion concentrations to fall, triggering water secretion and causing cells to shrink. Cell shrinkage mediates apoptotic volume decrease death (AVD) involving caspase-3. Cytosolic K+ concentration is a major regulator of caspase activation 86,87 . Other investigators have found that high extracellular K+ and inhibitors of BK and IK Ca2+-activated K+ channels block intrinsic and extrinsic apoptotic pathways88.

Low intracellular K+ concentrations activate caspase-1 via NLR oligomerization, which can mediate pyroptotic cell death73–75. Our data found that caspase-1 activation was blocked by high extracellular K+ and by specific inhibitors of voltage-gated K+ channels. The finding that caspase-1 activation did not seem to require the NLRP3 inflammasome adaptor ASC, suggests a novel K+ -dependent mechanism of caspase-1 activation by *E. histolytica* may exist*.* ASC was required for pro-caspase-1 autoproteolysis and IL-1β secretion by the NLRC4, NLRP3 and AIM2 inflammasomes but not the NLRP1b inflammasome89. Pro-caspase-1 can also be non-canonically activated by caspase-11 and appears to be involved an activation cascade during the inflammatory response 90,91. The finding that ASC deletion impaired cell killing and IL-1β secretion, but not caspase-1 activation may indicate that inflammasome activation by *E. histolytica* proceeds through non-canonical pathways, potentially via multiple NLRs.

Whole-genome pooled screens represent an efficient and powerful tool for identification of novel host genes involved in diverse processes. shRNAs provide stable, long term knockdown, do not induce the interferon response and make pooled screens possible. However, whole-genome shRNA screens suffer from several inherent limitations. These including off-target effects of shRNA constructs and incomplete knockdown of target genes. Thus validation of hits from the primary screen was critical. We selected a subset of hits identified in the primary pooled screen and validated these in a secondary screen using different RNAi technology and an independent assay for amebic cytotoxicity. Approximately 70% of the genes identified in the primary screen reduced cytotoxicity in the secondary screen, although some only marginally. Our primary screen was designed with multiple steps over several weeks, thus even small increases in resistance may have been advantageous as the population underwent selection. Interestingly, several knockdowns in the secondary screen significantly increased amebic cytotoxicity. This may be due to a to RNAi activation of gene transcription92 differences in the design of these assays and/or due to false-positive selection in the primary screen.

Another important consideration in our interpretation is that the majority of these studies were done in immortalized cultured human cells. The initial screening was performed in UMUC3 epithelial cells, while validation was performed in UMUC3 cells, HT-29 IECs, and THP-1 macrophages. We found that K+ efflux is a new and critical host mediator of amebic cytotoxicity in these cell types. Further confidence is added by validation of this pathway in across multiple cell lines from various tissues. K+ channels are the most complex class of ion channels in both structure and function, with diverse expression and function throughout the body. Our analysis found that K+ channels are highly expressed and regulated in the human colon during *E. histolytica* infection (Fig. 4).

K+ channel inhibitors have different effective concentrations across channel families. Our observation that some ion channel inhibitors only blocked amebic cytotoxicity at concentrations higher than reported IC50 values could be due to these cell-type specific effects due to tissue specific K+ channel expression. We observed that some inhibitors had differential efficacy in blocking amebic cytotoxicity in HT-29 IECs and THP-1 macrophages (Fig. 5, 7A). The specificity of some ion channel inhibitors is decreased with increasing concentration, potentiating broader inhibition of ion flux, which might explain the variability in effective concentrations. Blockade of BK Ca2+-activated K+ channels by paxilline was most effective in inhibiting cell killing of IECs by *E. histolytica*. Paxilline specifically inhibits KCNMA1, an apical K+ channel localized to goblet cells in the colon 59. Apical KCNMA1 is activated by Ca2+ 93 and notably KCNMA1 localization was extended along crypts in human ulcerative colitis 94. KCNMA1 overexpression mediated enhanced K+ secretion in experimental colitis 95 and deletion of KCNMA1 abolished luminal colonic K+ secretion in mice 96,97. KCNMA1 is also expressed on macrophages and regulates transcription of IL-698, which is supported by our finding that paxilline was also highly effective in blocking amebic killing of THP1 macrophages (Fig.5B). These studies in combination with our data suggest that KCNMA1 may be a critical regulator of inflammation in intestinal epithelial cells and immune cells.

In contrast to intestinal epithelial cells, AM 92016, a blocker of delayed rectifier K+ channels and CP 339818, a blocker of voltage-gated K+ channels were most effective for preventing amebic killing of THP1 macrophages. Both inhibitors block KCNA3, a hit from the RNAi screen. Immune cells express high levels of KCNA369 but KCNA3 is also localized to the epithelium99 and upregulation of KCNA3 is associated with Crohn’s disease100. This finding is consistent with our data that K+ channels mediate inflammasome activation in macrophages (Fig. 7A,B) and suggests that KCNA3 may be a target for anti-inflammatory drug development.

Several key questions remain about the role of ion flux induced during *E. histolytica* pathogenesis. The specific K+ channels that are activated in human colonic epithelial and infiltrating immune cells remain unknown. Our data suggest that *E. histolytica* activates K+ channels via increased cytosolic Ca2+ and possibly cAMP (Fig. 5B). It will be important to determine the mechanisms of cell death induced by K+ efflux in different cell types. Our current hypothesis is that K+ efflux leads to cell shrinkage and decreased intracellular K+ concentration. This can activate caspase-3 101, resulting in apoptotic cell death. As apoptosis minimizes intestinal inflammation, this may be adaptive for successful colonization of the intestinal epithelium by *E. histolytica*. However, our data indicate that K+ efflux activates pro-inflammatory cytokine production via a caspase-1 ASC-inflammasome in macrophages. The finding that ASC deficient cells were protected from amebic killing suggests that inflammasome activation mediates cell death via *E. histolytica* (Fig. 7B). Inflammasome activation and caspase-1 activation in macrophages also required K+ efflux, though caspase-1 activation apparently did not require the inflammasome adaptor ASC. We propose a model where decreased intracellular K+ concentration is a common signal that activates host caspases in a context and cell type dependent manner78. It will be critical to define how K+ efflux is involved in distinct forms of cell death during amebic infection. Finally, it will be clinically important to test the efficacy of K+ channel inhibitors in blocking both amebic cytotoxicity and *E. histolytica* activated secretion at the intestinal epithelium.

The unexpected finding that K+ efflux triggers cell killing, and activation of inflammatory cascades by the diarrheal pathogen *E. histolytica* is an important advance in the understanding of amebic pathogenesis. Host ion transport is a critically important area for future investigation as it also is the primary mechanistic cause of amebic diarrhea. Our work additionally indicates that K+ efflux activates inflammatory cascades in host immune cells. Therefore, specific inhibition of host ion efflux, in particular K+ efflux, may represent a novel, host-directed therapeutic intervention to block the damaging secretory and inflammatory responses caused by diarrheal pathogens.

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**TABLE 1 Sequencing results of selected libraries at round 6, 8 and 9 of selection.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Pool** | **TRCN Clones** | **Target Genes** | **Genes targeted by >1 shRNA** | **% of input library**  **(clones, genes)** |
| 6 | 5320 | 4314 | 760 | 6.6, 26.9 |
| 8 | 410 | 395 | 15 | 0.5, 2.5 |
| 9\* | 284 | 281 | 3 | 0.35, 1.7 |

\* Analysis was done using target genes identified in pool 9

**TABLE 2 Evidence score analysis of susceptibility candidate genes pool 9**

|  |  |
| --- | --- |
| **Factor** | **Score** |
| Independently selected shRNA clones | 1 point |
| Relative abundance (Sequence reads/gene) | 1 point >102  2 points 103-104  3 points >104 |
| Intestinal expression | 1 point |
| Regulated in response to *E. histolytica* | 3 point |
| Amebiasis KEGG pathway | 1 point |

**TABLE 3 Overrepresented functional categories in candidate susceptibility genes (Pool 9).**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Category** | **Term** | **Count** | **%** | **P-Value** | **Fold Enrichment** |
| BP | cell death | 25 | 9 | 0.00037 | 2.20 |
| MF | transcription activator activity | 17 | 6 | 0.00052 | 2.70 |
| BP | transmembrane transport | 21 | 8 | 0.00062 | 2.40 |
| MF | ion transmembrane transporter activity | 24 | 9 | 0.00065 | 2.20 |
| MF | substrate-specific transmembrane transporter activity | 26 | 9 | 0.00076 | 2.10 |
| MF | transmembrane transporter activity | 27 | 10 | 0.00130 | 2.00 |
| CC | intracellular organelle | 155 | 56 | 0.00260 | 1.20 |
| MF | gated channel activity | 12 | 4 | 0.00770 | 2.50 |
| MF | binding | 204 | 74 | 0.00850 | 1.10 |
| MF | cation transmembrane transporter activity | 17 | 6 | 0.01100 | 2.00 |
| MF | anion binding | 6 | 2 | 0.01300 | 4.30 |
| BP | transport | 56 | 20 | 0.01400 | 1.40 |
| MF | transferase activity | 39 | 14 | 0.01400 | 1.50 |
| MF | ion channel activity | 13 | 5 | 0.01500 | 2.20 |
| CC | integral to plasma membrane | 28 | 10 | 0.01700 | 1.60 |
| MF | substrate specific channel activity | 13 | 5 | 0.01800 | 2.20 |
| CC | apical part of cell | 8 | 3 | 0.02100 | 3.00 |
| CC | plasma membrane part | 45 | 16 | 0.02200 | 1.40 |
| CC | ion channel complex | 8 | 3 | 0.03300 | 2.60 |

BP-Biological Process, MF-Molecular Function, CC-Cellular Component

**TABLE 4 K+ channel candidate susceptibility genes in pool 9**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Gene name** | **Description** | **esiRNA** | **EXP, REG** | **Evidence score** |
| *KCNA3* | Voltage-gated, shaker-related | 84\*\*\* | ++++,  -0.07 | 5 |
| *KCNB2* | Voltage-gated delayed rectifier, Shab-related | 84\* | ++,  0.07 | 4 |
| *KCNIP4* | Voltage-gated interacting protein, binds Ca2+ | -24 \*\* | +,  -0.22 | 1 |
| *KCNJ3* | K+ inwardly-rectifying channel | 17 | +++,  -0.07 | 5 |
| *SLC24A3* | Solute carrier, Ca2+/Na+/K+ exchanger | -7 | ++++, -0.15 | 2 |

EXP/REG in amebiasis indicates the level of colonic expression and regulation of genes during human amebiasis. EXP of all *KCN* genes in the colon was determined and is indicated: (+) 0-25%, (++) 25-50%, (+++) 50-75%, (++++) 75-100%. Regulation is the difference in mean expression Day 1 (acute disease)- mean expression day 60 (recovery) (n=8).

esiRNA: % killing of esiRNA silenced target compared to control. 2-tailed *t* test *P* < 0.05, \*\* *P* < 0.005, \*\*\**P* < 0.001

**Table 5: Effect of channel inhibitors on amebic killing of UMUC3 cells**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | | **washout** | |
| **IC50 μm** | **ICmax μm** | **IC50 μm** | **ICmax μm** |
| ibutilide (IBU) | 4.4 | 47 (0%) | 4.7 | 47 (0%) |
| 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) | 4.6 | 50 (6%) | 14.9 | 94 (10%) |
| benzamil (BNZ) | 8.2 | 50 (1%) | 4.4 | 47 (7%) |
| tetraethylammonium chloride (TEA) | 45.4 | 234 (0%) | 37.2 | 234 (11%) |
| diltazem (DIL) | 25.9 | 50 (12%) | 57.6 | 750 (25%) |
| 4-aminopyrimadine (4-AP) | 1366 | 188 (44%) | 807 | 188 (44%) |
| procaine (PRO) | 1571 | 375 (32%) | 35.8 | 4.7(9%) |
| quinine (QUI) | 712 | 47 (51%) | 653 | 47 (46%) |

concentrations in μm

ICmax- concentration with maximum inhibition of amebic cytotoxicity, the % killing at the ICmax relative to untreated cells is shown in parenthesis

**Table 6: Effect of specific channel inhibitors on amebic killing intestinal epithelial cells (HT29) and macrophages (THP1)**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **HT29** | | | **THP1** | | |
| **IC50** | **95% CI** | **ICmax** | **IC50** | **95% CI** | **ICmax** |
| KCl | 6.8 mM | 5.2 - 8.9 | 50 mM (3.6%) | 11.9 mM | 8.4 - 16.8 | 12.5 mM (33.7%) |
| K2S04 | 9.1 mM | 5.2 - 16.1 | 12.5 mM (30.7%) | 13.8 mM | 6.2 - 30.8 | 25 mM (32.6%) |
| ChoCl | 52.2 mM | 34.2 - 79.8 | 50 mM (36.4%) | 29.2 mM | 16.3 - 52.3 | 12.5 mM (44.9%) |
| NaCl | 21.8 mM | 13.3 - 35.7 | 50 mM (11.6%) | 58.9 mM | 30.6 - 113.2 | 50 mM (41.5%) |
| clotrimazole  (CLO) | 5.9 μM | 4.0 - 8.8 | 6.25 μM (24.3%) | 13.9 μM | 7.4 – 26.0 | 6.25 μM (41.3%) |
| Paxilline (PAX) | 4.9 μM | 3.6 - 6.6 | 25 μM (16.0%) | 6.2 μM | 4.4 – 8.6 | 50 μM (14.4%) |
| Chromanol 293 (293B) | 6.9 μM | 4.2 - 11.3 | 25 μM (42.6%) | 7.8 μM | 5.1 – 12.1 | 25 μM (37.7%) |
| AM 92016 | 7.3 μM | 3.9 -13.8 | 12.5 μM (39.1%) | 4.1 μM | 2.5 – 6.7 | 12.5 μM (9.3%) |
| CP 339818 | 9.0 μM | 5.2 – 15.4 | 50 μM (35.8%) | 5.2 μM | 2.7 – 9.7 | 50 μM (13.5%) |
| TEA | 7.8 μM | 5.0 – 12.3 | 12.5 μM (36.1%) | 20.5 μM | 11.5 - 36.4 | 12.5 μM (53.8%) |

ICmax- concentration with maximum inhibition of amebic cytotoxicity and the % killing at the ICmax relative to untreated cells is shown in parenthesis (untreated-ICmax treated).

K2SO4 concentration reflects K+ ion concentration (2X mM K2SO4)

**FIGURE LEGENDS**

**FIGURE 1. Design and implementation of a whole genome RNAi screen to identify host factors required for amebic cytotoxicity** (a) Screening method for library cells were plated in culture dishes at low density and co-incubated with *E. histolytica* parasites for 2 hours. After selection, the parasites were removed and cells were cultured for 48-72 hours to expand resistant shRNA clones. The expanded pools selected in successive rounds at a ratio of 1 parasite to 5 host cells, except for the ninth and final round of selection, which was at a ratio of 1 parasite to 2 host cells. N = rounds of selection; P = pools of clones. (b) Selection increased resistance to amebic killing. Pool 6 of the RNAi library pool exhibited increased resistance relative to an empty vector pool selected in parallel after 6 rounds of selection. In the ninth and final round of selection each library was selected until no surviving cells were visible by microscopic examination. % survival of UMUC3 cells was determined by visual assessment of the monolayers after selection. The final round of selection (9) was done until no cells remained visible, cells did survive round 9 of selection and were used for further analysis (pool 9) (c) The selected RNAi library (RNAi) and the empty vector (EV) control from pool 9 of the screen were assayed for resistance to amebic killing. Cells were plated at a low density as in the screen and co-incubated with *E. histolytica* at a ratio of 1 parasite to 5 host cells. Cell killing was determined by measuring LDH in cellular supernatants. The mean of biological triplicates and SE is shown. \**P* < 0.01, \*\* < 0.001 by 2-tailed *t* test. (d) Bioinformatics analysis of candidate susceptibility genes. The top overrepresented biological processes are shown with their enrichment value. (e) The cluster of ion transport associated processes within pool 9 (similarity scores >0.3 with kappa scores shown). The bar graph represents the number of genes that are annotated for transport of a specific ion. Some transporters, for example, SLC24A3, have more than one substrate (K+/Ca2+/Na+).

**FIGURE 2. Validation of candidate susceptibility genes in a secondary screen.** 54 genes from pool 9 were selected for validation by esiRNA. Each knockdown was tested individually in a well-based assay of amebic cytotoxicity. Cytotoxicity was measured after 1 hour at a ratio of 1:5 parasites to host cells. Cytotoxicity was normalized to FLUC controls for each knockdown. K+ channels are bolded. The mean of three experimental replicates from at least three independent experiments is shown. Error bars represent the SE of the mean triplicate independent experiments. *P* values were calculated relative to FLUC cytotoxicity. \**P* < 0.01, \*\* < 0.001, \*\*\* < 0.001 by 2-tailed *t* test.

**FIGURE 3. Specific ion transport inhibitors blocked amebic cytotoxicity in UMUC3 cells.** Inhibitors of ion channels hits from the RNAi screen were tested for the ability to block amebic cytotoxicity *in vitro* in UMUC3 cells. Inhibitors were added to cells at the concentrations indicated above. After 30 minutes, inhibitors were removed from cells (+Washout +EH) or left in contact with cells (+Drug +EH). *E. histolytica* trophozoites were added at a ratio of 1:5 trophozoites to host cells. The addition of *E. histolytica* resulted in a 1:2 dilution of the inhibitors for the period of co-incubation (concentrations prior to dilution are shown on graph). LDH release was measured after 30 minutes. IC50 was determined by non-linear regression of the log10 of the inhibitor concentration vs. normalized cytotoxicity. IC50 determinations excluded drug concentrations that resulted in toxicity to UMUC3 cells in the absence of *E. histolytica.*

**FIGURE 4 *KCN* gene expression and regulation in the human colon in amebiais**. Average gene expression (day 60 – X axis) and regulation during acute amebiasis (Δ day 1- day 60 – Y axis) of the 94 annotated *KCN* genes. *KCN* was omitted from K+ channel gene names for clarity. *SLC24A3* and *CFTR* are shown for reference. *KCN* genes with documented intestinal expression are indicated with blue. Red indicates genes that were significantly differentially regulated in disease by 2-way *ANOVA* of acute amebiasis (day 1) expression vs. recovery (day 60) expression (n=8), *P<* 0.001. Quadrants divide the graph by mean expression (day 60) and mean regulation (Δ day 1- day 60) for gene expression of all probes on the microarray. The day 60 expression (recovered human colon, X-axis) was significantly correlated with the regulation during disease (Y axis), R=-0.54 *P >* 0.0001. (GEO accession GSE23750)40.

**FIGURE 5. K+ inhibitors blocked amebic cytotoxicity and K+ activation by *E. histolytica* in intestinal epithelial cells and macrophages**. (a) Chemical inhibition of ion transport blocked amebic cytotoxicity. Cells were switched to the indicated concentrations of chemicals immediately prior to the addition of *E. histolytica.* (b,c) Specific K+ channel inhibitors blocked amebic cytotoxicity. Cells were treated with inhibitors for 30 minutes prior to the addition of *E. histolytica*. (d) K+ channel activation by *E. histolytica.* Fluorescence values (F) correspond to thallium influx through open K+ channels. F values were normalized to the initial baseline value (F0). *E. histolytica* (+EH) or vehicle (-EH) was added after 40 seconds. The mean of 3 biological replicates with standard error for +EH is shown, -EH values are single measurements. (d) Inhibitors blocked K+ channel activation by *E. histolytica.* The area under the curve (AUC) of the fluorescence for each inhibitor and control were calculated in the presence (+EH) and absence of *E. histolytica* (-EH). Inhibitor concentrations were: (KCl - 25 mM, 293B-10 um CLO -10 um, PAX - 10 um, ChoCl – 25 mM). The AUC (% of control) for each inhibitor was normalized to the mean of the AUC media control (+EH was normalized to +EH control, -EH was normalized to –EH control). (+EH is the AUC of three biological replicates and error bars represent the SEM. *P*-values were calculated relative to untreated cells (\*, *P* < 0.001) by Fisher's LSD test.

**FIGURE 6. *E. histolytica* induced K+ efflux in IECs. (**a) Extracellular K+ ([K+ mM]E) concentrations were measured in supernatants of HT-29 cells incubated with or without *E. histolytica* at a ratio of 1 trophozoite to 5 host cells for 1 hour. LDH was measured concurrently in supernatants to measure cell killing. Symbols represent the mean of three independent experimental values with SEM shown. \*\**P* < 0.001 (+EH) vs. (-EH) by two-tailed fisher’s LSD test. (b) HT-29 cells were loaded with the K+-sensitive fluorescent dye PBFI to measure intracellular K+ concentration ([K+]I) upon interaction with *E. histolytica.* Cells showed a significant reduction in [K+]I after 30 minutes of contact with *E. histolytica.* The mean of 3 biological replicates for each experimental condition relative to the mean of untreated wild type cells is shown; error bars are the SEM of these values. *P*-values were calculated relative to untreated cells (\*, *P* < 0.05; \*\*, *P* < 0.005; \*\*\*, *P* < 0.001) by an unpaired two-tailed t test.

**FIGURE 7. Inhibition of K+ channel activity blocked amebic cytotoxicity in HT-29 IECs and amebic cytotoxicity and IL-1β production in THP-1 macrophages*.*** (a) IL-1β secretion was measured by ELISA in HT-29 cells and differentiated THP1 macrophages exposed to *E. histolytica* for 180 minutes (1 trophozoite to 5 cells). HT-29 cells did not secrete a detectable level of IL-1β (data not shown). Cytotoxicity of HT29 and THP1 cells was measured by LDH release. Inhibitors did not cause LDH release in the absence of *E. histolytica.* The mean of 3 experimental values per treatment was normalized and expressed as a percent of vehicle treated controls with standard error shown *P*-values were calculated relative to untreated cells (\*, *P* < 0.05; \*\*, *P* ≤ 0.008) by two-tailed Fisher's LSD test. (b) LDH, IL-1β and cleaved caspase-1 were measured WT and ASC-/- THP1 cells. WT cells were treated with KCl (50 mM), ChoCl (50 mM) AM92016 (10 μm) and YVAD (10 μm). The mean of each experimental condition relative to untreated wild type cells is shown; and error bars represent SEM. *P*-values were calculated relative to untreated cells (\*, *P* = 0.02; \*\*, *P* ≤ 0.002) by two-tailed Fisher's LSD test.

**FIGURE 8. Model for *E. histolytica* activation of K+ channels in host cell killing.** In both intestinal epithelial and immune cells, *E. histolytica* triggers increased intracellular Ca2+ and cAMP. In an intestinal epithelial cell (purple cell on the right), Cl- efflux is mediated by apical CFTR, while K+ efflux occurs at both apical and basolateral surfaces. Increased intracellular Ca2+ activates large conductance K+ channels in the apical and basolateral membranes. Cl- efflux may be activated directly by increased intracellular Ca2+ and/or cAMP or may occur secondarily to K+ efflux to regulate cellular charge polarization. As K+ and Cl- efflux occurs, water and intracellular ion concentrations fall which causes cells to shrink. Cell shrinkage and decreased intracellular K+ trigger caspase activation. In intestinal epithelial cells it appears that caspase-3 is activated leading to apoptotic death. In macrophages (blue cell on the left), decreased cytosolic K+ concentration mediated caspase-1 activation leading to inflammasome activation, IL-1β secretion and pyroptotic cell death.

**FIGURE S1. Validation and optimization of a whole genome RNAi screen to identify host factors important in amebic cytotoxicity.** (a) UMUC3 cells were susceptible to amebic cytotoxicity and killing was inhibited by 100 mM galactose at ratios of 1:5 and 1:10 parasite to UMUC3 cells. 100 mM glucose was tested as an osmotic control. \*\*\* *P*=0.0005. (b) Survival of UMUC3 cells at high (5 x 105 cells/ml) and low density (1 x 105 cells/ml) plating. UMUC3 cells at a low density were less susceptible to amebic killing at the same parasite to host cell ratio than were host cells plated at a high density. The lower density was used at a 1:5 ratio of parasites to host cells to minimize potential bystander effects on adjacent clones during *E. histolytica* killing. (c) Pools of resistant clones were sequenced to identify the shRNA construct expressed in each UMUC3 cell present in a selected pool. Sequence abundance of each clone (TRCN ID) was plotted on a logarithmic scale. shRNA sequences were normally distributed in the selected libraries. Clones with fewer than 10 sequencing read were excluded from the analysis.

**FIGURE S2**. **Inhibition of host caspases blocked amebic cytotoxicity in HT-29 IECs and amebic cytotoxicity and IL-1β production in THP-1 macrophages***.* IL-1β secretion was measured by ELISA in differentiated THP-1macrophages exposed to *E. histolytica* for 180 minutes. Cytotoxicity was measured by LDH release. For (A) and (B), % inhibition of cytotoxicity was calculated relative to untreated cells; the mean of 3 experimental values is shown; and error bars are the SEM of these values. *P*-values were calculated relative to untreated cells (\*, *P* < 0.05; \*\*, *P* < 0.005; \*\*\*, *P* < 0.001) using an unpaired two-tailed student’s t-test.

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