For consideration of publication in *Infection and Immunity*

**HIV-1 Tat Protein Enhances *Cryptosporidium parvum* -induced Apoptosis in Cholangiocytes via a Fas Ligand-dependent Mechanism**

**Running title:** Synergistic effect of Tat on *C. parvum*-induced apoptosis

Steven P. O’Hara, Aaron Small, Jeremy B. Nelson, Andrew D. Badley*, Xian-Ming Chen, Gregory J. Gores and Nicholas F. LaRusso*

Miles and Shirley Fiterman Center for Digestive Diseases, Division of Gastroenterology and Hepatology; #Infectious Diseases Research; Mayo Clinic College of Medicine, Rochester, MN 55905

This work was supported by National Institutes of Health Grants DK57993 and DK24031 (N.F.L), A1062261 (A.D.B), DK063947 (G.J.G.), A2071321 (X.C.) and by the Mayo Foundation.

* Address Correspondence: Miles and Shirley Fiterman Center for Digestive Diseases

Mayo Clinic College of Medicine
200 First Street, SW
Rochester, MN 55905
E-mail: larusso.nicholas@mayo.edu

Manuscript information: total text pages: 22; Figures: 10; tables: none
Word and character counts: abstract, 245 words; total characters, 49,151

The abbreviations used are: HIV-1, human immunodeficiency virus 1; AIDS, acquired immune deficiency syndrome; Tat, HIV trans-activator of transcription; FasL, Fas Ligand; NF-kB, Nuclear factor-kappa B
ABSTRACT

While *Cryptosporidium parvum* infection of the intestine has been reported both in immunocompetent and immunocompromised individuals, biliary infection is seen primarily in adult AIDS patients and is associated with development of AIDS-cholangiopathy. However, the mechanisms of pathogen-induced AIDS-cholangiopathy remain unclear. Since we previously demonstrated the involvement of the Fas/FasL system in paracrine-mediated *C. parvum* cytopathicity in cholangiocytes, we also tested the potential synergistic effects of HIV-1 Tat-mediated FasL regulation on *C. parvum*-induced apoptosis in cholangiocytes by semiquantitative RT-PCR, immunoblotting, immunofluorescence, and immunogold electron microscopy. H69 cells do not express CXCR4 and CCR5, receptors required for direct HIV-1 viral infection. However, recombinant biologically active HIV-1-associated Tat protein increased FasL expression in the cytoplasm of cholangiocytes without a significant increase of apoptosis. We found that *C. parvum*-induced apoptosis was associated with translocation of intracellular FasL to the cell membrane surface and release of full-length FasL from infected H69 cells. Tat significantly (*p < 0.05*) increased *C. parvum*-induced apoptosis in bystander cells in a dose-dependent manner. Moreover, Tat enhanced both *C. parvum*-induced FasL membrane translocation and release of full-length FasL. In addition, the FasL neutralizing antibody, NOK-1, and the Caspase-8 inhibitor, Z-IETD-fmk, both blocked *C. parvum*-induced apoptosis in cholangiocytes. The data demonstrate synergistic effects of HIV-1 Tat on *C. parvum*-induced cholangiocyte apoptosis via a paracrine-mediated, FasL-dependent mechanism. Our results suggest that concurrent active HIV replication, with associated production of Tat protein, and *C. parvum* infection
*parvum* infection synergistically increase cholangiocyte apoptosis and thus jointly contribute to AIDS-related cholangiopathies.

**Key words:** Apoptosis, HIV-1, AIDS-related cholangiopathies, Tat, *Cryptosporidium parvum*, Synergistic, Fas, Fas Ligand.
INTRODUCTION

Cryptosporidium parvum is an enteric pathogen and a common cause of diarrhea in humans and animals worldwide. In the immunocompetent, infection is usually limited to the intestine and causes an acute, self-limited disease. However, in the immunosuppressed, cryptosporidiosis may cause potentially fatal complications, including bile duct damage (12, 17, 21, 25, 30). AIDS-cholangiopathy, a group of biliary disorders including secondary sclerosing cholangitis and acalculous cholecystitis seen in AIDS patients, causes significant morbidity and mortality (35, 50). Recent studies indicate that development of this biliary syndrome involves opportunistic infection of the biliary tree. While a number of pathogens have been implicated, C. parvum is the single most common identifiable pathogen associated with this disease. Although biliary cryptosporidiosis is also seen in boys with X-linked immunodeficiency (18, 22), in adults, it has only been reported in patients with AIDS (12, 17, 21, 25, 30). In contrast, C. parvum infection of the intestine has been reported both in immunocompetent and immunocompromised subjects (40). Thus, unique synergistic pathologic effects on the biliary tree may exist between HIV-1 and C. parvum.

It is established that HIV-1 infects hepatocytes, Kupffer cells, and endothelial cells in the liver (8, 23); cholangiocyte infection has never been documented. During active replication, HIV-1 can also affect uninfected cells via release of HIV-derived soluble peptides from directly infected cells (27, 45, 46). One of these peptides, Tat (HIV-encoded trans-activator of transcription), circulates in the blood and is taken up by most cell types, including epithelial cells (44). Tat is essential for replication of the viral genome and is proposed to be an important factor in HIV-induced pathogenesis of AIDS
Exogenous Tat has a variety of effects on cellular processes in uninfected cells, including increased binding of NF-kB to DNA, increased production of cytokines, increased expression of cytokine receptors and modulation of cell survival and proliferation (2, 19, 31, 34, 42). Furthermore, studies have demonstrated that Tat increases apoptosis in a variety of cell types, including those not directly infected by HIV-1 (4, 42). Indeed, Tat synergizes with T-cell activating stimuli in the upregulation of Fas ligand (FasL), a molecule that activates the Fas death receptor resulting in apoptotic cell death (4, 48, 49).

Although the clinical features of biliary cryptosporidiosis in AIDS patients are well documented, it is not clear why individuals infected with HIV are susceptible to biliary cryptosporidiosis and associated cholangiopathies in contrast to individuals immunocompromised by other mechanisms. We previously demonstrated that C. parvum is cytopathic to bystander, uninfected cholangiocytes through a paracrine Fas/FasL-dependent apoptotic mechanism (9). It appears that C. parvum possesses a complex virulence capacity to initiate several signaling cascades within the infected host cell that may contribute to the development of disease, including the activation of nuclear factor-kappa B (NF-kB) signaling pathway resulting in apoptotic resistance in directly infected cells (14). Interestingly, C. parvum infection also upregulates FasL expression in infected cells and causes apoptosis in bystander non-infected cells via activation of the Fas/FasL death pathway (9). Highly active antiretroviral therapy (HAART) has decreased the incidence of AIDS-cholangiopathies and has dramatically improved survival rates of individuals with AIDS-cholangiopathies (29). However, C. parvum biliary infection and AIDS-cholangiopathy remain significant problems in
individuals that do not respond or do not have access to this therapy. How *C. parvum* activates the Fas/FasL death pathway and whether soluble factors released during active HIV-1 replication potentiate *C. parvum*-induced cholangiocyte death are unclear.

In the work described here, we show that apoptotic cell death in bystander cholangiocytes induced by *C. parvum* is associated with FasL membrane translocation and release of full-length FasL in infected cells. HIV-1 Tat protein increases FasL protein expression in the cytoplasm of cultured cholangiocytes without an increase of apoptosis. Moreover, Tat enhances *C. parvum*-induced FasL membrane translocation and release of full-length FasL in infected cells and consequently, apoptotic cell death in uninfected bystander cells. Therefore, Tat sensitizes cultured cholangiocytes to *C. parvum*-induced Fas/FasL-dependent apoptotic cell death in bystander cells, an observation which may explain why *C. parvum* damage of biliary epithelia occurs almost exclusively in patients with HIV.

**MATERIALS AND METHODS**

*C. parvum*. *C. parvum* oocysts harvested from calves inoculated with a strain originally obtained from Dr. Harley Moon at the National Animal Disease Center (Ames, IA) were purchased from a commercial source (Bunchgrass Farms, Troy Idaho). The oocysts were purified using a modified ether extraction protocol, suspended in PBS, and stored at 4 °C. Prior to cell culture infection, oocysts were treated with 1% sodium hypochlorite on ice for 20 min and then incubated in an excystation solution consisting of 0.75% taurodeoxycholate and 0.25% trypsin for 15 min at 37 °C. The treated oocysts were then washed in Dulbecco modified Eagle medium (DMEM)-F12 medium (Bio Whittaker,
Walkersville, Maryland) and the excysted sporozoites, intact oocysts, and empty oocysts were collected by centrifugation at 3000 rpm for 5 min. Sporozoite viability was assessed as described previously (7).

H69 cells and recombinant HIV-1 Tat protein. The human bile duct epithelia cell line, H69, is an SV40-transformed cell line originally derived from a normal liver harvested for transplant. The cells have been extensively characterized and continue to express cholangiocyte markers consistent with biliary function (20). Cells between passages 20 and 30 were used for the study. Recombinant full-length HIV-1 Tat protein (86 amino acid peptide) was purchased from Immunodiagnostics (Woburn, MA). A concentration of 100 to 500 ng/ml Tat showed no cytotoxic effects on H69 cells or on C. parvum sporozoites and was selected for the study. The concentration used throughout the study is consistent with other studies addressing physiological roles of Tat in tissues, where localized Tat concentration is expected to be slightly greater than that detected in serum from HIV-infected individuals (2 to 40 ng/ml) (43, 51).

Immunofluorescence and rtPCR for the HIV-1 co-receptors CXCR4 and CCR5. HIV-1 infection of cholangiocytes has never been documented or demonstrated; in fact cholangiocytes lack the HIV-1 receptor CD-4 (Paya, personal communication). We therefore attempted to localize the HIV-1 co-receptors CXCR4 and CCR5 to further demonstrate that HIV-1 cannot directly infect cholangiocytes. H69 cells and the human colonic adenocarcinoma cell line HT-29 (positive control) were grown to 70-80% confluence on 4 or 8 well slides and processed for immunofluorescence. Briefly, the
cells were fixed with 0.1 M PIPES (pH 6.95), 1 mM [ethylene-bis(oxyethylenenitrilo)]
tetraacetic acid, 3 mM MgSO4 and 2% paraformaldehyde. The cells were incubated
with mouse anti-human monoclonal antibodies against CXCR4 (MCA1619) or CCR5
(MCA1835) (Serotec, Raleigh, NC) followed by fluorescein-conjugated secondary anti-
mouse antibodies. Slides were then mounted with mounting medium (H-1000, Vector
Laboratories) and analyzed on a Zeiss LSM510 confocal microscope. rtPCR was also
performed to detect CXCR4 and CCR5 in control cells and H69 cells. Primer
sequences: CXCR4: forward primer: 5'-GGTGGTCTATGTTGGCGTCT-3'; reverse
primer: 5'-TGGAGTGTGACAGCTTGGAG-3'; CCR5: forward primer: 5'-
TAGTCATCTTGGGGCTGGTC-3'; reverse primer: 5'-TGTAGGGAGCCCAGAAGAGA-
3'. The amplicons were sequenced to confirm target amplification. QuantumRNA
Universal 18s primer pair (Ambion, Austin, TX) was used to confirm loading.

Infection assay. H69 cells were incubated prior to C. parvum infection for at least 4
hours in assay medium consisting of DMEM-F12 medium, 100 U/ml penicillin, and 100
µg/ml streptomycin (Life Technologies, Carlsbad, CA) in the presence or absence of Tat
at a concentration of 100 to 500 ng/ml. Freshly excysted C. parvum sporozoites were
resuspended in fresh media in the presence or absence of Tat protein and added to the
cell culture at a concentration of 5 X 10^5 sporozoites/well (9). The infected cell cultures
were incubated for 2 hours, fixed in 2% paraformaldehyde, and processed for
immunofluorescence using a C. parvum specific polyclonal antibody, anti-CP2, as we
previously reported (41). The infection percentage was determined by counting
infection sites per total cells in 20 fields at 400x magnification.
Apoptotic cytotoxicity. A co-culture system was used to address apoptotic cytotoxicity in an infected population of cells and uninfected, cocultured, bystander cells (9). Briefly, H69 cells were grown to 70-80% confluence in six-well Costar tissue culture inserts (Becton Dickinson Labware) with cells both on the inserts (upper chamber) and on the plates below the inserts (lower chamber). The two cell populations were separated by a polycarbonate membrane with a high-density (0.4 μm) pore size, which allows free movement of molecules (including Tat but not parasite) between the upper and lower chamber. Upon removal of H69 media both cell populations were washed once in DMEM-F12 media and resuspended in assay medium as described above. For Tat treated cells, recombinant Tat was added to the upper chamber and allowed to equilibrate between the upper and lower chambers for 3 hours to give a final concentration of 100-250 ng/ml in both the upper and lower chamber. 5 X 10⁵ sporozoites were then added to the upper chamber. After an overnight incubation (~14 hr), cells in the upper and lower chambers were fixed and apoptosis was quantified. Cells were stained with the nuclear staining dye DAPI (2.5 μM, 5 min) and fluorescein-labeled annexin V per manufacturers instructions (Pharmlingen) and viewed with a fluorescence microscope. The percentage of apoptotic cells was determined by counting the number of apoptotic cells per total cells in 20 fields at 400x magnification. The number of cells with both positive annexin V and nuclear morphology characteristic of apoptosis (i.e. condensation, margination, and/or fragmentation) were recorded (9). A control experiment was performed in which sporozoites were added to the upper chamber in the absence of H69 cells and cocultured with H69 cells grown on the plates...
in the lower chamber, the cells in the lower chamber were then assessed for apoptosis as above. Apoptosis inhibition assays were performed in 4-well slides in the presence or absence of Tat, the Caspase inhibitor Z-IETD-fmk (20mM), or the FasL antagonist antibody Nok1. Apoptotic cells were counted as above. To confirm apoptotic activity, cells were grown on a 96 well plate in the presence of Tat, *C. parvum*, and the FasL antagonist antibody Nok1. The Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, WI) was then performed per manufactures suggestion. The data represent experiments performed in triplicate.

**Intracellular FasL expression and membrane translocation by immunofluorescent and immunoelectron microscopy.** Scanning laser confocal microscopy was used to assess the distribution of FasL in *C. parvum* infected or uninfected H69 cells in the presence or absence of Tat protein. H69 cells were grown in 8 well chamber slides to 70-80% confluence in H69 media. The cells were subsequently washed in DMEM-F12 media and incubated in infection media or infection media plus Tat protein (100 ng/ml) for 4 hr. Upon infection, *C. parvum* sporozoites were suspended in fresh media or media plus Tat and added to individual wells at a concentration of 5 x 10^5 sporozoites/well. Infections were allowed to proceed overnight (14 hr). Non-permeabilized cells were fixed as above and processed for immunofluorescence to reveal membrane surface labeling of FasL. Permeabilized cells were fixed as above and subsequently treated with 0.2% Triton-X 100 for 5 minutes to reveal total FasL (both intracellular and membrane surface). The cells were then incubated with primary antibody against FasL (G247-4, Pharmingen; or NOK-1, BD Biosciences), and the C.
parvum specific polyclonal antibody, anti-CP2, followed by fluorescein and rhodamine-labeled secondary antibody, respectively. Slides were then mounted as described above followed by confocal scanning laser microscopy using identical parameters for each treatment, which was optimized for each antibody. Contrast and intensity for each image were manipulated uniformly using Adobe (Mountain View, CA) Photoshop software. Fluorescent intensity was determined using the Zeiss LSM510 software.

Briefly, individual cells (a minimum of 50 from each experimental condition) were traced and the mean value of pixel intensity was determined for the selected area. The average intensity was then determined for the population of cells from each condition.

For immunogold labeling, cells were fixed and processed as described previously (15). The relative distribution of FasL was determined by counting gold particles over cell profiles; totals are presented as gold particles per 10 μm². Plasma membrane labeling was quantitated by calculating the percentage of total gold particles associated with the plasma membrane. A total of five uninfected H69 control images and ten parasite infection sites were used for the analysis.

Immunoblot detection of FasL. H69 cells were grown to 70-80% confluence in T25 flasks and then exposed to C. parvum in media with or without 100-250 ng/ml Tat protein. After an overnight infection, cells were lysed and quantitative immunoblots were performed as previously described (9). Briefly, samples were separated by SDS-PAGE, and proteins were transferred to nitrocellulose membranes. Membranes were sequentially incubated with primary antibodies and then with 0.2 μg/ml horseradish peroxidase-conjugated secondary antibody and revealed by an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, England). The
FasL antibodies C-178 (Santa Cruz Biotechnology, Santa Cruz, CA) and Clone 33 (Transduction Laboratories) were used to confirm FasL reactivity in the blots. The data represent immunoblots from three separate experiments. Semi-quantitative rtPCR was also utilized to confirm expression in H69 cells. Primer sequences: FasL: forward primer: 5'-ACAACCTGCCCCTGAGCC-3'; reverse primer: 5'-AGTCTTCCTTTTCCATCCC-3'; Fas: forward primer: 5'-ACCAAGTGCAGATGTAAACC-3'; reverse primer: 5'-TTCCTTTCTCTTCACCCAAAC-3'. The intron-spanning amplicons were sequenced to confirm target amplification. QuantumRNA Universal 18s primer pair (Ambion, Austin, TX) was used to confirm equal loading.

To analyze for the release of FasL by H69 cells in response to *C. parvum* infection and/or Tat treatment, H69 cells were grown to 70-80% confluence in T25 flasks. After an overnight infection in the presence or absence of Tat protein, supernatants were collected for assays. To detect FasL in the media, the supernatants were spun at 8,000 rpm for 5 minutes, 100 μl aliquots were removed and concentrated approximately 10X using an ultra free concentrator with 10,000 molecular weight limits after 1 hour of spinning at 3,000 rpm at 4°C. The concentrated supernatants were then mixed with 5X Laemmli sample buffer, boiled, and loaded into wells for SDS-PAGE followed by immunoblot analysis with the FasL antibodies Clone 33 and G247-4 as described above.
Statistical analysis. All values are given as means ± SE. Means of groups were compared with Student’s (unpaired) t-test or ANOVA test where appropriate. p < 0.05 was considered statistically significant.

RESULTS

H69 cells do not express HIV-1 co-receptors. HIV-1 requires the cellular receptors CXCR4 (X4-tropic virus) or CCR5 (R5-tropic virus) for entrance into host epithelial cells (31). Using immunofluorescence microscopy, we were unable to detect either CXCR4 or CCR5 in our cultured human cholangiocytes while control HT29 cells, which are susceptible to HIV-1 infection, express both receptors (Fig. 1). Furthermore, using RT-PCR, we were not able to detect the mRNA for either of these receptors in human cholangiocytes (Fig. 1).

Tat protein does not affect C. parvum infection of cultured cholangiocytes. To determine if Tat protein affected C. parvum infection of cholangiocytes, H69 cells were pre-incubated for 4 hours with 100-250 ng/ml Tat protein and subsequently infected with C. parvum sporozoites. Tat administration had no effect on parasite invasion of cholangiocytes (23.2% of cultured cells infected [100 ng/ml Tat] and 21.4% [250 ng/ml Tat] vs 23.4% [no Tat], p > 0.05).

Tat protein potentiates C. parvum-induced apoptosis in bystander non-infected cholangiocytes. To test the potential effects of Tat treatment on C. parvum-induced apoptosis in bystander cholangiocytes, we used our co-culture system in which H69
cells grown in the upper chamber were exposed to *C. parvum*. While H69 cells in the lower chamber are not directly infected by *C. parvum*, molecules smaller than 0.4 μm (e.g., Tat) can pass through the filter separating the two cell populations as previously described (9). Approximately 2% of the cells from both the upper and lower chamber were found to undergo apoptosis after Tat protein treatment (either 100 or 250 ng/ml), similar to what was observed in non-treated control cells (Fig. 2A and B). Consistent with our previous study (14), a significant increase of apoptosis was detected in cells both in the upper chamber (7-fold, p < 0.001) and in the lower chamber (4-fold, p < 0.001) when only the cells in the upper chamber were exposed to *C. parvum* (Fig. 2A and B). Consistently more apoptosis was detected in the upper chambers than that in the lower chambers. The cells undergoing apoptosis in the upper chamber, which were directly exposed to *C. parvum*, were not those that were directly infected as assessed by confocal microscopy using anti-CP2 antibody to *C. parvum* (data not shown), confirming our previous observations (14). Importantly, when the cells in the upper chamber were first treated with Tat and then exposed to *C. parvum*, a significant increase in apoptosis was observed in cells both in the upper chamber (1.5 to 2-fold, p < 0.01) and in the lower chamber (1.3 to 1.8-fold, p < 0.01) in a Tat dose-dependent manner (Fig. 2A and B). In the upper-chamber, apoptosis was again observed predominantly in neighboring, non-directly infected cells (Fig. 2C). Taken together, these data suggest that Tat protein potentiates *C. parvum*-induced apoptosis in bystander non-infected cholangiocytes.
Tat protein upregulates FasL expression in the cytoplasm of cultured cholangiocytes. To test how Tat potentiates *C. parvum*-associated apoptosis in cholangiocytes, we assessed expression of Fas and FasL in H69 cells in the presence or absence of Tat protein. As previously reported, uninfected cholangiocytes expressed both Fas and FasL protein (Fig. 3A). Fas protein expression did not change in cells after treatment with Tat (Fig. 3A). However, using two FasL specific antibodies (C-178 and Clone 33), we observed a significant, dose-dependent increase in FasL protein expression in cell lysates in the presence of Tat compared to untreated cells (Fig. 3A). Quantitative analysis of Fas and FasL expression by Western blot is shown in Fig. 3B.

Expression of Fas and FasL mRNA was also assessed. As previously reported, uninfected cholangiocytes expressed mRNA for both Fas and FasL (Fig. 3c). Tat treatment had no effect of Fas mRNA expression but did cause a statistically significant dose dependent increase in FasL expression by RT-PCR (Fig. 3C). Semi-quantitative analysis of Fas and FasL mRNA expression for three experiments is shown in Fig. 3D.

To examine the cellular distribution of FasL in cholangiocytes, we stained H69 cells with and without Tat treatment using antibodies to FasL under two different conditions: cells with membrane permeabilization to stain both cytoplasmic and membrane distribution and cells without membrane permeabilization to stain membrane surface FasL only. No detectable FasL fluorescence was observed in cells without membrane permeabilization both in the presence or absence of Tat (Fig. 4A and C), suggesting no membrane translocation of FasL in both non-treated and Tat treated cells. In contrast, in membrane permeabilized cells which reflect both intracellular and membrane surface FasL, we observed a punctate, intracellular distribution of FasL in
cells in the absence of Tat (Fig. 4B). Importantly, a much stronger fluorescence was
observed in cells in the presence of Tat indicating an increase in FasL protein
expression (Fig. 4D). Quantification of fluorescent intensity of FasL in confocal images
is shown in Fig. 4E. These data suggest that cholangiocytes normally express a basal
level of FasL in the cytoplasm and that Tat protein increases intracellular FasL
expression without membrane translocation in cholangiocytes.

Tat protein enhances C. parvum-induced FasL membrane translocation and FasL
release in infected cells. Our previous studies demonstrated that C. parvum not only
upregulates FasL expression in infected cells but also caused a paracrine-mediated,
FasL-dependent apoptosis in bystander cells (9). To test how C. parvum activates
FasL-dependent apoptosis in bystander cells, we measured C. parvum-induced FasL
protein expression and membrane translocation in infected H69 cells in the presence
and absence of Tat. Consistent with our previous studies, an increase of FasL protein
expression was detected in lysates of C. parvum infected cell cultures by
immunoblotting (Fig. 5A and B). Moreover, a dose dependent, synergistic increase in
FasL expression was detected in C. parvum infected cell cultures in the presence of Tat
protein (Fig. 5A and B). Using confocal microscopy of cells without membrane
permeabilization, we detected a significant increase of FasL on the plasma membrane
in directly infected cells (Fig. 6B) compared with normal control cells (Fig. 6A) or
bystander, non-infected cells (asterisks in Fig. 6B). Additionally, a much stronger FasL
membrane surface labeling was detected in C. parvum-infected cells in the presence of
Tat compared with infected cells without Tat (Fig. 6C). Quantitative analysis of FasL
membrane labeling is shown in Fig. 6D.
To test whether *C. parvum*-induced FasL membrane translocation results in release of FasL from infected cells, we assessed FasL in the supernatants from cells following *C. parvum* infection. Cell culture supernatants were harvested after overnight infection, concentrated 10-fold, and immunoblotted for FasL expression. Two FasL specific antibodies were used to detect FasL in the media from infected cell cultures. No FasL was detected in the media of uninfected cells (lane 1 in Fig. 7). A detectable amount of full length was found in the supernatants from *C. parvum*-infected cells (lane 2 in Fig. 7). Minimal FasL was detected in the media of cells after treatment with Tat alone (lane 3 in Fig. 7A). However, significantly more FasL was detected in the supernatants from cells exposed to *C. parvum* and Tat compared to those exposed to *C. parvum* without Tat (lane 4 vs. lane 2 in Fig. 7). These data suggest that Tat protein enhances *C. parvum*-induced FasL membrane translocation and FasL release in infected cells. Immunogold electron microscopy was used to visualize the distribution of FasL in infected cells. In accordance with our fluorescence observations, very little labeling was observed in uninfected cells (Fig. 8A and B), while infected cells exhibited extensive FasL labeling on the plasma membrane and on membranous protrusions (Fig. 8C and D). Quantitative analysis of gold particle distribution is shown in Fig. 8E and F. The immunogold data suggest that *C. parvum* infection not only upregulates FasL, but induces membrane translocation in infected cells.

**Inhibition of the Fas/FasL pathway inhibits Tat-enhanced, *C. parvum*-induced apoptosis.** Having established that an increase of FasL protein expression and FasL membrane translocation is associated with *C. parvum* infection and Tat-treatment, we
then asked whether inhibition of the Fas-mediated apoptotic pathway would decrease
*C. parvum*-induced, Tat potentiated cell death. Cells were grown to 70% confluence, incubated in the presence or absence of Tat, and the NOK1 FasL antibody, a neutralizing FasL antibody that blocks FasL-induced apoptosis via inhibition of FasL binding to Fas receptors (39). Cells were then infected overnight and assessed for apoptosis using DAPI nuclear stain and Annexin V fluorescence. The administration of 2.5 μg/ml of the NOK-1 antibody significantly inhibited *C. parvum*-induced apoptosis in the presence and absence of Tat (Fig. 9A). The percentage of apoptotic cells dropped to levels not significantly different from control Tat-untreated, uninfected cells in the presence of 5 μg/ml NOK-1 antibody for the Tat 100 ng/ml treated cells. This observation is supported by Nok1 antibody suppression of Caspase 3/7 activity (compared to isotype control IgG) in Tat treated *C. parvum* infected cell cultures as determined by fluorometric analysis (Fig. 9B). To further assess the anti-apoptotic effects of Fas/FasL pathway inhibition, we performed the same assay in the presence or absence of the caspase-8 specific inhibitor, Z-IETD-fmk, to block the function of caspase-8, a downstream effector of the Fas death signaling pathway involved in *C. parvum*-induced apoptosis in bystander cells as previously reported (9). In the presence of 20 μM Z-IETD-fmk, apoptosis in *C. parvum* infected cells in the presence or absence of Tat was not significantly different than control tat-untreated, uninfected cells (Fig. 9C).
DISCUSSION

The results of our studies provide the first evidence suggesting that HIV-1-associated Tat protein enhances microbial-induced apoptotic cell death. Using a human immortalized but non-malignant cholangiocyte cell line, we have shown that: i) apoptotic cell death in bystander uninfected cholangiocytes induced by C. parvum is associated with membrane translocation in and release of full-length FasL from infected cells; ii) whereas HIV-1-associated Tat protein alone does not cause apoptosis in uninfected cholangiocytes, Tat protein increases FasL protein expression in the cytoplasm of uninfected cholangiocytes and enhances apoptotic cell death induced by C. parvum in bystander uninfected cells; and iii) C. parvum-induced FasL membrane translocation and release of full-length FasL in cholangiocytes are significantly enhanced in the presence of Tat peptide. Thus, synergistic effects between HIV-1 Tat and C. parvum biliary infection potentiate cholangiocyte injury via paracrine-mediated, Fas/FasL-dependent epithelial cell apoptosis, a process potentially involved in the pathogenesis of AIDS-related cholangiopathies.

HIV-1 infects cells via direct binding to membrane surface receptors such as CD4 and CXCR4 and CCR5 (5). Although HIV-1 infects epithelial cells in the intestine and kidney via the CXCR4 and CCR5 receptors (33), our data indicate that cholangiocytes do not express these receptors and, therefore, are not susceptible to direct HIV-1 infection. Indeed, direct HIV-1 infection of cholangiocytes in humans has not been reported. Meanwhile, we, as well as others, have successfully infected cholangiocytes with C. parvum in vitro (13, 47), suggesting that C. parvum infection of cholangiocytes does not require HIV cholangiocyte infection. Here, we show that Tat, a soluble 16 kDa
biologically active peptide released from HIV-1 infected T-cells and macrophages and 
essential for viral replication (2), does not affect C. parvum infection of cultured 
cholangiocytes. However, Tat significantly increases apoptotic cell death in uninfected 
cholangiocytes induced by C. parvum. Thus, whereas HIV infection may not be a 
prerequisite for C. parvum infection in the biliary tree, synergistic pathologic effects 
between HIV-1 infection and C. parvum biliary infection exist and result in cholangiocyte 
injury via apoptosis. This finding may explain not only why C. parvum damage of biliary 
epithelia occurs almost exclusively in patients with HIV but also why HAART therapy is 
associated with a decreased incidence of AIDS cholangiopathy.

Apoptotic cell death in AIDS patients has been reported in several cell types, 
including lymphocytes, neurons, endothelial and epithelial cells, and hematopoietic 
derived cells (38). Numerous mechanisms of HIV-induced cell death have been 
demonstrated, including the sensitization of uninfected cells by soluble factors released 
from HIV-1 infected cells, including gp120, Nef, and Tat (2, 27, 44, 46). Tat appears to 
upregulate FasL and induces apoptosis in several cell types including epithelial cells (4, 
42). In our work, we observed a Tat-induced increase in FasL message and protein 
expression in uninfected cholangiocytes. Tat enhances FasL transcription through 
interactions with NF-kB binding sites within the FasL promoter (32). Here, we further 
confirmed that Tat increases FasL expression in cholangiocytes. However, Tat alone did 
not cause apoptosis in uninfected cholangiocytes. Moreover, cellular distribution of FasL 
in Tat-treated cells as assessed by immunofluorescence revealed a cytoplasmic 
distribution; no increase of FasL on the cell surface or in the culture medium from Tat-
treated cells was detected. This observation could explain why Tat alone does not
cause apoptosis in cultured cholangiocytes, since Fas/FasL-mediated cell apoptosis requires FasL membrane translocation (6, 26).

We have previously demonstrated that *C. parvum* infection induces apoptosis in cholangiocytes via a paracrine-mediated, Fas/FasL-dependent mechanism (9). In accordance with previous reports (14, 37), our data suggests that *C. parvum* induces pro-apoptotic activity in non-infected, neighboring cells. While the report by Mele et al., 2004 demonstrates diminished apoptosis at 24-hours post-infection compared to earlier time points, those cells undergoing apoptosis at 24-hours were non-infected neighboring cells. Our current study extended our previous observations and provided direct evidence of FasL membrane translocation and release of full-length FasL from directly infected cells. Membrane translocation of FasL can activate Fas receptors on adjacent cells resulting in cell death (proximal paracrine pathway), whereas FasL released from cells can activate Fas receptor and cause apoptosis in distant cells (distal paracrine pathway) (9); our co-culture model can distinguish these two processes. Directly infected cells in the upper chamber can cause apoptosis in bystander cells via both proximal and distal paracrine pathways, whereas only released FasL activates apoptotic cell death in the lower chambers (only the distal paracrine pathway). Interestingly, our data suggest that while HIV-1-associated Tat protein alone does not cause apoptotic cell death in uninfected cholangiocytes, it increases FasL expression in the cytoplasm of cholangiocytes and potentiates *C. parvum*-induced apoptosis in bystander cholangiocytes. More importantly, Tat significantly enhances *C. parvum*-induced FasL membrane translocation and release of FasL from infected. Thus, Tat protein upregulates FasL expression but not translocation and sensitizes cholangiocytes
to *C. parvum*-induced apoptotic cell death, suggesting a synergistic effect of Tat protein with *C. parvum* on cholangiocyte injury via Fas/FasL-dependent apoptosis.

The mechanisms by which FasL is translocated to the membrane and released from infected cells are unclear and currently under investigation. *C. parvum* activates kinase pathways in infected cells (10, 11, 16); we recently demonstrated the recruitment of transporters and channels to the site of infection (15), consistent with the active recruitment of vesicles to the plasma membrane. It therefore seems reasonable that *C. parvum* induced initiation of vesicular trafficking and membrane insertion may culminate in the shedding of FasL-bearing membrane in directly infected cells. Indeed, we observed a punctate, vesicular-like intracellular distribution of FasL in Tat-treated cholangiocytes. Immunonogold electron microscopy was used to visualize the distribution of FasL in infected cells. In accordance with our fluorescence observations, very little labeling was observed in uninfected cells, while infected cells exhibited extensive FasL labeling on the plasma membrane and on membranous protrusions. Similarly, membrane release of FasL via FasL-bearing microvesicles has been reported in other cell types, including cytolytic T-cells, NK cells, epithelial ovarian cancer cells, and early trophoblast cells (1, 3, 24, 28, 36).

In summary, using an *in vitro* model of biliary cryptosporidiosis, we demonstrate that HIV-1-associated Tat protein increases FasL expression in the cytoplasm of cultured cholangiocytes. *C. parvum*-infection induces FasL membrane translocation and release of full-length FasL in cholangiocytes and thus causes apoptotic cell death in bystander uninfected cholangiocytes, a process significantly enhanced in the presence of Tat protein (Fig. 10). Thus, HIV-1-associated Tat protein enhances *C. parvum*-induced
apoptosis in cholangiocytes, a process that may contribute to the development of AIDS-
cholangiopathies and be relevant to other opportunistic infections in AIDS patients in
general. Future studies will focus on the molecular mechanisms of Tat-induced FasL
upregulation, and the parasite initiated events responsible for the membrane
translocation and release of active FasL in infected cells.

ACKNOWLEDGMENTS

We thank Splinter PL and Tietz PS for helpful and stimulating discussions; and
Mrs. D. Hintz for secretarial assistance.
REFERENCES:


activates nuclear factor kappa B in biliary epithelia preventing epithelial cell apoptosis. Gastroenterology 120:1774-1783.


FIGURE LEGENDS

Figure 1. Expression of the HIV-1 receptors CXCR4 and CCR5 in cultured human cholangiocytes (H69 cells) observed by immunofluorescent microscopy. A and B, No obvious staining for both the CXCR4 and CCR5 receptors was observed in H69 cells. C and D, Human colonic adenocarcinoma epithelial cells (HT-29 cells) were used as the positive control, showing strong reaction to both CXCR4 and CCR5 antibodies as reported by others. E and F, Using RT-PCR we could not detect CXCR-4 and CCR-5 message in H69 cells. Our previous studies also indicate that cholangiocytes do not express CD4, another receptor required for direct HIV-1 infection. Bar = 10 μm.

Figure 2. Recombinant HIV-1 Tat protein potentiates C. parvum-induced apoptosis in H69 cells in the upper and lower (uninfected bystander cells) chamber of the co-culture system. A, Cells in the upper chamber of the co-culture system have both a C. parvum-infected population (approximately 25%) and an uninfected population of cells. Tat potentiates apoptosis in the upper chamber in a dose-dependent manner. B, Cells in the lower chamber do not interact directly with C. parvum; however, small molecules and components can freely pass through the filter separating the two populations of cells and affect cells in the lower chamber. Again, we observed a significant increase of apoptosis in cells co-cultured with cells exposed to C. parvum in the upper chamber. Tat treatment further enhanced apoptosis in those cells. The cells in the upper chamber consistently had an increased incidence of apoptotic cells compared to the lower
chamber for each condition. The data represent 3 separate experiments for each condition. C, Confocal immunofluorescent microscopy of apoptotic, uninfected bystander cells. The cells in which a C. parvum infection site could be demonstrated by immunofluorescence (arrows, red), in the presence or absence of Tat protein, were resistant to apoptosis. Neighboring uninfected cells were sensitive to apoptosis as noted by nuclear morphology (DAPI staining) and Annexin V staining (arrowheads, green).

Figure 3. Tat increases FasL, but not Fas, protein and mRNA expression in cultured cholangiocytes in a dose-dependent manner. A, Fas and FasL immunoblots of cultured cholangiocyte lysates after an overnight incubation in the presence of two concentrations of recombinant Tat peptide. No detectable difference was observed in the expression of Fas receptor under these conditions. Using two FasL antibodies (Clone 33, Transduction Labs shown) we detected a dose-dependent increase in FasL expression in Tat-treated cells. B, Quantitative analysis of both FasL and Fas of immunoblots from three experiments. Overnight incubation in 100 ng/ml of Tat peptide resulted in a nearly 6-fold increase in FasL expression over untreated control cells. A dose-dependent increase in FasL expression is observed in cells treated overnight in 250 ng/ml recombinant Tat. Quantitative analysis of immunoblots of Fas receptor from three separate experiments revealed no significant difference in expression of this receptor. C, Semi-quantitative RT-PCR of Fas and FasL in the presence of two concentrations of recombinant Tat peptide. No difference was observed in the
expression of Fas message under these conditions. A dose dependent increase in FasL message was observed in Tat-treated cells.  

D, Semi-quantitative analysis of both Fas and FasL RT-PCR from 3 experiments. While no difference in expression was observed in Fas message with either concentration of Tat peptide, a significant increase in FasL message was observed in both cells treated with 100 and 250 ng/ml Tat peptide.

Figure 4. Tat increases intracellular FasL expression but not membrane surface expression of FasL in cultured cholangiocytes. A and C, Non-membrane-permeabilized control H69 cells (A) and Tat-treated H69 cells (C) reveal minimal and similar FasL labeling on the cell surface. B, Membrane-permeabilized control H69 cells (without Tat treatment) showed FasL fluorescence both in the cytoplasm and membrane surface. D, Cells were treated with 100 ng/ml recombinant Tat peptide overnight and then permeabilized with Triton X-100 prior to immunofluorescence. A significant increase of FasL labeling was observed in those cells (70% increase, p < 0.01). Coupled with data from non-permeabilized Tat-treated cells, these data suggest that Tat increases intracellular FasL expression but does not effect membrane translocation of FasL. E, Quantitative analysis of fluorescence intensity for non-permeabilized cells reveals no significant difference between Tat-untreated and Tat-treated cells. Fluorescence intensity for permeabilized cells reveals a significant increase in fluorescence intensity for those cells treated with 100 ng/ml recombinant Tat. Bar = 10 μm.
Figure 5. Tat enhances *C. parvum*-induced FasL expression in cultured cholangiocytes. **A**, FasL immunoblots of cultured cholangiocyte lysates after an overnight infection in the presence or absence of recombinant Tat peptide. Two antibodies were used to assess FasL expression (Clone 33, Transduction Labs shown) and both antibodies showed an increase in FasL expression in cells exposed to *C. parvum* compared to uninfected control cells. *C. parvum*-induced FasL expression is further enhanced in the presence of Tat peptide. Actin was blotted as a loading control. **B**, Quantitative analysis of immunoblots from three experiments. A modest, yet significant increase in FasL expression was observed in *C. parvum*-infected cells compared to uninfected control cells. The expression of FasL was significantly enhanced when the infection was performed in the presence of recombinant Tat peptide.

Figure 6. Tat peptide enhances membrane translocation of FasL in *C. parvum*-infected cholangiocytes as observed by confocal microscopy. Cells were processed for immunofluorescent staining without membrane permeabilization. **A**, Uninfected control cholangiocytes exhibit minimal FasL surface expression. **B**, *C. parvum* infected cells, in the absence of Tat, exhibit aggregates of FasL fluorescence in directly infected cells. Aggregates of FasL fluorescence are observed in directly infected cells (infection sites denoted by arrowheads). **C**, Tat-treated, *C. parvum*-infected cultured cholangiocytes exhibit robust FasL immunofluorescence (*C. parvum* in red; FasL in green). **D**, Quantitative analysis of fluorescence intensity for each condition confirms the increased
fluorescence of *C. parvum*-infected cells, which is potentiated by the presence of recombinant Tat peptide. Bar = 5 μm.

**Figure 7.** Tat increases release of full-length FasL from *C. parvum*-infected cell cultures. Representative western blot for FasL in the supernatants after concentration with ultrafree concentrator. An increase of full-length FasL (∼37 kDa) is detected in the cell culture media from *C. parvum*-infected cells compared to uninfected control cells (Lane 2 vs. Lane 1). *C. parvum* infection in the presence of 100 ng/ml recombinant Tat peptide increases the amount of detectable full-length FasL compared to *C. parvum*-infected cells in the absence of Tat (Lane 4 vs. Lane 2). Minimal FasL was detected in cell culture media from Tat treated cells (Lane 3).

**Figure 8.** Immunoelectron microscopy. **A,** uninfected cholangiocytes reveal minimal FasL labeling throughout the cytoplasm and plasma membrane. **B,** boxed region of **A.** **C,** infected cholangiocytes reveal FasL labeling throughout the cytoplasm and plasma membrane. Numerous gold particles (arrows) are observed on membrane extensions near the parasite. **D,** boxed region of **C.** **E,** total gold particles per 10 μm² were counted for uninfected and infected cells. *C. parvum* infected cells exhibited an increase of total FasL as observed by immunogold EM. **F,** the percentage of total gold particles localized to the plasma membrane is significantly greater in infected cells compared to uninfected cells. The data represent analysis of five H69 uninfected control images and ten *C. parvum* infection sites.
Figure 9. Inhibition of Fas/FasL death pathway blocks inhibits *C. parvum*-induced, Tat-potentiated apoptosis. After an overnight culture in the presence or absence of Tat, as well as in the presence or absence of the FasL antagonist antibody, Nok1, to neutralize FasL function and Z-IETD-fmk, a specific inhibitor to caspase-8, to block activation of Fas death signaling, *C. parvum*-induced apoptosis was analyzed by Annexin 5 labeling and DAPI staining. A, in the absence of Nok1, Tat potentiates *C. parvum*-induced apoptosis. However, when cultured overnight in the presence of Nok1, the number of *C. parvum*-induced apoptotic cells decreased significantly for both the Tat-untreated and Tat-treated populations. The administration of 5 μg/ml Nok1 to both the Tat-untreated and Tat-treated populations decreased the incidence of apoptosis to levels not significantly different from control cells. B, cells treated with 100 ng/ml Tat in the absence of Nok1 antibody exhibit significantly greater Caspase-3/7 activity compared to cells cultured with Nok1 antibody. C, in the absence of Z-IETD-fmk, Tat potentiates *C. parvum*-induced apoptosis. However, when cultured overnight in the presence of 20 μM Z-IETD-fmk, the number of *C. parvum*-induced apoptotic cells decreased significantly for both the Tat-untreated and Tat-treated populations to levels not significantly different from control cells. The data represent experiments performed in triplicate.

Figure 10. Working model of Tat-*C. parvum* synergistic increase in cholangiocyte apoptosis. Based on earlier studies and observations made in this manuscript, we propose that *C. parvum* induces an increase in FasL expression in directly infected cells.
and promotes the release of FasL, resulting in an increased apoptosis in non-infected bystander cells via activation of both Fas/FasL induced proximal and distal paracrine pathways. While HIV-1 cannot infect cholangiocytes directly, HIV-1 infected T-cells can release biologically active Tat which potentiates *C. parvum*-associated apoptosis via upregulation of FasL and membrane translocation/release of FasL in infected cells.
----- Figure 1 -----
Figure 2

(A) Upper Chamber

- Absence of Tat
- Tat 100 ng/ml
- Tat 250 ng/ml

* P < 0.01

(B) Lower Chamber

- Absence of Tat
- Tat 100 ng/ml
- Tat 250 ng/ml

* P < 0.05

(C) C. parvum

Tat (100 ng/ml) + C. parvum
Fold increase over control H69

--- Figure 3 ---
--- Figure 4 ---

**Non-permeabilized**

A. Absence of Tat

B. Absence of Tat

C. Tat

D. Tat

**Permeabilized**

E. Absence of Tat

F. Absence of Tat

---

*Significance: *, p < 0.01

Fluorescence intensity (arbitrary units)
**Figure 5**

Panel A shows a Western blot analysis of FasL and Actin expression in the presence of C. parvum with and without Tat. The figure indicates a significant increase in FasL expression with Tat stimulation.

Panel B presents a bar graph illustrating the fold increase in FasL expression over control for uninfected and C. parvum-infected cells, with and without Tat. The graph shows a significant increase in FasL expression with Tat treatment, particularly at 250 ng/ml Tat.

Statistical significance is indicated with *p < 0.05 and **p < 0.01.
Figure 6
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat (100 ng/ml)</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parvum</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FasL Clone 33</td>
<td></td>
<td></td>
<td></td>
<td>37 kDa</td>
</tr>
</tbody>
</table>

----- Figure 7 -----
----- Figure 8 -----
----- Figure 9 -----

A

![Bar Chart A](image)

Uninfected control
No Tat  Tat 100 ng/ml
C. parvum

- Absence of Nok1
- 2.5 μg/ml Nok1
- 5 μg/ml Nok1

* p > 0.05

B

![Bar Chart B](image)

No Nok1  5µg Nok1
C. Parvum Tat 100 ng/ml

- Caspase 3/7 fluorescence (arbitrary units)

C

![Bar Chart C](image)

Uninfected control
No Tat  Tat 100 ng/ml
C. parvum

- Absence of Z-IETD-fmk
- Z-IETD-fmk (20mM)

* p > 0.05
Apoptosis

Fas

C. parvum

----- Figure 10 -----