Contribution of Epithelial Apoptosis and Subepithelial Immune Responses in *Campylobacter jejuni*-Induced Barrier Disruption

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Contribution of *Campylobacter jejuni* is a widespread zoonotic pathogen and the leading bacterial cause of foodborne gastroenteritis in humans. Previous infection studies showed disruption of intercellular contacts, induction of epithelial apoptosis, and immune activation, all three contributing to intestinal barrier dysfunction leading to diarrhea. The present study aims to determine the impact of subepithelial immune cells on intestinal barrier dysfunction during *Campylobacter jejuni* infection and the underlying pathological mechanisms. Infection was performed in a co-culture of confluent monolayers of the human colon cell line HT-29/B6-GR/MR and THP-1 immune cells. Twenty-two hours after infection, transepithelial electrical resistance (TER) was decreased by 58 ± 6% compared to controls. The infection resulted in an increase in permeability for fluorescein (332 Da; 4.5-fold) and for FITC-dextran (4 kDa; 3.5-fold), respectively. In contrast, incubation of the co-culture with the pan-caspase inhibitor Q-VD-OPh during the infection resulted in a complete recovery of the decrease in TER and a normalization of flux values. Fluorescence microscopy showed apoptotic fragmentation in infected cell monolayers resulting in a 5-fold increase of the apoptotic ratio, accompanied by an increased caspase-3 cleavage and caspase-3/7 activity, which both were not present after Q-VD-OPh treatment. Western blot analysis revealed increased claudin-1 and claudin-2 protein expression. Inhibition of apoptosis induction did not normalize these tight junction changes. TNFα concentration was increased during the infection in the co-culture. In conclusion, *Campylobacter jejuni* infection and the consequent subepithelial immune activation cause intestinal barrier dysfunction mainly through caspase-3-dependent epithelial apoptosis. Concomitant tight junction changes were caspase-independent. Anti-apoptotic and immune-modulatory substances appear to be promising agents for treatment of campylobacteriosis.

**Keywords:** apoptosis, caspase, epithelial barrier, tumor necrosis factor alpha, *Campylobacter*, epithelial cell, immune cell co-culture, tight junction
INTRODUCTION

Diarrheal disease is a major cause of morbidity and mortality worldwide. Campylobacter jejuni (C. jejuni) is a frequent commensal bacterium in poultry and wild birds and the leading cause of bacterial diarrhea in humans. As a zoonotic pathogen being highly contagious via the fecal-oral route, C. jejuni infection occurs by consumption of raw or undercooked meat, raw dairy products or contaminated water. The symptoms of the campylobacteriosis vary from fever, aches, and dizziness to severe manifestations with abdominal cramps and bloody diarrhea. The disease is self-limiting and antibiotic treatment is only recommended in chronic or severe cases. Nevertheless, C. jejuni infection result in very large health costs (Hoffmann et al., 2012; Tam and O’Brien, 2016) and can lead to complications such as post-infectious reactive arthritis and Guillain-Barré syndrome.

The pathogenesis of intestinal barrier dysfunction in the C. jejuni infection is not completely understood. During the infection, bacteria adhere to the mucus and transmigrate through the mucus layer and the epithelium (Backert et al., 2013) by invasion of enterocytes (Konkel et al., 1999; Song et al., 2004) or paracellularly with no changes in epithelial integrity (Boehm et al., 2012). Subsequent epithelial barrier impairment and activation of the innate inflammatory response was described in vivo in human cell cultures (Jones et al., 2003; Hu et al., 2006). These processes are also observed in vivo in C. jejuni patients (Spiller et al., 2000; Bückert et al., 2018) and in experimentally infected immune-deficient mice (Fox et al., 2004; Bereswill et al., 2011). In the pathogenesis of epithelial barrier dysfunction, apart from immune cell infiltration, tight junction changes, focal leaks and sodium malabsorption, the C. jejuni-induced epithelial cell death accompanies the pathological changes in the C. jejuni infected mucosa.

In previous studies on the related bacteria Arcobacter butzleri or Campylobacter concisus, we were able to show that epithelial cell death and in particular apoptosis induction and not the compromised tight junction alone can lead to the epithelial barrier defect during infection (Bückert et al., 2009; Nielsen et al., 2011).

Two canonical pathways of apoptosis activation have been elucidated - the extrinsic pathway and the intrinsic pathway. Induction of these pathways results in activation of initiator caspases, leading to apoptosis commitment. The extrinsic pathway is triggered by ligand binding to the tumor necrosis factor (TNF) receptor superfamily members. The intrinsic pathway involves the release of caspase-activating factors by mitochondria in response to intracellular injuries such as DNA damage. Initiator caspases are then able to cleave pro-caspases and thus turn on downstream effector pro-caspases -3, -6, and -7, which in turn activate or inhibit target proteins, leading to apoptotic cell death (Delhalle et al., 2003). Apoptosis in the gut is associated with intestinal cell shedding - extrusion of enterocytes at the surface as a result of the migration from the base of the crypt to the top of the epithelium (Bullen et al., 2006). This process promotes a continuous turnover of the intestinal cells achieved without loss of intestinal barrier function.

Epithelial apoptosis is a physiological process, but if stimulated it can exceed the regenerative capacity of the mucosa and the epithelium cannot sustain a proper barrier function. Increased apoptosis causes (i) a reduction of the transepithelial resistance (TER), (ii) loss of water and electrolytes as well as (iii) increased permeability for macromolecules leaking into the lumen (leak-flux diarrhea) or (iv) increased antigen uptake from the lumen into the organism (leaky gut) (Bojarski et al., 2001). Enhanced antigen presentation to the submucosal immune cells, being part of the “leaky gut” phenomenon, reinforces the inflammation. This signifies that the disease enters a vicious circle, with the result that the intestinal tissue damage may rise to extremes.

We hypothesize induction of apoptosis as possible pathomechanism, induced indirectly by cytokines or together with direct C. jejuni effectors, affecting cellular viability and epithelial integrity. Although an increase of epithelial apoptosis in C. jejuni-infected tissue is evident, its mechanisms and impact on epithelial barrier function have not been elucidated yet and was not taken under consideration of the immune response in an in vitro model.

In the present study, we applied a recently described C. jejuni infection model in a co-culture of HT-29/B6-GR/MR epithelial and THP-1 immune cells to investigate the mechanisms leading to intestinal barrier disruption during the infection, such as epithelial cell death and tight junction changes, as well as the impact of subepithelial immune activation.

MATERIALS AND METHODS

Co-culture of Human Epithelial Cells and Macrophage-Like Immune Cells

We performed the infection experiments in a co-culture of HT-29/B6-GR/MR epithelial cells and THP-1 immune cells as recently described (Lobo de Sá et al., 2019) with the modification of the filter insert with larger pore size to allow bacterial translocation. Briefly, HT-29/B6-GR/MR cells (Bergann et al., 2011) were cultivated in 25 cm² culture flasks for 7 days in RPMI 1640 culture medium (Sigma Aldrich, St. Louis, MO, United States) supplemented with 10% fetal calf serum (FCS; Gibco, Carlsbad, CA, United States), 1% penicillin/streptomycin (Corning, Wiesbaden, Germany), G418 (300 µg/ml; Invitrogen, Carlsbad, CA, United States) and hygromycin B (200 µg/ml; Biochrom GmbH, Berlin, Germany). For experimental use, cells were grown on 3 µm pore size Millicell PCF filters membranes (Merck Millipore, Billerica, MA, United States) at a density of 10⁶ cells cm⁻² with a medium change every 2 days for 9 to 11 days till confluence. On the day of the experiment, the cells were washed three times and incubated for at least 1 h in antibiotic-free culture medium in the presence of 10% heat-inactivated FCS. THP-1 cells were incubated in 12-well plates with the antibiotic-free medium in the presence of 10% heat inactivated FCS. THP-1 cells were removed, adhesion and differentiation state of THP-1 cells were controlled under a light microscope. The co-culture was started by placing the PCF filters with HT-29/B6-GR/MR cells.
into 12-well plates with adherent THP-1 immune cells at the bottom of the plate (Figure 1).

**Pharmacological Inhibitors**

For the inhibition of apoptosis, we incubated the *C. jejuni* infected and not infected co-culture of HT-29/B6-GR/MR and THP-1 cells with 10 μM Q-VD-OPh hydrate ((3S)-5-(2,6-difluorophenoxo)-3-[(2S)-3-methyl-1-oxo-2-[(2-quinolinylcarbonyl) amino]butyl]amino]-4-oxo-pentanoic acid hydrate, Calbiochem, San Diego, CA, United States) solved in DMSO (Sigma Aldrich, St. Louis, MO, United States). Staurosporine (Sigma Aldrich, St. Louis, MO, United States) was used for the induction of apoptosis at the concentration of 1 μM. The culture medium was supplemented with the given pharmacological inhibitors for at least 1 h before the infection and during the whole duration of the experiment.

**C. jejuni Infection of the Co-culture and Bacterial Transmigration**

*Campylobacter jejuni* 81-167 reference strain was cultivated for 2 days on blood agar plates (Columbia Agar with Sheep Blood; Oxoid, Wesel, Germany) in an impermeable plastic container at 37°C with Oxoid CampyGen gas packs (Thermo Scientific, Waltham, MA, United States) to establish microaerobic conditions. For infection, we harvested bacterial colonies using an inoculation loop and resuspended them in the antibiotic-free cell-culture medium. After at least 2.5 h of further incubation in microaerobic conditions, the bacteria were gently centrifuged at 5000 × g, 10°C for 2 min, in favor of bacterial viability. The bacteria pellet was resuspended in Dulbecco’s phosphate buffered saline (DPBS; Sigma Aldrich, St. Louis, MO, United States) for quantification of the infection dose. The number of bacteria was estimated using optical density measurement and adjusted to OD$_{600}$ = 2. The HT-29/B6-GR/MR cells in the co-culture setting were infected on the apical side with a multiplicity of infection (MOI) of 350 (Figure 1).

For quantification of bacterial transmigration, 25 μl of medium were removed from the basolateral compartment of the 12-well plates at the time points of 6, 12, and 24 h post-infection. Samples were diluted in 10-fold steps with antibiotic-free culture medium, sufficiently vortexed and incubated on the blood agar plates for 36 h. The number of colony-forming units (CFU) was counted and adjusted by the dilution coefficient to calculate the number of transmigrated bacteria.

**Cytometric Bead Array and Flow Cytometry**

At the timepoint of 22 h after infection we collected the medium from basolateral compartment of the 12 well plate to analyze the secretion of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α) during the infection using a human cytometric bead array kit and manufacturers protocol (CBA; BD Biosciences human Th1, Th2, Th17 Kit, Flex Set IL-1β, Franklin Lakes, NJ, United States). Flow cytometric measurement were performed with FACS CantoII (BD Biosciences; Franklin Lakes, NJ, United States) and analyzed with FACP Array software v3.0 (BD Biosciences, Franklin Lakes, NJ, United States).

**Measurement of Transepithelial Electrical Resistance**

The transepithelial electrical resistance (TER) was assessed before and 22 h after the infection. We performed the measurement with a chopstick electrode set (STX2, World Precision Instruments, Sarasota, FL, United States) and an epithelial volt-ohm meter (Institute of Clinical Physiology, Charité, Berlin). Electrodes were washed with 80% ethanol and phosphate buffered saline (PBS; Sigma Aldrich, St. Louis, MO, United States) in between the measurements. In pre-tests we have measured the TER of infected monolayers at multiple time points over the incubation.

![FIGURE 1 | Schematic sideview on the co-culture infection model. Epithelial cells HT-29/B6-GR/MR grown on filter supports were placed in a 12-well plate containing stimulated THP-1 immune cells. The co-culture was inoculated with C. jejuni 81-176 reference stain from the apical side. The infection experiments were performed under microaerobic conditions for 22 h with or without the apoptosis inhibitor Q-VD-OPh. After 22 h electrophysiological measurements were performed to assess the epithelial barrier integrity. Epithelial cells and basal medium were then harvested. The samples were further processed to evaluate the effects of the infection as well as the pathological mechanisms of epithelial barrier dysfunction caused by C. jejuni.](image-url)
period to determine the earliest onset of the barrier effect (22 h post-infection). For the experiments, TER was measured in the epithelial monolayers before infection, then the co-cultures were placed in microaerobic atmosphere in favor of the bacteria. The monolayers were measured again after the incubation period of 22 h.

**Transepithelial Permeability**

For the measurement of the epithelial permeability 10 µl of fluorescein (332 Da, 100 mM, Fluorescein sodium salt, Sigma Aldrich, St. Louis, MO, United States) or fluorescein isothiocyanate-dextran solution (FITC-dextran, 4 kDa, 20 mM, Sigma Aldrich, St. Louis, MO, United States) dissolved in the culture medium was added to the apical side of the cell monolayer on PCF filter membranes placed in 12-well plates. The basal medium was removed at three subsequent time points every 15 (fluorescein) or 30 (FITC-dextran) minutes for the fluorescence measurement in a spectrophotometer (Infinite, Tecan GmbH, Männedorf, Switzerland). The standard for the fluorescent molecule concentration was determined in a dilution series. The permeability of the cell monolayer for the macromolecules was calculated from flux divided by concentration difference.

**Apoptosis Staining and Tight Junction Immunofluorescence**

We analyzed the epithelial apoptotic rate using the TUNEL protocol (In situ Cell Death Detection Kit, Roche, Mannheim, Germany). Cells grown on filter supports were fixed with 2% paraformaldehyde for 30 min at a time point of 22 h after _C. jejuni_ infection, thereafter permeabilized with 0.5% Triton X-100. Cell monolayers were incubated with TUNEL reagent at 37°C, repeatedly washed with blocking solution containing 5% goat serum in DPBS. 4′,6-Diamidino-2-phenylindole (DAPI) was applied as a nuclear counterstain. Apoptosis-positive cells were visualized with confocal laser scanning microscopy (Zeiss LSM780, Jena, Germany) and counted per high-power field.

For immunostaining of tight junction proteins, the epithelial monolayers were washed with PBS, permeabilized with 0.5% Triton X-100 (Sigma Aldrich, St. Louis, MO, United States) for 7 min, and blocked for 10 min with 1% goat serum. Then the cells were incubated with the primary antibodies anti-occludin (1:100; Invitrogen, Carlsbad, CA, United States) and anti-ZO-1 (1:100; Invitrogen, Carlsbad, CA, United States), and anti-caspase-3-cleaved (1:1000; Cell Signaling Technology, Denver, MA, United States) overnight, and anti-β-actin (1:10000; Sigma Aldrich) over 4 h at 4°C. Afterward the membranes were washed with TBST buffer and incubated for 2 h at the room temperature with peroxidase conjugated secondary antibodies goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch, Ely, United Kingdom) prepared with 1% milk powder in TBST. Nitrocellulose membranes were then placed in the SuperSignal West Pico PLUS chemiluminescent peroxidase substrate (Thermo Scientific, Waltham, MA, United States) for 2 or 5 min for anti-β-actin and other antibodies, respectively. The chemiluminescence was measured using Fusion FX7 (Vilber Lourmat, Eberhardzell, Germany). ImageJ 1.52o quantification software was used for densitometric analysis. Signal intensity values were normalized by the loading control (β-actin) (Schneider et al., 2012). In parallel, the activity of caspase-3/7 was assessed using the Caspase-3/7 activity assay kit following manufacturer’s protocol (SensoLyte; AnaSpec, Fremont, CA, United States). Cell lysates were incubated with caspase-substrate Ac-DEVAD-AFC and the fluorescence intensity of the fluorogenic indicator product was measured at Ex/Em = 380 nm/500 nm (Tecan, Männedorf, Switzerland).

**Statistical Analysis**

All data are expressed as mean values ± standard error of the mean (SEM). Statistical analysis was performed using two-way unpaired Student’s _t_-test with Bonferroni-Holm adjustment for multiple comparison. Significance level was set at _α_ = 0.05.

**RESULTS**

Subepithelial Immune Cells Caused Epithelial Barrier Dysfunction at an Early Time Point of _C. jejuni_ Infection by Means of Apoptosis Induction

_Campylobacter jejuni_ is known to cause epithelial barrier dysfunction. Transepithelial electrical resistance (TER) served as a functional parameter to measure the integrity of the epithelial cell monolayer. As in prior studies, we confirmed that a barrier...
defect of C. jejuni-infected HT-29/B6 cell monolayer occurred only after 40 to 48 h after exposure to the cell mono-culture (Bücker et al., 2018) with 41 ± 4% of initial TER ($p < 0.001$, $n = 7–8$, data not shown). In line with this, at an earlier incubation time point of 22 h after infection, the TER did not drop (Figure 2A). Also, the apoptosis inhibitor Q-VD-OPh did not result in any significant alteration of the TER in the HT-29/B6-GR/ MR cell culture on the first day of incubation. Since the mucosal immune activation in campylobacteriosis is a key feature of the disease, a co-culture of HT-29/B6/GR-MR and activated THP-1 immune cells was used for the infection assay. The contribution to the TER effect by the subepithelial THP-1 cells in an early incubation period was shown here in comparison to the unaffected mono-culture (Figures 2A,B). 22 h after exposure of the co-culture with C. jejuni, the infection caused a reduction in TER of 58 ± 6% ($p < 0.001$) compared to the controls (Figure 2B). Whereas, the co-culture incubated with the pan-caspase inhibitor Q-VD-OPh showed no changes in TER during the infection with C. jejuni. Surprisingly, the developing decrease in TER was completely blocked by Q-VD-OPh. Thus, the inhibition of apoptosis led to a full recovery of the barrier deficit.

Inhibition of Epithelial Apoptosis Normalized the Paracellular Permeability for Macromolecules in C. jejuni Infection

Besides TER measurements that rather reflect the ional permeability, we performed flux measurements of macromolecules in order to assess the barrier function for the paracellular leak pathway in our model. The permeability of the cell monolayer in the co-culture setting for small and mid-sized macromolecules changed during the C. jejuni infection. 22 h after infection, the fluorescein (332 Da) translocation from apical to the basal compartment increased 4.5-fold in infected samples compared to controls ($p < 0.001$), but remained steady under Q-VD-OPh treatment (Figure 3A). Similar to the fluorescein permeability, the FITC-dextran (4 kDa) translocation increased 3.5-fold during the infection ($p < 0.001$). Also, Q-VD-OPh incubation of the infected samples led to a full recovery of the FITC-dextran permeability (Figure 3B). The inhibition of apoptosis completely prevented the development of epithelial leaks and sufficiently sealed the epithelial barrier against the passage of macromolecules.

C. jejuni Infection Changed the Expression of Tight Junction Proteins

As molecular or cellular correlate to the barrier defect by C. jejuni, expression changes of tight junction proteins and/or epithelial cell damage (cell death) are thinkable. Therefore, we performed western blotting of treated epithelial cell monolayers. In the densitometric analysis of the Western blots, we discovered an almost 2-fold increase of an integral tight junction protein occludin ($p < 0.05$) and the barrier-forming claudin-1 protein ($p < 0.01$) expression. The claudin-1 effect was not diminished by apoptosis inhibition (Figure 4A). Cells incubated with Q-VD-OPh during the infection had similar increase in claudin-1 expression ($p < 0.01$) but only a change in occludin expression by trend. The channel-forming claudin-2 was also induced in HT-29/B6-GR/MR cells during the infection with ($p < 0.01$) and without ($p < 0.05$) concurrent apoptosis inhibition. Claudin-5 and claudin-8 show a reduction by trend that could develop an influence on the barrier integrity at a later time point of infection, whereas tricellulin and claudin-4 expression remained stable during the first hours of infection (Figure 4A). Tight junction expression changes were not prevented by apoptosis inhibition and presumably have a caspase-independent regulator. As the subcellular distribution of tight junction proteins can influence the epithelial barrier function, we analyzed immunofluorescent stainings of the treated monolayers. In confocal micrographs the intact co-localization of tight junction protein occludin together with zonula occludens protein-1 (ZO-1) was observed, without any re-distribution of tight junction protein signals.
to intracellular compartments in treated or infected cells (Figure 4B). At most a zigzag pattern of the biccular junction became visible in infected monolayers, which might indicate the beginning of the following impact of C. jejuni on tight junction protein redistribution at later stages of infection.

Epithelial Apoptosis in C. jejuni Infection Caused by Increased Caspase Activity

Although apoptosis can be initiated by different pathways, it ends up with a common final sequence of a few effector caspases. Therefore, we investigated the activation of caspase-3 in our infection model. The infection of the co-culture with C. jejuni resulted in an increased caspase-3 cleavage. In the densitometry analysis of Western blots, infected samples showed a 9-fold increased band signal of the 19 kDa caspase-3 cleavage product (p < 0.01). This increase of the effector caspase was sufficiently blocked by the pan-caspase inhibitor Q-VD-OPh (p < 0.01) (Figure 5A). These changes correspond to the results of the caspase-3/7 activity assay. Here, a substantial rise of the effector caspase activity following the C. jejuni infection (p < 0.001) was measured with a completely inhibited activity, when incubated with Q-VD-OPh (p < 0.001) (Figure 5B). The effect of the changed number of apopotoses on the integrity of the cell monolayer was investigated after TUNEL staining by confocal laser scanning microscopy. C. jejuni infection led to a 5-fold increase in the number of apoptotic cells in the cell culture. The increase in the apoptotic rate was prevented by incubation of the cell culture with the pan-caspase inhibitor Q-VD-OPh during the infection (p < 0.001) (Figures 5C,D). Thus, the predominant mechanism of the barrier defect in the early phase of infection was shown to be more caspase-dependent apoptosis induction than tight junction disruption.

Cytokine Secretion Was Induced in C. jejuni-Infected Co-culture

Increased apoptosis induction can develop from direct bacterial contact and/or subepithelial cytokine release. To understand the mechanism of epithelial cell apoptosis, we further investigated the secretion of cytokines from THP-1 cells in the infected co-culture. C. jejuni infection resulted in a 2.5-fold caspase-independent increase of TNFα concentration (p < 0.05). Immune activation was not affected by apoptosis inhibition with Q-VD-OPh (Figure 6A). Other pro-inflammatory cytokines such as IFN-γ, IL-1β, IL-2, IL-6 or IL-17 were not induced by C. jejuni or influenced by apoptosis inhibition after 22 h in the used infection setting (Figures 6B–F). Production of TH2 cytokines IL-10 and IL-13 also remained unchanged (Figures 6G,H). Therefore, the cell death receptor pathway of TNFα might play the major role in our experimental setup.

C. jejuni Transmigration Across the Epithelium Was Not Limited by Apoptosis Inhibition

The translocation of C. jejuni and/or lipo-oligosaccharides (LOS) from the apical side through the epithelial monolayer, reaching the immune cells, is supposed as a pivotal step of the following aggravated outcome of the C. jejuni infection in vivo, leading to enhanced barrier disruption and again potentiated antigen influx into the subepithelium (leaky gut concept). In the transmigration experiment, we observed that inhibition of apoptosis resulted not in a decrease, as expected, but in a 5.5-fold increase of bacterial translocation during the first 6 h of infection (p < 0.05, n = 6). After 12 h, the number of the bacteria, that reached basolateral compartment through the Q-VD-OPh incubated HT-29/B6-GR/MR cells, was only 3.5-times higher compared to the control infection in the co-culture (p < 0.05, n = 6) (Figure 7). At the time point of 24 h post-infection there was no significant difference between the two groups. Thus, the transmigration of C. jejuni was not limited by Q-VD-OPh, whereas the unrestricted passage of solutes and macromolecules could be diminished. Tight junction changes alone cannot explain the drop in resistance and increase in permeability for fluorescein and 4 kDa dextran. The increased apoptotic ratio in

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**FIGURE 3 | Epithelial permeability toward macromolecules in C. jejuni-infected co-cultures 22 h post-infection. Co-cultures of HT-29/B6-GR/MR and THP-1- cells were infected with C. jejuni with or without apoptosis inhibitor Q-VD-OPh. (A) Fluorescein flux and (B) FITC-Dextran flux across the epithelial monolayer were measured after 22 h of C. jejuni infection. The flux measurements were performed in the cell monolayer filter supports and are presented as mean values ± SEM. n = 6, ***p < 0.001, unpaired Student’s t-test with Bonferroni-Holm correction for multiple comparisons.**
**FIGURE 4** | Expression of tight junction proteins in C. jejuni-infected co-cultures 22 h post-infection. Co-cultures of HT-29/B6-GR/MR and THP-1- cells were infected with C. jejuni with or without apoptosis inhibitor Q-VD-OPh. (A) Western blot was performed on the cell lysates 22 h after infection. Expression level of tight junction proteins was normalized with β-actin. Immunoblots were subjected to densitometric analysis. The dashed line represents control value set to 100%. Q-VD-OPh control treatment did not change the expression level of the described tight junction proteins compared to untreated controls (data not shown). Data are presented as mean values in percent of the control values, ± SEM. n = 7–9, *p < 0.05, **p < 0.01, unpaired Student’s t-test with Bonferroni-Holm adjustment for multiple comparisons. (B) The cell monolayers were analyzed by confocal microscopy after immunofluorescence staining. The micrographs show the localization of the tight junction proteins occludin (green) and zonula occludens protein-1; ZO-1 (red) at cell borders. The merge (yellow) image represents the co-localization of the tight junction proteins in a single plane of a Z-stack. White arrows indicate zigzag pattern of bicellular cell-cell contacts in C. jejuni-infected monolayers.

The monolayers seems to be the only barrier-relevant factor in the first phase of infection.

**DISCUSSION**

The perturbation of the intestinal barrier is considered to be a key mechanism for the development of C. jejuni-induced diarrhea. While apoptosis was hitherto insufficiently analyzed in the C. jejuni-infected epithelial cell culture, it is commonly referenced in C. jejuni-infected mice, apes and human tissues (Russell et al., 1993; Haag et al., 2012; Bücker et al., 2018). The role of apoptosis in epithelial barrier dysfunction was discussed but have never been investigated for the C. jejuni infection. Hence, we aimed on the effect of apoptosis induction on the barrier impairment caused by C. jejuni in an immune and epithelial cell co-culture approach to alleviate the diarrheal outcome of the disease. This novel method enabled us to study the interplay...
of \textit{C. jejuni} with the epithelial monolayer and immune cells, reflecting an infection model for the delineation of the diarrheal symptoms of the infection.

\section*{Q-VD-OPh as Inhibitor of \textit{C. jejuni}-Triggered Barrier Dysfunction}

For the first time we present a substance, the pan-caspase inhibitor Q-VD-OPh, which targets epithelial monolayers and completely inhibited the epithelial barrier dysfunction in \textit{C. jejuni} infection. In the co-culture setting, it mitigated the decrease of the TER completely. No other compound was able to produce an outcome of this scale. In pre-tests, we targeted on the host cell signaling with the kinase inhibitors Y-27632 (PI3K) or ML-7 (MLCK) as well as blocking experiments on NF-κB activation (BAY 11-7082), but could not achieve any barrier-relevant protection effects (data not shown). In the literature, the improvement of barrier function was reported for use of vitamin D and PI3-kinase inhibitor LY294002 (Wine et al., 2008; Bücker et al., 2018). Their effects, ranging from mild to substantial, did not prevent from the loss of the barrier function, although \textit{C. jejuni}-dependent activation of PI3K, ERK, p38, and NF-κB as well as impairment of actomyosin signaling were reported (Jin et al., 2003; Krause-Gruszczynska et al., 2007; Li et al., 2011).
FIGURE 6 | Immune activation during *C. jejuni* infection. Co-cultures of HT-29/B6-GR/MR and THP-1 cells were infected with *C. jejuni* with or without apoptosis inhibitor Q-VD-OPh. The secretion of the barrier relevant cytokines such as TNF-α, INF-γ, and IL-1β (A–C) as well as further interleukins (D–H) were assessed in the infected co-culture after 22 h. Cytokine concentration was measured in the culture medium from basal compartment. The concentrations are presented as mean values ± SEM, n = 6, *p* < 0.05, unpaired Student’s t-test with Bonferroni-Holm adjustment for multiple comparisons.

Recently published data on curcumin as a preventive drug against *C. jejuni*-induced barrier disruption showed a complete recovery of the barrier function of treated epithelial monolayers. Curcumin effects relied on immune regulation with a decreased level of cytokine production, protecting the enterocytes. In the same time frame, direct anti-apoptotic properties of curcumin were discussed (Lobo de Sá et al., 2019). Regarding the success of anti-apoptotic treatment with Q-VD-OPh, we consider apoptosis as a crucial pathomechanism for epithelial barrier dysfunction in the early stage of the *C. jejuni* infection.

Inhibition of Apoptosis as Mechanism for Reducing Macromolecule Passage

Inhibition of apoptosis resulted in the recovery of the increased epithelial permeability toward macromolecules after
infection to the level of control values. It is known that single-cell epithelial defects rapidly close by an actomyosin contraction “purse string” mechanism (Florian et al., 2002). As possible explanation for persistent barrier dysfunction, C. jejuni actively triggers signaling pathways to stimulate their own uptake by host cells using activation of the Rho GTPase, which in combination with induction of TNFα and INFγ pathways can lead to increased restitution time of single-cell lesions or increased numbers of cell extrusions (Günzel et al., 2006; Cróinín and Backert, 2012). Such mechanisms are also known for Shigella, Salmonella, and enterohaemorrhagic Escherichia coli during the initial stages of infection (Gudipaty and Rosenblatt, 2017). Increased epithelial permeability by single cell apoptosis facilitates unwanted loss of solutes and uptake of noxious agents (Gitter et al., 2000). One can conclude that apoptosis not only lead to barrier dysfunction in C. jejuni infection but can also be a part of the diarrheal mechanism and the cause of an excessive immune response, by increased antigen entry into the subepithelial compartment.

**No Downregulation of Barrier-Relevant Tight Junction Proteins**

Tight junction proteins are the main determinants for sealing the paracellular pathway. Their impairment causes the leak flux mechanism by increased paracellular permeability (Barmeyer et al., 2017). Considering the TER reduction and increased macromolecular leak flux caused by C. jejuni, we investigated the expression of tight junction proteins. Surprisingly, no downregulation of barrier-relevant tight junction proteins was observed. While the increased expression of claudin-2, a tight junction protein forming a channel for small cations and water, could partially explain the decrease in TER, it could not be responsible for the translocation of larger molecules. The contribution of claudin-2 upregulation under inflammatory conditions is described in vitro and in vivo (Heller et al., 2005; Zeissig et al., 2007; Luettig et al., 2015).

In our co-culture model the reduced TER and increased paracellular flux could not be explained by the expression change of occludin. Increased expression of occludin was reported to strengthen the barrier (McCarthey et al., 1996), but without a sealing effect in TER by occludin per se, since occludin knockout mice did not develop a barrier impairment (Schulzke et al., 2005). Here, claudins with sealing properties maintain the paracellular barrier. However, it is a matter of debate whether in an occludin knockout animal or cell model, other tight junction proteins can functionally compensate a loss of occludin (Saitou et al., 2000; Schulzke et al., 2005). Also, in a Caco-2 occludin knockout cell monolayer, the integrity of the epithelial barrier was intact, likewise in the wildtype Caco-2 cells, as indicated by a stable TER. Interestingly, in the Caco-2 model the cleavage of occludin by the secreted C. jejuni protease HtrA was shown, that facilitated the paracellular movement of the bacteria between the host cells (Harrer et al., 2019). Occludin was described as a molecular target of C. jejuni, as well as other pathogens and bacteria or viruses, leading to a direct or indirect disruption of occludin. In our experiments, confocal micrographs of the C. jejuni-infected monolayers revealed no protein redistribution of occludin to intracellular compartments and the occludin signal was clearly present in the tight junction domain. In the same time a zigzag pattern of cell-cell contacts was visible that points to morphological changes or the pre-stage for the following tight junction changes (re-distribution or scattering) in the further course of infection.

In general, upregulation of claudin-1 increases the epithelial resistance and decreases paracellular permeability to macromolecules (Inai et al., 1999). We also observed a claudin-1 upregulation in C. jejuni infection, the claudin-1 paradox, claudin-1 increase while the TER is decreased, as also shown before for C. fetus and C. jejuni in vivo. (Bückler et al., 2017, 2018; Lobo de Sá et al., 2019). The overall increase of claudin-1 was associated with retraction of the protein from tight junction strands and localization in the basolateral membrane and intracellular compartments during the infection (Bückler et al., 2018). Both claudin-1 and claudin-2 upregulation is shown to be mediated by TNFα signaling (Amasheh et al., 2010). The TNFα level was increased in our C. jejuni infection model. Claudin-1 level is known to be strongly increased in apoptosis inducer-treated HT-29/B6 cells (Bojarski et al., 2004), but was caspase independent in our infection model. Although we could show a change in the expression of the measured tight junction proteins, similar expression change was observed in infected monolayers after Q-VD-OPh treatment that showed sufficient barrier function in TER measurement and no increase of paracellular permeability for macromolecules. Therefore, tight junction changes alone cannot explain the disturbed barrier function caused by C. jejuni infection and its recovery. As an example of the intact tight junction meshwork pattern we showed occludin in co-localization with ZO-1 in
controls, infected and Q-VD-Oph-treated monolayers in our co-culture setting. We propose that ongoing changes in tight junction protein expression and distribution, with impact on barrier function as reported (Bück et al., 2018; Lobo de Sá et al., 2019) arise later on, with epithelial apoptosis being the main manifestation in the beginning of the infection, as shown here.

Impaired Barrier Function Results From Induction of Epithelial Apoptosis

The increased epithelial apoptosis was caspase-3-dependent, mediated by C. jejuni infection, and the subsequent increase in TNFα. While the related pathogens Campylobacter concisus and Arcobacter butzleri directly initiate epithelial cell death (necrosis and apoptosis) that were partially barrier relevant with significant effects on TER and molecule marker fluxes (Bück et al., 2009; Nielsen et al., 2011), we did not find such evidence for C. jejuni in the literature. Hence, we could show for the first time that epithelial apoptosis has a main effect on epithelial barrier function in C. jejuni infection, and in the early phase (first day of infection) it is the predominant reason for barrier impairment.

Cytotoxic Mechanism Causing Epithelial Apoptosis

Regarding the inflammatory input, TNFα was the main cytokine increased during the infection. TNFα-induced barrier dysfunction was mostly caused by single—cell apoptotic events, increasing focal conductivity of the epithelium (Schulzke et al., 2006). Nevertheless, the immune response to the interaction with C. jejuni caused barrier impairment and induced overlapping effects of apoptosis and tight junction changes (Lobo de Sá et al., 2019). We proposed that C. jejuni possesses direct pro-apoptotic effectors. Previously, several bacterial proteins were associated with epithelial cell death. Cytolysin distending toxin prepared from C. jejuni was reported to cause cell cycle arrest and apoptosis in HeLa and epithelial Caco-2, or mononcyc 28SC cells, while having no impact on T84 cells (Whitehouse et al., 1998; Kalischuk et al., 2007; Jain et al., 2009). The Campylobacter serine protease HtrA was shown as a virulence factor in mouse models with increased caspase-3 activity in both gnotobiotic IL-10-deficient mice and infant mice infected with C. jejuni wild type compared to htrA-deficient mutants (Heimesaat et al., 2014a,b; Schmidt et al., 2019). In both models, higher levels of pro-inflammatory cytokines such as TNFα, IL-6, and IFNγ in the colonic mucosa were measured in HtrA+C. jejuni infection. Thus, indirect effects on the epithelial cell death are conceivable. Cytokines secretion, e.g., IL-13, IL-1β, IFN-γ, IL-10, IL-6, IL-17A, and IL-2, did not differ from control values in our co-culture, with an exception of TNFα. Moreover, gamma-glutamyl transeptidase was found to inhibit epithelial cells proliferation in Caco-2 and AGS cell lines but was not responsible for cellular apoptosis (Floch et al., 2014). Hence, although no bacterial factor produced by C. jejuni was reported to induce defined apoptosis in intestinal epithelial cells, defined cytotoxicity was shown before. Interestingly, cytokine cocktails composed of IFNγ, TNFα, IL-13, and IL-1β, found to be the main barrier relevant cytokines in C. jejuni-infected human biopsies, substantially aggravated the C. jejuni-mediated epithelial defects (Bück et al., 2018). Treatment with TNFα, a combination of TNFα and IFNγ, as well as solely IL-13 was shown to cause barrier dysfunction not only by tight junction alterations but also by the means of epithelial apoptosis in HT-29/B6 cells and rat colon (Grotjohann et al., 2000; Gitter et al., 2006; Heller et al., 2008). Therefore, we consider synergistic effects of cytokines and pathogen-host interaction described in previous studies (Rees et al., 2008; Bück et al., 2018) as the leading mechanism to epithelial apoptotic events. Thus, the co-culture setting as an infection model is particularly advantageous to further investigate these mechanisms.

Apoptosis as a Limiting Mechanism of C. jejuni Translocation

We assume that apoptosis inhibition not only tightens the barrier for solutes and macromolecules but also limits the translocation of C. jejuni via the paracellular route. The ability of C. jejuni to translocate across epithelial cell monolayer by paracellular and transcellular routes, is a crucial step of the infection. By reaching the subepithelial matrix, pathogen initiates the antigen presentation to the mucosal immune cells and reaches receptors located at the basolateral cell membrane of epithelial cells, like integrin receptors, which were shown to be necessary for cellular invasion (Backert et al., 2013). Surprisingly, transmigration of C. jejuni increased in Q-VD-Oph incubated samples. We suggest that the transcellular translocation capacity was increased with the higher number of viable epithelial cells. Additionally, the sealing of the focal leaks caused by apoptosis requires cytoskeletal transformation (Florian et al., 2002). Actin rearrangement and/or microtubule dynamics were also involved in the bacterial invasion process (Krause-Gruszczynska et al., 2007). Hence, this process might interfere after repair of apoptotic single cell lesions, resulting in impaired bacterial translocation.

The importance of epithelial cell death suggests the use of anti-apoptotic substances for prevention or treatment of the C. jejuni enteritis. Although medical use of caspase-inhibitors like Q-VD-Oph is unreasonable, substances with pronounced anti-apoptotic and immune-modulatory effect, e.g., vitamin D, curcumin and myrrh, have already been proven to their effectiveness in cell culture or mouse models (Rosenthal et al., 2017; Bück et al., 2018; Lobo de Sá et al., 2019; Mousavi et al., 2019). These compounds represent a potent alternative to the antibiotic treatment of C. jejuni infection.

CONCLUSION

Caspase-dependent epithelial apoptosis, caused either by TNFα or by direct bacterial cytotoxicity, are the main mechanisms of the C. jejuni-induced barrier dysfunction in the early state of infection. Epithelial apoptosis caused leakage of macromolecules...
from apical to the subepithelial compartment but was not associated with tight junction changes or increased bacterial transmigration. Induction of apoptosis is a key mechanism for the development of leak flux diarrhea. These mechanisms provide further insight into new therapeutic approaches for the campylobacteriosis.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the findings of this article will be made available by corresponding author, RB, or first author, EB, to any qualified researcher upon reasonable request.

**AUTHOR CONTRIBUTIONS**

EB and RB: conceptualization. EB, FL, and PN: data curation and Formal analysis. RB: funding acquisition, methodology, project administration, and supervision. EB: investigation and Writing the original draft. FL: resources. FL, PN, and RB: writing, review, and editing.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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