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The effects of *Lawsonia intracellularis*, *Salmonella enterica* serovar Typhimurium and co-infection on IL-8 and TNFα expression in IPEC-J2 cells

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ABSTRACT

Lawsonia intracellularis is among the most important enteric pathogens of swine and has been shown to be a risk factor for increased *Salmonella enterica* shedding. *S. enterica* serovar Typhimurium, in addition to being a significant pathogen of swine, also remains one of the most common causes of foodborne illness worldwide. Inflammation and the expression of IL8 and TNFα are an important process in the establishment of *S.* Typhimurium infection. Yet the effect of *L. intracellularis* on the expression of these cytokines by enterocytes, the niche both pathogens occupy during infection, is poorly understood. In this study we compared cytokine gene expression between singly and dually infected IPEC-J2 cells, a non-transformed porcine enterocyte cell line. Our results show that *L. intracellularis* leads to increased expression of IL8 and TNFα and has an additive effect on their expression in co-infection. The increase in expression of inflammatory cytokines may be one mechanism by which *L. intracellularis* favors *S.* Typhimurium infection.

1. Introduction

Lawsonia intracellularis is an obligate intracellular pathogen that infects mitotically active enterocytes and causes porcine proliferative enteropathy (PPE), a disease that leads to decreased weight gain, diarrhea and can lead to death of animals (Vannucci and Gebhart, 2014). A hallmark of PPE lesions is the absence or minimal infiltration of inflammatory cells to sites of infection (Vannucci and Gebhart, 2014). The transcriptome of infected enterocytes has been described (Vannucci et al., 2013a,b) and of note there was no increase of any cytokines including IL-1β, IL-8 and IL-18. These pro-inflammatory cytokines are secreted by enterocytes responding to the engagement of pathogen recognition receptors (PRRs) and lead to the recruitment of inflammatory cells (Mogensen, 2009). *L. intracellularis* is a Gram-negative bacterium that possess a polar flagellum and thus has several pathogen associated molecular patterns (PAMPs) that should be recognized by PRRs of the enterocyte to lead to the expression of pro-inflammatory cytokines and chemokines, yet this response remains ill defined.

Salmonella enterica is another significant enteric pathogen not only for swine but for humans. Non-typhoidal *Salmonella* including *Salmonella enterica* serovar Typhimurium remain a leading cause of foodborne illness worldwide (Kirk et al., 2015). Inflammation is known as a crucial part of *S. enterica* infection, which it utilizes to establish infection in the host (Kim and Isaacson, 2017) and the cytokines IL-8 and TNFα are important in mediating this process (Boyen et al., 2008; Drumo et al., 2016). It has also been shown that co-infection with *L. intracellularis* can increase the shedding of *S. enterica* in co-infected animals (Beloeil et al., 2004). Thus, this study had the objective of characterizing the response of IPEC-J2 cells, a porcine enterocyte cell line, to *L. intracellularis* infection by detecting expression of IL8 and TNF; as well as to investigate the impact of co-infection with both pathogens on the expression of these inflammatory cytokines.

2. Materials and methods

2.1. Cell line and culture conditions

The IPEC-J2 cell line, a non-transformed columnar epithelial cell line isolated from a piglet mid-jejunum, was cultured in antibiotic free media following previously described protocols (Brosnahan and Brown, 2012). The IPEC-J2 cells were from passages that ranged from 26-30. Cells were seeded on to 24 mm, 6 well, 0.4 μm size pore Transwell plates (Corning) at a concentration of 15×10^4 cells per well to allow for a confluence of 40–50% after overnight incubation. These culture conditions were used since growing cells and non-confluent conditions are needed for *L. intracellularis* culture (Vannucci et al., 2012).

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2.2. Bacteria for cell culture challenge and LPS

The *L. intracellularis* strain used was PHE/MN1-00 grown in IPEC-J2 cells following previously described protocols and of passage 47–55 (Vannucci et al., 2012). *L. intracellularis* used in assays was frozen in culture media and quantified by serial dilution and immunohistochemistry staining (Guedes et al., 2002). The *Salmonella enterica* serovar Typhimurium strain 798 was used in this study (Wood et al., 1989). It was grown to logarithmic phase in LB Miller broth for infection of IPEC-J2 cells, and the inoculum was quantified by serial plating on LB agar and using OD_{600} measurements (Schmidt et al., 2008). The LPS used was from *Salmonella enterica* serovar Typhimurium (Sigma, L6143).

2.3. IPEC-J2 cell infection and exposure to LPS

IL8 and TNFα gene expression by IPEC-J2 cells was measured at three different time points after infection with *L. intracellularis*. A one hour incubation time was used to directly compare the response of IPEC-J2 cells with the one hour incubation time of *S.* Typhimurium or LPS. The protocol was adapted from previously optimized protocols (Skjolaas et al., 2007; Arce et al., 2010). The four hour and three day incubation times were used to allow time for *L. intracellularis* to enter and replicate in the IPEC-J2 cells. It has previously been shown that *L. intracellularis* can enter IPEC-J2 cells within 10 min after exposure, become internalized within a membrane bound vacuole within 3 h and actively replicate freely in the cytoplasm within 2 days (McOrist et al., 1995). To directly compare and maintain all treatments similarly, each well was seeded with 15×10^4 IPEC-J2 cells. Following an overnight incubation, 10^7 *L. intracellularis* organisms were added to some of the wells and incubated for the three days in 10% hydrogen, 10% carbon dioxide, and 80% nitrogen atmosphere conditions to allow for *L. intracellularis* replication (Vannucci et al., 2012). After three days, the following exposures occurred: *S.* Typhimurium (10^7) was added to uninfected IPEC-J2 cells for one hour or to IPEC-J2 cells that were infected three days earlier with *L. intracellularis* (co-infection), LPS (1 μg/ml) was added to uninfected IPEC-J2 cells for one hour, and *L. intracellularis* (10^7) added to uninfected IPEC-J2 cells for one hour or four hours and cell culture media alone was added to serve as a negative control. The cells were incubated at 37 °C in an atmosphere of 5% CO_2. Immediately following the incubations, cells were collected and suspended in RNAlater following the manufacturer's instructions (Thermo Scientific). Each experiment was repeated twice, once in duplicate and another time in triplicate. Infection with *L. intracellularis* was confirmed by performing immunohistochemistry of infected IPEC-J2 cells using polyclonal antibody specific to *L. intracellularis* following a previously described protocol (Guedes et al., 2002). A parallel culture of *L. intracellularis* was also prepared to confirm infectivity and replication and monitored using immunohistochemistry.

2.4. RNA extraction and real time quantitative PCR

RNA extraction was performed using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. RNA quality and quantity were assessed with Nanodrop (Thermo Scientific) and RNA concentrations were normalized between all samples before proceeding to genomic DNA elimination and reverse transcription with the QuantiTect Reverse Transcription Kit (Qiagen). Real time PCR was performed using the Perfecta SYBER Green FastMix (Quanta Biosciences) with each primer at 300 nM. PCR cycling conditions consisted of an initial cycle at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 45 s. The primers used were: TNFα (5′-CGCCCACGTTGTAGCCAA TGT, 5′-CAGATAGTCGGGCAGGTTGATCTC) and IL8 (5′-TTCGATGCC AGTGCATAAATA, 5′- CTGTACAACCTTCTGCACCCA) (Arce et al., 2010). The housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-ACACTCACTCTTCTACCTTTG, 5′-CAAATTCAT TGTCGTACCAG) (Nygard et al., 2007) and beta-actin (5′- CAGACTAC CTCATGAAGATCC, 5′-ATCTGCTGGAAGGTGGACAG) were used. The beta-actin primers were designed using Primer 3 (Untergasser et al., 2012). Quantification of expression was performed with both housekeeping genes using the ΔΔCq method, with the average ΔCq of cells from control wells as the reference expression (Skjolaas et al., 2007). Negative control wells did not express TNFα, therefore a Cq value of 36 was attributed to these cells to allow for the quantification of gene expression.

2.5. Statistical analysis

The Wilcoxon Rank Sum test was performed to compare fold changes and Cq values between the different treatments in R (version 3.3.3 (2017-03-06)). P values were corrected for multiple comparisons and values below 0.05 were considered significant.

3. Results

3.1. The effect of Lawsonia intracellularis and co-infection with S. Typhimurium on IL8 expression

To determine if *L. intracellularis* induced IL8 expression in IPEC-J2 cells, we exposed cells to *L. intracellularis* for 1, 4 or 72 h. LPS and *S.* Typhimurium were used as positive controls following an adapted protocol previously described (Skjolaas et al., 2007; Arce et al., 2010). LPS induced an average fold change of 2.31 relative to negative control after one hour incubation and *S.* Typhimurium a fold change of 8.47 (Fig. 1). *L. intracellularis* exposure for one hour led to an average fold change of 0.93 and when incubated for four hours a fold change of 1.17. *L. intracellularis* incubation for 72 h led to an average fold change of 5.46 which was significantly higher than incubation for 1 h, 4 h or LPS treatment ($p < 0.05$) (Fig. 1).

Since *L. intracellularis* enhances shedding of *S. enterica*, we wondered if *L. intracellularis* infection of IPEC-J2 cells would alter expression of IL8 induced by *S.* Typhimurium. Adding *S.* Typhimurium to IPEC-J2 cells that had been infected with *L. intracellularis* three days earlier led to an average 15.00 fold increase in expression compared to the negative control cells (Fig. 1). This was significantly higher than both single *L. intracellularis* incubation for 72 h or *S.* Typhimurium infection ($p < 0.05$). Normalizing using either of the housekeeping genes, beta-actin or GAPDH, yielded very similar results. However, beta-actin was used to present this data since it had less variation among the different treatment groups compared to GAPDH. There was statistical significance between co-infection and single *S.* Typhimurium infection

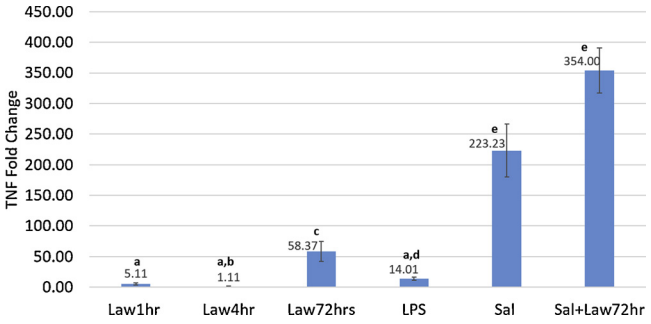

Fig. 1. IL8 fold change compared to negative control. Error bars represent standard error of the mean, values on top of bars represent treatment average, different letters indicate statistical significance ($p < 0.05$, Wilcoxon rank sum test). Law1hr = *L. intracellularis* incubation for 1 h; Law4hr = *L. intracellularis* incubation for 4 h; Law72hr = *L. intracellularis* incubation for 3 days; LPS = *S.* Typhimurium LPS treatment for one hour; Sal = *S.* Typhimurium incubation for one hour; Sal + Law72hr = *L. intracellularis* incubation for 3 days with a one hour *S.* Typhimurium incubation.

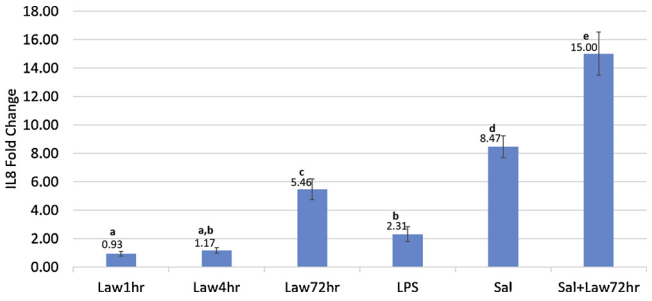

Fig. 2. TNF fold change compared to negative control. Error bars represent standard error of the mean, values on top of bars represent treatment average, different letters indicate statistical significance ($p < 0.05$, Wilcoxon rank sum test). Different letters indicate statistical significance ($p < 0.05$, Wilcoxon rank sum test). Law1hr = *L. intracellularis* incubation for 1 h; Law4hr = *L. intracellularis* incubation for 4 h; Law72hr = *L. intracellularis* incubation for 3 days; LPS = *S.* Typhimurium LPS treatment for one hour; Sal = *S.* Typhimurium incubation for one hour; Sal + Law72hr = *L. intracellularis* incubation for 3 days with a one hour *S.* Typhimurium incubation.

using beta-actin ($p < 0.05$) to normalize the data. Using GAPDH as the house keeping gene this comparison led to a p value greater than 0.05 (data not shown). Immunohistochemistry confirmed that *L. intracellularis* indeed did infect the IPEC-J2 cells (data not shown).

3.2. The effect of Lawsonia intracellularis and co-infection with S. Typhimurium on TNFα gene expression

We then investigated the effects of the various treatments on TNFα expression. The fold change measured when IPEC-J2 cells were exposed to LPS or *S.* Typhimurium were 14.01 and 223.23, respectively (Fig. 2). Incubation of IPEC-J2 cells for one hour with *L. intracellularis* led to an average fold change of 5.11 while incubation for four hours led to an average fold change of 1.11, these differences were not statistically significant. Incubating IPEC-J2 cells with *L. intracellularis* for 72 h significantly increased the expression of TNFα compared to the two earlier time points and to LPS with an average fold change of 58.37 ($p < 0.05$, Fig. 2). Co-infection with both *L. intracellularis* and *S.* Typhimurium led to an average fold change of 354. This was a higher expression compared to single *L. intracellularis* infection for 72 h ($p < 0.05$) and single *S.* Typhimurium infection (Fig. 2).

4. Discussion

IPEC-J2 cells have been shown to be a useful model for the study of pathogen-host interactions in swine (Brosnahan and Brown, 2012). As an enterocyte cell line, these cells represent the initial niche that both *L. intracellularis* and *S.* Typhimurium occupy to cause infection (Vannucci and Gebhart, 2014; Kim and Isaacson, 2017). A hallmark of *S.* Typhimurium infection is the release of pro-inflammatory cytokines from infected enterocytes, of which IL-8 is considered among the most crucial (Boyen et al., 2008). This chemokine leads to the recruitment of large numbers of neutrophils that amplify the inflammatory response (LaRock et al., 2015). *S.* Typhimurium has developed a metabolic predilection towards inflammation that enhances its competitive advantage over competing bacteria in inflammatory conditions (Kim and Isaacson, 2017). Thus, it is likely that with increased inflammation, *S.* Typhimurium would be favored in the gut.

An immune suppressive mechanism has been proposed in *L. intracellularis* infection due to the fact that infiltration of inflammatory cells is not a major feature of this infection (Vannucci and Gebhart, 2014). This observation is a bit surprising considering this bacterium possess flagella which should stimulate the PRR toll like receptor (TLR) 5. Being a Gram negative bacterium it also should stimulate other PRRs including NOD (nucleotide-binding oligomerization domain)-like receptors and TLR-4 which binds to LPS (Mogensen, 2009). When these PRRs are triggered they lead to the secretion of pro-inflammatory cytokines and chemokines including TNFα and IL8 that promote the migration of leukocytes (Mogensen, 2009). Some pathogens, however, have developed ways to suppress host responses and the secretion of inflammatory cytokines. For instance, while *S. enterica* flagellin is strongly recognized by TLR5, other bacteria such as *Helicobacter pylori* have a different flagellin composition that is not detected by TLR5 (Andersen-Nissen et al., 2005). Another example is *Staphylococcus aureus* whose beta-hemolysin can specifically inhibit IL8 expression and migration of neutrophils (Tajima et al., 2009). In this study, we found that exposure of IPEC-J2 cells to *L. intracellularis* for one or four hours induced minimal levels of TNFα and IL8. However when given a longer time to replicate, we observed a significant increase in the expression of these cytokines in infected cells ($p < 0.05$, Figs. 1 and 2). This indicates that *L. intracellularis* likely does not actively inhibit the enterocyte innate immune host response when given a longer time to replicate, at least as measured by these inflammatory cytokines. An immune suppressive mechanism, however, is still possible since *L. intracellularis* alone for one or four hours did not lead to much induction of IL8 or significant TNFα expression as compared to the other treatments, and time points beyond three days were not investigated. It must also be noted that the passage of *L. intracellularis* used (passage 55) is less virulent than the same strain with less passages (Vannucci et al., 2013a,b). Interestingly, comparing the one hour incubation time of IPEC-J2 cells with *L. intracellularis* to a one hour incubation time with *S.* Typhimurium demonstrates the stark difference between both pathogens. Within one hour, *S.* Typhimurium readily led to a large and significant increase of TNFα and IL8 while it took between four and 72 h for *L. intracellularis* to induce comparable amounts of gene expression. It must be considered, however, that *L. intracellularis* was thawed prior to the one hour infection whereas *S.* Typhimurium was already in log phase. Since *L. intracellularis* growth cannot be monitored by OD like *S.* Typhimurium, bacteria had to be quantified after propagation in culture and stored at −80 °C until used in the experiment.

Skjolaas et al., 2007 demonstrated that previous exposure of IPEC-J2 cells to *Bacillus licheniformis* led to a significant decrease in secretion of IL-8 after subsequent exposure to *S.* Typhimurium; whereas no difference was observed with previous exposure to *Lactobacillus reuteri*. Probiotic treatment with *B. licheniformis* has recently been found to significantly reduce the shedding and colonization of *S. Typhimurium* in experimentally challenged pigs (Barba-Vidal et al., 2017). This demonstrates that different bacteria can have different effects on the host response to *S.* Typhimurium. Co-infection of *S.* Typhimurium with *L. intracellularis* did not decrease IL8 expression but rather increased it (Fig. 1). This suggests that *L. intracellularis* may increase inflammation which is favorable to *S.* Typhimurium infection. Infection with *L. intracellularis* has been shown to increase *S. enterica* shedding and vaccination against *L. intracellularis* has been shown to decrease *S.* Typhimurium shedding in co-infected animals (Beloeil et al., 2004; Leite et al., 2018). It is important to consider that co-infection cytokine levels were also increased compared to single *L. intracellularis* infection. This suggests that *S.* Typhimurium likely also changes the host's response to *L. intracellularis*. Confirmation of these cytokine changes at the intestinal level with co-infection *in vivo* would strengthen these findings. Recently, an inflammatory signature was found in animals with progressed lesions infected with *L. intracellularis*. The same study found TNF to be a significant and activated upstream regulator of gene expression in intestinal tissue (Leite et al., 2019). This finding correlates with the increase in TNF expression induced by *L. intracellularis* observed here and the suggestion that inflammation may be involved in the favoring of one pathogen by another.

This study characterized the IPEC-J2 enterocyte response to two crucial cytokines involved in intestinal inflammation. We found that *L. intracellularis* does not seem to suppress the enterocyte innate immune host response, but rather induces significant IL8 and TNF expression

when allowed to replicate for a longer period. This is important information to better comprehend its pathogenesis. The finding that *L. intracellularis* can increase IL8 and TNFα gene expression in co-infection by *S.* Typhimurium suggests both pathogens may act in favor of each other to induce inflammation as measured by these cytokines at the enterocyte level.

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