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***Salmonella enterica* serovar Choleraesuis infection of the porcine jejunal Peyer's patch rapidly induces IL-1 β and IL-8 expression**

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Abstract

Salmonella enterica serovar Choleraesuis is an enteric pathogen of swine, producing septicemia, enterocolitis, pneumonia, and hepatitis. The initial molecular events at the site of *Salmonella* infection are hypothesized to be critical in the initiation of innate and adaptive immune responses; however the acute immune response elicited by porcine intestinal tissues is not well understood. To address this need, we employed explants of jejunal Peyer's patch (JPP) mucosa from pigs to examine *Salmonella*-induced immune responses under controlled conditions as well as to overcome limitations of whole animal approaches. JPP explants mounted in Ussing chambers maintained normal histological structure for 2 h and stable short-circuit current and electrical conductance for 2.5 h. After *ex vivo* luminal exposure to *Salmonella* serovar Choleraesuis, JPP responded with an increase in mRNA expression of IL-1 β and IL-8, but not TNF α . Increased IL-1 β and IL-8 expression were dependent on efficient *Salmonella* adhesion and internalization, whereas mutant *Salmonella* did not induce inflammatory cytokine expression. Commensal enteric bacteria, present in some experiments, also did not induce inflammatory cytokine expression. These findings indicate that *Salmonella* uptake by Peyer's patch is important in the induction of an innate response involving expression of IL-1 β and IL-8, and that *ex vivo* intestinal immune tissue explants provide an intact tissue model that will facilitate investigation of mucosal immunity in swine.

Keywords

animal models; swine; cytokines; interleukins; mucosal immunology

1.1.1.2 Introduction

Salmonella enterica represents a serologically diverse group of enteric pathogens capable of infecting both animals and humans. *Salmonella* serovar Choleraesuis is a major enteric pathogen in swine which produces septicemia, enterocolitis, pneumonia, and hepatitis, and is a common cause of systemic disease (Schwartz, 1999). Ingested *Salmonella* invade epithelial cells of the small intestine, including those of the gut-associated lymphoid tissue (GALT). Peyer's patches and mesenteric lymph nodes constitute a part of the organized GALT which provides specific, acquired immune defense against bacterial pathogens (Mowat, 2003).

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To date, the innate mucosal immune response to *Salmonella* has not been described in pigs. In this species, studies have focused primarily on vaccine development (Kennedy et al., 1999; Maes et al., 2001) and the characteristics of *Salmonella* infection in different intestinal regions (Bolton et al., 1999; Meyerholz and Stabel, 2003). Within 10 minutes of *Salmonella* serovar Choleraesuis or Typhimurium exposure in porcine ileal and jejunal loop models, bacteria adhere to the brush border of enterocytes and invade epithelial cells. After 1 to 2 h, *Salmonella* are detected within epithelial cells and the lamina propria (Meyerholz and Stabel, 2003; Schauser et al., 2004). Studies of porcine Peyer's patches indicate that the follicle-associated epithelium within jejunal Peyer's patches preferentially takes up inert particles in comparison to the neighboring absorptive epithelium (Beier and Gebert, 1998; Liebler et al., 1995). Peyer's patches constitute the main portal of entry early in infection by *Salmonella* serovar Typhimurium (Jones et al., 1994; Schauser et al., 2004).

Virulent *Salmonella* inject protein effectors via a type III secretion system to induce actin rearrangements and macropinocytosis by epithelial cells (Cossart, 2004). *Salmonella* flagellin is translocated to the epithelial basolateral membrane and binds to type 5 Toll-like receptors (TLR5) (Gewirtz et al., 2001a; Gewirtz et al., 2001b). Binding of flagellin to TLR5 activates NF- κ B and MAPK signaling pathways and induces expression of inflammation-associated genes, including IL-8 and other chemokines (CXCL8) (Elewaut et al., 1999; Gewirtz et al., 2001a; Medzhitov, 2001). Injection of SipB protein by *Salmonella* serovar Typhimurium or Dublin induces caspase-1-mediated cell death of macrophages and dendritic cells (Hersh et al., 1999; van der Velden et al., 2003; Watson et al., 2000). Caspase-1 enzymatically cleaves IL-1 β and IL-18 to their bioactive forms, and IL-1 β additionally activates NF- κ B-mediated transcription of chemokines (Lee et al., 2004; Monack et al., 2000). The combined secretion of IL-8 (CXCL8), MIP-2 (CXCL2), and MIP-3 α (CCL20) induces chemotaxis of neutrophils and dendritic cells to the site of *Salmonella* invasion (Coates and McColl, 2001; McCormick et al., 1993; Sierro et al., 2001; Zhang et al., 2003).

The early, innate host response to infection is critical for the development of an effective adaptive immune response (Medzhitov and Janeway, 2000). Clarification of the acute molecular and cellular events occurring in response to enteric infection is important in understanding the mechanisms underlying innate immune responses to *Salmonella*. Although simplified cell culture systems are useful in identifying *Salmonella* virulence factors required for invasion of host cells (McCormick, 2003), the complexity of the Peyer's patch cannot be readily recreated *in vitro*. In addition, many studies utilize cell lines originating from outside of the intestine, and thus may exhibit markedly different phenotypes compared to cells residing in the GALT. For example, intestinal macrophages do not function like monocytes in their responses to lipopolysaccharide (Spottl et al., 2001). In addition, epithelial cells exhibit polarized expression of surface proteins (Gewirtz et al., 2001a), and *Salmonella* infection of non-polarized epithelial cell layers may induce responses which would not occur *in vivo*.

An investigation of intact mucosal tissues may reveal integrated signaling between cells residing in the Peyer's patches which would not be evident when studying isolated cell types. Therefore, we have examined acute cytokine responses of intact porcine JPP mucosa to *Salmonella* exposure *ex vivo* in an Ussing chamber. Intestinal tissues were obtained from pigs conventionally colonized with commensal microflora, as a competent intestinal immune system was necessary in these studies. Enteric commensal bacteria are critical to the development of Peyer's patches (Pabst and Rothkotter, 1999), production of antibodies (Butler et al., 2002; Cukrowska et al., 2001), and migration of B and T lymphocytes to the Peyer's patches (Yamanaka et al., 2003). Our findings indicate that intact JPP respond rapidly to *Salmonella* infection by increased expression of IL-1 β and IL-8 mRNAs, and efficient *Salmonella* adhesion and internalization are required to induce these changes in mRNA expression.

1.1.1.3 Materials and Methods

Animals

Weaned Yorkshire-Landrace outbred pigs of each sex (5 – 8 weeks of age) were clinically healthy, had continuous access to water and nonmedicated pig feed, were not fasted prior to sacrifice, and were under veterinary care. Randomly selected pigs were further screened for *Salmonella* by an *invA*-based PCR assay and by bacterial isolation from intestinal tissues. All procedures were conducted on animals anesthetized by an intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol®; 8 mg/kg; Fort Dodge Laboratories, Inc, Fort Dodge, IA) in combination with xylazine, and subsequently euthanized with intravenous Beuthanasia®-D Special (0.5 ml/kg; Schering-Plough Animal Health, Union, NJ) in accordance with approved University of Minnesota IACUC protocols. Segments of jejunum containing Peyer's patches were removed within 15 min after death and placed in ice-cold, oxygenated Krebs' bicarbonate buffer (composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 0.5 mM MgCl₂, 25 mM NaHCO₃, 1.0 mM NaH₂PO₄, and 11.0 mM D-glucose). Intestinal segments were incised longitudinally along the mesenteric border and stripped of the circular and longitudinal muscle layers by blunt dissection.

Bacterial phenotypes, growth and ex vivo inoculation

Salmonella serovar Choleraesuis strain χ 3246 is a virulent porcine field isolate (Kennedy et al., 1999). *Salmonella* serovar Choleraesuis strain χ 4522 is Δ *cya* Δ (*crp-cdt*) *pmi*-3834 *vpl*⁺. Both strains of *Salmonella* were grown statically overnight in Luria-Bertani broth (LB) at 37 °C ($\geq 10^8$ CFU ml⁻¹). Overnight cultures of strain χ 3246 were diluted 1:100 in fresh LB media and allowed to grow in static culture at 37°C for two h until they reached the early logarithmic phase of growth ($\leq 1 \times 10^8$ CFU ml⁻¹).

Bacterial quantitation

To quantitate intracellular bacteria, the follicle-containing region of the tissue was incubated at 37°C under 5% CO₂ in PBS containing 100 µg/ml gentamicin for 80 min to kill extracellular bacteria (Elsinghorst, ; Green et al., 2003). For assessment of total bacteria (adherent and intracellular), the follicle-containing JPP tissue was washed in three separate aliquots of PBS. Tissues were homogenized, serially diluted, and spread-plated in triplicate on differential media, XLD and Brilliant Green (BG) agar (Becton Dickinson, Sparks, MD), to differentiate *Salmonella* from enteric rapid lactose fermenting bacteria. Comparison of growth on XLD and BG additionally differentiated wild type and mutant *Salmonella* strains. Strain χ 3246 grew equally well on XLD and BG and formed black, H₂S positive colonies on XLD, while strain χ 4522 grew better on BG and formed red, H₂S negative colonies on XLD. The amount of adherent *Salmonella* was determined by calculating the total of adherent and intracellular *Salmonella* minus the number of intracellular *Salmonella*, in matched samples.

Ussing chamber and measurement of transepithelial ion transport

Explants of proximal jejunum containing Peyer's patches with smooth muscle layers removed, as described above, were mounted in Ussing chambers with a flux area of 2 cm² and short circuited (O'Grady, 1996). Between four and nine tissue pieces from one animal were analyzed in each experiment. Tissues were bathed on both sides in a physiological salt solution (composition: 130 mM NaCl, 6 mM KCl, 3 mM CaCl₂, 0.7 mM MgCl₂, 0.29 NaH₂PO₄, and 1.3 mM Na₂HPO₄; pH 7.4) that was circulated in water-jacketed reservoirs maintained at 39 °C (porcine core temperature) and gassed with 95% O₂/ 5% CO₂ by gas lift. Ten mM mannitol or D-glucose was added to the luminal or contraluminal sides of the tissue, respectively. The antibiotics polymyxin B (50 µg/ml) and trimethoprim (50 µg/ml) (Fisher, Pittsburgh, PA) were added to the contraluminal bathing medium. Preliminary experiments demonstrated that

addition of contraluminal antibiotics decreased the numbers of internalized commensal bacteria, but had no effect on *Salmonella* internalization (data not shown).

Tissues were incubated for 30–45 min for stabilization of baseline electrical parameters before bacteria were added to the luminal reservoir and permitted to interact with the mucosa for periods from 1 to 3.5 h. Short-circuit current (I_{sc} , in $\mu A/cm^2$), a measure of active ion transport, was measured continuously in each JPP tissue by an automatic voltage clamp apparatus (Model TR100, JWT Engineering, Overland Park, KS). Tissues were subjected to a 5 mV pulse every 100 sec to permit determination of tissue electrical conductance (G_t , in mS/cm^2), a measure of ionic permeability, as previously described (O'Grady, 1996). Mock infected JPP controls were incubated in Ussing chambers for identical time intervals in the absence of luminal *Salmonella*. To assess tissue viability, active glucose-stimulated Na^+ absorption was measured at the end of each experiment by the rapid elevation in I_{sc} occurring in response to the luminal addition of 10 mM glucose. Electrophysiological data were collected and analyzed by PowerLab Chart software (Version 4.0, ADInstruments, Colorado Springs, CO).

Histology

Tissues containing JPP follicles were excised from the flux area, fixed in 10% buffered formalin, embedded in paraffin, sectioned (5–7 μm) and stained with hematoxylin and eosin. Two to three sections from each of two or three JPP samples from 2 pigs were examined at each time point.

RNA isolation

Tissues were stored in RNAlater (Ambion, Austin, TX) at $-20^\circ C$ until total RNA isolation with RNeasy midi columns (Qiagen, Valencia, CA). RNA quality was determined by Agilent 2100 Bioanalyzer analysis with a RNA Nano chip (Agilent Technologies, Palo Alto, CA) and RNA 6000 ladder (Ambion, Austin, TX).

Real time quantitative RT-PCR

The relative expression of messenger RNA transcripts from specific genes was measured in triplicate by real-time quantitative RT-PCR. Reverse transcriptase reactions were performed following a modified Superscript II RNaseH- protocol (Invitrogen, Carlsbad, CA) with 2 μg of total RNA and random hexamer primers (Applied Biosystems, Foster City, CA). Approximately 30 ng of converted total RNA, diluted in 10 mM Tris-HCl, 1 mM EDTA, was mixed with the SYBR Green I Master Mix kit (Applied Biosystems, Foster City, CA), and porcine specific primers designed by Primer3 (Rozen and Skaletsky, 2000) (Table 1). The reactions were denatured at $95^\circ C$ for 10 minutes followed by 50 cycles of $95^\circ C$ for 15 seconds and $60^\circ C$ for 1 minute in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR efficiencies were calculated by linear regression of the log transformed, background subtracted relative fluorescence values (LinRegPCR program, ver. 7.4) (Ramakers et al., 2003), then outlier efficiency values were excluded (Bar et al., 2003), and the mean efficiency (E) was calculated (Table 1). Relative starting template fluorescence (R_0) was determined using the method of Peirson et al. (Peirson et al., 2003). R_0 values for specific genes were normalized by dividing with the R_0 values of cyclophilin A or β_2 -microglobulin, and these normalized values were expressed relative to those of untreated control samples, pooled from the JPP of three pigs. To confirm amplicon specificity, the PCR products were subjected to melting curve analysis (Schmittgen et al., 2000) at $95^\circ C$ for 15 seconds, $60^\circ C$ for 20 seconds, then ramping from $60^\circ C$ to $90^\circ C$ over 20 minutes or were analyzed by agarose gel electrophoresis.

Statistics

Statistical analyses of data were performed using the PRISM computer software program (Version 3.03; GraphPad Software, Inc., San Diego, CA), and analyzed by an unpaired t-test, ANOVA, or correlation where appropriate. P values less than 0.05 were considered significant.

1.1.1.4 Results

Electrophysiological and morphological characteristics of the ex vivo model

JPP tissues maintained in Ussing chambers displayed no statistically significant changes in I_{sc} or G_t over time (Table 3). Although the difference between G_t of mock-infected and *Salmonella*-exposed JPP was statistically significant (Table 3), there was no significant difference in G_t before and after the addition of the *Salmonella* culture to the luminal reservoir (15.3 ± 0.9 mS/cm², 15 min in the Ussing chamber, compared to 16.8 ± 0.8 mSiemens/cm² at 1.0 h, unpaired t-test). Luminal infection of JPP with *Salmonella* serovar Choleraesuis did not alter I_{sc} for periods up to 2.5 h (Table 3, two-way ANOVA). These data indicate that spontaneous active ion transport and ionic permeability were intact during this time period and *Salmonella* infection had no overt effect on tissue function. Histological examination of mucosal tissue indicated that after 2 h in the chamber, the epithelium was intact (Figure 1). Peak elevations in I_{sc} after luminal addition of 10 mM glucose were not significantly different in tissues exposed to *Salmonella* (20.5 ± 4.0 μ A/cm², 19 JPP/ 5 pigs) or mock-infected (19.7 ± 4.7 μ A/cm², 9 JPP/ 6 pigs) at time points from 1.5 to 2.5 h in the chamber.

Bacterial internalization and the innate response of isolated JPP

Salmonella grown to early log phase and exposed to JPP at a density of 10^4 – 10^6 CFU/ml were internalized, but mRNA levels of inflammatory cytokines were not greatly altered relative to mock-infected JPP (Figure 2, low inoculum of 10^6 CFU/ml). *Salmonella* serovar Choleraesuis strain χ 3246, both after early log and overnight growth, invaded JPP and induced cytokine responses which were dependent on the time of exposure. Incubation of tissue with more *Salmonella* (10^7 to 10^8 CFU/ml) in either log or stationary growth phase for 3.5 h maximally increased mRNA levels of IL-1 β , IL-6 and IL-8 in JPP (Figure 2).

Conditions of overnight growth and a high amount (10^7 CFU/ml) of *Salmonella* were selected to further characterize the interaction of *Salmonella* and JPP. *Salmonella* serovar Choleraesuis strain χ 3246 was significantly internalized by JPP after contact with the luminal aspect for 2 h compared to strain χ 4522, $p < 0.05$ (Figure 3A). Strain χ 3246 also adhered to JPP at a greater level than strain χ 4522, although these differences were not statistically significant (3 JPP preparations from 3 pigs) (Figure 3B). No internalized *Salmonella* were detected in mock-infected or uninfected JPP (15 and 12 JPP/ 8 pigs, respectively). After 2 h incubation in the luminal bathing media, numbers of *Salmonella* serovar Choleraesuis strain χ 3246 and χ 4522 were increased two-fold compared to the initial inoculum (7.3 ± 0.1 log₁₀ CFU/ml at time 0 and 7.6 ± 0.1 log₁₀ CFU/ml at 2 h, 26 JPP/ 7 pigs). These data indicate that the bathing media supported minimal, if any, *Salmonella* replication.

Indigenous enteric bacteria were detected from tissues incubated in Ussing chambers (48% of JPP) and in untreated JPP (50% of JPP). However, there was no relationship between the cytokine mRNA levels in mock-infected JPP and the numbers of commensal bacteria after 2 h incubation in the Ussing chamber, indicating that the presence of commensal bacteria did not induce changes in cytokine mRNA (Figure 4). Moreover, the numbers of internalized commensal bacteria measured in JPP were not significantly altered by the presence (3.3 ± 1.4 log₁₀ CFU/g commensal bacteria) or absence (3.5 ± 1.1 log₁₀ CFU/g commensal bacteria) of *Salmonella*.

Infection with *Salmonella* serovar Choleraesuis strain χ 3246 (14 JPP/ 7 pigs) induced a significant increase in IL-1 β and IL-8 mRNA levels compared to cytokine mRNA levels in mock-infected JPP (13 JPP/6 pigs) (t-test, $p < 0.01$) (Figure 5). Luminal exposure to the same amount of mutant strain χ 4522 (7 JPP/4 pigs) induced an increase in cytokine mRNA expression, which was comparable to that of mock-infected controls. Changes in TNF α gene expression were insignificant in all treatments (Figure 5).

Discussion

In the present study, we have shown that IL-1 β and IL-8 are important components of the innate response of porcine jejunal Peyer's patches to *Salmonella* infection. Induction of IL-1 β and IL-8 mRNA in ileal PP correlated with migration of neutrophils to the mucosa, 6 h after intragastric inoculation of pigs with *Salmonella* serovar Choleraesuis χ 3246 (Hyland et al., unpublished data). *Salmonella*-mediated induction of IL-1 β and IL-8 has been demonstrated in epithelial cell lines and in the jejunum of chickens, which do not have Peyer's patches (McCormick et al., 1993; Withanage et al., 2004; Zeng et al., 2003). *Salmonella* serovar Typhimurium infection of ligated intestinal loops in mice increases mRNA levels of IL-1, TNF α , and IL-6 along with secretion of TNF α protein (Arnold et al., 1993; Klimpel et al., 1995). However, in this study, TNF α mRNA levels were not significantly increased after *ex vivo* infection, indicating that TNF α may not be as critical to the early response to *Salmonella* in pigs as it is in mice. In the *ex vivo* model, we observed an increase in IL-6 mRNA levels in both mock- and *Salmonella*-infected JPP. Thus, it may represent a tissue response to the nonspecific stress of incubation in the Ussing chamber similar to its induction in keratinocytes after skin damage for enhanced wound healing (Wang et al., 2004).

After luminal exposure to JPP *ex vivo*, virulent *Salmonella* serovar Choleraesuis strain χ 3246 was internalized intracellularly within one hour of exposure. Attenuated strain χ 4522 was selected since it did not protect pigs against challenge with virulent *Salmonella* serovar Choleraesuis (Kennedy et al., 1999). *Salmonella* serovar Choleraesuis strains such as χ 4522, which lack cyclic AMP (cAMP) and the cAMP receptor protein (CRP) global regulatory system, are defective in transport and catabolism of carbohydrates, amino acids and peptides as well as diminished in their ability to attach to and invade cells (Botsford and Harman, 1992; Kelly et al., 1992). Although strain χ 4522 is attenuated in both growth characteristics and invasion of cells, it is likely that cell adhesion and invasion are more important early in the pathogenic process. The numbers of both *Salmonella* strains in the luminal bathing medium did not change significantly during 2 h, increasing on average only 2-fold. In addition, growth of *Salmonella* serovar Typhimurium inside cells is inhibited, with one division occurring every 1.5 to 2.5 h in peritoneal macropages (Lowrie et al., 1979).

These data indicate that a high level of *Salmonella* adhesion and internalization was necessary for the induction of IL-1 β and IL-8. These results suggest that *Salmonella* strains with mutations in invasion-associated genes would not be expected to induce an effective innate or adaptive response. This knowledge should aid in efforts directed toward rational vaccine design since inflammatory cytokine expression is important for the induction of adaptive immune responses (Murtaugh and Foss, 2002).

For several decades, active and paracellular solute transport has been investigated across sheets of intestinal mucosa mounted in Ussing chambers (Lindemann, 2001). In previous studies of human intestinal mucosa maintained in Ussing chambers, I_{sc} and G_t were stable for at least 2 h and the epithelial surface remained histologically intact for at least 2.5 h (Larsen et al., 2001; Soderholm et al., 1998). Because epithelial degradation and edema occur after several hours of incubation (Figure 2 and (Larsen et al., 2001; Soderholm et al., 1998), experiments in the Ussing chamber were limited to 2 to 3 h. Glucose-stimulated Na⁺ absorption at the end

of the incubation period demonstrated that the JPP was functionally intact (Larsen et al., 2001).

Salmonella serovar Choleraesuis exposure did not alter electrophysiological parameters, I_{sc} or G_t . By contrast, *Salmonella* serovars Typhimurium and Dublin decrease transepithelial resistance (TER) across T84 or MDCK monolayers (Otte and Podolsky, 2004; Tafazoli et al., 2003). However, *Salmonella* serovar Typhimurium exposure did not significantly alter short circuit current or conductance of porcine ileal absorptive epithelial mucosa during a 90 min period (K.L. Scheiber and D.R. Brown, unpublished observations). Exposure of porcine JPP to *Salmonella* serovar Choleraesuis strain SC-54 also did not significantly alter tissue conductance (Green et al., 2003). Comparisons of *Salmonella* serovars Choleraesuis, Typhimurium and Dublin exposure to porcine ligated intestinal loops indicated that serovar Dublin induced maximum tissue damage, while serovar Typhimurium induced greater amounts of epithelial swelling than serovar Choleraesuis (Bolton et al., 1999; Meyerholz and Stabel, 2003). It has been suggested that the minimal cell damage induced by *Salmonella* serovar Choleraesuis may allow it to more easily enter the systemic circulation due to a reduced inflammatory response (Meyerholz and Stabel, 2003). These observations are consistent with and help explain the lack of effect of *Salmonella* serovar Choleraesuis on the electrophysiological responsiveness of intact mucosal tissue.

An *ex vivo* model such as the one thus described provides a controlled setting to investigate early mucosal immune responses to *Salmonella* and other enteric pathogens. The Ussing chamber model reduces physiological and animal-to-animal variation within each experiment, as JPP from an individual animal can be exposed to different treatment regimens. The presence of intracellular rapid lactose fermentors, which likely represent nonpathogenic intestinal commensal bacteria, in the JPP did not interfere with the studies. Mock-infected JPP demonstrated a slight increase in cytokine mRNA levels relative to untreated JPP as shown in Figure 5, but there was no relationship between the presence or absence of internalized commensal bacteria and changes in cytokine mRNA.

Inflammatory cytokine responses, which are essential to the induction of effective adaptive immunity, were rapidly increased after infection of jejunal Peyer's patches *ex vivo* with virulent *Salmonella* serovar Choleraesuis. IL-1 β and IL-8 mRNA levels were both consistently increased after *Salmonella* exposure, but TNF α was not changed. These results indicate that the *ex vivo* JPP tissue preparation retains the ability to respond innately with specific cytokine and chemokine signaling. In addition, the expression of IL-6 under all conditions of tissue incubation is consistent with a stress response that must be considered in the model.

Abbreviations

AE, absorptive epithelium; I_{sc} , short circuit current; GALT, gut-associated lymphoid tissue; G_t , conductance; JPP, jejunal Peyer's patches; IL, interleukin; LB, Luria-Bertani broth.

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Figure 1.

Histology of Peyer's patch mucosa mounted in Ussing chambers and bathed in oxygenated physiological salt solution. Representative photomicrographs (40X magnification) of H & E-stained jejunal Peyer's patches in cross-section immediately after isolation (A), and after incubation in Ussing chamber for 2 h (B). Photomicrographs are representative of observations in 2–3 tissues from two pigs.

Figure 2.

Virulent *Salmonella* serovar Choleraesuis strain χ 3246 induced time dependent changes in cytokine gene expression of JPP. *Salmonella* grown to early log phase were added at a low inoculum ("low", 10^6 CFU/ml) and *Salmonella* grown to early log or overnight growth were added at a 10-fold higher inoculum ("high", 10^7 CFU/ml) for 1 or 3.5 h. Levels of IL-1 β , IL-6, IL-8, and TNF α mRNA transcripts were measured by real-time quantitative PCR, and fold changes are expressed relative to cyclophilin A mRNA levels and an untreated control JPP. The results are representative of experiments in two pigs. Commensal bacteria were detected at $3.6 \pm 0.3 \log_{10}$ CFU/g (mean $\pm$ SE) in 13 of 19 JPP from two pigs.

Figure 3.

Virulent *Salmonella* serovar Choleraesuis strain χ3246 is internalized by JPP more efficiently than attenuated strain χ4522 after exposure 2 h *ex vivo*. **A.** Tissues were exposed on their luminal aspect to $7.3 \pm 0.1 \log_{10}$ CFU/ml (mean $\pm$ SE) of strain χ3246 (21 JPP/10 pigs) or strain χ4522 (11 JPP/ 7 pigs) for 2 h. * $p < 0.05$. Commensal bacteria were detected in 9 of 31 *Salmonella* -infected JPP from 10 pigs, with a mean of $3.1 \pm 0.5 \log_{10}$ CFU/g (mean $\pm$ SE). **B.** *Salmonella* serovar Choleraesuis strain χ4522 adheres to and is internalized poorly by JPP tissue. Tissues were exposed on their luminal aspect to $6.7 \pm 0.1 \log_{10}$ CFU/ml (mean $\pm$ SE) of strain χ3246 or strain χ4522, both grown overnight in LB (3 JPP/3 pigs) for 2 h. Values are CFU/g tissue after 2 h, expressed as a percentage of the *Salmonella* inoculum in the luminal bathing medium at time zero, and the error bars show standard error. The open bar (□) represents adherent bacteria and the solid bar (■) represents internalized *Salmonella*.

Figure 4.

Virulent *Salmonella* serovar Choleraesuis strain χ 3246 infection for 2 h induces increased expression of IL-1 β and IL-8 mRNA compared to mock infect JPP and attenuated strain χ 4522. Tissues were not mounted in the Ussing chamber (8 JPP/6 pigs untreated JPP), or mock-infected by mounting in the Ussing chamber for 2 h (13 JPP/6 pigs JPP), or luminally exposed to $7.5 \pm 0.1 \log_{10}$ CFU/ml (mean $\pm$ SE) of strain χ 3246 (16 JPP/6 pigs) or strain χ 4522 (7 JPP/4 pigs) for 2 h. Levels of IL-1 β , IL-6, IL-8, and TNF α mRNA transcripts were measured by real-time quantitative PCR, and fold changes were expressed relative to β 2-microglobulin mRNA levels and to pooled untreated control JPP. The error bars indicate standard error. Commensal bacteria were detected in 22 of 44 JPP from 6 pigs, with a mean of $3.2 \pm 0.3 \log_{10}$ CFU/g (mean $\pm$ SE). * $p < 0.01$ compared to mock-infected by unpaired t-test.

Figure 5.

JPP mounted in the Ussing chamber for two h express the similar amounts of IL-1 β , IL-6 and IL-8 mRNA when commensal bacteria were present or not. JPP were mounted in the Ussing chamber for two h and the numbers of intracellular commensal bacteria were determined. Levels of IL-1 β , IL-6, and IL-8 mRNA transcripts were measured by real-time quantitative PCR, and fold changes are expressed relative to β 2-microglobulin mRNA levels and to pooled untreated control JPP. Error bars represent standard error of 5 JPP without any detectable commensal bacteria ($\square$) and 10 JPP with greater than 50 CFU/g commensal bacteria ($\blacksquare$).

Table 1

Porcine primer sequences for real-time quantitative RT-PCR analysis.

Gene	Sequence 5' to 3'	GenBank Accession No.	Final conc. (nM)
β 2 microglobulin	F ^I TCTACCTTCTGGTCCACACTGAG	L13854	30
	R ^I TCATCCAACCCAGATGCA		65
cyclophilin A	F GCGTCTCCTTCGAGCTGTT	AY008846	100
	R CCATTATGGCGTGTGAAGTC		100
IL-1 β	F CCTCCTCCCAGGCCTTCTGT	M86725	30
	R GGGCCAGCCAGCACTAGAGA		65
IL-6	F ATTAGTACCAAAGCACTGATCC	M86722	100
	R TGAGAATGATCTTGTGTCTTC		100
IL-8	F AACTGAGAGTGATTGAGAGTGGA	M86923	100
	R GCTGTTGTTGTTGCTTCTCAGTT		100
TNF α	F TTCCAGCTGGCCCTTGAGC	X57321	70
	R GAGGGCATTTGGCATACCCAC		30

^IF= forward, R= reverse

Table 2
PCR reaction efficiency and basal gene expression levels in untreated porcine jejunal Peyer's patches

Gene	Size (bp) ¹	Product Tm (°C) ²	PCR Reaction Efficiency ³	Relative Template Abundance ⁴	Number of Pigs
β2 microglobulin	161	81.77 ± 0.02	1.92 ± 0.01	2,607.0 ± 558.5	5
cyclophilin A	160	80.46 ± 0.04	1.61 ± 0.01	2,894.0 ± 405.4	2
IL-1β	178	81.20 ± 0.03	1.87 ± 0.01	19.3 ± 5.1	7
IL-6	148	82.89 ± 0.03	1.57 ± 0.01	8.5 ± 1.3	7
IL-8	157	79.08 ± 0.03	1.87 ± 0.01	218.7 ± 45.8	7
TNF α	146	83.78 ± 0.07	1.79 ± 0.01	46.9 ± 12.6	6

¹ PCR product size

² T_m (mean ±SE, n= 26-55 samples), determined experimentally by melting curve analysis (Schmittgen et al., 2000).

³ PCR reaction efficiency (mean ±SE, n= 24-53 samples)(Ramakers et al., 2003)

⁴ Relative gene abundance in 10⁹ fluorescence units (mean ±SE, n= 2-7 pigs) (Peirson et al., 2003)

Table 3
Electrophysiological parameters of JPP after *Salmonella* exposure

Condition	Time in the Ussing chamber			n/N ²
	t=30 min	t=1 h	t=2 h	
<i>Salmonella</i> ¹ , G _i (mS/cm ²)	16.6 ± 0.7 *	16.8 ± 0.8 *	16.7 ± 0.9 *	34/ 8
Control, G _i (mS/cm ²)	14.2 ± 0.7 *	14.7 ± 1.0 *	14.7 ± 0.8 *	23/ 10
<i>Salmonella</i> ¹ , I _{sc} (μA/cm ²)	14.8 ± 2.4	15.7 ± 2.6	14.0 ± 2.0	30/ 9
Control, I _{sc} (μA/cm ²)	16.9 ± 4.1	16.7 ± 4.3	14.1 ± 4.3	20/ 11

¹ After a 30- minute equilibration period, *Salmonella* serovar Choleraesuis strain SC-54 or strain χ 3246 were added to the luminal aspect of the tissue

² Number of tissues (n) examined from N pigs for each condition.

* p < 0.0001, control vs. *Salmonella*-exposed at each time point indicated, two-way ANOVA