EXPRESSION OF TOLL-LIKE RECEPTORS IN PORCINE

IMMUNE CELLS AND TISSUES

by

THOMAS EDWARD BURKEY

B.A., Rockford College, 1996
M.S., Kansas State University, 2003

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

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Abstract

Toll-like receptors (TLR) are instrumental in discriminating between pathogenic and commensal bacteria and act as mediators, along with downstream chemokines, of subsequent innate and adaptive immune responses. However, little is known about the expression and regulation of TLR or chemokines in swine. The objectives of the experiments described herein were to characterize the expression of porcine TLR and to identify regulatory patterns in these receptors in the presence of live *Salmonella enterica* serovar Typhimurium (ST) or Choleraesuis (SC). The first two experiments evaluated the in vivo and in vitro expression of TLR2, 4, 5 and 9. Our results indicate that TLR2, 4, 5 and 9 are constitutively expressed in vitro in a porcine jejunal epithelial cell line (IPEC-J2), porcine mononuclear phagocytes (pMPs) and in vivo in the distal ileum. In IPEC-J2 cells, ST elicited an increase in TLR2 mRNA ($P < 0.05$), and both ST and SC increased TLR2 mRNA in pMPs ($P < 0.05$). In vivo, oral challenge with ST increased ($P < 0.05$) both TLR2 and TLR4 mRNA in the distal ileum. In addition, the second experiment evaluated interleukin 8 (IL8) and CC chemokine ligand 20 (CCL20) expression in IPEC-J2 cells in response to ST or purified bacterial flagellin (Flag). TLR5 was constitutively expressed in the ileum and in IPEC-J2 and pMP cells. Interestingly, IL8 and CCL20 mRNA and protein were increased ($P < 0.05$) by ST and Flag, even in the absence of changes in TLR5. In the third experiment, the expression of TLR and chemoattractive mediators were evaluated in a panel of tissues obtained from pigs challenged with ST and SC. All genes of interest were constitutively expressed; however, the effects of treatment were limited to isolated tissues and genes. Taken together, the data indicate that TLR and chemoattractive mediators are expressed in porcine tissues and cells and that the observations described represent novel evidence that pig pathogens may regulate TLR expression and activate chemokine secretion.

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Dedication

I would like to dedicate this body of work to my family. The love and commitment you have showed over the last five years has provided me with the motivation and will to complete this endeavor. THANK YOU!

MAKE A DUST

My son, remember you have to work.

Whether you handle pick or wheelbarrow or a set of books,

digging ditches or editing a newspaper,

ringing an auction bell or writing funny things,

you must work.

Don't be afraid of killing yourself by overworking on the sunny side of thirty.

Men die sometimes,

but it is because they quit at nine p.m.,

and don't go home until two a.m.,

it's the intervals that kill,

my son.

The work gives you appetite for your meals,

it lends solidity to your slumber;

it gives you a perfect appreciation of a holiday.

There are young men who do not work,

but the country is not proud of them.

It does not even know their names;

it only speaks of them as old So-and-So's boys.

Nobody likes them;

the great, busy world doesn't know they are here.

So find out what you want to be and do.

Take off your coat and make dust in the world.

The busier you are,

the sweeter will be your sleep,

the brighter your holidays,

and the better satisfied the whole world will be with you.

Unknown

CHAPTER 1 - Expression and regulation of porcine Toll-like receptors

The gastrointestinal immune system

Introduction

The primary function of the immune system is to identify and eliminate pathogens. In vertebrates, the immune system is subdivided into the innate and adaptive arms of immunity. In a broad sense, the innate immune system is composed of anatomic, physiologic, phagocytic and inflammatory barriers. The aforementioned barriers enable the innate immune system to provide the first line of defense against infectious disease. Because of the vast surface area of the gastrointestinal tract (GIT) and the constant exposure to commensal and pathogenic microorganisms, the gastrointestinal immune system has been the subject of great interest for the past several years.

The mucosal surface of the GIT forms an intricate collaboration with the intestinal lumen. The diverse milieu of antigenic dietary components as well as commensal and pathogenic bacteria within the GIT has facilitated the need for an evolving and sophisticated gastrointestinal immune system. Much of the burden on gastrointestinal immunity is shouldered by intestinal epithelial cells (IEC). The IEC monolayer provides anatomic and physiologic barriers designed to maintain homeostasis within the GIT. Taken together, along with the idea that the GIT must fulfill its primary absorptive function, it is imperative that the mucosal immune system of the gut effectively discriminate and respond appropriately to enteropathogens as well as harmless food antigens or antigens from commensal organisms. Failure to deal with antigenic stimuli appropriately can result in chronic inflammation, decreased digestive function and decreased rate of growth.

The importance of mucosal immunity is clear when one considers that the gut contains greater than 10^{12} lymphocytes and has a greater concentration of antibodies than any other site in the body (Mayer, 2000). The intestinal immune system is adequately equipped to generate a protective immune response directed at harmful pathogens, but it also has the capability to be tolerant of the ubiquitous dietary antigens and normal microbial flora while maintaining the ability to permit the absorption of nutrients. In addition to thorough reviews of the mammalian gastrointestinal immune system (Brandtzaeg and Pabst, 2004; James, 1993; Kagnoff, 1993; Par, 2000), there have also been reviews published regarding the porcine immune system (Blecha,

2001) and reviews specific to the porcine gastrointestinal immune system (Mayrhofer, 1984; Stokes et al., 2001). Therefore, this section will provide a brief description of mucosal immunity as well as key components of the gastrointestinal immune system highlighting specific functions and differences between swine and other mammalian systems.

Mucosal immunity

Confronted with a large array of antigens, the immune system faces a considerable challenge in its efforts to maintain local tissue homeostasis in the intestinal mucosa. For example, at least 400 different species of bacteria contribute to a total of approximately 10^{14} microbes that are distributed throughout the GIT (Gorbach et al., 1967; Suau et al., 1999). The mucosal immune system must prevent the dissemination and proliferation of these potentially harmful agents while sparing the vital structures and function of the intestine. In order to carry out this daunting task, the intestinal mucosa, complete with a single layer of epithelial cells, provides a barrier to the pathogenic and nonpathogenic bacteria present within the gastrointestinal milieu (Neutra et al., 2001). In addition to the physical barrier that the epithelia provide, the mucosal immune system also employs other gut-associated lymphoid tissues (GALT) to protect the organism and to mediate subsequent innate and adaptive immune responses. The importance of interactions between the microbiota, the gut epithelium and the GALT were emphasized by Falk et al. (1998) whom suggested that an important 'trialogue' exists between these components that shapes the intestinal ecosystem.

The communication within the mucosal immune system is carried out by a large and highly specialized collection of tissues and cells within the GIT. In fact, the intestine is considered to be the largest lymphoid organ and contains more immune cells than any other organ including the spleen and liver. The immune cells within the GIT are highly compartmentalized in the GALT and its associated components, which will be briefly described in the following sections.

Gut-associated lymphoid tissue

The GALT provides specific host defense and encompasses the largest collection of immune cells in the body (Mowat and Viney, 1997). The GALT is the focal point of the mucosal immune system and is generally divided into functional compartments known as inductive or effector sites. There is some discrepancy regarding the classification of the

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structures and tissues that comprise the GALT because there is no absolute distinction between the functional compartments. For the purpose of this review we will consider the inductive portion of the GALT as comprising the appendix, isolated lymphoid follicles and Peyer's patches (Brandtzaeg and Pabst, 2004). The lamina propria is generally considered an effector site within the GALT and is referred to as a compartment containing cells outside of the Peyer's patches (Nagler-Anderson, 2001).

Generally speaking, GALT represents a site where B and T lymphocytes interact with intestinal antigens. However, before antigens reach the GALT, antigens must breach the intestinal epithelial monolayer. Separating the GALT from the intestinal lumen is an intestinal epithelial cell (IEC) monolayer. The IEC monolayer provides both intrinsic and extrinsic barriers to potentially harmful pathogens and antigens (reviewed by Pitman and Blumberg, 2000). The intrinsic mechanism hinges on the establishment of a physical barrier via the highly organized IEC monolayer that facilitates selective transfer of lumenal contents to the underlying GALT. IEC that line mucosal surfaces also function extrinsically by secreting proteins (e.g. mucins, antimicrobial peptides and immunoglobulins) that limit interaction of potential pathogens with the gut mucosa. However, antigens and pathogenic microorganisms do in fact circumvent the physical barrier provided by IEC. For example, antigen may be taken up by microfold (M) cells found within the follicle-associated epithelium of Peyer's patches (Tyrer et al., 2006). In addition, antigen may be sampled directly by dendritic cells that open tight junctions between IEC in order to extend dendrites into the intestinal lumen (Rescigno et al., 2001), and certain species of bacteria overcome the epithelial barrier by using specialized invasion strategies such as the Type III secretion system (Hapfelmeier et al., 2005). Pathogens and other antigens within the gut lumen that traverse the IEC barrier eventually interact with phagocytic cells (e.g. macrophages and dendritic cells) as well as B and T lymphocytes within the GALT. These interactions provide the necessary signals for the initiation of an adaptive immune response and the generation of effector mechanisms (Pasare and Medzhitov, 2005). Effector cells then proceed to the mesenteric lymph nodes where the immune response is amplified. Activated lymphocytes are then passed into the blood stream via the thoracic duct and travel to the gut in order to carry out their specific effector functions. Taken together, the IEC monolayer and GALT combine to initiate and carry out innate and adaptive immune responses.

In the following sections we will discuss the specific GALT compartments of mammals, their functional significance and specific attributes as it applies to the domestic pig.

Appendix

Considered as the beginning of the large intestine and part of the colon, the cecum is a pouch-like structure at the at the end of the small intestine that is separated from the ileum by the ileocecal valve. The appendix, similar in structure and form to the cecum, is a diverticulum that extends from the cecum. The appendix is highly vascular, is lymphoid-rich, and produces immune cells that are normally attributed to the GALT (Somekh et al., 2000; Spencer et al., 1985). It has been hypothesized that the appendix may have exocrine, endocrine, and neuromuscular functions. However, limited evidence suggests that the most likely function of the appendix is as part of the gastrointestinal immune system (Dasso and Howell, 1997; Pospisil and Mage, 1998; Shanahan and O'Sullivan, 1997). Hypothesized functions attributed to the appendix have not been unequivocally proven, and the most prominent functional period of the appendix probably exists in the developing fetus and the neonatal animal (Dasso et al., 2000). In the domestic pig, the significance of the appendix becomes irrelevant as the appendix cannot be found within the porcine gastrointestinal anatomy (Schantz et al., 1996; Simic and Ilic, 1976).

Isolated lymphoid follicles and intestinal cryptopatches

Isolated lymphoid follicles and cryptopatches are small lymphoid aggregates that represent another component of the GALT. Isolated lymphoid follicles are lymphoid aggregates in the antimesenteric wall of the small intestine that have been described in mice (Hamada et al., 2002) and humans (Moghaddami et al., 1998). Similar to Peyer's patches, isolated lymphoid follicles contain germinal centers with segregated B and T cell areas and an overlying follicle-associated epithelium complete with M cells (Hamada et al., 2002). In regard to the function of isolated lymphoid follicles, Lorenz and Newberry (2004) have provided evidence that these lymphoid aggregates are inductive sites for antigen-specific mucosal immune responses. Kanamori et al. (1996) described murine cryptopatches as small aggregates of lymphocytic cells in the basal lamina propria of the small and large intestine. This group also characterized the cells within cryptopatches as lineage-negative and expressing the stem cell factor known as c-kit. In regard to cryptopatch function, Suzuki et al. (2000) has provided evidence that cryptopatches develop progenitor T cells for extrathymic intraepithelial lymphocyte descendants. However,

Pabst et al. (2005) has provided evidence that argues against separate isolated lymphoid follicle and cryptopatch lymphoid aggregations. These authors conclude that there are no reasonable distinctions between isolated lymphoid follicles and cryptopatches. Moreover, Pabst et al. (2005) were unable to detect cryptopatches in human, rat, or pig intestine.

Peyer's patches

Islands of discrete, organized lymphoid tissue with areas populated by B and T lymphocytes located in the small intestine are known as Peyer's patches and have been thoroughly reviewed elsewhere (Heel et al., 1997; Mayrhofer, 1984). Briefly, unlike the adjacent absorptive epithelium, Peyer's patches are overlaid with a specialized follicle-associated epithelium. The follicle-associated epithelium has a filamentous brush border glycocalyx but lacks membrane-associated hydrolytic enzymes characteristic of the absorptive epithelium (Neutra et al., 2001). In addition, the follicle-associated epithelium harbors specialized antigen sampling M cells that are interdigitated within the epithelium (Owen and Jones, 1974). Underlying the follicle-associated epithelium, mucosal follicles of Peyer's patches contain large B cell follicles with adjacent T cell areas surrounding a germinal center with supporting follicular dendritic cells. Generally speaking, Peyer's patches are sites of antigen sampling and have a role in the induction of mucosal immune responses. However, differences between Peyer's patch development, structure and function between species have been reported (Andersen et al., 1999; Griebel and Hein, 1996).

Peyer's patches have been described in the domestic pig and are known to reside in the small (jejunum and ileum) and large (spiral colon) intestine (Binns and Licence, 1985; Chu and Liu, 1984). Within the small intestine of pigs, discrete Peyer's patches are found in the jejunum and upper ileum (jPp) and a continuous Peyer's patches is evident along the terminal ileum (iPp) (Rothkotter et al., 1990). Even though, jPp and iPp are morphologically similar, differences in B and T cell distribution and quantity vary between jPp and iPp (Pabst et al., 1988). In addition, while human and mouse iPp have been established as secondary lymphoid organs, there is evidence to suggest that iPp in sheep, cattle and swine have exhibited properties consistent with a function as primary B-cell lymphoid organs (Andersen et al., 1999; Parng et al., 1996; Reynolds, 1987).

Taken together, the Peyer's patch is a complex lymphoid aggregate that has multiple functions. The Peyer's patch has a role in decreasing antigen translocation across the mucosal epithelium via selective uptake by M cells, yet it also must recognize luminal antigen in order to direct subsequent immunological responses. The role of M cells in microbial recognition has not been fully elucidated due to difficulties establishing suitable in vitro models but recent work has provided some insight into M cell function. For example, Tyrer et al. (2006) has provided evidence that pattern recognition receptors (e.g. Toll-like receptors; TLR) are important for M cell recognition and induction of mucosal immune responses to Gram-negative bacteria. Due to the fact that the in vitro model established by this group consisted of human epithelial cells cocultured with murine Peyer's patch cells, these observations may or may not be applicable to swine. However, recent observations by Shimosato et al. (2005) and Tohno et al. (2005) has provided evidence that TLR are expressed on porcine M cells and contribute to ligand specific transcytosis which is consistent with the hypothesis that Peyer's patches may be responsible for the induction of immune responses. Subsequent to immune induction, the lamina propria has proven to function as the regulator of immune responses in the intestine (Makala et al., 2001).

Lamina propria

The gastrointestinal lamina propria is comprised of smooth muscle cells, fibroblasts, blood vessels and lymphatics that make up a highly vascular layer of loose connective tissue underlying and supporting the mucosal epithelium. In addition, the lamina propria also contains macrophages, dendritic cells, neutrophils, mast cells and lymphocytes that participate in lamina propria effector functions. Following induction in the Peyer's patch, mature T and B cells migrate to and collect in the lamina propria where T cells can directly eliminate pathogens and T and B cells can participate in the production of cytokines and immunoglobulins (e.g. IgA). In humans, the majority of lamina propria T cells are CD4+ and express the αβ T cell receptor (TCR) (Brandtzaeg et al., 1998). Lamina propria T cells differ from peripheral T cells in that they have a higher threshold of activation, produce high levels of cytokines upon stimulation, and have a phenotype associated with immunologic memory (Wittig and Zeitz, 2003). In addition, most species express CD25 and isoforms of CD45 which are consistent with antigen recognition and immunologic memory, respectively (Haverson et al., 1999). Between species, the population of lymphocytes that reside in the lamina propria has been classified as

heterogeneous and the organization of these cells is classified as random (Bailey et al., 2005). Collectively, these characteristics are consistent with the effector function of lamina propria lymphocytes that enable these cells to participate in immunosurveillance and to actively respond to potential pathogens. However, there are important differences in lamina propria lymphocytes between humans and swine that may relate to the function of these compartmentalized cells.

In the small intestine of pigs, Pabst and Rothkötter (Pabst and Rothkotter, 1999) categorized lymphocytes as diffuse or organized. As is the case for most species, intraepithelial lymphocytes and lymphocytes contained in the lamina propria are considered diffuse lymphocytes. However, in contrast to most other species, the lamina propria of the pig has a greater degree of organization (Wilson et al., 1996). For example, Vega-Lopez et al. (1993) observed that plasma cells are preferentially localized to the intestinal crypts and T cells to the intestinal villi. Vega-Lopez et al. also observed a spatial separation between $CD4^+$ and $CD8^+$ T cells within the lamina propria of intestinal villi. In addition, researchers have also observed differences in cytokines secreted by activated lamina propria T lymphocytes (Bailey et al., 1994; Harriman et al., 1992). The significance of the differences that exist in pigs has not been fully elucidated. However, it has been suggested that lamina propria lymphocytes, in addition to their effector function, also have a role in immunoregulation (Bailey et al., 2001).

Intraepithelial lymphocytes

Intraepithelial lymphocytes represent a large, heterogeneous subclass of T cells that are integrated in the epithelial layer of many tissues (reviewed by Hayday et al., 2001; Mowat and Viney, 1997). Functionally, lines of evidence have portrayed human and murine intraepithelial lymphocytes as having cytolytic and immunoregulatory properties that can be quickly summoned to maintain epithelial integrity and to protect host tissues from infectious agents. Intraepithelial lymphocytes are T lymphocytes that can be differentiated from circulating and lamina propria T lymphocytes. For example, circulating T cells are subdivided into similar proportions of $CD4^+$ and $CD8^+$ T cell populations, whereas the majority of intraepithelial lymphocytes are $CD8^+$ (Gebert et al., 1996). In addition, intraepithelial lymphocytes can be αβ and γδ TCR^+ with the $γδ^+$ cells having abundant expression of the CD8αα homodimer (Gebert et al., 1996). Intraepithelial lymphocytes also have a greater proportion of TCRγδ+ cells than what has been found in the circulation of birds and mice (Bucy et al., 1988; Goodman and Lefrancois,

1988). Another defining feature of intraepithelial lymphocytes is their ability to bind to E cadherin on IEC which is facilitated by the expression of αEβ7 integrin (Cepek et al., 1994). Researchers have also noted that characteristics such as morphology, size and sedimentation density contribute to the heterogeneity of lymphocytes categorized as intraepithelial lymphocytes (Hayday et al., 2001).

Because of this heterogeneity within intraepithelial lymphocytes populations, Hayday et al. (2001) has recently proposed that intraepithelial lymphocytes be classified into two subgroups: Type a and type b. Intraepithelial lymphocytes that are thymus-dependent, activated within the peripheral circulation, that express the αβ TCR, and that recognize antigen in the context of MHC I or II would be included as type a intraepithelial lymphocytes. Type b intraepithelial lymphocytes are thymus-independent cells that are TCRγδ$^+$, TCRγδ$^+$ CD8αα$^+$ or TCRαβ$^+$CD8αα$^+$. Both types of intraepithelial lymphocytes are cytolytic effectors that secrete cytokine and chemokine mediators. However, Hayday et al. (2001) argues that type a intraepithelial lymphocytes are more indicative of an adaptive response whereas type b intraepithelial lymphocytes are "revertants" to the innate response. The role of type b intraepithelial lymphocytes is also supported by evidence summarized by Havran et al. (2005) that indicates intraepithelial γδ$^+$ T cells are involved in tissue repair, lysis of damaged epithelial cells and inflammatory cell recruitment. Consistent with the heterogeneous nature of intraepithelial lymphocytes, there is evidence to suggest that intraepithelial lymphocyte populations vary between species.

Similar to humans and mice, pig intraepithelial lymphocytes also express CD2 and have a high proportion of CD8$^+$ cells (Stokes et al., 2001). However, neonatal pigs are mostly CD2$^-$CD4$^-$CD8$^-$ and CD8$^+$ intraepithelial lymphocytes cannot be recognized until the animal matures. It has also been demonstrated that phenotypic changes in porcine intraepithelial lymphocytes are influenced by exposure to environmental antigens (Pabst and Rothkotter, 1999). Vega-Lopez et al. (2001) observed similar developmental changes in intraepithelial lymphocytes and proposed that the delayed maturation of intraepithelial lymphocytes might be positively correlated to the increased disease susceptibility of young pigs. Even though there is more to learn about intraepithelial lymphocytes, particularly in domestic animal species, their location among the intestinal epithelium is indicative of their importance as immune regulators and effectors at the lumenal-epithelial interface.

Intestinal epithelial cells

Kagnoff (1993) theorized that because of the diverse environment within the gut lumen, IEC have evolved mechanisms that comprise an effective anatomical and immunologically active barrier. One mechanism of the IEC barrier is the innate recognition and differentiation of commensal and pathogen associated molecular patterns via pattern recognition receptors such as the TLR family (Didierlaurent et al., 2002). Within the GIT, the IEC are compartmentalized, particularly in the small intestine where different populations of IEC form a vertical crypt-villus axis (Turner, 2003). In most species, stem, goblet, secretory, enteroendocrine and Paneth cells populate the crypt-villus axis. The intestinal villi comprise absorptive enterocytes and goblet cells while Paneth cells can be found in the villus crypts. In addition, specialized M cells are interspersed among the follicle-associated epithelium that overlays Peyer's patches. M cells are important for the immune surveillance function of the GIT (Gewirtz and Madara, 2001; Neutra et al., 2001). Taken together, along with the idea that the GIT must fulfill its primary absorptive function, it is imperative that the gastrointestinal immune system effectively discriminate and respond appropriately to enteropathogens and harmless food antigens or antigens from commensal organisms. Failure to deal with antigenic stimuli appropriately can result in chronic inflammation and decreased digestive function. Here we provide a brief review of IEC with specific emphasis on the immunological aspects of the porcine epithelia.

IEC and intraepithelial lymphocytes comprise the epithelial layer of the intestine and are separated from the underlying lamina propria by the basal lamina. The crosstalk between the gut lumen, IEC and the lamina propria provide the information that directs the gastrointestinal immune system. In addition to the physical barrier that the IEC monolayer provides, Christ and Blumberg (1997) suggested that IEC have immunological functions that can be broadly categorized as follows: 1) secretion of soluble protein factors; 2) regulators of immune responses; 3) immunosurveillance; and 4) as targets for immune effectors. Theses immunological functions of the IEC monolayer are closely related to its structural organization.

A critical component of the barrier function attributed to the IEC monolayer is the formation of epithelial tight junctions (for review see Gumbiner, 1987). Tight junctions contribute to the highly selective IEC monolayer and participate in the polarization of the epithelial cell into apical and basolateral domains. Thus, the formation of tight junctions and the IEC monolayer is vitally important for separating the mucosa from lumenal components while

allowing for the absorption of nutrients. Therefore, the IEC monolayer, coated by mucus secreted from goblet cells, provides a nonspecific physical barrier that prevents invasion by commensal and pathogenic bacteria that reside within the gastrointestinal tract (GIT).

In addition to its barrier function, IEC can be stimulated to secrete cytokines, chemokines and antimicrobial proteins that aid in the protection of the organism and act to regulate subsequent immune responses. For example, our laboratory group and others have observed highly polarized secretion of chemokines and cytokines (e.g. IL8 and TNFα) from IEC in vitro in response to bacterial invasion (Eckmann et al., 1993; Burkey et al., submitted; Skjolaas et al., 2006). In addition, a variety of antimicrobial peptides are secreted by IEC (for review see Ganz, 2003). According to Ganz (2003), defensins are abundant in Paneth cells and are the most prominent group of antimicrobial peptides in humans. In swine, twelve defensins have been characterized in pigs (Zhang et al., 1998; Song et al., 2006); however, there is some controversy surrounding the presence of paneth cells in pigs (Dekaney et al., 1997; Myer, 1982; Obremski et al., 2005). Additional antimicrobial peptides, such as cathelicidins and protegrins, have been identified in swine (reviewed by Zhang et al., 2000). The synthesis and secretion of cytokines, chemokines and antimicrobial peptides is dependent on the ability of IEC to decipher information received from the intestinal milieu.

Another important immunologic function of IEC is immunosurveillance. This role of IEC is largely accomplished via TLR (Bogunovic et al., 2000; Philpott et al., 2001; Takeda and Akira, 2003). Recognition and detection of bacteria and bacterial products by TLR initiates a signaling cascade that culminates in the activation of the transcription factor nuclear factor kappa B (NF-κB) and transcription of proinflammatory cytokines (Ghosh et al., 1998; Medzhitov et al., 1997). Similar to cytokine and chemokine mediators, the expression of antimicrobial peptides has also been linked to signaling via TLR (Vora et al., 2004). The immunosurveillance role of IEC is not restricted to TLR. The expression of major histocompatability complex (MHC) molecules on IEC in some species has lead to the hypothesis that IEC can function as non-professional antigen presenting cells (Christ and Blumberg, 1997). However, MHC II is not present on porcine intestinal epithelial cells (Dvorak et al., 1987; Schierack et al., 2005). Therefore, at least in pigs, the role of antigen presenting cell may not be applicable.

As regulators of the immune response, there is evidence to indicate that IEC have mechanisms to avoid deleterious immune responses while retaining the ability to mediate an

adaptive immune response. As we have already mentioned, the IEC is constantly bathed with commensal and pathogenic bacteria. TLR recognition and expression patterns may help the gut to be tolerant of the antigenic load within the GIT. One example is underlined by the specificity and expression of TLR5. TLR5 is specific for bacterial flagellin and its expression is highly polarized to the basolateral surface of IEC (Gewirtz et al., 2001). Therefore, not only is TLR5 specific to a particular ligand, but ligand-receptor interactions may only occur in the case of epithelial injury or bacterial invasion. Taken together, TLR are central to innate recognition and immunity, contribute to immune regulation and lead to the initiation of adaptive immune responses (for review see Pasare and Medzhitov, 2005; Werling and Jungi, 2003).

Knowledge regarding the intricacies of the gastrointestinal immune system as it applies to the inductive and effector sites is particularly important in pigs due to the development of these sites as the pig matures. Neonatal pigs are immunologically incompetent until about 4 wk of age (Blecha, 2001). The sections above outlined the inductive and effector sites of the gastrointestinal immune system and included a brief summary of the contributions of IEC to this system with particular emphasis on the domestic pig. The following sections will review what is currently known about TLR expression and regulation in vertebrates with emphasis on what is currently known about TLR in the domestic pig.

Toll-like receptors

Introduction

Innate immune defense mechanisms are antigen-nonspecific, exist prior to antigenic exposure and are responsible for the prevention of infection and the elimination of microbes. An important aspect of the innate immune system is the recognition and discrimination of potential pathogens from non-pathogenic, commensal microorganisms. Receptors of the innate immune system that have a large role in recognition events include NK activating receptors, scavenger receptors, mannose receptors and TLR. Arguably, the most important of these receptors are the TLR, which represent a class of pattern recognition receptors. Germline-encoded pattern recognition receptors are responsible for the innate recognition of pathogen associated molecular patterns (Janeway and Medzhitov, 2002). Pathogen associated molecular patterns include structures that are essential for microbial survival and include, for example, lipopolysaccharide

(LPS), peptidoglycan, and flagellin. Following the detection of potential pathogens, the innate immune system is set in motion to contain or eliminate pathogenic threats and to provide mediators that direct the adaptive immune response. For the purpose of this review, this section will provide a synopsis of the discovery of TLR, describe the structure, signaling and function of the known TLR, and review what is currently known about TLR with regard to swine.

Toll-like receptor discovery and background

The discovery of Toll-like receptors and their role as mediators of mammalian host defense can be attributed to discoveries made in the fruit fly, *Drosophila melanogaster* (for review see Lemaitre, 2004). The Toll protein was originally identified as a component important for embryonic development in *Drosophila* (Anderson and Nusslein-Volhard, 1984; Hashimoto et al., 1988). Subsequent to this discovery, Lemaitre et al. (1996) discovered a link between the Toll protein and the expression of *Drosophila* antimicrobial peptides. However, the exact function of *Drosophila* Toll was not completely understood. Two other discoveries in invertebrates completed the link between Toll and the induction of antimicrobial peptides and fueled the efforts to identify the mammalian version of the Toll protein. The first discovery was made by Fehlbaum and colleagues (Fehlbaum et al., 1994) and provided the first description of an inducible antimicrobial peptide in insects. The second, made by Lemaitre et al. (1995), was the discovery of the *Drosophila* immune deficiency (imd) mutation and lead to the observation that imd-mutant flies were vulnerable to infection by Gram-negative organisms.

One year after the discovery of the *Drosophila* Toll, Janeway and colleagues discovered the human homologue of the Toll protein (Medzhitov et al., 1997), a protein later to be designated as TLR4. There were three features of TLR4 that linked this protein with innate immunity and intracellular signaling. First, because the Drosophila Toll participates in an anti-fungal response in the adult fly, it was hypothesized that mammalian homologues would participate in similar innate immune responses. Second, the Drosophila Toll participated in a signal transduction pathway leading to the activation of the transcription factor Dorsal, the fly homologue of NF-κB. Third, the intracellular domain of *Drosophila* Toll has significant homology with the type I IL-1 receptor, the known mammalian TLR, and the cytosolic adapter protein MyD88. Taken together, these findings suggest that mammalian Toll proteins function, as is the case with *Drosophila* Toll proteins, in host immune responses. Subsequent to the

discovery of mammalian Toll, a great deal of effort has been spent on elucidating the protein structure, different forms of the Toll protein and the ligands that bind to TLR.

Toll-like receptor structure and ligands

Toll-like receptors are a family of type I transmembrane proteins that, to date, include at least 13 family members in mammals. Because of considerable homology in their cytoplasmic regions, TLR are members of a large superfamily that includes the interleukin-1 receptor (IL-1R). However, TLR are distinguished from other members of the IL-1R superfamily by their extracellular regions. IL-1R family members contain three immunoglobulin-like domains, whereas TLR can be characterized by extracellular leucine-rich repeats. In addition, TLR have a highly homologous cytoplasmic Toll/IL-R domain, a short transmembrane region, and a ligand-binding ectodomain with cysteine-rich regions (Medzhitov et al., 1997). The Toll/IL-R domains vary in size and consist of 150-200 amino acids and mediate protein-protein interactions that are crucial for signal transduction (Kopp and Medzhitov, 1999; Slack et al., 2000). The extracellular leucine-rich repeat domains of TLR form a horseshoe shaped structure that is essential for recognition of various pathogen associated molecular patterns (Bell et al., 2003). Remarkably, considering the extent of structural homology among the different TLR, these receptors retain the ability to detect a wide array of ligands (Janeway, Jr. and Medzhitov, 2002).

The consensus ligands of TLR 1-11 have been summarized in several reviews (Akira and Takeda, 2004; Takeda et al., 2003; Werling and Jungi, 2003; Barton and Medzhitov, 2002) and are represented here in Table 1.1. Briefly, TLR1 is thought to be the receptor for microbial lipopeptides (Takeuchi et al., 2002) while TLR2 recognizes a broad range of microbial products including peptidoglycan and lipopeptides from Gram-positive bacteria (Schwandner et al., 1999; Takeuchi et al., 1999) and zymosan from yeast (Underhill et al., 1999). In addition, TLR2 has been shown to pair with at least TLR1 and TLR6 to recognize lipopeptides, and this cooperation may affect the specificity of the pairing (Wyllie et al., 2000). TLR3 has been shown to function as the receptor for double-stranded RNA, a product of many viruses (Alexopoulou et al., 2001). TLR4 recognizes Gram-negative LPS (Poltorak et al., 1998), and in addition, may also recognize endogenous ligands including heat shock proteins (Vabulas et al., 2002). The only known ligand for TLR5 is flagellin, a primary component of bacterial flagella (Hayashi et al., 2001). Recent work has shown that ligands for TLR7 and 8 include synthetic compounds (Jurk et al., 2002) as

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well as single stranded RNA in a species dependent manner (Heil et al., 2004). Bacterial DNA has immunostimulatory properties because of the presence of unmethylated cytidine-phosphate-guanosine (CpG) motifs and is recognized by TLR9 (Hemmi et al., 2000). The specific ligands for TLR10 and 11 have not been determined although TLR11 has been associated with uropathogenic bacteria (Zhang et al., 2004). Interactions between the TLR and their consensus ligands leads to activation of complex signal transduction pathways that culminates in the activation of transcription factors and synthesis of proteins important for mediating the subsequent immune response.

Toll-like receptor signaling

TLR-mediated recognition of pathogen associated molecular patterns leads to intracellular signaling that ultimately controls and shapes the patterns of innate and adaptive immunity. TLR signaling in human and murine systems has been thoroughly reviewed (Akira, 2003; Akira and Takeda, 2004); thus, we provide a brief overview of the central signaling pathways of TLR with specific attention to signaling as it pertains to the intestinal epithelium.

TLR engagement by their respective ligands induces dimerization of the receptor and conformational changes that permit the recruitment of the adapter protein MyD88 to the cytoplasmic Toll/IL-R portion of the TLR. MyD88 was first identified by Lord et al. (1990) and was later determined to be essential for IL-1R (Wesche et al., 1997) and TLR (Medzhitov et al., 1998) signaling. Following ligand stimulation and recruitment, the activated MyD88 interacts with IL-1R associated kinase (IRAK) via their death domains. The interaction of MyD88 and IRAK triggers the autophosphorylation of IRAK and activates TNF receptor-associated factor (TRAF) 6. The IRAK-TRAF complex then dissociates from the TLR complex and interacts with transforming-growth factor-β-activated kinase (TAK1) and the TAK1 binding proteins (TAB1 and TAB2). At this point, the activity of TAK1 causes the signaling pathway to diverge. Separately, TAK1 phosphorylates mitogen-activated protein (MAP) kinases and the inhibitor of nuclear factor-κB (IκB)-kinase complex. The MAP kinases are serine/threonine kinases that have been observed to influence the status of transcription factors that regulate key components of the immune response (Schroder et al., 2001). Phosphorylation of IκB leads to its proteolytic degradation as well as the translocation of NF-κB to the nucleus culminating in the expression of genes that encode proinflammatory cytokines. The above description outlines the general TLR

signaling pathway. However, it is important to note that there are components of the TLR signaling pathway that remain to be elucidated.

The discovery of homologues to MyD88 has given way to an additional TLR signal transduction pathway known as the MyD88-independent signaling pathway (Kawai et al., 1999). In addition to contributing to the defense against pathogenic microorganisms, the MyD88-independent signaling pathway has also been attributed to the control of endogenous host microflora (Bjorkbacka et al., 2004). While both the MyD88-dependent and -independent pathways mediate signaling generated from the interaction of LPS and TLR4 (Kawai et al., 1999), TLR2 does not use the MyD88 independent pathway and TLR3 uses the MyD88-independent pathway preferentially (Alexopoulou et al., 2001). The differences in signaling pathways have been attributed to the presence of the aforementioned MyD88 homologues. The MyD88 homologues include Toll/IL-R -domain-containing adaptor protein, Toll/IL-R-domain-containing adaptor protein inducing IFN-β (TRIF), and TRIF-related adaptor molecule. The presence of multiple ligands, receptors, adaptor molecules and possible pathways adds specificity to the TLR signaling network. In addition, the subtle differences in TLR signaling pathways expression patterns will certainly reveal how TLR signaling directs innate and adaptive immune responses and will open more avenues for therapeutic intervention in disease states.

Toll-like receptor expression and regulation

To date, a number of studies have evaluated the expression of TLR in a variety of tissues and cell types. The majority of information regarding TLR expression has been gathered from murine and human model systems. The characterization of TLR in normal and diseased states has helped us to explore settings in which TLR may play a pivotal role in disease and disease prevention. Here we provide a brief discussion of mammalian TLR expression and instances of TLR differential regulation.

The expression of TLR mRNA is ubiquitous in vertebrate tissues and cells. Zarember and Godowski (2002) have observed mRNA expression of TLR 1-10 in a panel of human tissues, leukocyte subpopulations, and peripheral blood granulocytes and monocytes. In addition these authors suggested that TLR mRNA is expressed in greater abundance in settings prone to host-microbe interaction (i.e. leukocytes, spleen, intestine, and lung). Consistent with this idea, Muzio et al. (2000) observed that monocytes and macrophages express mRNA for most TLR

with the exception of TLR3. Additional researchers have also observed high levels of TLR expression in peripheral blood leukocytes (Medzhitov et al., 1997) as well as in monocytes, dendritic cells, B cells and T cells (Akashi et al., 2000; Muzio et al., 2000). Even though there is evidence for TLR mRNA in many tissues and cell types, there is also a body of evidence that suggests TLR expression may vary in specific subpopulations and with cell maturity. For example, subpopulations of human dendritic cells express distinct TLR mRNA (Kadowaki et al., 2001) and the level of expression varies between immature and mature cells (Visintin et al., 2001). Thus, there is a high degree of TLR expression in cells of the immune system and this is consistent with the idea that TLR are important as sentinels of infection and mediators of an appropriate immune response. The mucosal surface of the GIT is another venue in which the recognition and detection of commensal and pathogenic microorganisms by TLR is vitally important for maintaining homeostasis.

Because of the presence of large numbers of commensal and pathogenic bacteria at the interface between the intestinal lumen and IEC, it follows that TLR would have significant role in host defense. Most TLR can be detected in human intestinal epithelial cell lines (Gewirtz, 2003); however, specific TLR may have a more prominent role at mucosal surfaces than other TLR. For example, TLR5 is present in IEC and has been observed to be highly polarized to the basolateral surface of the epithelium (Gewirtz et al., 2001). In addition, TLR2, 3 and 4 have been detected in human intestinal epithelial cell lines (Cario et al., 2000; Cario and Podolsky, 2000) but there is some controversy regarding the level of detection as well as the localization of the receptor within the IEC. Cario et al. (2002) has provided evidence that certain TLR may be able to traffic within the epithelial cell in response to bacteria to avoid activation. Other examples of physiologic regulation of TLR are also available. For example, the physiologic regulation of TLR expression has been explored in murine and human models. For instance, Matsumura et al. (2000) observed differential regulation of TLR2 and 4 mRNA in tissues taken from mice treated with LPS or proinflammatory cytokines. Differential regulation of TLR2 and 4 has also been observed in humans by Hausmann et al. (2002). In this particular experiment, TLR2 and 4 mRNA and protein expression was differentially regulated in macrophages isolated from the intestines of human patients with inflamed mucosa. In addition, several groups have reported in vitro experiments in which TLR may be regulated by specific cytokines (Staege et

al., 2000; Miettinen et al., 2001) and in vivo experiments where differential TLR regulation was associated with the immune response to microbial pathogens (Krutzik et al., 2003).

To date, most of what is known about TLRs and their ligands has been delineated through research in human and murine cell lines and tissues. The patterns of TLR expression and regulation are not fully appreciated, particularly in the presence of live, enteric pathogens. Any contribution regarding TLR expression and regulation may increase our understanding of the interaction between the lumenal contents of the gastrointestinal tract and the underlying immune cells and tissues. Hopefully, a greater understanding of the gastrointestinal immune system and pattern recognition receptors such as TLR will lead to new intervention strategies, including novel therapeutics, to combat pathogens that threaten young animals.

Porcine Toll-like receptors

Introduction

Research involved with elucidating the presence and role of TLR in the domestic animal species is still in its infancy. It is probable that all of these TLR exist in domestic animals but only a few have been characterized at the molecular and functional levels. For example, human orthologues of TLR have been used to determine the existence of TLR 1-7 and 10 in chickens (Iqbal et al., 2005), and TLR1-10 in cattle and sheep (Griebel et al., 2005; Menzies and Ingham, 2005). However, only TLR 2 and 4 have been characterized at the molecular and functional levels in cattle (Werling and Jungi, 2003) and chickens (Fukui et al., 2001; Leveque et al., 2003). In the porcine species, TLR 1, 2, 4, 5, 6, 9 and 10 have been characterized at the molecular level (Alvarez et al., 2006; Griebel et al., 2005; Muneta et al., 2003; Shinkai et al., 2005; Thomas et al., 2006). The following is a review of what is currently known about porcine TLR in terms of their expression and regulation.

Toll-like receptor expression in swine

Tohno et al. (2005) has provided evidence of TLR2 mRNA and protein expression in tissues of adult swine. Using real-time quantitative PCR, these researchers detected TLR2 mRNA expression in a panel of porcine tissues (heart, thymus, lung, spleen, liver, kidney, skeletal muscle, duodenum, jejunum and ileum) with the greatest levels of expression observed

in the Peyer's patches and mesenteric lymph nodes (MLN). In addition, using anti-swine TLR2 polyclonal antibodies, this group also observed high levels of TLR2 protein in Peyer's patches and MLN. Investigating further, Tohno et al. (2005) used flow cytometry to detect a high level of TLR2 expression in T cells from Peyer's patches and MLN as well as in cells positive for cytokeratin 18 expression. The latter observation is important as cytokeratin 18 is a marker for M cells and would be indicative of the potential for TLR2 to participate in the detection and transcytosis of microorganisms in the GALT via M cells. TLR2 protein has also been observed by Muneta et al. (2003) in porcine alveolar macrophages.

In regard to the TLR4 gene, two different groups have reported the determination of its full-length genomic sequence (Alvarez et al., 2006; Thomas et al., 2006). Thomas et al. (2006) also established that TLR4 could be detected by RT-PCR in swine liver, spleen and lymph nodes. Alvarez et al. (2006) reported the cloning of porcine TLR4 gene from alveolar macrophages and also determined that this gene could also be detected by RT-PCR in porcine cells (dendritic cells and monocytes) and tissues (thymus, lymph node, spleen, brain, liver, kidney and ovary).

The coding sequence for TLR5 has not been published in a peer reviewed scientific journal; however, the sequence for this gene has been submitted (GenBank® accession number AB208697). A review of the literature has provided one published report revealing evidence for TLR5 expression in swine. Raymond and Wilkie (2005), using real-time PCR, observed TLR5 expression in porcine monocytes and monocyte derived dendritic cells.

Shimosato et al. (2003) have determined the genomic sequence of swine TLR9 from the genetic material of porcine Peyer's patches. These researchers have also verified the presence of TLR9 transcript by real-time quantitative PCR in a panel of porcine tissues. Most currently, Shimosato et al. (2005) has confirmed the presence of TLR9 mRNA in neonatal and adult swine tissues as before as well as provided evidence for high levels of TLR9 protein in the Peyer's patches and MLN of adult swine. In addition, Shimosato et al. (2005) also used immunohistochemistry and flow cytometry to observe preferential expression of TLR9 on M cells of the follicle-associated epithelium.

The molecular characterization of porcine TLR is incomplete. However, of the TLR that have been characterized, TLR expression has been found in nearly every tissue in which it has

been probed for. The significance of TLR expression remains to be determined. Currently, we do not have a clear understanding of how TLR are regulated.

Regulation of Toll-like receptors in swine

Although little evidence regarding the regulation of porcine TLR expression can be found in full-length, peer reviewed journal publications, several groups have provided preliminary evidence suggesting that TLR may be differentially regulated by various stimuli. Eicher et al. (2004) reported increased TLR2 and 4 mRNA in porcine blood leukocytes and lung tissue obtained from heat-stressed barrows. Willing and Van Kessel (2005), using a gnotobiotic pig model, observed high levels TLR2 mRNA expression in small intestinal tissue and IEC from the small intestine. This group also reported that TLR2 and 4 gene expression was upregulated in germ free pigs that were inoculated with sow feces or Gram-negative bacteria. Another example of TLR2 mRNA expression in pigs was provided by Liu et al. (2001). In this study, TLR2 mRNA was increased in peripheral blood monocytes obtained from pigs infused with LPS. However, detection of porcine TLR mRNA was conducted using a human TLR2 probe; therefore, even though there is a high degree of homology between species, caution is warranted when interpreting this result. Taken together, these results indicate that TLR mRNA may be differentially regulated in the presence of various stimuli.

The regulation of TLR mRNA has also been investigated in swine models of infectious disease. Nishi et al. (2005) utilized a model where pigs were infected with *Toxoplasma gondii* to observe significant increases in TLR2 and 4 mRNA as well as the inflammatory cytokine IL1β in the MLN, liver, jejunum and ileum. Most recently, Raymond and Wilkie (2005) used real-time PCR to investigate the effects of specific pathogen associated molecular patterns on the expression of TLR mRNA in cultured porcine monocytes (Mo) and monocyte derived dendritic cells (MoDC). In cultured Mo, expression of TLR4, 5 and 9 mRNA was significantly increased compared to untreated control cells by lipoteichoic acid (LTA), LPS, and CpG oligodeoxynucleotide (CpG), for TLR4, 5 and 9, respectively. TLR expression in cultured porcine MoDC was observed to be regulated by a more diverse sampling of pathogen associated molecular patterns. Specifically, TLR4 was significantly upregulated by LPS and LTA, TLR5 was significantly increased by CpG and a dsRNA mimic (polyIC), and TLR9 was significantly increased by LPS, polyIC, CpG and LTA. Currently, the consensus ligands for TLR4, 5 and 9

are LPS, flagellin and CpG, respectively. Raymond and Wilkie (2005) proposed that the ability of pathogen associated molecular patterns, other than what is considered to be the consensus pathogen associated molecular pattern, to increase TLR expression may be due to the ability of pathogen associated molecular patterns to induce transcription factors capable of inducing various TLR mRNA. However, it is also possible that the discrepancies reported in this experiment may be due to primer design in this experiment. In this study TLR primers were designed from published human and murine sequences. Once again, even though there is a high degree of homology between human, murine and porcine TLRs, it is possible that these primers may not have been specific to the individual TLR. However, the experiments by Raymond and Wilkie (2005) do illustrate the differential regulation of TLR mRNA by microbial constituents and provide a basis for investigating the effects of specific swine pathogens on TLR mRNA expression.

Recent experiments in our own lab have been designed to elucidate the patterns of TLR mRNA expression and regulation in vitro and in vivo using quantitative real-time PCR. Our experiments have been designed to elucidate the effects of pathogen associated molecular patterns and swine-specific pathogens on TLR mRNA expression in vitro using cultured mononuclear phagocytes (pMP) and a porcine jejunal epithelial cell line (IPEC-J2) and in vivo in pigs experimentally infected with *Salmonella enterica* Typhimurium (ST) or Choleraesuis (SC). The effects of ST and SC on swine health and performance have been well documented (see Baumler et al., 2000; Fedorka-Cray et al., 2000) but little is known about the relationship of these organisms and TLR in swine.

In an initial experiment, tissue segments obtained from the distal ileum of pigs inoculated with ST, were evaluated for changes in TLR mRNA expression at 0, 4, 8, 24 and 48 h post-inoculation. TLR2 and 4 mRNA was significantly increased by 24 h post-inoculation, TLR5 mRNA was numerically increased within the same time frame, and TLR9 mRNA was not affected by ST. In a subsequent experiment, TLR mRNA expression was evaluated in a panel of tissues obtained from pigs following chronic exposure to ST or SC. Tissues were obtained 14 d following the initial dose of bacteria. In this experiment, TLR mRNA was largely unresponsive to ST or SC. There were no significant effects of treatment for TLR2 and 4 in any of the tissues analyzed (tonsil, jejunum, ileum, colon, MLN, spleen and liver). Similar effects were observed for TLR5 and 9 except significant effects of treatment were observed in the jejunum and colon.

Specifically, ST and SC induced significant increases in TLR5 and 9 mRNA compared to uninfected control tissue. Conversely, TLR5 and 9 mRNA expression was significantly decreased in the colon obtained from SC treated pigs.

In terms of TLR mRNA expression in pigs, we have observed a constitutive level of TLR2, 4, 5 and 9 mRNA in IPEC-J2 and pMP cells in vitro. In addition, our in vivo experiments have provided evidence for TLR2, 4, 5 and 9 expression in various tissues including: skeletal muscle (unpublished observations), fetal tissues (intestine, liver, lung and heart) (Burkey et al., 2005), as well as the tonsil, jejunum, ileum, colon, MLN, spleen and liver (Burkey et al., 2006).

In addition to the expression data, we have also investigated the differential regulation of TLR mRNA by specific pathogen associated molecular patterns and *Salmonella* ssp. Specifically, in IPEC-J2 cells, ST or SC failed to induce changes in TLR4 or 9 expression; however, ST did induce increased expression of TLR2 and 5 mRNA compared to control cells (Burkey et al., 2004). In pMPs, numerical and significant changes in mRNA were observed for each TLR. For TLR2, both ST and SC induced significantly greater mRNA expression than control cells. In regard to TLR4, 5 and 9 in pMPs, each of the TLR were numerically increased by ST and SC. LPS did not elicit changes in any of the TLR in IPEC-J2 cells. In separate experiments, flagellin was used as the specific pathogen associated molecular pattern to investigate its effect on TLR5 expression in IPEC-J2 cells. Once again, although no statistically significant effects were observed, numerical increases in TLR5 mRNA were observed in IPEC-J2 cells that were cultured in the presence of various concentrations of purified bacterial flagellin from ST. Although, TLR5 mRNA was not significantly increased by flagellin, concomitant and significant increases in IL8 and CCL20 mRNA and IL8 protein were observed and presumably, initiated via the ligation of TLR5. We have also observed variable levels of TLR mRNA regulation in vivo in pigs experimentally infected with ST or SC.

Conclusion

There remains a great deal of mystery regarding the functions of the multifaceted gastrointestinal tract. The ability to efficiently and appropriately deal with the enormous antigenic load within the gut lumen allows the host to maintain homeostasis and absorb required nutrients. The experiments described in the following chapters may contribute to the growing body of information regarding the expression and regulation of TLR in the domestic pig.

Presumably, this information may then lead to a greater understanding of the TLR system in pigs and how *Salmonella* ssp. elicit responses specific to particular hosts. In the future, fundamental understanding of TLR, their ligands, and their signal transduction systems may help in the search of alternatives to antimicrobials and lead to the development of more efficient disease interventions.

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Chapter 1 - Figures and Tables

Table 1.1. Example ligands of Toll-like receptors (TLRs)

TLRs	Ligands
TLR2	Peptidoglycan, lipopeptides, zymosan
TLR3	Viral dsRNA
TLR4	LPS of Gram-negative bacteria,
TLR5	Bacterial flagellin
TLR7	ssRNA, synthetic compounds
TLR8	ssRNA, synthetic compounds
TLR9	CpG DNA
TLR10	Not determined
TLR11	Uropathogenic bacteria
Heterodimers:	
TLR1/TLR2	Lipopeptides
TLR6/TLR2	Lipopeptides

CHAPTER 2 - Expression of porcine Toll-like receptors 2, 4 and 9 in response to lipopolysaccharide and *Salmonella enterica* serovars Typhimurium and Choleraesuis

Abstract

Salmonella enterica serovar Typhimurium (ST) and Choleraesuis (SC) are among the most frequently isolated salmonellae serovars causing enteric disease in swine. Enteric disease in young pigs is of major concern in modern production systems due to the potential negative implications on animal health, food safety, and economic return. Epithelial cells express Toll-like receptors (TLR) that are instrumental in the discrimination between pathogenic and commensal bacteria and that act as mediators of subsequent innate and adaptive immune responses. However, little is known about the expression and regulation of TLR in swine. The objectives of the present experiments were to characterize the expression of porcine TLR2, 4 and 9 and to identify regulatory patterns of these receptors in the presence of live ST or SC. Our results indicate that TLR2, 4 and 9 are constitutively expressed in vitro in a porcine jejunal epithelial cell line (IPEC-J2), porcine mononuclear phagocytes (pMPs), and the distal ileal gut wall (including the continuous Peyer's patch). In IPEC-J2 cells, ST elicited an increase in TLR2 mRNA ($P < 0.05$), and both ST and SC increased TLR2 mRNA in pMPs ($P < 0.05$). In contrast, neither TLR4 nor TLR9 were affected by bacteria. In vivo, oral challenge with ST increased both TLR2 and TLR4 mRNA in the distal ileum of pigs at 24 and 48 h after treatment ($P < 0.05$ for both TLR at both times). However, the mRNA for both TLR2 and 4 returned to pre-challenge levels by 144 h. Taken together, the data indicate that TLR2, 4 and 9 are constitutively expressed in swine gut epithelia and pMPs, and that in the presence of a specific invasive swine pathogen in vivo, there is evidence for transient upregulation of mRNA for TLR2 and TLR4.

Keywords: Pigs, Toll-like Receptors, Salmonella enterica

1. Introduction

The mammalian gastrointestinal tract is lined with a single layer of epithelial cells that not only form a physical barrier, providing protection from luminal bacteria (pathogenic and commensal), but also provide an interface between the external and internal environments. As an interface, the epithelial monolayer has the remarkable responsibility of distinguishing between and directing an appropriate immune response to pathogenic and non-pathogenic antigens and microorganisms. The functionality of the intestinal epithelia is particularly important in neonatal swine as this period of time represents a significant vulnerability to pathogens such as ST and SC. These serovars have proven to be of great importance to the swine industry in terms of overall animal health and economic return, particularly in light of the fact that there is ongoing pressure to eliminate in-feed antibiotics that are commonly used in swine production systems.

Epithelial cells along the gastrointestinal tract express TLR which may help in the process of discriminating between pathogenic and commensal bacteria (Bogunovic et al., 2000; Philpott et al., 2001; Takeda et al., 2003). Activation of TLRs by their respective ligands initiates a signaling cascade that results in the activation of the cellular transcription factor nuclear factor-κB (NF-κB) and subsequent upregulation of costimulatory molecules and pro-inflammatory cytokines (e.g. IL-8, IL-6, IL-1 and TNFα) (Ghosh et al., 1998; Medzhitov et al., 1997). Ligands for TLRs are diverse, both in terms of structure and origin. However, several commonalities have surfaced regarding TLR ligands. Most TLR ligands are pathogen-associated molecular patterns that signal infection, recognize several structurally unrelated ligands, may require accessory proteins, and are recognized by direct binding (Medzhitov, 2001). Ligands have not been completely elucidated for each TLR, but many ligands are known. For example, TLR2 has been observed to recognize gram-positive and mycobacterial pathogen associated molecular patterns such as lipoteichoic acid and peptidoglycan (Schwandner et al., 1999), TLR4 recognizes LPS (Hoshino et al., 1999), and TLR9 recognizes unmethylated cytidine-phosphate-guanosine DNA motifs (Hemmi et al., 2000). For further reference, several recent reviews (Akira et al., 2004; Takeda et al., 2003; Werling et al., 2003; Barton et al., 2002) have summarized the ligands for TLR1 through TLR11.

Expression of swine TLRs in response to invasive *Salmonella* spp. have not been characterized. In fact, most of what is currently known regarding TLR expression, signaling and regulation has been discovered utilizing human and murine models. In domestic animals there have been recent reports documenting TLR expression patterns in avian (Iqbal et al., 2005) as well as bovine and ovine species (Menzies et al., 2005). Therefore, the broad objective of the current experiments was to characterize TLR expression in porcine in vitro and in vivo experimental settings in response to relevant swine pathogens. With that broad goal in mind, experiments were designed to characterize the expression of TLR2, 4 and 9 *in vitro* in porcine epithelial and monocytic cell populations, as well as in vivo in intestinal tissue from pigs to elucidate possible regulatory patterns in the presence of live *Salmonella* serovars using quantitative real-time PCR.

2. Materials and Methods

2.1. Culture and treatment of porcine neonatal jejunal epithelial cells (IPEC-J2)

We employed the neonatal jejunal epithelial cell line IPEC-J2 which was derived from a single animal less than 12 h old (Rhoads et al., 1994) (obtained from Dr. Bruce Schultz, Department of Anatomy and Physiology, Kansas State University, U.S.A.). Cell cultures were maintained in 50% DMEM - 50% F12 medium (Invitrogen, Carsbad, CA) supplemented with insulin/transferrin/Na selenite media supplement (1%; Sigma-Aldrich Co., St. Louis, MO), epidermal growth factor (5 ng/ml; Invitrogen), streptomycin/penicillin (1%; Invitrogen), with FBS (5%; Hyclone, Logan, UT). For experimentation, IPEC-J2 cells were seeded onto six-well Costar Snapwells™ (Corning Inc., Corning, NY) and maintained in the above mentioned media. The cells were allowed to adhere for 24 h before being washed and re-fed every other day for 7 d to allow the formation of a model epithelium (average cell density was 2.5×10^5 per well and transepithelial resistance was approximately 4000 ohm·cm^2). Twenty four hours before experimentation, cells were washed and replacement media was as above but devoid of antibiotics.

2.2. Tumor necrosis factor alpha (TNFα) protein secretion

Measurements of TNFα production from IPEC-J2 cells treated with LPS, SC and ST were performed by a swine specific ELISA (Biosource International, Camarillo, CA). Treatment media was obtained for analysis from both the apical and basolateral compartments in the IPEC-J2 polarized culture system at 1.5, 3 and 6 h after exposure to LPS, SC and ST.

Salmonella enterica serovars Typhimurium (ST) and Choleraesuis (SC) were isolates from swine origin (obtained from Dr. Jerome Nietfeld, Diagnostic Medicine/Pathobiology, Kansas State University, KS). Identification of *Salmonella* serotypes was verified by the National Veterinary Services Laboratory (Ames, IA). Both strains were grown in Luria Bertani medium at 37°C, for 24 h, at which point bacterial populations were estimated by spectrophotometry at 600 nm optical density (Current Protocols in Molecular Biology (2002), Unit 1.2.1-1.2.2). Bacteria were then pelleted and resuspended in DMEM/F12 or RPMI growth media (for IPEC-J2 and mononuclear phagocyte experiments, respectively) devoid of FBS and antibiotics.

2.3 Culture and treatment of porcine mononuclear phagocytes

Porcine mononuclear phagocytes (pMPs) were isolated from porcine peripheral blood (adapted from Goff et al., 1996). Briefly, peripheral blood was obtained from six, first parity gilts by jugular venipuncture and cells were isolated from the buffy coats by use of Accu-Paque™ Lymphocytes (Accurate Chemical and Scientific Corp., Westbury, NY) density gradient. Following isolation, pMPs were washed in PBS (Invitrogen) and resuspended in RPMI supplemented with FBS (7%; Hyclone) and antibiotic/antimycotic (1%; Invitrogen). Cells were seeded onto 24-well plates (Corning Inc.) at a concentration of 1×10^6 cells/well and incubated overnight (37 °C, 5% CO_2) in order to allow pMPs to adhere to the plate. The following day, nonadherent cells were discarded and the residual adherent cells were incubated for a further 2 h in medium alone or in the presence of LPS (10 ng/ml), ST or SC (1×10^8 CFU/well). After 1 h to allow for bacterial invasion, cells were washed to remove extracellular bacteria, and media containing 50 μg/ml gentamicin was added to kill any remaining extracellular bacteria.

2.4 Experimental animals and tissue collection

Twenty crossbred barrows (typical of U.S.A. commercial pigs), approximately 5 wk of age, were used and the experimental protocol was approved by the Kansas State University

Institutional Animal Care and Use Committee. These pigs showed no clinical signs or laboratory evidence of salmonellosis or other enteric diseases. Pigs were penned in an environmentally controlled isolation facility at 25°C and under constant light with *ad libitum* access to feed and water. After an acclimation period of 7 d, pigs were challenged orally with 3×10^9 CFU of ST whereas the control group received sterile medium. Samples of the entire gut wall (that included the continuous Peyer's patch) were excised from the ileocecal fold back to the ileocecal junction and the digesta flushed with ice cold sterile PBS. Samples were obtained at 0 (four pigs given only media), 8, 24, 48, and 144 h following oral ST challenge (n = four pigs/sacrifice time). Ileal tissue samples were placed in cold RNA*later*® (Ambion Inc., Austin, TX) until the RNA could be extracted as described below.

2.5 RNA isolation and real-time RT-PCR analysis

Total RNA was extracted using TRI® Reagent (Sigma-Aldrich Co.) according to the manufacturer's protocol. Following total RNA isolation, DNA-*free™* (Ambion Inc.) was used to remove contaminating DNA from all RNA samples. Samples were reconstituted in nuclease-free water (Ambion Inc.) and frozen in 25 to 50 μl aliquots for further analysis. RNA quality was assessed by agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. RNA quantity was determined by spectrophotometry using an optical density of 260 nm. Reverse transcription was carried out using TaqMan® (Applied Biosystems, Foster City, CA) reverse transcription reagents. Briefly, reverse transcription was carried out in a 50 μl final volume that included 25 mM $MgCl_2$, 500 μM dNTP's, 2.5 μM random hexamers, 0.4 U/μL Rnase inhibitor, 50 U/μL MultiScribe reverse transcriptase, and TaqMan RT buffer. The reverse transcription mixture was incubated at 25°C for 10 min, heated to 37°C for 60 min, and inactivated at 95°C for 5 min. The resultant cDNA was stored (-20°C) until used. Real-time quantitative PCR was utilized to quantify the genes of interest (TLR2, 4, 5, and 9) relative to the quantity of 18S rRNA. The PCR reactions were carried out in 96-well plates with the appropriate forward and reverse primers (900nM), the appropriate TaqMan® TAMRA probe (200 nM), PCR Mastermix (Applied Biosystems), and 3.5 μL of the cDNA sample. The porcine specific TLR primers and detection probes (Table 1) were synthesized from published GenBank® sequences using PrimerExpress® software (Applied Biosystems). Commercially available eukaryotic 18S rRNA (Applied Biosystems) primers and probe were used as an

endogenous control. PCR reactions, run in triplicate wells, were carried out with the ABI
PRISM® 7700 Sequence Detection System (Applied Biosystems) using 50 cycles of
amplification with alternating 15 s 95°C denaturation and 1 min 60 °C anneal/extension.

2.6 Statistical analyses

Relative abundance of TLRs in cultured jejunal enterocytes and pMPs were calculated
with the ΔΔCT method using the average ΔCT values of cells from control wells as the reference
expression. The ΔΔCT values were expressed as $2^{-\Delta\Delta CT}$ to obtain relative abundance values. The
relative abundance values were analyzed using the PROC MIXED procedure of SAS (SAS Inst.
Inc., Cary, NC) to determine the effect of time after ST on TLR relative expression. Real-time
PCR data from ileal samples were handled similarly except that average pre-inoculation ΔCT (0
h) values were used as the reference expression (n = tissue from four pigs at each time point).
Data bars in Figure 1 represent six observations per treatment obtained from duplicate wells of
cells from three independent in vitro experiments. The model included effects of treatment, time
and their interaction. Polarized secretion of TNFα from cultured cells (Fig. 2) was analyzed with
effects of treatment, time and location (apical or basolateral) and their interactions in the model.
Secretion of TNFα was expressed as pg/well to account for the considerably greater volume of
media present in the basolateral compartment (2.5 ml) compared to the apical compartment (0.5
ml).

3. Results

3.1 TLR expression and secretion of TNFα from porcine jejunal epithelial cells

Initially, we sought to characterize the time-course of TLR expression in IPEC-J2 cells
when treated with LPS or the important swine pathogens ST or SC. Figure 1 depicts the relative
abundance of mRNA for TLR2, TLR4, and TLR9 in IPEC-J2 cells treated with LPS, ST or SC.
Treatment of IPEC-J2 cells with ST elicited increased (P < 0.05) TLR2 mRNA at 3 h post-
inoculation compared to control and LPS-treated cells, and at 6 h post-inoculation when
compared to all other treatments (Figure 1, panel A). For TLR4 and TLR9, there were no
significant treatment × time interactions or main effects of time after LPS or bacterial exposure.
However, treatment with ST and SC increased TLR4 compared to control and LPS-treated cells

when averaged across all time points (P < 0.05) and TLR9 mRNA was increased by SC compared to all other treatments (P < 0.05; Figure 1, panels B and C, respectively).

Following apical treatment with LPS, SC and ST, media was collected from the IPEC-J2 cultures depicted in Figure 1, and the concentration of TNFα in the apical and basolateral compartments was determined (Figure 2). Although there was no treatment × time × position (apical versus basolateral direction) interaction noted for secretion of TNFα, a treatment × position interaction was observed (P < 0.05; Figure 2). From this interaction there are two comparisons worthy of note. First, when averaged across all treatments and time points, TNFα secretion was polarized in the basolateral direction (P < 0.0001). Secondly, within the basolateral compartment (Figure 2, panel D) secretion of TNFα was greater (P < 0.05) for cells treated with ST compared to control or SC-treated cells when means are averaged across all time points.

3.2 TLR expression and regulation in porcine mononuclear phagocytes

A second in vitro experiment was conducted using pMPs with a similar design as the experiments described above using IPEC-J2 cells. Relative expression of TLR2 mRNA was increased at 1.5 h post-inoculation by both ST and SC (Figure 3, panel A; P < 0.05 compared to LPS-treated and control cells). For TLR4 and TLR9 mRNA, only a significant time effect was observed (Figure 3 panels B and C, respectively) with the greatest relative expression at 1.5 h post-inoculation (P < 0.05).

3.3 TLR expression in the porcine distal ileum following ST challenge

For this experiment, the relative expression of TLR mRNA in gut wall containing continuous Peyer's patches obtained from the porcine distal ileum was determined after oral ST challenge (Figure 4). Treatment with ST (P < 0.05) increased the relative abundance of both TLR2 and TLR4 mRNA by 24 h post-inoculation compared to controls (P < 0.05), and the greatest increase for TLR2 and TLR4 was observed by 48 h post-inoculation (P < 0.05), representing approximately 3- and 2-fold increases in mRNA for TLR2 and 4, respectively.

4. Discussion

Salmonellosis remains a source of economic burden to the swine industry due to losses associated with reduced feed conversion, depressed growth, and increased mortality in young

pigs. In the past several years, our group characterized the pathophysiology following acute oral challenge with *Salmonella enterica* serovar Typhimurium. Our findings using this enteric disease model have lead to the following general conclusions following a single dose of ST: 1) within 24 h post-inoculation these animals experienced a febrile response and inappetence ultimately resulting in decrease growth performance (Balaji et al., 2000; Burkey et al., 2004a; Turner et al., 2002a; Turner et al., 2002b); 2) ST elicited activation of the endocrine stress axis as evidenced by increased cortisol (Balaji et al., 2000) and decreased serum concentrations of insulin-like growth factor-1 (Balaji et al., 2000; Jenkins et al., 2004); and 3) ST caused self-limiting enteritis which typically resolved itself within 1 wk post-inoculation without evidence of elevated systemic inflammatory mediators (Balaji et al., 2000; Burkey et al., 2004a; Fraser et al., 2005). The observation that ST produced overt symptoms of enteric disease in the absence of engagement of peripheral inflammatory cells and systemic elevations in TNFα, IL-1β or IL-6 suggested to us a key role of the enteric mucosal immune system in containing the response to ST. Therefore, the current experiments focused on delineating the expression and regulation patterns of evolutionarily conserved pattern recognition receptors within the porcine gastrointestinal tract and porcine systemic immune cells in response to salmonellae serovars.

Direct apical exposure of the model swine jejunal epithelium in vitro to ST produced over a 20-fold increase in the expression of TLR2 mRNA by 6 h after treatment, whereas TLR4 and TLR9 remained largely unaffected by LPS or the invasive enteric pathogens. At least two important points are worth noting relative to the interpretation of these observations. First, although relative abundance of TLR4 mRNA wasn't affected by either LPS or bacteria, it appears to be expressed constitutively at very high levels in IPEC-J2 cells compared to other TLRs based on the Ct values obtained from real-time PCR (data not shown). This observation is not apparent when the relative expression is computed using the ΔΔCT method. Moreover, we confirmed in an independent in vitro experiment that the lack of change in relative abundance of TLR4 mRNA could not be explained by the lack of a source of LPS binding protein that could be provided by the presence of serum in the media (data not shown). This is relevant because the fully functional TLR4 signaling complex requires the presence of additional proteins (LPS binding protein, CD14 and MD-2) (Shimazu et al., 1999). CD14, along with LPS binding protein, facilitates delivery of LPS to TLR4 and is expressed on the surface of cells of the

myeloid lineage and also exists as a soluble plasma protein (Pugin et al., 1993). However, the presence of CD14 on epithelial cells remains uncertain (Cario et al., 2000; Suzuki et al., 2003).

The second and perhaps most significant observation from the in vitro experiments with IPEC-J2 cells is the impressive increase in relative abundance of TLR2 in response to ST, but not SC or LPS. This effect on TLR2 in IPEC-J2 cells is generally consistent with our parallel experiment in mononuclear phagocytic cells (Figure 3) where only TLR2 mRNA was increased by salmonellae, albeit to a lesser magnitude and with a different timecourse. The hyporesponsiveness of intestinal epithelial cells to LPS was not surprising in that this has been reported by our laboratory for swine cells (Skjolaas et al., 2006) and also for other intestinal epithelial cell lines (Abreu et al., 2001; Otte et al., 2004). The effect of ST to increase TLR2, but not the swine adapted serovar SC is consistent with contrasting effects of the serovars in driving IL-8 secretion (Skjolaas et al., 2006), and may be related to the fact that SC was found to be far less invasive to this swine epithelial cell line (Schierack et al., 2005). This effect of invasive Gram-negative bacteria on TLR2 is intriguing and our observations are corroborated by those of Tötemeyer et al. (2003). These authors, using an in vivo model of ST infection in mice, observed similar increases in TLR2 and TLR4 mRNA and hypothesized that the increase in TLR2 mRNA is TLR4 dependent. This phenomenon could also be, in the in vivo model, explained by infiltration of immune cells into the gut wall during infection. However, further work is required to verify these possibilities in the domestic pig.

In previous experiments with IPEC-J2 cells, we demonstrated that apical treatment with ST produced an unmistakable IL-8 secretory response that was heavily polarized in the basolateral direction (Burkey et al., 2004b). This observation provided evidence of functional TLR activation and engagement of NFκB signaling pathway (Abreu et al., 2003; Ozato et al., 2002). In view of this observation, we hypothesized that ST might broadly activate inflammatory cytokine secretion and this would be reflected in polarized secretion of TNFα. However, although overall ST increased TNFα statistically compared to untreated wells (see Figure 2, panel D), the effect was modest. The most striking characteristic of TNFα secretion from this model epithelium however was not the response to treatment. Rather, even in untreated cells, TNFα secretion was almost entirely directed basolaterally. Assuming this observation provides insight into the situation in vivo, it suggests the epithelium directs a basal level of TNFα toward

cells in the lamina propria. The functional significance of such an arrangement remains to be determined.

The in vivo oral exposure of pigs to ST compliment and extend our in vitro observations suggesting the interaction of the gut with ST works to increase relative mRNA abundance of both TLR2 and TLR4 in the ileal gut wall. Although these are the first data to suggest such an effect of ST in the pig, it is not clear from our observations whether the enhanced expression represents an effect on the mucosal epithelium or other cellular elements in the lamina propria or submucosa because we sampled the entire gut wall. Although the effect of oral ST waned after 48 h, the time domain of the increase correlates very closely to the anti-inflammatory arm of the acute phase response, namely the peripheral secretion of cortisol (Balaji et al., 2000). It is tempting to speculate that upregulation of the pro-inflammatory arm of the innate response represented by enhanced expression of CCL20 (Skjolaas et al., 2006), TLR2 and TLR4 (data from these studies) is fairly quickly brought into check by anti-inflammatory counter measures provided by neuroendocrine activation and the secretion of cortisol (Balaji et al., 2000). Finally, it is worth noting that TLR mRNA expression does not necessarily provide irrefutable evidence for the presence of active, competent receptor molecules. Future studies and the availability of porcine reagents will allow us to determine if in fact these results can be confirmed at the protein level.

Taken together, these findings provide new insights into the expression and regulation of TLRs in swine epithelial and immune cells, and in the porcine small intestine when challenged with invasive enteric bacteria. The cross-talk between the intestinal mucosa and the underlying lamina propria are paramount to proper and coordinated defense against bacteria and bacterial products, and warrant further detailed study.

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Chapter 2 - Figures and Tables

Figure 2.1 Relative abundance of Toll-like receptor 2 (TLR2; panel A), Toll-like receptor 4 (TLR4; panel B), and Toll-like receptor 9 (TLR9; panel C) mRNA from cultured porcine jejunal epithelial cells (IPEC-J2) in the absence of FBS and treated with media alone (control), 10 ng/ml lipopolysaccharide (LPS), 10^8 CFU/well *Salmonella enterica* serovar Choleraesuis (SC), or 10^8 CFU/well *Salmonella enterica* serovar Typhimurium (ST). Total RNA extracted at 1.5, 3.0 and 6.0 h post treatment. Each bar represents the least square mean (± SEM) of six observations. Within time periods, bars without common superscripts differ (P < 0.05).
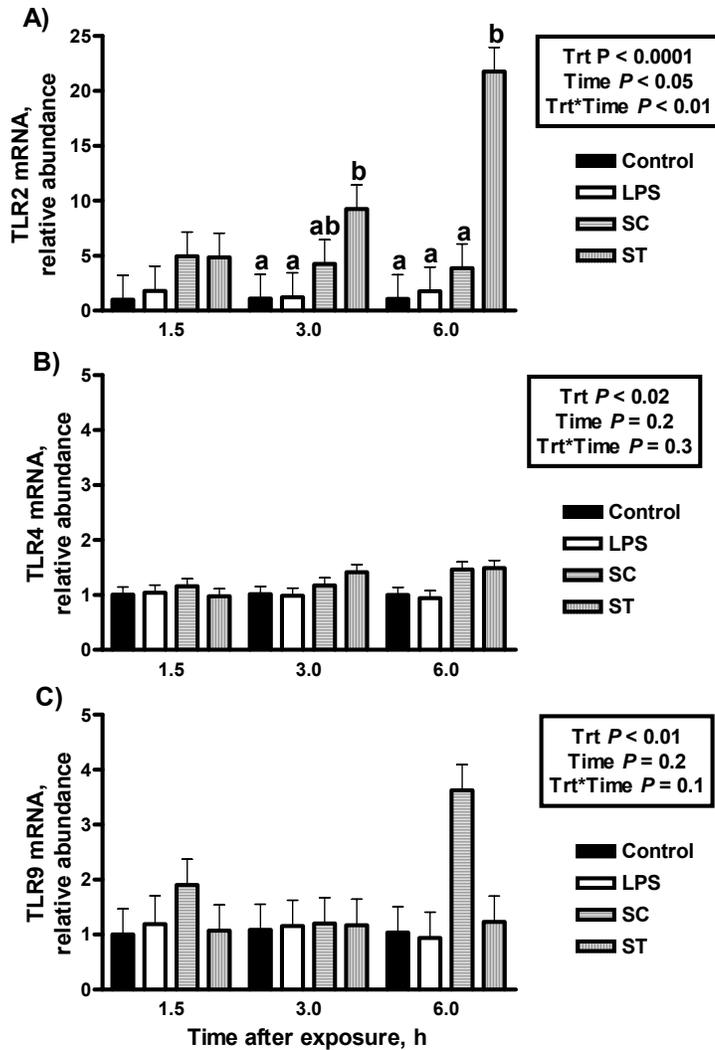
Figure 2.2 Polarized tumor necrosis factor alpha (TNFα) secretion by confluent porcine jejunal epithelial cells (IPEC-J2) monolayers treated with media alone (control; CON), 5 ng/well (10 ng/ml) lipopolysaccharide (LPS), 10^8 cfu/well *Salmonella enterica* serovar Choleraesuis (SC), or 10^8 cfu/well Typhimurium (ST) in the apical (AP) compartment for 1 h. Treatment media were removed and replaced with media containing gentamicin. Media from the AP and basolateral (BL) compartments were collected and assayed for cytokines at 1.5 (panel A), 3.0 (panel B), and 6.0 h (panel C) after the onset of LPS or bacterial treatment. Each bar represents the least square mean (± SEM) of four observations. Panel D depicts the main of effects of treatment when means were averaged across all time points. Bars without common superscripts differ (P < 0.05).
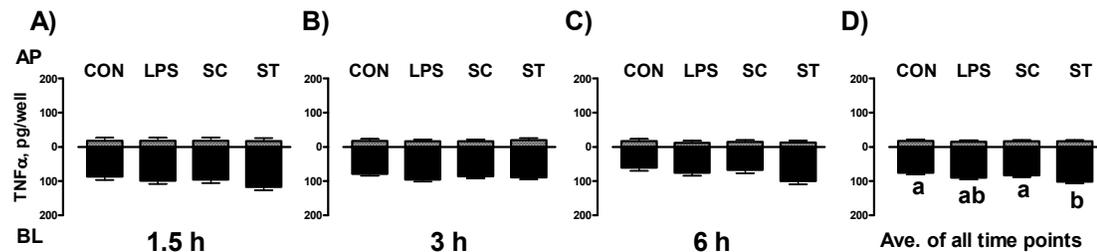
Figure 2.3 Relative abundance of Toll-like receptor 2 (TLR2; panel A), Toll-like receptor 4 (TLR4; panel B), and Toll-like receptor 9 (TLR9; panel C) mRNA from cultured porcine mononuclear phagocytic cells (pMPs) treated with media alone (control), 10 ng/ml lipopolysaccharide (LPS), 10^8 CFU/well *Salmonella enterica* serovar Choleraesuis (SC), or 10^8 CFU/well *Salmonella enterica* serovar Typhimurium (ST). Total RNA extracted at 1.5, 3.0 and 6.0 h post treatment. Each bar represents the least square mean (± SEM) of six observations. Within time periods, bars without common superscripts differ (P < 0.05).
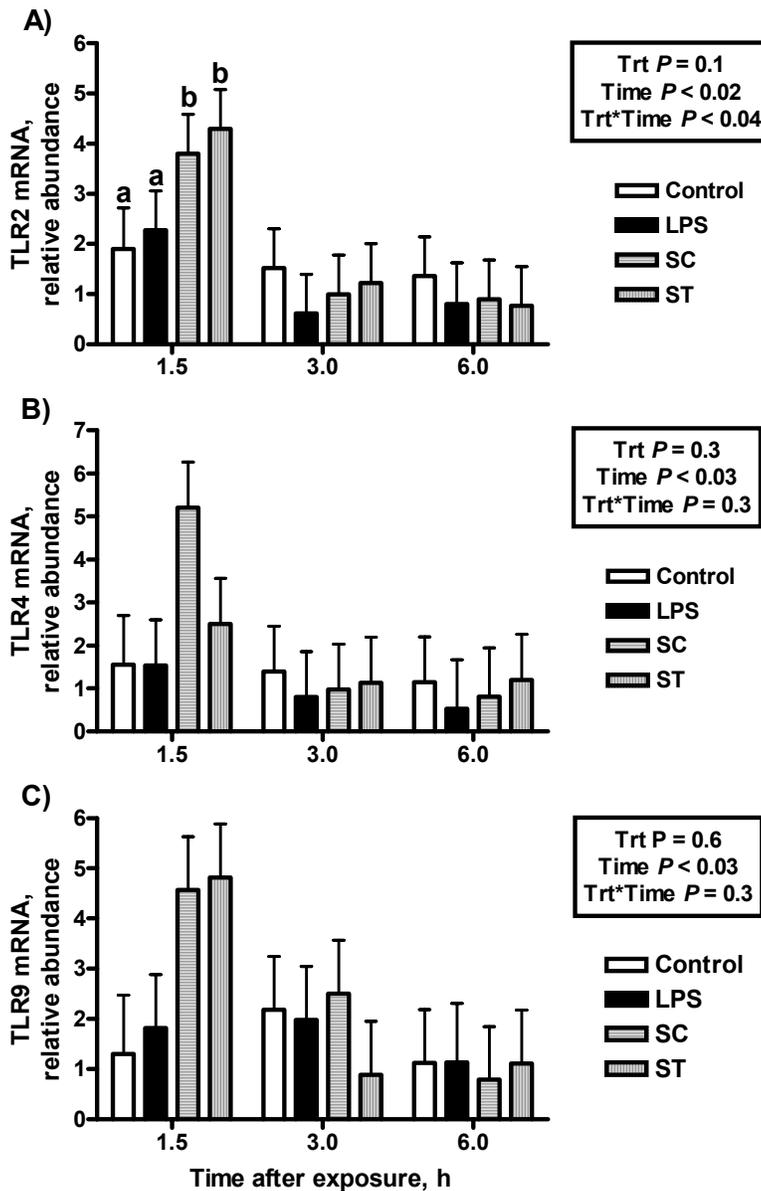
Figure 2.4 Relative abundance of Toll-like receptor 2 (TLR2; panel A), Toll-like receptor 4 (TLR4; panel B), and Toll-like receptor 9 (TLR9; panel C) mRNA from porcine distal ileum isolated at 0, 8, 24, 48, and 144 h after 10^9 CFU oral _Salmonella enterica_ serovar Typhimurium challenge. Each time point represents tissue obtained from four pigs. Bars without common superscripts differ (P < 0.05).
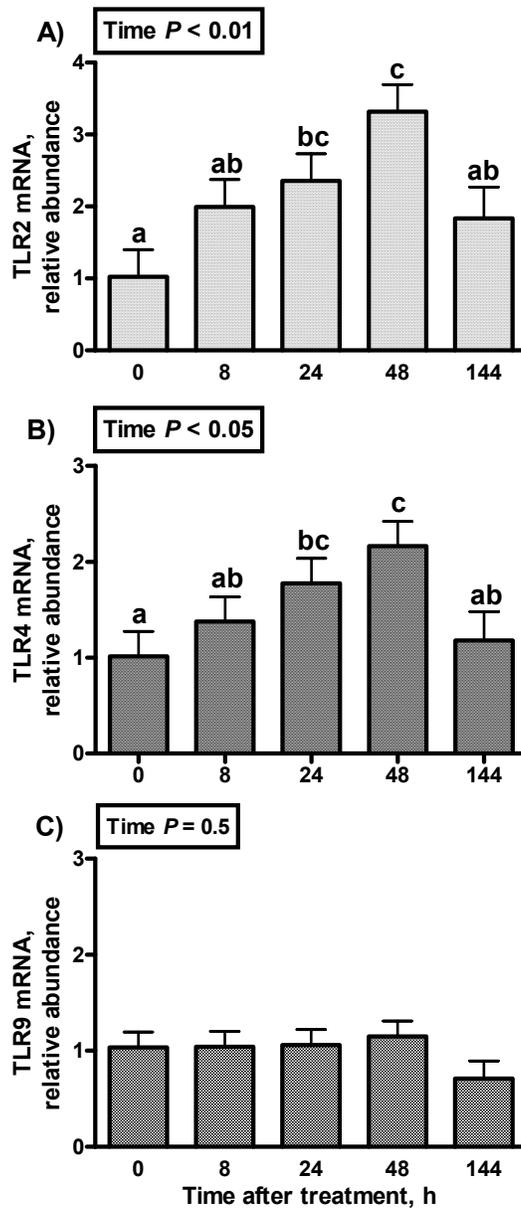
Table 2.1 Primer and probe sequences used for TLR mRNA quantification by real-time RT-PCR.

Primer set	Product length	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Probe sequence (5'-3')[1]
TLR2	84	CAG CAC GAG AAT ACA CAG TTT AAC C	AAC GAG TTG AGA TTG TTA TTG CTA ATA TCT	ATT GGC TTC CCC AGA CCC TGG AAG T
TLR4	71	TGT GGC CAT CGC TGC TAA C	GGG ACA CCA CGA CAA TAA CCT T	TCA TCC AGG AAG GTT TCC ACA AAA GTC G
TLR9	80	CAA TGA CAT CCA TAG CCG AGT GT	TCA GAT CGT TGC CGC TAA AGT	AGC AGC TCT GTA GCG CCT CAC TGT GC

[1]All probes were synthesized with 6-FAM as the 5' fluorophore and TAMRA as the 3' quencher dye.

CHAPTER 3 - Effects of flagellin and *Salmonella enterica* serovar Typhimurium on Toll-like receptor 5 and chemokine expression in swine

Abstract

Toll-like receptor (TLR) 5 represents one member of a class of pattern recognition receptors important in detecting and mediating the immune response to microbial invaders via pathogen associated molecular patterns. One specific pathogen associated molecular pattern secreted by both commensal and pathogenic bacteria (e.g. *Salmonella* ssp.) is flagellin, the principal constituent of bacterial flagella. Recent work has shown that monomeric flagellin, in addition to playing a role in bacterial adhesion, can act as a proinflammatory/immune activator via TLR5. In addition, it has been observed that TLR5 is preferentially expressed on the basolateral aspect of human and murine intestinal epithelial cells (IECs). Taken together, the interplay between flagellin, TLR5 and IECs represents an important component of innate mucosal and adaptive immunity. Therefore, the objective of the current study was to determine the in vivo and in vitro expression and regulation of TLR5, interleukin 8 (IL8) and CC chemokine ligand 20 (CCL20) in vivo in porcine ileum, and in vitro in porcine jejunal epithelial (IPEC-J2) and mononuclear phagocytic (pMP) cells in response to *Salmonella enterica* serovar Choleraesuis (SC), serovar Typhimurium (ST), or flagellin derived from ST. Quantitative real time PCR assays were used to determine the relative expression of TLR5, IL8 and CCL20 target genes. Our findings show that porcine TLR5 was constitutively expressed in vivo in samples from the ileal gut wall and in vitro in IPEC-J2 and pMP cells. However, TLR5 was not significantly regulated by exposure to ST or purified flagellin derived from ST, even though expression of chemokine mediators (i.e. IL8 and CCL20) indicated activation of the inflammatory response. Specifically, ST (10^8 CFU/well) and purified flagellin (10, 100 and 325 ng/ml) provoked upregulation of IL8 and CCL20 ($P < 0.02$) mRNA in IPEC-J2 cells within 1.5 h after exposure. Moreover, IL8 secretion from IPEC-J2 cells was increased ($P < 0.05$) and highly polarized toward the basolateral direction when these cells were exposed to ST (10^8 CFU/well) and purified flagellin (1.0, 10, 100 and 325 ng/ml). Collectively, our results indicate that porcine TLR5 is constitutively expressed in vivo and in vitro and that this pattern recognition receptor is not consistently upregulated when the inflammatory cascade is activated by live SC, ST or purified flagellin derived from ST.

1. Introduction

TLR5, as well as the other known TLR, can be classified as classical pattern recognition receptors that have the ability to recognize conserved microbial structures known as pathogen associated molecular patterns (Janeway, Jr. and Medzhitov, 2002). TLR are type I integral transmembrane glycoproteins that include a cytoplasmic Toll−interleukin 1 receptor domain and an extracellular domain characterized by leucine-rich repeat motifs. The insertion of different amino acids in the extracellular leucine-rich repeat domain may explain the ability of TLR to recognize various ligands (Bell et al., 2003) and in the specific case of TLR5, may confer its ability to recognize bacterial flagellin.

Flagellin, an approximately 40-60 kDa protein encoded by the gene *fliC*, is the major constituent of the bacterial flagellar filament (Hayashi et al., 2001) and it has the capacity to induce innate and adaptive immune responses (Honko and Mizel, 2005). Even though the idea that flagellin could elicit an adaptive response has been appreciated for several decades (Ada and Byrt, 1969), its partnership with TLR5 has only been recently appreciated as a key component of the innate response to bacterial pathogens (Reichhart, 2003).

Our interest in the interactions between TLR5, flagellin and IECs stems from the fact that *Salmonella* ssp. are of particular importance to the swine industry. Swine salmonellosis poses health and economic risks in pork production systems. The onset and early stages of mammalian salmonellosis are characterized by the initial contact between the bacterium and the apical surface of the host epithelial cell and the induction of a classical acute inflammatory reaction (McGovern and Slavutin, 1979). Flagellin may be exclusively responsible for the activation of NF-κB in intestinal epithelial model systems (Tallant et al., 2004). In addition, Gewirtz et al. (1999; 2001b) provided two observations that has revealed further complexity to the flagellin-TLR5 axis. First, apically applied flagellin does not elicit a cytokine response in polarized human epithelial cells; and second, translocation of apical flagellin to the basolateral surface is independent of bacterial invasion.

To our knowledge, there are no previous published reports of the expression or regulation of porcine TLR5 in response to bacterial flagellin or important swine serovars SC and ST.

Therefore, the objectives of the current studies were to investigate the expression and regulation of porcine TLR5 in response to SC, ST or flagellin derived from ST.

2. Materials and Methods

2.1. Experimental Animals and Tissue collection

Twenty crossbred barrows, typical of commercial pigs and approximately 5 wk of age, were used. These pigs showed no clinical signs or laboratory evidence of salmonellosis or any other enteric diseases. Pigs were penned individually in an environmentally controlled isolation facility at 25°C and under constant light with *ad libitum* access to feed and water. After an acclimation period of 7 d, pigs were challenged orally with 3 x 10^9 CFU ST (n=16) whereas the control group (n = 4) received only sterile medium. The ST was a primary isolate from a clinical case of salmonellosis in pigs and was confirmed to be ST at the National Veterinary Services Laboratory (Ames, IA). Samples of the entire gut wall were obtained including the continuous Peyer's patch. A sample containing the entire ileum was excised from the ileocecal fold back to the ileocecal junction and the digesta flushed with ice cold sterile PBS. For this experiment, the distal ileum was obtained at 0, 8, 24, 48, and 144 h following oral ST challenge (n = four pigs/sacrifice time). Ileal tissue samples were placed in cold RNA*later*® (Ambion, Inc., Austin, TX) until the RNA could be extracted as described below.

2.2. Culture of porcine neonatal jejunal epithelial cells (IPEC-J2)

The neonatal jejunal epithelial cell line IPEC-J2 was derived from a single animal less than 12 h old (Rhoads et al., 1994). These cells were a gift from Dr. Bruce Schultz, Anatomy and Physiology, Kansas State University. Cell cultures were maintained in 50% DMEM - 50% F12 medium (Invitrogen, Carsbad, CA) supplemented with insulin/transferrin/Na selenite media supplement (1%; Sigma-Aldrich Co., St. Louis, MO), epidermal growth factor (5 ng/ml; Invitrogen), streptomycin/penicillin (1%; Invitrogen), and FBS (5%; Hyclone, Logan, UT). For experimentation, IPEC-J2 were seeded (2.5 x 10^5 to 4.0 x 10^5/well) onto six-well Costar Snapwells™ (Corning Inc, Corning, NY) and maintained in the previously mentioned media. The cells were allowed to adhere for 24 h before being washed and re-fed every other day for 7 d of growth to allow for confluency and tight junction formation (average cell density was 2.5 x

10^5 per well and transepithelial resistance was approximately 4000 ohm·cm^2). Twenty-four hours before experimentation, cells were washed and replacement media was as above but devoid of antibiotics.

2.3 Bacteria for in vitro challenge studies

The *Salmonella* isolates used in this study were obtained from clinical cases of porcine salmonellosis (ST as noted above and SC, also provided by Dr. Jerome Nietfeld, Diagnostic Medicine Pathobiology, Kansas State University). Identification of the SC isolate, as for ST, was verified by the National Veterinary Services Laboratory (Ames, IA). Both strains were grown in Luria Bertani medium at 37°C, for 24 h, at which point bacterial populations were estimated by spectrophotometry (OD 600 nm). Bacteria were then pelleted and resuspended in DMEM/F12 or RPMI growth media, as appropriate for the experiment, that was devoid of FBS and antibiotics.

2.4 Exposure of IPEC-J2 cells to flagellin, LPS and bacteria

Two separate experiments utilizing IPEC-J2 cells were conducted. The first experiment (Figure 2) included IPEC-J2 cells that were exposed to media alone (control), purified flagellin from ST (1.0, 10, 100, 325 ng/ml; Alexis Biochemicals, San Diego, CA), or ST (included at 10^8 bacteria/well). The second experiment (Figure 4A) included IPEC-J2 cells exposed to media alone (control), LPS (10 ng/ml; Sigma Chemical Co., St. Louis, MO), SC or ST (included 10^8 bacteria/well for both SC and ST). Confluent IPEC-J2 cells, as described above, were washed twice with PBS and 0.5 ml control media, LPS or bacteria were added to the top (apical) wells, while 2.5 ml of control media or flagellin (in the case of experiments where flagellin was included as a treatment) was added to the bottom (basolateral) wells. IPEC-J2 cells were subsequently incubated at 37°C, 5% CO$_2$ for 1 h. Cells from all treatments were then washed with sterile PBS and both apical and basolateral media were replaced with fresh control media containing 50 μg/ml gentamicin. In the flagellin experiments, gentamicin was added without washing the cells. Media was removed from apical and basolateral compartments at the indicated times for quantitation of IL8 secretion (swine specific IL8 sandwich ELISA, Biosource International, Camarillo, CA), and RNA was extracted from the cells according to the RNA extraction procedures described below.

2.5 Culture and treatment of porcine mononuclear phagocytes

Porcine mononuclear phagocytes (pMPs) were isolated from porcine peripheral blood as previously described (adapted from Goff et al., 1996). Briefly, peripheral blood was obtained from six, first parity healthy gilts by jugular venipuncture and cells were isolated from the buffy coats by use of Accu-Paque™ Lymphocytes (Accurate Chemical and Scientific Corp., Westbury, NY) density gradient. Cells were seeded onto 24-well Costar plates at a concentration of 10^6 cells/well and incubated overnight (37 °C, 5% CO_2) in order to allow pMPs to adhere to the plastic. The following day, nonadherent cells were discarded and the residual adherent cells were incubated for a further 2 h in medium alone or in the presence of LPS (10 ng/ml), SC or ST (1 x 10^8 CFU/well for both SC and ST). After 1 h to allow for bacterial invasion, cells were washed to remove extracellular bacteria, and media containing 50 μg/ml gentamicin was added to kill any remaining extracellular bacteria.

2.6 RNA isolation and real-time RT-PCR analysis

Total RNA was extracted from cells or tissues at the indicated times using TRI® Reagent (Sigma-Aldrich Co.) according to the manufacturer's protocol. Following total RNA isolation, DNA-*free*™ (Ambion Inc.) was used to ensure removal of contaminating DNA from all RNA samples. Samples were reconstituted in nuclease-free water (Ambion Inc.) and frozen in 25-50 μl aliquots for further analysis. RNA quality was verified by agarose gel electrophoresis and visualization of the 28S and 18S ribosomal RNA. RNA was quantified by spectrophotometry (OD 260/280 nm). Reverse transcription was carried out using TaqMan® (Applied Biosystems, Foster City, CA) reverse transcription reagents. Briefly, reverse transcription was carried out in a 50 μl final volume that included 25 mM $MgCl_2$, 500 μM dNTP's, 2.5 μM random hexamers, 0.4 U/μL RNase inhibitor, 50 U/μL MultiScribe reverse transcriptase, and TaqMan RT buffer. The reverse transcription mixture was incubated at 25°C for 10 min, heated to 37°C for 60 min, and inactivated at 95°C for 5 min. The resultant cDNA was stored (-20°C) until used. Real-time quantitiative PCR was utilized to quantify the genes of interest (TLR5, IL8 and CCL20) relative to the quantity of 18S ribosomal RNA in total RNA isolated from porcine ileal samples, cultured IPEC-J2 cells and pMPs. The PCR reactions were carried out in 96-well plates with the appropriate forward and reverse primers (900nM), the appropriate TaqMan® TAMRA probe (200 nM), PCR Mastermix (Applied Biosystems), and 3.5 μL of the cDNA sample. The porcine

specific primers and detection probes were synthesized from published GenBank® sequences using PrimerExpress® software (Applied Biosystems). Sequences for IL8 and CCL20 were as previously published (Skjolaas et al., 2006). For TLR5, the primers (5'→3' forward primer: CAG CAC GAG AAT ACA CAG TTT AAC C; 5'→3' reverse primer: AAC GAG TTG AGA TTG TTA TTG CTA ATA TCT) and probe (5' 6-FAM fluorophore; 3' TAMRA quencher dye; ATT GGC TTC CCC AGA CCC TGG AAG T) were designed to detect a 100 base product. Commercially available eukaryotic 18S ribosomal RNA (Applied Biosystems) primers and probe were used as an endogenous control. Assays using non-template controls and samples were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems). Thermal cycling parameters were utilized according to manufacturer recommendations and included 50 cycles of 15 s at 95°C and 1 min at 60°C.

2.7 Statistical analyses

Relative abundance of target gene mRNA in ileal samples was determined using the ΔΔCT method using the average pre-inoculation ΔCT (0 h) as the reference expression (n = tissue from four pigs at each time point). The ΔΔCT values were expressed as $2^{-\Delta\Delta CT}$ to obtain relative abundance values. The relative abundance values were analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) to determine the effect of time. Real-time PCR data from cultured jejunal enterocytes and pMPs were handled similarly except that relative abundance values were calculated relative to the average ΔCT of cells from control wells. The model included effects of treatment, time and their interaction. The polarized secretion of IL8 from cultured cells was analyzed with effects of treatment, time and position (apical or basolateral) in the model. Secretion of IL8 was expressed as pg/well to account for the considerably greater volume of media present in the basolateral compartment (2.5 ml) compared to the apical compartment (0.5 ml).

3. Results

3.1 In vivo challenge with Salmonella enterica serovar Typhimurium

This experiment was a time dependent study designed to evaluate TLR5 mRNA expression in ileal gut wall samples containing the continuous Peyer's patch obtained from pigs

orally challenged with ST (Figure 1). Although TLR5 expression was evident in all samples evaluated, the expression of TLR5 mRNA was not affected by oral ST challenge. However, slight numeric increases in TLR5 expression were observed by 48 h after infection.

3.2 In vitro challenge of porcine gut epithelium with flagellin and Salmonella enterica serovar Typhimurium

Because the in vivo challenge described above included cells within the entire width of the gut wall and not just the epithelial layer, we next evaluated TLR5 and chemoattractive mediators in a model gut epithelium. In addition, we sought to compare the effects of live ST to purified flagellin derived from ST. To accomplish this, in vitro experiments were designed to evaluate the responses of IECs to flagellin as well as live bacterial challenge. The effects of flagellin and ST on TLR5, IL8 and CCL20 gene expression in IPEC-J2 cells are illustrated in Figure 2. In this experiment, no treatment × time interaction was noted for TLR5 mRNA (Figure 2A). However, there was a significant main effect of time ($P < 0.02$) where TLR5 mRNA was more abundant at 1.5 h than 5 h when averaged across all treatments. In addition, there was also a strong tendency ($P = 0.06$) for TLR5 mRNA to be increased by ST and flagellin (10 ng/ml) compared to the control cells when means were averaged across both time points. Both IL8 and CCL20 mRNA (Figures 2B and 2C, respectively) demonstrated treatment × time interactions ($P < 0.004$ and $P < 0.0001$ for IL8 and CCL20, respectively). At 1.5 h following treatment, flagellin (10, 100 and 325 ng/ml) and ST increased ($P < 0.05$) IL8 mRNA compared to untreated control cells, with ST eliciting the greatest change. At 5 h following treatment, IL8 mRNA was similar among control cells and flagellin treated cells, whereas ST treated cells expressed greater IL8 mRNA than cells among any of the other treatment groups ($P < 0.05$). Similarly, at 1.5 h following treatment, CCL20 mRNA was increased by flagellin (10, 100 and 325 ng/ml) and ST compared to untreated control cells ($P < 0.05$). In addition, cells treated with 100 and 325 ng/ml flagellin elicited greater ($P < 0.05$) CCL20 mRNA expression than all other treatments. At 5 h following treatment, flagellin (10 and 100 ng/ml) elicited greater CCL20 mRNA expression than untreated control cells ($P < 0.05$) while ST elicited greater CCL20 mRNA expression than all other treatments ($P < 0.05$).

Media from both the apical and basolateral compartments were collected at 1.5 and 5 h after treatment and the concentration of IL8 was determined using a porcine-specific ELISA

(Figure 3). The concentration in each compartment was adjusted to reflect the greater volume in the basolateral compartment and expressed as picograms/well. All main effects of treatment, time, position (apical vs. basolateral) and their interactions were highly significant ($P < 0.001$). Therefore, only the treatment × time × position means were compared further. No differences in IL8 secretion among treatments were noted at 1.5 h in either the apical or basolateral compartments. However, within the apical compartment at 5 h, flagellin (100 and 325 ng/ml) and ST treated cells had greater IL8 secretion than untreated controls ($P < 0.005$ and $P < 0.04$; respectively for 100 and 325 ng/ml flagellin vs. control) with ST eliciting greater IL8 secretion than all other treatments ($P < 0.0001$). Within the basolateral compartment at 5 h, all flagellin concentrations were observed to increase ($P < 0.05$) IL8 secretion compared to untreated controls with flagellin at concentrations of 10, 100 and 325 ng/ml similarly increasing IL8 over untreated control cells and IPEC-J2 cells exposed to 10 ng/ml flagellin. Similar to the apical compartment at 5 h, ST stimulated greater increases in IL8 secretion within the basolateral compartment compared to all other treatments ($P < 0.05$).

3.3 In vitro challenge of porcine gut epithelium and mononuclear phagocytes with LPS and Salmonella enterica serovars Typhimurium and Choleraesuis

In addition to the previous in vitro experiment, we also sought to investigate the effects of LPS, SC or ST on TLR5 mRNA expression in IPEC-J2 cells (Figure 4A) as well as pMPs (Figure 4B). Although TLR5 mRNA was not affected by the main effects of time, treatment, or their interaction in these two experiments, impressive numerical increases (approximately 35-fold increases) in TLR5 mRNA were observed in response to ST in IPEC-J2 cells and pMPs at 1.5 h following initial treatment of the cells. In addition, it is important to note that neither of the cell types (IPEC-J2 and pMPs) was responsive to LPS.

4. Discussion

In previous work from our laboratory, a single dose of ST produced transient enteric disease, including fever, inappetence, slowed growth (Balaji et al., 2000; Turner et al., 2002a, 2002b; Burkey et al., 2004b), activation of the endocrine stress axis (Balaji et al., 2000), and disruption of the endocrine growth axis (Balaji et al., 2000; Jenkins et al., 2004; Davis et al., 2005). Despite these unmistakable pathophysiologic changes, most of these effects resolved

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within approximately the first week following oral inoculation, and remarkably, these changes occurred completely in the absence of ST-induced elevations of systemic TNFα (Balaji et al., 2000; Fraser et al., 2005), IL1β (Fraser et al., 2005), or IL6 (Burkey et al., 2004a). Collectively, these observations suggested to us that the inflammatory sequelae provoked by ST in the pig were effectively confined by the gut mucosal immune system. In the current experiments, we sought to characterize relationships between TLR5 and flagellin in model systems that might closely represent events associated with the innate immune response to salmonellae serovars of relevance to pigs. In addition to other TLR, TLR5 is a part of a sophisticated recognition system that has evolved to identify and appropriately respond to pathogenic and commensal stimuli present within the gastrointestinal tract. It has been established that TLR5 is expressed in a variety of human and murine colonic cell lines (Otte et al., 2004; Rhee et al., 2004) and that it is preferentially expressed on the basolateral surface of IECs (Gewirtz et al., 2001a).

Here, we report for the first time in swine tissues that TLR5 is expressed constitutively, both in vivo and in vitro. Moreover, in a related study (Skjolaas et al., 2006), we reported that ST enhanced CCL20 and tended to increase IL8 in samples of distal ileum obtained from the same animals reported in the current study in Figure 1. In addition, mRNA for TLR2 and TLR4 (but not TLR9) were upregulated in these same tissue extracts, reaching approximately threefold and twofold increases, respectively, by 48 after oral ST inoculation (Burkey et al., submitted). Thus, it appears that epithelial and (or) immune cells in the ileal gut wall were activated by oral ST in vivo (CCL20 and to a lesser extent, IL8 from Skjolaas et al., 2006; TLR2 and TLR4 from Burkey et al., submitted), whereas mRNA for TLR5 was not affected as reported here. Although it is not apparent when relative abundance of mRNA is expressed as $\Delta\Delta Ct$, it is clear from the Ct values from the real-time PCR assays that far less mRNA for TLR5 was present in ileal samples (and from cultured IPEC-J2 swine epithelial cells; data not shown) than other TLRs. This observation is generally consistent with observations from other commonly used gastrointestinal epithelial cell lines (A.T. Gewirtz, personal communication). Thus it seems reasonable to conclude that the mRNA for TLR5 is generally expressed at relatively low levels and, based on our results here, appears to be fairly resistant to in vivo upregulaton in pigs by the invasive pathogen ST (except perhaps very early after exposure, as discussed below).

Our experiments with a model porcine gut epithelium resulted in responses generally consistent with those discussed above. We observed that basolateral flagellin induced increases

in IL8 (Figure 2B) and CCL20 (Figure 2C) mRNA expression within 1.5 h following exposure with similar increases induced by live apical ST. The effects of flagellin and live bacteria on IL8 gene expression were followed by highly polarized secretion of IL8 protein (Figure 3) into the culture media. The polarization of IL8 secretion in the direction of the basolateral compartment has been observed by others (McCormick et al., 1995) and represents a mechanism by which polymorphonuclear cells may be directed through the lamina propria to the subepithelial sites of salmonellae invasion. In addition, there are several reports where in vitro transcription and secretion of proinflammatory cytokine mediators are increased when exposed to flagellin or flagellated bacteria in the absence of concomitant increases in TLR5 gene expression. For example, IL8 (Gewirtz et al., 2001a; Tallant et al., 2004; Zeng et al., 2003) and CCL20 (Sierro et al., 2001) secretion has been observed in polarized model intestinal epithelial cells exposed to purified flagellin. Gewirtz et al. (2001a) also demonstrated that the proinflammatory cascade in IECs in response to purified basolateral (but not apical) flagellin is mediated via the flagellin-TLR5 ligand-receptor complex and subsequent activation of NF-κB. In addition, Tallant et al. (2004) also demonstrated that purified flagellins and wild-type flagellated salmonellae elicit similar inflammatory responses from intestinal epithelial cells.

In the final set of experiments communicated in the current report, we evaluated the timecourse of TLR5 mRNA relative expression in the IPEC-J2 model swine epithelium (Figure 4A) and in pMPs (Figure 4B). In these in vitro studies, we included LPS as a negative control. The fact that TLR5 mRNA in response to LPS was essentially identical to that of cells exposed only to media and wash steps was expected as this is consistent with the response to LPS in other intestinal epithelial cell lines reported previously (Abreu et al., 2001). Our findings here extend those observations to include pMPs among cells in which TLR5 mRNA is not affected by LPS. However, there are two additional compelling suggestions that emerge from this set of experiments. The first is that there were impressive numerical increases in TLR5 mRNA elicited by ST in IPEC-J2 cells and pMPs (Figure 4A and B, respectively), and that most of this effect appears to be at 1.5 h after apical exposure to ST and is similar to the effect observed in our previous experiments with IPEC-J2 cells (Figure 2A). (The effect had unmistakably waned at 3 and 6 h after ST). Referring back to Figure 2A, a similar trend can be seen when comparing ST at 1.5 h to that of mRNA for TLR5 in control cells at 1.5 h. However, the fold increase was less in that study and the lack of significant treatment × time interaction precludes more definitive

statements relative to the early response of TLR5 mRNA in response to ST. The second conclusion to be drawn is more definitive and perhaps more far reaching. That is, the swine adapted serovar SC failed to stimulate TLR5 mRNA, even early on, in either IPEC-J2 cells or pMPs. This observation is generally consistent with a previous report from our laboratory indicating IPEC-J2 cells to be generally unresponsive to SC as evidenced by the lack of IL8 secretory response following apical exposure to this serovar (Skjolaas et al., 2006). Collectively, these observations are completely consistent with the recent report of SC being less capable of invading IPEC-J2 cells than ST (Schierack et al., 2005). These collective in vitro observations (Schierack et al., 2005; Skjolaas-Wilson et al., 2006; and the current study) may point to a preference of SC to invade mucosal surfaces other than gastric epithelium, a contention supported by the report that SC could easily establish disease in pigs in vivo when administered intranasally (Gray et al., 1995). Taken together, these reports point to fundamental differences in the interactions of these two salmonellae serovars with swine mucosal surfaces. These observations are important given that SC and ST are serovars of greatest economic importance to the swine industry worldwide. They too help to further define the so-called host adapted nature of SC.

In conclusion, the current studies provide important new information relative to the regulation of TLR5 in swine cells and tissues and point to important contrasting effects in response to relevant swine salmonellae serovars.

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Chapter 3 - Figures

Figure 3.1 Relative abundance of Toll-like receptor 5 (TLR5) mRNA from porcine distal ileum isolated at 0, 8, 24, 48, and 144 h after 10^9 CFU oral *Salmonella enterica* Serovar Typhimurium challenge. Each time point represents tissue obtained from four pigs.
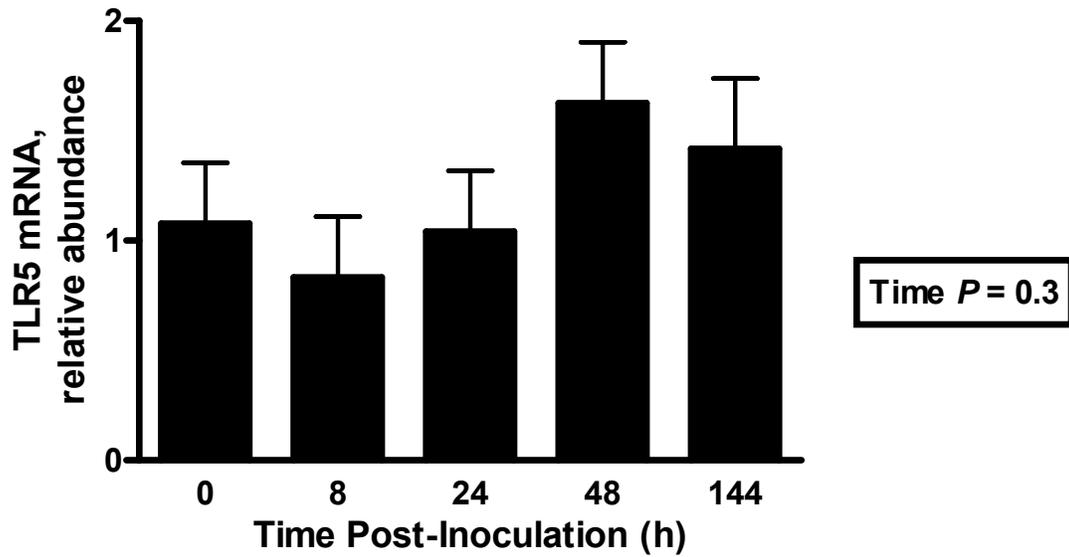
Figure 3.2 Relative abundance of Toll-like receptor 5 (TLR5; panel A), interleukin 8 (IL8; panel B), and CC chemokine ligand 20 (CCL20; panel C) mRNA from cultured porcine jejunal epithelial cells (IPEC-J2) treated with media alone (control), 1.0, 10.0, 100.0, or 325 ng/ml flagellin, or 10^8 CFU/well *Salmonella enterica* Serovar Typhimurium (ST). Total RNA was extracted at 1.5 and 5.0 h post treatment. Each bar represents the least square mean (± SEM) of four observations. Within time periods, bars without common superscripts differ (P < 0.05).
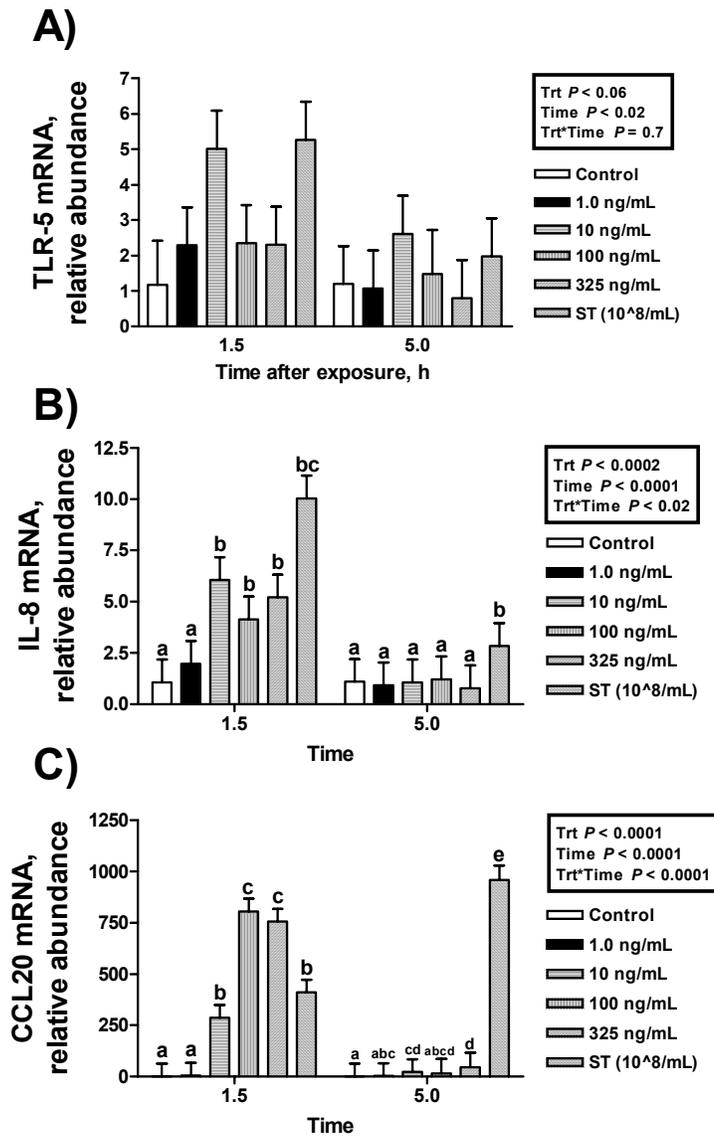
Figure 3.3. Polarized interleukin 8 (IL8) secretion by confluent porcine jejunal epithelial cells (IPEC-J2) monolayers treated with media alone (control), 1.0, 10, 100, or 325 ng/ml Flagellin in the basolateral compartment, or 10^8 CFU/well *Salmonella enterica* serovar Typhimurium (ST) in the apical (AP) compartment for 1 h. After 1 h of exposure all wells were subjected to the addition of media containing gentamicin. Media from the AP and basolateral (BL) compartments were collected and assayed for cytokines at 1.5 and 5.0 h after the addition of the respective treatments. Each bar represents the least square mean (± SEM) of four observations. Within time periods and position, bars without common superscripts differ (P < 0.05).
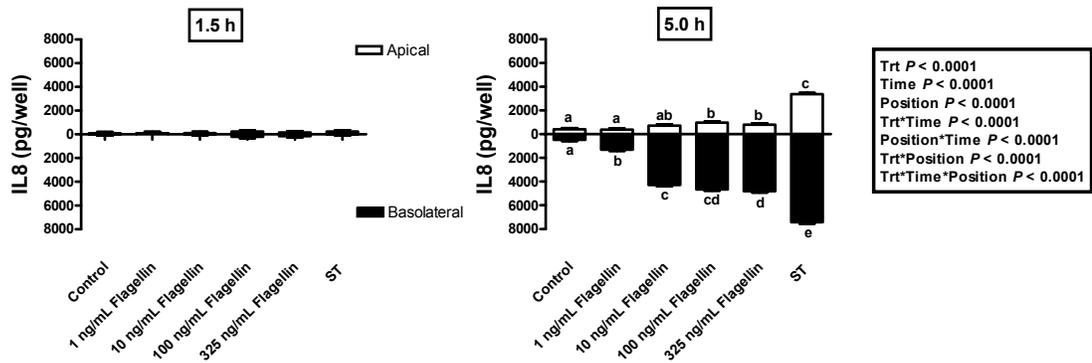
Figure 3.4 Relative abundance of Toll-like receptor 5 (TLR5) mRNA from cultured porcine jejunal epithelial cells (IPEC-J2; panel A) and cultured porcine mononuclear phagocytic cells (pMPs; panel B) treated with media alone (control), 10 ng/ml lipopolysaccharide (LPS), 10^8 CFU/well *Salmonella enterica* serovar Choleraesuis (SC), or 10^8 CFU/well *Salmonella enterica* serovar Typhimurium (ST). Total RNA extracted at 1.5, 3.0 and 6.0 h post treatment. Each bar represents the least square mean (± SEM) of at least six observations.
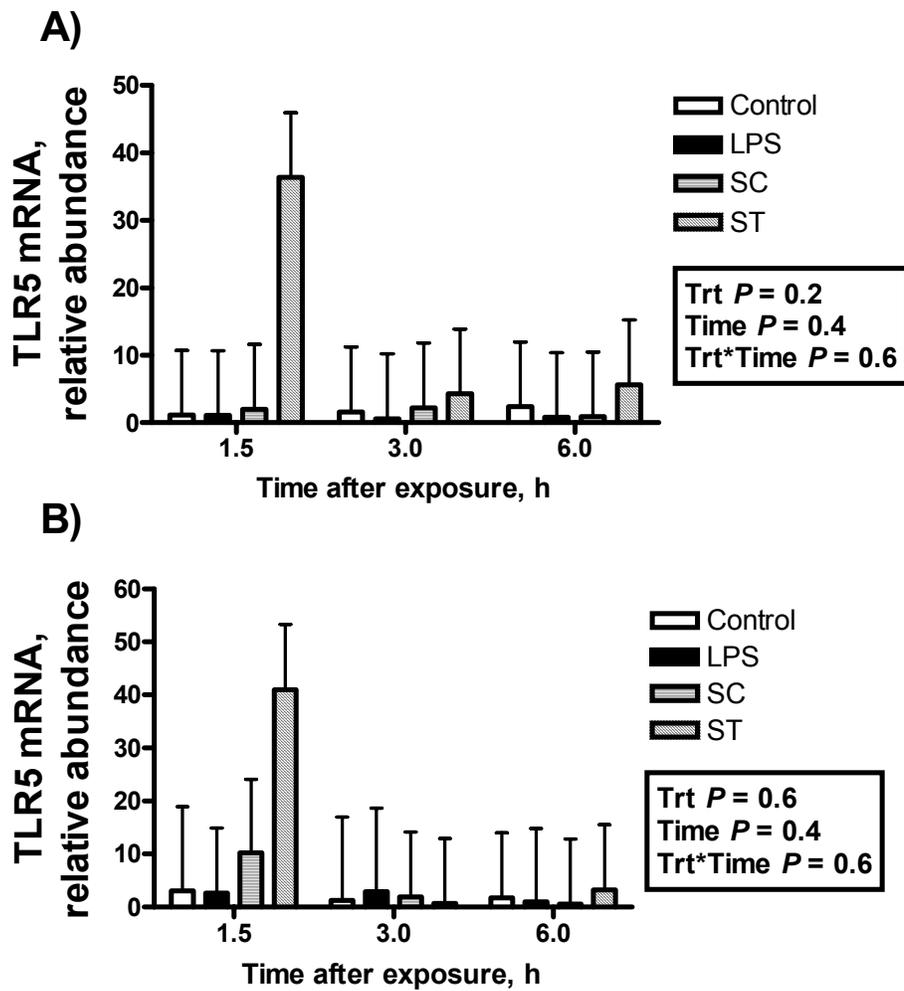
CHAPTER 4 - Expression of Toll-like receptors, interleukin 8, macrophage migration inhibitory factor, and osteopontin in tissues from pigs challenged with *Salmonella enterica* serovar Typhimurium or serovar Choleraesuis

Abstract

Two serovars of *Salmonella enterica*, namely serovar Typhimurium (ST) and serovar Choleraesuis (SC) account for the vast majority of clinical cases of swine salmonellosis worldwide. These serovars are thought to be transmitted among pigs in production settings mainly through fecal-oral routes. Yet, few studies have evaluated effects of these serovars on expression of innate immune targets when presented to pigs via repeated oral dosing in an attempt to model transmission in production settings. Thus, a primary objective of the current experiments was to evaluate expression of Toll-like receptors (TLR) and selected chemoattractive mediators (interleukin 8, IL8; macrophage migration inhibitory factor, MIF; and osteopontin, OPN) in tissues from pigs exposed to ST or SC that had been transformed with kanamycin resistance and green (STG) or red (SCR) fluorescent protein to facilitate isolation from pen fecal samples. In vitro studies confirmed that STG and SCR largely (though not completely) retained their ability to upregulate IL8 and CC chemokine ligand 20 (CCL20) in cultured swine jejunal epithelial cells. Transformed bacteria were then fed to pigs in an in vivo study to determine tissue specific effects on mRNA relative expression. Pigs were fed cookie dough inoculated with bacteria on days 0, 3, 7, and 10 with 10^8 CFU STG (n=8) or SCR (n=8), while control (CTL) pigs (n = 8) received dough without bacteria. Animals were sacrificed 14 d from the initial bacterial challenge and samples of tonsil, jejunum, ileum, colon, mesenteric lymph node (MLN), spleen, and liver were removed for subsequent RNA isolation. Expression of mRNA in tissues was determined using real-time quantitative PCR and expressed relative to 18S rRNA. Within CTL pigs, when expressed relative to the content in liver, mRNA for all targets demonstrated substantial tissue effects (P < 0.001 for all TLR; MIF, and OPN; and P < 0.05 for IL8). Feeding STG and SCR resulted in significant (P ≤ 0.05) tissue specific effects for TLR5, TLR9, IL8, MIF and OPN. However, aside from STG stimulated increase in IL8 in MLN (approximately ten-fold increase relative to CTL; P < 0.05), significant changes in other molecular targets were generally less than one-fold. Results suggest that transformed bacteria may be useful in modeling chronic oral exposure of pigs to economically important salmonellae serovars. However, although statistically significant effects of bacterial feeding were observed in

selected tissues for some targets, most changes in mRNA were generally incremental in magnitude.

1. Introduction

Salmonella is an enteric pathogen that is both an economic burden in swine production systems and a threat to safety of pork products. In the United States, clinical porcine salmonellosis is almost solely due to infection with *Salmonella enterica* serovar Choleraesuis (SC) or Typhimurium (ST) (Fedorka-Cray et al., 2000). SC is a swine host-adapted serovar that causes severe, often fatal disease and ST represents a nonhost-adapted serovar that is often associated with less severe gastroenteritis (Isaacson, 1996). Key components of porcine salmonellosis include the following: 1) bacterial attachment and adhesion to the mucosal epithelium; 2) invasion through the mucosa; and 3) localized survival of bacteria within enterocytes, endothelial cells and the lamina propria or systemic dissemination of bacteria via neutrophils and macrophages (Isaacson and Kinsel, 1992). Differences in clinical signs between nonhost- (ST) and host- (SC) adapted serovars may depend on specific serotype virulence factors, natural and acquired host resistance, the route of infection or dose of the bacteria (Gray et al., 1996; Meyerholz and Stabel, 2003). The clinical signs consistent with porcine salmonellosis include fever and diarrhea for SC and ST infected pigs, with the additional onset of septicemia resulting in enterocolitis and pneumonia for pigs infected with SC (Roof et al., 1992).

Intestinal epithelial cells form a physical barrier to commensal and pathogenic microbiota within the gastrointestinal tract. Toll-like receptors, one family of germ-line encoded pattern-recognition receptors, are expressed on intestinal epithelial cells and a variety of other cell types of immune lineage (Takeda et al., 2003). These receptors function as sentinels of infection via recognition of pathogen-associated molecular patterns and by directing appropriate innate and adaptive immune responses to invading microorganisms (Didierlaurent et al., 2002). Specifically, TLR2, TLR4, TLR5 and TLR9 detect microbial products such as peptidoglycan, LPS, flagellin, and unmethylated Cpg motifs, respectively (Takeda et al., 2003). Intestinal epithelial cells are instrumental in generating chemotactic signals in response to enteric pathogens (e.g. ST) (Eckmann et al., 1997), presumably following detection by TLR. Ligation of TLR initiates a signaling cascade that results in the activation of the transcription factor NF-κB and subsequent upregulation of costimulatory molecules as well as inflammatory cytokines and chemokines. This signaling cascade is presumed to initiate neutrophil migration in the

direction of mucosal sites that have been invaded by enteric pathogens (Rothkotter et al., 1999). Recruitment of immune cells has been attributed to various chemoattractive mediators. CXC chemokine ligand 8 (also known as CXCL8 or IL8) is involved in neutrophil chemotaxis and is secreted by IEC after invasion by various bacteria (Eckmann et al., 1993; Thelen 2001; Burkey et al., submitted). Macrophage migration inhibitory factor (MIF), known to affect macrophage movement, was rapidly increased after *Salmonella dublin* challenge of Caco-2 cells (Maaser et al., 2002). Osteopontin (OPN) is a key mediator of recruitment and retention of macrophages and T cells to sites of inflammation (Mazzali et al., 2002).

Information regarding tissue-specific mRNA expression of TLRs and selected chemoattractive mediators (IL8, MIF, OPN) could provide important insight from which possible management and therapeutic interventions could arise. Hence, we chose to investigate TLR and IL8, MIF and OPN because these molecular targets may represent important markers in the pathogenesis of porcine salmonellosis.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Wild-type *Salmonella enterica* serovars Typhimurium (ST) and Choleraesuis (SC) isolates from swine origin were obtained from Dr. Jerome Nietfeld, Diagnostic Medicine Pathobiology, Kansas State University, and identification of *Salmonella* serotypes was verified by the National Veterinary Services Laboratory (Ames, IA). Wild-type SC was transformed with red fluorescent protein (kanamycin resistant pDsRed-Express-1 vector, catalog no. 6994-1, BD Biosciences Clontech, Palo Alto, CA; lac Z promoter cloned into this vector upstream of DsRed-Expression coding sequence) and ST was transformed with green fluorescent protein by first modifying pDsRed-Express-1 vector (BD Biosciences) to generate a GFPuv vector for use in ST transformation. To modify the above pDsRed-Express-1 vector, the DsRed-Express coding sequence was excised and the GFPuv coding sequence from the pGFPuv vector (BD Biosciences Clontech) was inserted into pDsRed-Express-1 vector. *Salmonella* transformation was performed by electrotransformation as per Sanderson et al. (1995). STG and SCR were grown in Luria Bertani medium at 37°C for 24 hr, at which point bacterial populations were

estimated by spectrophotometry (OD 600 nm). Bacteria were then pelleted and resuspended accordingly for the in vitro and in vivo experiments described below.

2.2. In vitro bacterial challenge with wild-type and transformed Salmonella

In order to verify that the transformed *Salmonella* spp. used in the in vivo challenge model retained its ability to elicit an inflammatory response, wild-type (ST and SC) bacteria were compared to transformed (STG and SCR) *Salmonella* spp. by exposing a porcine neonatal jejunal epithelial cell line (IPEC-J2) to the serovars. Our group has utilized IPEC-J2 to characterize the innate mucosal response to invasive bacterial pathogens (Skjolaas et al., 2006) and this cell line has recently been characterized by others (Schierack et al., 2005). Briefly, IPEC-J2 were seeded (2.5×10^5 to 4.0×10^5/well) onto six-well Costar Snapwells™ (Corning Inc, Corning, NY) and maintained in 50% DMEM - 50% F12 medium (Invitrogen, Carsbad, CA) supplemented with insulin/transferrin/Na selenite media supplement (1%; Sigma-Aldrich Co., St. Louis, MO), epidermal growth factor (5 ng/ml; Invitrogen), streptomycin/penicillin (1%; Invitrogen), and FBS (5%; Hyclone, Logan, UT). The cells were grown to confluency ($\sim$ 7 d) and twenty-four hours before experimentation, cells were washed and media devoid of antibiotics was added to all cells. Treatments included uninfected control cells, SC, ST, SCR and STG (bacteria added at 1.0×10^8 bacteria/well). Confluent IPEC-J2 cells, as described above, were washed twice with PBS and 0.5 ml of media alone (CTL) or bacteria containing media were added to the top (apical) wells, while 2.5 ml of media was added to the bottom (basolateral) wells and plates were further incubated at 37°C, 5% CO_2 for 1 h. Then, cells from all treatments were washed and both apical and basolateral media were replaced with fresh media containing 50 μg/ml gentamicin. Media were removed from both the apical and basolateral compartments 6 h after the initial bacterial exposure for determination of IL8 secretion (swine specific IL8 sandwich ELISA catalog KSC0181, Biosource International, Camarillo, CA). The concentration of IL8 in each compartment was adjusted to reflect the greater volume of media in the basolateral chamber and was expressed as picograms/well. In addition, total RNA was extracted with TRI® Reagent (Sigma-Aldrich Co., St. Louis, MO) according to the manufacturer's instructions.

2.3 Animals and experimental protocol

The experimental protocol used in this study was approved by the Kansas State University Institutional Animal Care and Use Committee. A total of 24 weaned pigs (initially 6.8 ± 1.3 kg) were blocked by weight and randomly allotted to one of three treatment groups in a 14 d study. Each group (n = 8) included a total of four pens with two pigs/pen. All pigs were housed under constant illumination in two similar environmentally controlled rooms. Pens contained one self-feeder and one nipple waterer to provide *ad libitum* access to feed and water. Pigs were fed a standard corn/soy bean meal nursery diet with no added antimicrobials. Before the start of the study, fecal samples were obtained and cultured to ensure that all pigs were not shedding *Salmonella*. The three treatment groups in this study consisted of the following: uninfected controls (CTL), pigs challenged with STG or SCR.

2.4 In vivo bacterial challenge and tissue collection

After an acclimation period of 4 d, pigs were fed cookie dough balls on days 0, 3, 7, and 10 that contained 10^8 CFU STG (n=8) or SCR (n=8), while CTL pigs (n = 8) received dough without bacteria. Pigs were initially fed laboratory-derived STG or SCR. Subsequently, fecal samples were pooled across pens and within treatments and STG or SCR isolates containing the appropriate fluorescent and kanamycin resistant plasmids we re-fed to pigs. Animals were sacrificed 14 d from the initial bacterial challenge by sodium pentobarbital injection and samples of tonsil, jejunum, ileum, colon, mesenteric lymph node (MLN), spleen, and liver were removed. Upon collection, all tissue samples were immediately frozen by immersion in liquid nitrogen (N_2) and stored (-80°C) for subsequent RNA isolation.

2.5 RNA extraction and reverse transcription (RT) PCR

Approximately 100 mg of each frozen tissue sample was finely ground using a liquid N_2-cooled mortar and pestel. Following grinding, 2 ml TRI® Reagent (Sigma-Aldrich Co.) was added and grinding continued until the mixture had thawed. The liquefied tissue was then transferred to 1.5 ml microcentrifuge tubes and RNA isolation was completed as per the manufacturer's protocol. Following total RNA isolation from all tissues and cells, DNA-*free*™ (Ambion Inc., Austin, TX) was used to ensure removal of contaminating genomic DNA. Samples were reconstituted in Nuclease-Free Water (Ambion Inc.) and frozen for further analysis. RNA quality was verified by agarose gel electrophoresis and visualization of the 28S

and 18S ribosomal RNA. RNA was quantified by spectrophotometry (OD 260/280 nm).
Reverse transcription was carried out using TaqMan® (Applied Biosystems, Foster City, CA)
reverse transcription reagents. Briefly, reverse transcription was carried out in a 50 or 100 µl
final volume that included 25 mM $MgCl_2$, 500 µM dNTP's, 2.5 µM random hexamers, 0.4 U/µl
Rnase inhibitor, 50 U/µl MultiScribe reverse transcriptase, and TaqMan RT buffer. The reverse
transcription mixture was incubated at 25°C for 10 min, heated to 37°C for 60 min, and
inactivated at 95°C for 5 min. The resultant cDNA was stored (-20°C) until used for real-time
quantitative PCR.

2.6 Real-time quantitative PCR

Real-time quantitative PCR was utilized to quantify TLR (2, 4, 5 and 9) and
chemoattractive mediators (IL8, MIF and OPN) mRNA expression relative to the quantity of 18S
rRNA in total RNA isolated from samples of porcine tonsil, jejunum, ileum, colon, MLN, spleen
and liver (only CCL20 and IL8 mRNA were analyzed in the in vitro IPEC-J2 experiment). The
PCR reactions were carried out in 96-well plates with the appropriate forward and reverse
primers (900nM), the appropriate TaqMan® TAMRA probe (200 nM), PCR Mastermix
(Applied Biosystems), and 3.5 µL of the cDNA sample. The porcine specific primers and
detection probes for all TLR and chemokines were synthesized from published GenBank®
sequences using PrimerExpress® software (Applied Biosystems) (Skjolaas et al., 2006; Burkey
et al., submitted). Commercially available eukaryotic 18S rRNA (Applied Biosystems) primers
and probe were used as an endogenous control. Assays using non-template controls and samples
were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems).
Thermal cycling parameters were utilized according to manufacturer recommendations and
included 50 cycles of 15 s at 95°C and 1 min at 60°C.

2.7 Statistical analyses

Relative abundance of target gene mRNA was determined for control pigs alone and in
comparison to STG and SCR challenged pigs using the $\Delta\Delta CT$ method. Initially, the relative
abundance of target gene mRNA was determined using the average liver ΔCT as the reference
expression (n = tissue from eight pigs). The liver was chosen as the reference point because it
had the greatest average ΔCT (i.e. the lowest expression of all the tissues sampled). In order to

compare the relative abundance of target gene mRNA expression in STG and SCR challenged pigs to uninfected CTL pigs, a similar analysis was performed using the average control ΔCT (of CTL pigs) as the reference expression. A similar analysis was used to compare wild-type (SC or ST) and transformed (SCR or STG) bacteria to uninfected control cells in the in vitro IPEC-J2 experiment. The ΔΔCT values were expressed as $2^{-\Delta\Delta CT}$ to obtain relative abundance values. The relative abundance values generated from the in vivo challenge study were square root transformed to ensure homogeneity of variance and were then analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) to determine the relative abundance of target gene expression in uninfected tonsil, jejunum, ileum, colon, MLN, and spleen compared to the expression in the liver. The PROC MIXED procedure was used to determine the main effects of treatment (STG or SCR) on target gene expression in the aforementioned tissues compared to uninfected controls. To facilitate depiction of the data, relative abundance values from the in vivo challenge study were back transformed and are represented in Figures 3 and 4 (representing fold changes in TLR and chemoattractive mediators from CTL tissues only) and Tables 1 and 2 (representing fold changes in TLR and chemoattractive mediators in tissues resulting from bacterial treatment).

3. Results

3.1 In vitro challenge of swine gut epithelial cells with wild-type and transformed Salmonella enterica serovars Typhimurium and Choleraesuis

Salmonellae used in this experiment were transformed with plasmids containing fluorescent red or green proteins. The fluorescent markers and the antibiotic resistance conferred by the plasmids provided two phenotypic markers by which to re-isolate the bacteria in pooled pen fecal samples to culture for subsequent oral inoculations and to differentiate them from potential environmental salmonellae. However, because bacterial transformation with fluorescent plasmids was reported to decrease invasiveness of salmonellae (Knodler et al., 2005), we first sought to confirm the ability of transformed bacteria to provoke inflammatory chemokine secretion (IL8; Figure 1) and gene expression (IL8 and CCL20; Figure 2) in model porcine intestinal epithelial cells. Wild-type (SC and ST) and transformed (STG) *Salmonella* elicited greater basolateral IL8 secretion than uninfected control cells (P < 0.01 for SC and P <

0.0001 for ST and STG). In the apical compartment, ST and STG increased IL8 relative to controls (P < 0.0001). The abundance of IL8 mRNA was similar between control, SC and SCR exposed cells (Figure 2A) whereas both ST and STG exposure (Figure 2B) resulted in greater levels of IL8 mRNA than controls (P < 0.03). Both SC and SCR (Figure 2C) increased CCL20 mRNA relative to controls (P < 0.004 and P < 0.05 for SC and SCR, respectively). Cells exposed to ST and STG (Figure 2D) also had greater CCL20 mRNA expression than control cells (P < 0.0001 and P < 0.001 for ST and STG, respectively).

3.2 Toll-like receptor and chemoattractive mediator expression in tissues from healthy swine

We utilized the steady state expression of mRNA in CTL pigs to determine the constitutive expression of mRNA for TLRs (Figure 3) and chemoattractive mediators (Figure 4) among tissues in healthy swine. For these calculations, expression in the liver was chosen as a reference point because targets of interest were expressed in the least abundance (i.e. highest ΔCT values) in liver. In general, TLR2 (Figure 3A), 4 (Figure 3B), 5 (Figure 3C) and 9 (Figure 3D) were expressed in all tissues (tonsil, jejunum, ileum, colon, MLN, spleen and liver) analyzed, and the main effect of tissue was highly significant (P < 0.001) for each of the TLR indicating differences in expression between tissues. Specifically, for TLR2 (Figure 3A), all tissues had greater gene expression than the liver (P < 0.05). TLR2 was expressed in the greatest abundance in the colon and was greater than all other tissues except for the ileum (P < 0.05). A similar trend was observed for TLR4 mRNA (Figure 3B). All tissues, except for the jejunum, had greater TLR4 expression when compared to the liver, whereas the colon and the spleen had greater abundance of the receptor than all other tissues (P < 0.05). Once again, similar to TLR2 and TLR4, mRNA for TLR5 (Figure 3C) revealed that the colon had greater abundance than all other tissues (P < 0.05). The relative abundance of TLR5 was similar between the tonsil, jejunum, MLN, spleen and liver, while expression in the ileum was intermediate between these tissues and the colon. Finally, the relative abundance of TLR9 mRNA was greater in all tissues compared to the liver (P < 0.05). However, contrary to the other TLRs, TLR9 expression was greatest in the tonsil, ileum and MLN and greater than TLR9 mRNA in the colon (P < 0.05).

IL8 had the greatest abundance in the colon and was also greater in the MLN, spleen, and tonsil when compared the liver (P < 0.05). The greatest abundance of MIF and OPN mRNA was

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observed in ileal tissue with OPN mRNA greater than all other tissues. In addition, MIF mRNA was greater in the tonsil and colon compared to the spleen and liver ($P < 0.05$). Expression of OPN mRNA in the tonsil, jejunum, and MLN were greater when compared to the colon, spleen and liver ($P < 0.05$).

3.3 Toll-like receptor and chemoattractive mediators in tissues from swine infected with Salmonella enterica serovar Typhimurium or Choleraesuis

Steady state expression of TLR and selected chemoattractive mediators was next evaluated in response to chronic exposure to swine salmonellae serovars STG and SCR. For these comparisons, the average control pig ΔCT (for each tissue and target mRNA of interest) was used as the reference point in order to compare tissues from pigs exposed to salmonellae. Relative abundance of mRNA for TLRs (2, 4, 5 and 9) and chemoattractive mediators (IL8, MIF, OPN), expressed as $\Delta\Delta CT$ values, are represented in Tables 1 and 2, respectively. Table 3 summarizes trends among tissues for TLR, IL8, MIF, and OPN mRNA when compared to CTL animals.

Exposure to STG did not affect relative abundance of TLR2, whereas TLR2 mRNA tended to be decreased by SCR in both the jejunum and ileum ($P < 0.06$ and $P < 0.09$ for the jejunum and ileum, respectively). Similarly, TLR4 mRNA tended to be decreased in the colon and spleen by SCR ($P < 0.1$). Both STG and SCR increased TLR5 and TLR9 mRNA in jejunal samples. In contrast, TLR5 and TLR9 mRNA were decreased ($P < 0.05$) in the colon by SCR and STG, respectively. Oral exposure to STG increased IL8 mRNA compared to CTL in MLN ($P < 0.05$). In addition, mRNA for MIF was decreased ($P < 0.01$) in the colon and MLN of pigs inoculated with STG and SCR, while SCR tended to decrease MIF expression in the ileum ($P < 0.1$). Finally, SC tended to decrease OPN mRNA in the colon ($P < 0.06$) and decreased OPN mRNA in the MLN ($P < 0.05$).

4. Discussion

Salmonella enterica serovars Typhimurium and Choleraesuis are serovars that are often implicated as the primary causative agents leading to salmonellosis in swine (Schwartz, 1999). Salmonellosis in swine has recently attracted the attention of research efforts due to its potential as a zoonotic agent; because of negative implications related to the efficiency and economics of

swine production systems; and because of the implications in pork safety. Recently, our research group conducted a series of experiments characterizing the pathophysiological consequences of oral ST challenge in young pigs (reviewed in Johnson et al., 2005). In general, pigs given a single oral dose of ST showed transient enteric disease characterized by fever, inappetence, retarded growth activation of the endocrine stress axis, and disruption of the endocrine growth axis. Despite these pathophysiological consequences, salmonellosis resulting from ST inoculation is self-limiting and resolves itself without evidence of ST-induced changes in peripheral TNFα, IL1β or IL6. Collectively, these observations suggested to us that the inflammatory sequelae provoked by ST in the pig were effectively confined by the gut mucosal immune system. In the current study, we utilized an alternative approach to model repeated oral exposure of weaned pigs to SC and ST in an effort to simulate the putative avenue of movement of bacteria among pigs in commercial settings, and to help define tissue specific changes in mRNA immune targets provoked by chronic exposure to the two serovars.

To facilitate isolation from pens and re-feeding, salmonellae serovars in the current study were transformed with fluorescent green or red proteins and kanamycin resistance in order to provide phenotypic markers by which STG and SCR could be identified. However, salmonellae serovars transformed with fluorescent plasmids appear to have reduced invasion (Knodler et al., 2005). Thus, prior to their in vivo use in the current study, we tested the ability of STG and SCR to interact with swine intestinal epithelial cells and to provoke chemokine secretion and signaling. Based upon those in vitro studies, we concluded that the transformed bacteria were inflammatory based upon their effects on IL-8 and CCL20. However, STG did, in deed, have reduced ability to provoke IL-8 secretion both apically and basolaterally in our model epithelial system. In contrast, the inability of SC and SCR to provoke changes in IL8 mRNA is completely consistent with differences between the wild-type serovars we have observed previously (Skjolaas et al., 2006).

The expression of TLR mRNA is broadly evident among human tissues and a variety of cell types (Zarember and Godowski, 2002). Variable expression of TLR mRNA and protein has been reported in response to LPS, inflammatory cytokines, microbial pathogens and cases of mucosal inflammation (Hausmann et al., 2002; Krutzik et al., 2003; Matsumura et al., 2000; Miettinen et al., 2001; Staege et al., 2000). Evidence for the ubiquitous nature of TLR mRNA expression in pigs is also emerging (Shimosato et al., 2003; Shimosato et al., 2005; Thomas et

al., 2006; Tohno et al., 2005). From the observations of the current studies among tissues in CTL pigs, it can be concluded that: 1) expression of TLRs in tissues of the gastrointestinal tract is generally greater than that of the liver; and 2) the colon appears to expresses more prominent relative mRNA levels of TLR2, TLR4, and especially TLR5. In addition to TLRs, downstream chemoattractive mediators may have significant roles in the immune response to enteric pathogens. CCL20 expression has been observed in human and porcine intestinal epithelial cell lines and, like TLR, there is evidence in support of its regulation by inflammatory cytokines as well as bacterial pathogens (Izadpanah et al., 2001; Skjolaas et al., 2006). From the current study, aside from generally lower steady-state levels of mRNA for IL-8, MIF, and OPN in liver, the only other obvious general conclusion in healthy animals is that OPN is expressed most prominently in tonsil and small intestine relative to other tissues evaluated.

Among the TLRs examined following exposure to 14 d of feeding STG and SCR, significant effects ($P \leq 0.05$) of both salmonellae serovars were limited only to TLR5 and TLR9, with only trends observed for downregulation of TLR2 by SCR in selected tissues. In the jejunum, both serovars upregulated TLR5 and TLR9, whereas SCR decreased TLR5 in the colon, and STG decreased TLR9 in the colon. Following a single oral dose of ST in weaned pigs, both TLR5 (Burkey et al., submitted) and TLR9 (Burkey et al., submitted) mRNA remained essentially unchanged in the gut wall of the distal ileum, at least through 144 h after bacterial challenge. In contrast, it appears both TLR5 and TLR9 are upregulated in the jejunal gut wall following chronic exposure to repeated oral doses of both salmonellae serovars used in the current study. Considering the flagellated nature of swine salmonellae serovars, including those used in the current study and given that flagellin is the ligand for TLR5 (Gewirtz, 2003), upregulation of TLR5 might be expected, although the mRNA for this TLR remained unchanged or even downregulated in the colon (by SC). Perhaps more surprising is the observation that TLR9 was also upregulated in this tissue by both serovars, again, in view of the currently understood ligand for TLR9 (Hemmi et al., 2000). On the other hand, it should be emphasized that statistically significant effects of salmonellae serovars on TLR5 and TLR9 in the current study were approximately one-fold or less in either direction relative to controls, and this may reflect the movement of tissue expression back to a slightly altered steady state in the face of repeated bacterial challenge over the 14 d treatment.

Of the chemoattractive mediators evaluated in the current study, the effect of STG to increase IL8 in MLN was among the effects with the greatest magnitude increase. This effect is generally consistent with effects of a single oral dose of ST on IL8 relative abundance in the distal ileum of pigs (Skjolaas et al., 2006). In addition, although STG showed slightly diminished ability to provoke IL8 secretion in our in vitro system, increased IL8 mRNA in the MLN provides evidence that the transformed bacteria likely penetrated the gut mucosal epithelium. On the other hand, the effects of both STG and SCR to decrease MIF in the colon and MLN, although consistent among the two serovars, represent incremental declines all less than approximately one-fold.

In the current study, we report new information regarding the relative expression of selected TLRs and chemoattractive mediators among tissues of healthy swine gastrointestinal tract, MLN, and spleen relative to steady state expression in the liver. In addition, results of the current study suggest that transformed bacteria may be useful in modeling chronic oral exposure of pigs to economically important salmonellae serovars. These transformed isolates appear to largely retain their inflammatory properties, at least in vitro. Finally, although statistically significant effects of bacterial feeding were observed in selected tissues, it should be noted that, in large part, changes in mRNA were generally incremental and represent a limited subset of potential immune and metabolic targets in pigs that may be affected by chronic exposure to salmonellae.

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Chapter 4 - Figures and Tables

Figure 4.1 Polarized interleukin 8 (IL8) secretion by confluent porcine jejunal epithelial cell (IPEC-J2) monolayers treated with media alone (CTL), wild-type *Salmonella enterica* serovar Choleraesuis (SC) or Typhimurium (ST), SC transformed with red fluorescent protein (SCR), or ST transformed with green fluorescent protein (STG). All bacteria were added to the apical compartment at 10^8 CFU/well. After 1 h of exposure all wells were subjected to the addition of media containing gentamicin. Media from the apical and basolateral compartments were collected and assayed for IL8 6 h after the addition of the respective treatments. Each bar represents the least square mean (± SEM) of four observations.
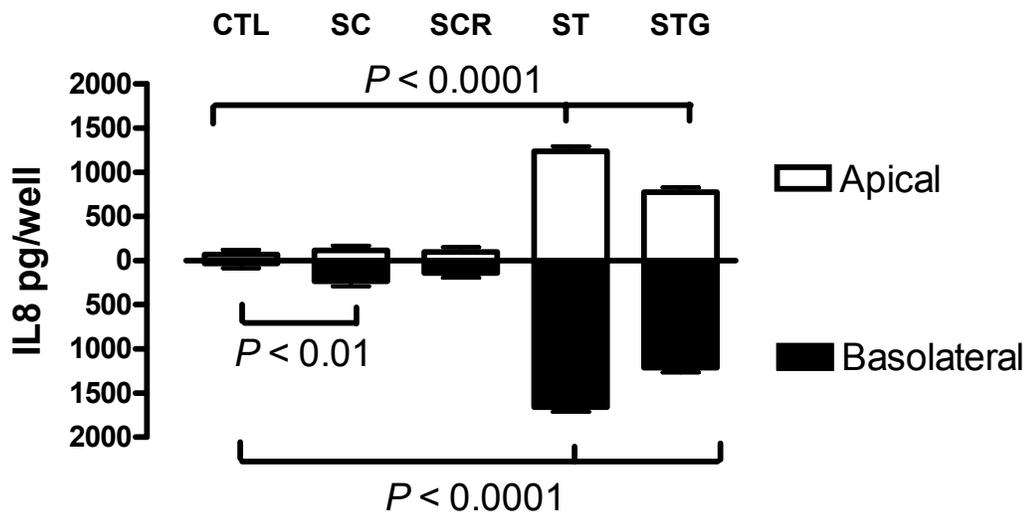
Figure 4.2 Relative abundance of interleukin 8 (IL8; panel A and B) and CC chemokine ligand 20 (CCL20; panel C and D) mRNA from cultured porcine jejunal epithelial cells (IPEC-J2) treated with media alone (CTL), wild-type *Salmonella enterica* serovar Choleraesuis (SC) or Typhimurium (ST), SC transformed with red fluorescent protein (SCR), or ST transformed with green fluorescent protein (STG). All bacteria were added to the apical compartment at 10^8 CFU/well. Total RNA was extracted 6.0 h post treatment. Each bar represents the least square mean ($\pm$ SEM) of four observations. Letters above bars denote significant differences between treatments.
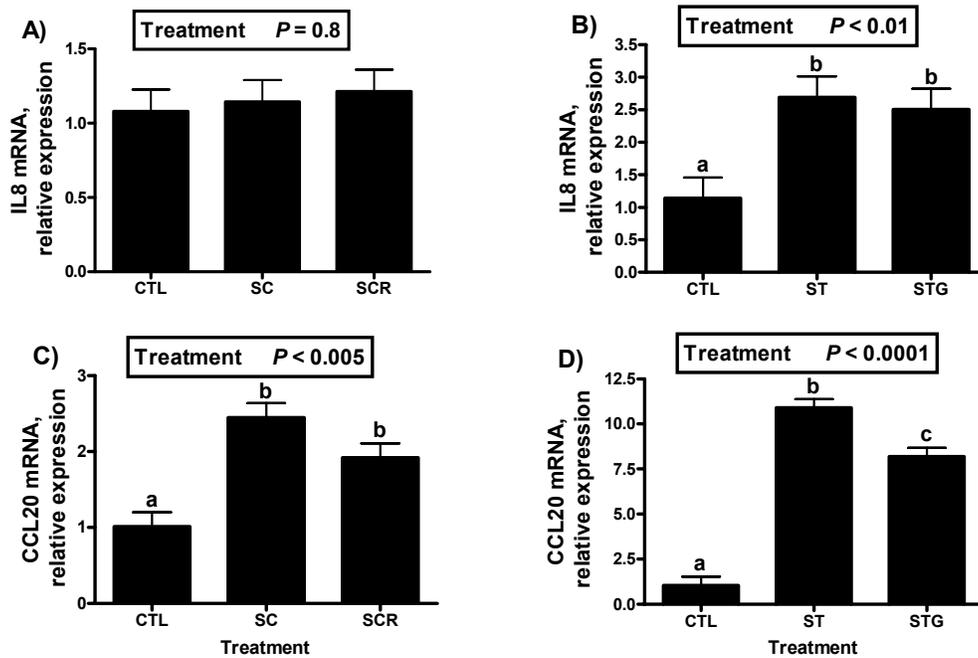
Figure 4.3. Relative abundance of Toll-like receptor 2 (TLR2; panel A), Toll-like receptor 4 (TLR4; panel B), Toll-like receptor 5 (TLR5; panel C) and toll-like receptor 9 (TLR9; panel D) mRNA from tonsil, jejunum, ileum, colon, mesenteric lymph node (MLN), spleen and liver tissues obtained from control pigs. Total RNA extracted 14 d from the initiation of the experiment. Each bar represents the least square mean (± SEM) of eight observations. Bars without common superscripts differ (P < 0.05).

TLR2: Tissue $P < 0.0001$ TLR4: Tissue $P < 0.0001$
TLR5: Tissue $P < 0.0005$ TLR9: Tissue $P < 0.0001$
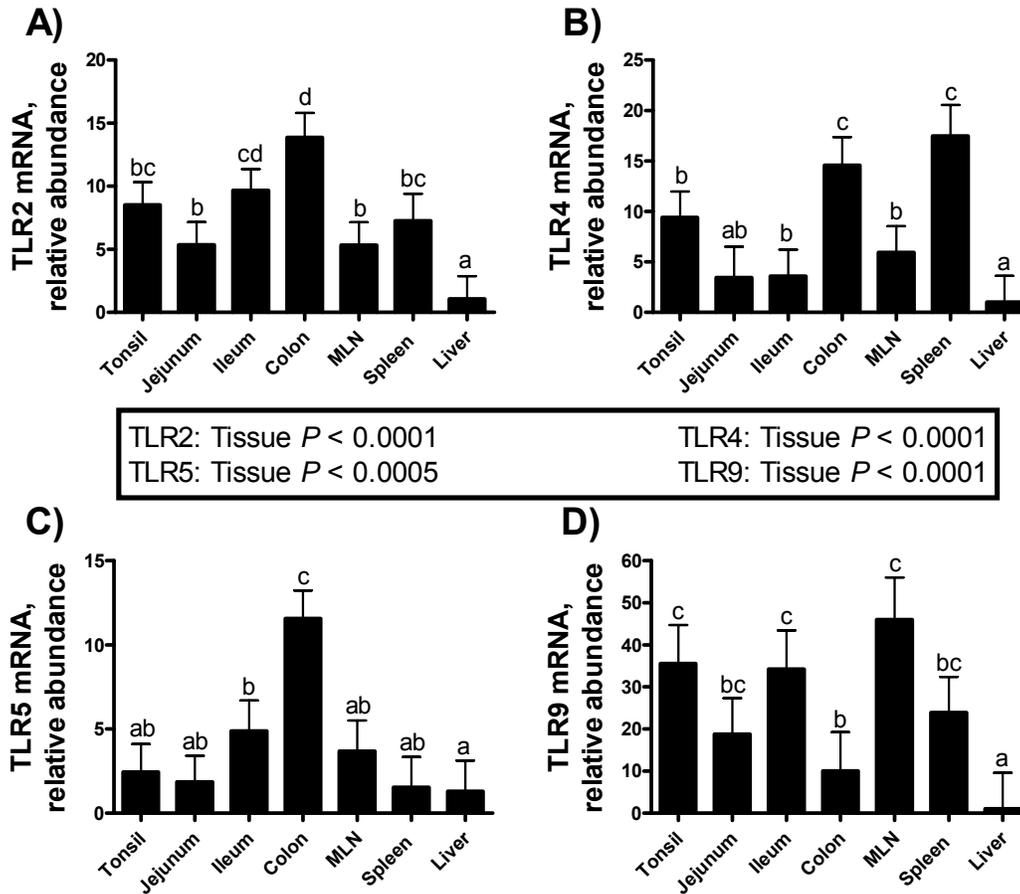
Figure 4.4 Relative abundance of interleukin 8 (IL8; panel A), macrophage migration inhibitory factor (MIF; panel B), and osteopontin (OPN; panel C) mRNA from tonsil, jejunum, ileum, colon, mesenteric lymph node (MLN), spleen and liver tissues obtained from control pigs. Total RNA extracted 14 d from the initiation of the experiment. Each bar represents the least square mean (± SEM) of eight observations. Bars without common superscripts differ (P < 0.05).
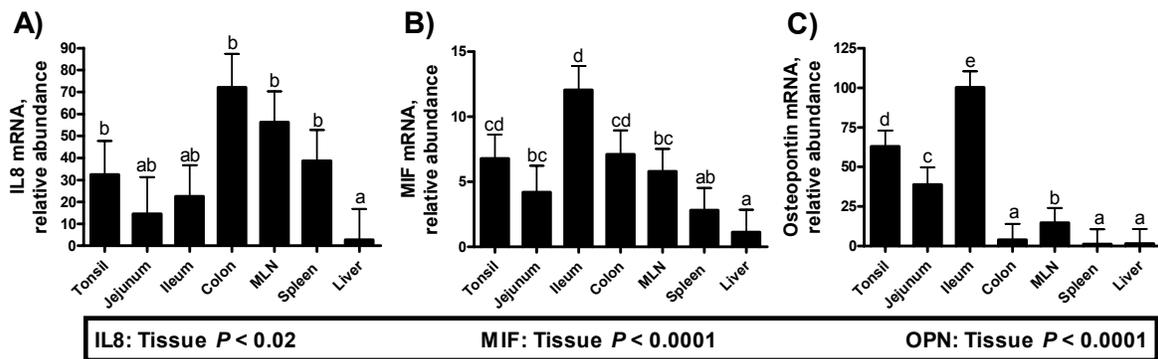
Table 4.1 Toll-like receptor (TLR) mRNA relative abundance in tissues from control (CTL) pigs or pigs exposed to transformed *Salmonella enterica* serovar Typhimurium (STG) or serovar Choleraesuis (SCR).

Treatment:	TLR2				TLR4				TLR5				TLR9			
Tissue:	CTL	STG	SCR	P^a	CTL	STG	SCR	P^a	CTL	STG	SCR	P^a	CTL	STG	SCR	P^a
Tonsil	1.23 ± 0.3	1.03 ± 0.3	1.24 ± 0.3	0.87	1.64 ± 0.6	1.23 ± 0.6	1.41 ± 0.6	0.93	1.28 ± 2.9	6.21 ± 2.7	5.37 ± 2.7	0.47	1.09 ± 0.5	1.29 ± 0.4	1.99 ± 0.4	0.40
Jejunum	1.11 ± 0.2	1.16 ± 0.2	0.63 ± 0.2	0.06	1.18 ± 0.2	1.27 ± 0.1	1.23 ± 0.1	0.70	1.16 ± 0.3	1.99 ± 0.3	2.53 ± 0.4	0.02	1.02 ± 0.1	1.80 ± 0.1	1.72 ± 0.2	0.01
Ileum	0.64 ± 0.3	0.84 ± 0.3	0.20 ± 0.3	0.09	1.01 ± 0.5	2.34 ± 0.5	1.08 ± 0.5	0.16	1.33 ± 1.7	4.92 ± 1.7	0.63 ± 1.7	0.21	1.35 ± 1.0	4.03 ± 1.1	0.76 ± 1.1	0.11
Colon	0.84 ± 0.2	0.56 ± 0.2	0.40 ± 0.1	0.16	1.94 ± 0.3	0.72 ± 0.3	0.29 ± 0.3	0.10	0.58 ± 0.5	0.70 ± 0.5	0.18 ± 0.4	0.05	0.68 ± 0.2	0.76 ± 0.2	0.22 ± 0.2	0.02
MLN[b]	1.10 ± 0.3	1.46 ± 0.3	1.11 ± 0.3	0.44	1.07 ± 0.2	1.14 ± 0.2	1.21 ± 0.2	0.30	0.96 ± 0.4	1.18 ± 0.3	1.26 ± 0.3	0.94	0.84 ± 0.2	0.93 ± 0.2	0.79 ± 0.2	0.61
Spleen	1.03 ± 0.2	1.10 ± 0.1	1.13 ± 0.1	0.68	1.03 ± 0.1	1.17 ± 0.1	0.88 ± 0.1	0.09	0.91 ± 0.4	1.08 ± 0.4	0.92 ± 0.3	0.27	1.48 ± 0.2	0.84 ± 0.2	0.79 ± 0.2	0.73
Liver	1.30 ± 0.8	2.90 ± 0.7	1.10 ± 0.8	0.37	1.06 ± 0.5	2.11 ± 0.5	1.43 ± 0.5	0.40	1.07 ± 0.6	1.74 ± 0.6	1.03 ± 0.6	0.63	1.40 ± 1.0	3.32 ± 0.9	2.13 ± 1.0	0.34

[a] Probability of treatment effect from the analysis of variance.
[b] Mesenteric lymph node.

Table 4.2 Interleukin 8 (IL8), macrophage migration inhibitory factor (MIF), and osteopontin (OPN) mRNA relative abundance in tissues from control (CTL) pigs or pigs exposed to transformed *Salmonella enterica* serovar Typhimurium (STG) or serovar Choleraesuis (SCR).

Treatment:	IL8				MIF				OPN			
Tissue:	CTL	STG	SCR	P^a	CTL	STG	SCR	P^a	CTL	STG	SCR	P^a
Tonsil	1.75 ± 1.2	2.35 ± 1.2	2.19 ± 1.3	0.61	1.07 ± 0.2	0.90 ± 0.2	0.94 ± 0.2	0.74	1.05 ± 0.3	0.95 ± 0.2	1.58 ± 0.2	0.21
Jejunum	0.10 ± 0.4	1.60 ± 0.4	1.80 ± 0.5	0.15	1.01 ± 0.1	0.90 ± 0.1	0.79 ± 0.1	0.31	1.21 ± 0.4	1.50 ± 0.3	1.20 ± 0.4	0.84
Ileum	2.44 ± 2.6	6.35 ± 2.6	2.12 ± 2.8	0.56	1.24 ± 0.6	1.85 ± 0.6	0.32 ± 0.6	0.07	1.16 ± 1.8	3.87 ± 1.7	0.43 ± 1.7	0.20
Colon	1.36 ± 0.7	2.10 ± 2.8	0.26 ± 0.7	0.15	1.16 ± 0.2	0.59 ± 0.2	0.36 ± 0.2	0.01	1.17 ± 0.3	1.40 ± 0.3	0.52 ± 0.2	0.06
MLN	3.13 ± 4.5	14.3 ± 4.8	2.50 ± 4.2	0.03	1.23 ± 0.3	0.96 ± 0.3	0.34 ± 0.2	0.01	1.25 ± 0.5	1.47 ± 0.5	0.26 ± 0.4	0.05
Spleen	4.21 ± 1.1	2.62 ± 1.0	1.68 ± 1.1	0.39	1.02 ± 0.1	0.92 ± 0.1	0.86 ± 0.1	0.52	1.26 ± 0.3	0.77 ± 0.3	1.29 ± 0.3	0.45
Liver	2.74 ± 10.7	26.5 ± 11.5	0.64 ± 10.7	0.25	1.12 ± 0.2	1.33 ± 0.2	1.18 ± 0.2	0.78	1.41 ± 2.3	1.85 ± 2.4	6.85 ± 2.3	0.12

[a] Probability of treatment effect from the analysis of variance.

Table 4.3 Summary of changes in Toll-like receptor (TLR) mRNA relative abundance (from Table 1), and in interleukin 8 (IL8), macrophage migration inhibitory factor (MIF) and osteopontin (OPN) mRNA relative abundance (from Table 2) expression in tissues from pigs exposed to transformed *Salmonella enterica* serovar Typhimurium (STG) or serovar Choleraesuis (SCR).

Treatment:	TLR2		TLR4		TLR5		TLR9		IL8		MIF		OPN	
Tissue:	STG	SCR	STG	SCR	STG	SCR	STG	SCR	STG	SCR	STG	SCR	STG	SCR
Tonsil	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
Jejunum	↔	↓c	↔	↔	↑b	↑b	↑a	↑a	↔	↔	↔	↔	↔	↔
Ileum	↔	↓c	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓c	↔	↔
Colon	↔	↔	↔	↓c	↔	↓b	↓b	↔	↔	↔	↓a	↓a	↔	↓c
MLN	↔	↔	↔	↔	↔	↔	↔	↔	↑b	↔	↓a	↓a	↔	↓b
Spleen	↔	↔	↔	↓c	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
Liver	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔

↔ Indicates no change in mRNA expression in response to STG or SCR versus control tissue.
↑ Indicates an increase in mRNA expression by treatment versus control tissue.
↓ Indicates a decrease in mRNA expression by treatment versus control tissue.
[a] Indicates highly significant increase or decrease by treatment at $P \leq 0.01$.
[b] Indicates significant increase or decrease by treatment $P \leq 0.05$.
[c] Indicates a tendency for increased or decreased expression by treatment $P \leq 0.1$.