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Characterizing the porcine immune response to an environmental and pathogenic challenge: swine barn dust and Salmonella infection

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Characterizing the porcine immune response to an environmental and pathogenic challenge: swine barn dust and *Salmonella* infection

by

Susan Marie Knetter

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Immunobiology

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Iowa State University
Ames, Iowa
2013

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DEDICATION

I dedicate this dissertation to Hollingsworth Road. It is where I began my love of God, agriculture and science; and where I came to understand that they are really all one and the same.
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So let’s go.
ABSTRACT

Pork is the most highly consumed meat worldwide, and the swine industry generates $34.5 billion annually to the U.S. economy alone. Maintaining pork safety and minimizing production losses associated with swine disease impacts profitability, food safety and animal health. As such, the research presented herein focused on characterizing aspects of the porcine immune response to two challenges that impact swine health: swine barn dust exposure and *Salmonella* infection.

In the first set of experiments, swine barn organic dust extract (ODE) treatment negatively affected porcine macrophage phagocytosis, intracellular bacterial killing and NF-κB translocation. Further, ODE stimulated production of inflammatory cytokines and increased surface expression of CD163, the cellular receptor targeted by porcine reproductive and respiratory syndrome virus. Together, these results demonstrated ODE suppresses macrophage function, and implicate barn dust as a potential underlying cause of swine respiratory disease outbreaks.

Similar to the respiratory tract, the gastrointestinal tract is an interface of routine immune system exposure to a myriad of foreign antigens and pathogens. *Salmonella enterica* serovar Typhimurium enters the swine gut and can cause enteric disease, resulting in varied levels of fecal shedding from infected pigs. As these animals are often asymptomatic, the risk of disease transmission and pork
contamination at slaughter is increased. The second set of experiments identified distinct immune response differences between low shedding (LS) and persistently shedding (PS) pigs experimentally inoculated with *Salmonella enterica* serovar Typhimurium (ST). The PS pigs had longer pyrexia and increased serum interleukin (IL) -1β, TNF-α, interferon (IFN) -γ, and IL-12p40 at 2 days post-inoculation compared to non-inoculated controls, with up-regulation of genes in the blood involved in the STAT1, IFNB1 and IFNG regulation networks. The LS pigs had brief pyrexia, decreased fecal shedding more rapidly, and up-regulated genes involved in negative immune regulation as a component of their response.

The third set of experiments defined additional differences, characterizing the effects of *ex vivo* endotoxin stimulation in blood from LS and PS pigs. Endotoxin stimulation elicited similar inflammatory profiles from the blood of LS and PS pigs prior to inoculation with ST, with some differences in cytokine and gene expression responses. Differences between LS and PS were more evident on day 2 post-inoculation, as blood from PS pigs increased plasma IL-1β, TNF-α, IFN-γ, CXCL8 and IL-10 as well as the expression of genes and networks involved in inflammation. Blood from LS pigs appeared to have an attenuated response to endotoxin stimulation post-inoculation, increasing only plasma TNF-α, CXCL8 and IL-10. Further, blood from the LS pigs had a dampened plasma IL-1β response when compared to stimulation prior to inoculation, and up-regulated only 3 genes in response to endotoxin. These results demonstrate
distinct differences in the blood response to ex vivo stimulation with endotoxin from pigs that differ in fecal ST shedding, providing potential tools for biomarker development to reduce swine disease and limit food safety risk.

Taken together, these three sets of experiments provide new findings to enhance the understanding of porcine immune response to an environmental and pathogenic challenge. Additionally, they provide insight into how these responses may shape disease outcome, with potential to assist in diagnostic and biomarker development as tools to improve swine health.
CHAPTER 1

GENERAL INTRODUCTION

Introduction

Understanding of the porcine immune system has grown considerably in the last 30 years as reagent development advances and the need for human models expands beyond rodents. Since the development of the first porcine-specific monoclonal antibodies to immune cell markers in 1983, the number of publications regarding the porcine immune system has quadrupled (1). The recent publication of the porcine genome (2) promises to enhance our research tools and our understanding of porcine biology. Moreover, the economic importance of the swine industry has provided a foundation for continued research in maintaining swine health and understanding disease. The number of researchers using the pig as a biomedical model also continues to rise, and the physiological similarity to humans make it a relevant model for studies in transplantation, nutrition, cardiology and gastroenterology among others (3).

Although swine research has advanced dramatically, the exploration of porcine molecular immunology is still in early stages, and much has yet to be defined. Thus, the overall objective of this research dissertation was to advance the understanding of several aspects of the porcine immune response to environmental and pathogenic challenges common to the swine industry.
Thesis Organization

This dissertation is comprised of five chapters, three of which are written in the format required by individual journal submission. The first chapter is a review of the literature, encompassing the topics of porcine immunology, the impacts of agricultural dust on respiratory health, and swine salmonellosis. The following three chapters are manuscripts either submitted or to be submitted to *Veterinary Immunology and Immunopathology*, *Innate Immunity* and the *Journal of Innate Immunity*. The fifth and final chapter is composed of general conclusions and future research directions for all areas discussed. Relevant references for each chapter are included within the chapter. Author contributions include:

Chapter 2: Knetter, Wannemuehler, Tuggle and Ramer-Tait conceived of and designed the research experiments; Knetter conducted experiments, analyzed the data and wrote the manuscript; Ramer-Tait had final responsibility for manuscript content. All authors reviewed and approved the manuscript.

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Chapter 4: Bearson, Nettleton, Wannemuehler, Ramer-Tait Lunney and Tuggle conceived of and designed the research experiments; Knetter, Bearson, Ramer-Tait, Kurkiewicz, Schroyen, Nettleton, Berman, and Cohen performed the experiments; Knetter, Bearson, Kurkiewicz, and Nettleton analyzed the data; Knetter wrote the manuscript; Tuggle had final responsibility for manuscript content.

Review of the Literature

**The Porcine Immune System**

**Anatomy**

Similar to other mammals, the thymus is a primary lymphoid organ and the location of T cell development and maturation in the pig. It consists of loosely packed epithelial cells organized into lobules and surrounded by an outer cortex and a dense basement membrane. Lymphocytes aggregate in the outer cortex and decrease in number toward the inner medulla. Corpuscles secreting thymic proliferative and survival cytokines are also found in the medulla. Although the majority of lymphocytes in the thymus are T cells, antibody-secreting B cells have also been observed in the porcine thymus, producing both immunoglobulin (Ig) G and IgA (4). The thymus involutes and atrophies as the animal ages, eventually becoming minimally active. The other primary lymphoid organ in the pig is the bone marrow, and it is here where the majority of post-natal hematopoiesis occurs (5). Similar to the thymus, stromal cells of the bone
marrow produce survival and support factors to facilitate growth of undifferentiated stem cells. Transcriptional changes within these cells polarize them toward lineage differentiation, however lymphocytes leave the bone marrow as immature (6). Chemokine gradients ligate distinct surface receptors on migrating cells and direct them to different tissues for further differentiation and maturation.

The pig spleen acts to clear senescent erythrocytes from circulation in the red pulp portion, and to regulate lymphocyte circulation and leukocyte interactions in the white pulp portion. B cells aggregate near the corona of lymphoid follicles, and T cells near the periarteriolar lymphoid sheath through which they enter from the blood (7). They also aggregate around B cell follicles, providing cytokine support to germinal centers where B cells undergo proliferation and development. As the spleen directly filters the blood, it is a main site for soluble antigen sampling (7).

Porcine lymph nodes have a structure known as inverted, and share this rarity with the rhinoceros, dolphin and elephant (5). The majority of mammalian lymph nodes have an outer cortex containing a paracortex that surrounds a large medulla (Figure 1 A), where the inverted lymph nodes feature an opposite organization: an outer medulla that surrounds nodules of paracortex, each containing a cortex (Figure 1 B). In both structure types, B cell follicles are located within the cortex, and T cells home to the paracortex. Lymphocytes enter the lymph node via either lymph flowing through the afferent lymphatics, or
directly from the blood through high endothelial venules (HEV). Whereas most lymph node structures allow lymphocytes to exit via the efferent ducts into the thoracic duct, the density of the outer medulla in the inverted structure inhibits lymphocytes exiting in the lymph. Instead they leave the lymph node via the HEVs, resulting in largely acellular lymph emptying into the thoracic duct of pigs. This circulation pattern also results in peripheral blood providing an accurate window to the porcine immune response.
The gut associated lymphoid tissue (GALT) of the porcine ileum consists of lymphoid follicles that are either single or grouped into Peyer’s patches (PP).
In the terminal ileum, the ileal Peyer’s patch (IPP) consists of a series of continuous follicles ~2 m long at birth, but involutes at several weeks of age (8). The IPP is dominated by B cells undergoing apoptosis, with only 5-13% of them entering circulation. It has been previously reported that, similar to sheep, the swine IPP is a primary lymphoid organ, however recent studies have demonstrated that B cell lymphogenesis does not take place in the IPP, nor is it required for the maintenance of the B cell population (8). Studies indicate that antigenic stimulation plays a role in IPP involution, as the IPP of germ-free piglets increases in size after birth (9). The jejunal Peyer’s patches (JPP) are homologous to the conventional PP of humans; they are not considered continuous, have a greater number of T cells than the IPP, and their development depends on intestinal colonization by the commensal microbiota (8). An individual PP consists of an M cell dome separating lymphoid follicles from the gut lumen. M cells are capable of macropinocytosis, and can directly take up antigen and protein from the gut lumen for delivery to antigen presenting cells for antigenic priming of B and T cells (10). Lymphocytes enter the PP via venules that integrate lymphoid nodules. Intraepithelial lymphocytes, as well as antigen presenting cells (APCs) and lymphocytes of the lamina propria, are also important immune modulators located in the gut (5).

The tonsils sit at the opening of both the respiratory and gastrointestinal tracts, and due to the rooting behavior characteristic of the species, are a major site of antigenic sampling in swine (11). Lymphocytes enter the tonsils via HEVs
in parafollicular areas, similar to lymph nodes. The majority of T cells found here express $\alpha/\beta$ TCRs (5). Lymphocytes are aggregated into follicles, and the uptake of antigen by resident macrophages and dendritic cells (DCs) primes their activation, as in other lymphoid tissue. Similar to the gut, the tonsil mucosa is adjacent to a commensal microbial community, and developing immune tolerance to this community also takes place within the lymphoid follicles through antigenic priming in the presence of tolerizing cytokines (12).

The respiratory tract has a large surface area that is virtually in constant and direct contact with the outside environment. Antigen can reach the lungs either through the blood or from the bronchial tree, where many resident macrophages and DCs are present for antigen uptake (13). It has been reported that pulmonary intravascular macrophages cover 16% of the lung capillary surface by 30 days of age (14). Antigenic clearance also takes place via mucociliary transport where particles are removed through mucus secretion and active movement of mucus along the tract via ciliary action (15). Host defense peptides and soluble Ig also are prevalent in the respiratory tract mucosa, serving to neutralize and destroy inhaled pathogens (16). Alveolar macrophages make up >94% of white blood cells in bronchoalveolar lavage fluid (BALF), and are a main mechanism for pathogen and debris phagocytosis, destruction and antigen presentation in lung (13). Porcine respiratory epithelia are also capable of sensing antigen and secreting inflammatory mediators. Lymphocytes enter the lung via the blood, and marginate into either the bronchial lamina propria, the
bronchial epithelium or the interstitial space for antigenic priming (17). Humans, rats and several other species require a period of pulmonary development known as alveolarization, or the achievement of the full number of alveoli and functionality of pulmonary defenses (18). It was reported by Dickie, et al. (2009) that after only one week of age, piglets had completed alveolarization, based on a relatively high number of alveolar macrophages (18). These alveolar macrophages were also capable of generating reactive oxygen species and of phagocytosis, two of their main functions in immune defense.

Fetal and neonatal development

The porcine placenta is epithelio-chorial, separating the fetal and maternal blood supply by six layers of tissue *in utero*. As such, there is no maternal transfer of immune cells or their larger products, such as Igs, to the fetus. Colostrum and milk are therefore the main mechanisms by which passive transfer of Igs, cytokines, chemokines and growth factors takes place (10). Gut closure occurs 24-36 h post-parturition and inhibits the direct absorption of these macromolecules ingested in colostrum (19).

Immune system development begins as early as d 16 in the 114 d gestation with the start of hematopoiesis in the yolk sac. B cell receptor rearrangement begins here even in the absence of antigenic stimulation as early as d 20. Although thymic and splenic tissue are observed on days 21 and 22 respectively (5), the functional loss of the yolk sac at ~ d 25 shifts hematopoiesis
to the fetal liver where IFNα/β-secreting cells can be identified (20). Thymocytes begin migrating to the thymus at approximately d 40, where they are exclusively γ/δ T cells (21). B cells begin appearing in the periphery at approximately the same time, and hematopoiesis shifts to the bone marrow. Unlike mice and humans, porcine B cells rearrange the λ light chain first, followed by the κ light chain later in gestation (22). At this time, fetal porcine B cells also begin secreting a small amount of IgM and isotype switching occurs, resulting in the production of IgM, IgG3 and IgA in the absence of antigen stimulation (22).

Natural killer (NK) cells can be detected in the blood, spleen and tissues at d 45, and although the population reaches stable numbers at ~ d 70, they have been reported to lack functional cytotoxic capabilities (20). At d 45, CD4+ and CD8+ T cells have been observed in the thymus. At ~ d 50, α/β T cells begin appearing in the periphery and B cells in the thymus are capable of spontaneous isotype switching from IgM to IgG, unlike many other species. It is not until d 60-80 however that the majority of lymphopoiesis occurs and lymphocytes migrate in large numbers to the lymph nodes and spleen. In late gestation, nearly all cell types and tissues of the immune system can be identified. The receptor rearrangement and isotype switching that occurs \textit{in utero} without antigenic stimulation, as well as the presence of antibody-secreting B cells in the thymus, are phenomena which contradict paradigms set by human and rodent studies.

\textbf{Innate immunity}
Overview

Innate immunity comprises not only the cellular mechanisms of the innate immune system, but also the physical and chemical barriers and defenses that protect the animal from pathogens. Physical barriers such as the skin and gut mucosa, as well as competitive exclusion provided by commensal microbiota, provide a first line of defense. Chemical defenses such as proteases and defensins in secretions also contribute to the initial defense against pathogens. However, once these barriers are breached, the animal depends on cellular defenses of the innate immune system as the next line of defense to combat invaders. The classic innate response is rapid, non-specific and short-lived. Innate immune cells such as phagocytic macrophages take up and destroy microbes, secrete inflammatory agents and prime the adaptive immune system for a more long-lasting and recurrent defense. In this way, they are the so-called first responders to many pathogens, and are often capable of controlling infection without an adaptive immune response.

Neutrophils

Porcine neutrophils are similar to those described in other species in phenotype and function. They are polymorphonuclear, short-lived cells that are highly phagocytic. They are the major blood leukocyte in circulation and their cytoplasm is rich in granules containing antimicrobial peptides (23). The expression of adhesion molecules by the endothelium of the blood vessel wall is
induced in inflammation by cytokines, and these molecules bind to integrins on
the surface of passing neutrophils, mediating their extravasation and diapadesis
into tissues. Neutrophils also express chemokine receptors that direct them
along a chemokine gradient toward sites of inflammation. The combination of
integrin binding and stimulation by chemokines and inflammatory cytokines such
as CXCL8 (also known as interleukin-8) and tumor necrosis factor-α (TNF-α)
activate neutrophils (6). Neutrophil granules contain a variety of enzymes,
including lysozyme to disrupt microbial cell walls, myeloperoxidase to generate
respiratory bursts, elastase for connective tissue degradation, cathepsins that
activate other resident cell types, and lactoferrin which sequesters iron from
pathogens (24). Neutrophils can engulf pathogens by several means, including
receptor-specific binding to antibody or complement-coated pathogens. They
also have a unique capability to form neutrophil extracellular traps (NETs) which
consist of chromatin decorated with granules released by the neutrophil in
response to CXCL8 (25). These NETs form a fibrous network around the
pathogen to trap and destroy it. Upon phagocytosis of the pathogen, neutrophils
employ several mechanisms for destruction including an oxygen-mediated
respiratory burst that forms lytic reactive oxygen intermediates (25). They also
form an intracellular phagosome around the ingested microbe that fuses with
their intracellular granules to aid in pathogen destruction.

Early studies of porcine neutrophils found them to be less chemotactic
than those of other species, responding only to complement-mediated
mechanisms and not bacterial chemotactic factors (26). However, *in vivo* infection studies have demonstrated that porcine neutrophils respond strongly to interleukin (IL)-1β and CXCL8 produced by gut epithelial cells, migrating to PP in response to infection with *Salmonella* species (27). Porcine neutrophils are also the major source of PR-39, a cathelicidin that has perhaps the broadest array of functions of all porcine host defense peptides (23). It promotes wound repair, angiogenesis, and it is a chemotactic factor to other neutrophils. It’s capacity to interfere with pathogen protein and DNA synthesis has identified PR-39 as a main defense against a variety of important swine pathogens, such as *Actinobacillus pleuropneumoniae* (28).

**Mast Cells, Eosinophils & Basophils**

Studies of porcine basophils, eosinophils and mast cells have largely focused on the pig as a model for human allergy. These cells have granular cytoplasms, and release these granules after triggers of inflammation. Mast cells are found mostly in connective tissues such as the submucosa and dermis, and contain large granules with chemoattractant and inflammatory properties (6). They have high-affinity receptors for the constant region (FcR) of IgE and upon ligation, activate gene transcription and degranulation. The release of histamine, serotonin, prostaglandins and leukotrienes largely mediate vasodilation and the release of IL-4,-5,-6,-13 and TNF-α to promote inflammation, pyrexia and a Th2 response (29).
Eosinophils are attracted from the bone marrow by Th2 cells and mast cell degranulation via the blood by chemoattractants such as IL-5, histamine and CXCL8 (30). They release two types of granules: small primary granules containing peroxidase and acid phosphatase, and large secondary granules containing eosinophil-derived neurotoxin, eosinophil peroxidase, and eosinophil cationic protein (10). These proteins are especially effective at killing IgE-coated parasites. Eosinophils also contribute to inflammation by the release of IL-3,-4,-5,-6,-13 and TNF-\(\alpha\). Swine leukocyte antigen (SLA) is the porcine major histocompatibility complex, and in the lamina propria of the pig small intestine, a significant proportion of SLA-II+ cells are eosinophils with a capacity for antigen presentation (31). Porcine basophils are less well studied, and are the least numerous granulocyte. They have cytoplasmic granules that contain molecules similar to those found in mast cells, and are thought to exert a similar function (29).

**Monocytes and Macrophages**

Macrophages are the most well-studied innate cell type in pigs, as they play a pivotal role in both protection from and susceptibility to important economic swine diseases (1). Monocytes are produced in the bone marrow and circulate in the blood until entering the tissues via diapedesis and chemokines gradients, much the same as neutrophils. Upon tissue entry, they are termed macrophages, and differentiate further based on the tissue environment they
enter and by what mechanism they’re activated. They are located throughout a variety of tissues to not only phagocytose foreign material, but also to assist in immune regulation, take up dead cells and debris and to promote inflammation and the recruitment of other macrophages (32). The termed used for many tissue macrophages is dependent upon where they are located; i.e. Kupffer cells are macrophages of the liver, osteoclasts are macrophages found in bone, and microglia are macrophages of the central nervous system. They express pathogen recognition receptors (PRRs) that are ligated by highly conserved structures expressed by multiple microbes known as pathogen associated molecular patterns (PAMPs). Gordon (2003) reviewed the classification of macrophages based on their mechanism of activation. After ligation by PAMPs, classically activated macrophages enhance their phagocytic and endocytic capabilities and take up the foreign material. They secrete pro-inflammatory cytokines such as Type I interferons (IFN), IFN-γ and TNF-α, as well as enhance their expression of co-stimulatory molecules and SLA-II for antigen presentation (33). Macrophages also express FcRs and can therefore sense Ig-coated pathogens for uptake. Through classical activation, CD4+ T cells can activate macrophages by secretion of IFN-γ, stimulating their intracellular microbicidal capabilities and the synthesis of pyrogenic cytokines IL-1β, IL-6 and TNF-α. Macrophages that are activated alternatively, sometimes referred to as M2 macrophages, are those that are programmed for wound repair, promoting humoral immunity and immunosuppression. These macrophages are often
activated in the presence of IL-4, TGF-β or IL-10, or by the uptake of apoptotic host cells or debris.

Porcine macrophage and B cell studies led to the discovery that the actions of IL-4 were different in the pig than in mice and humans. Instead of stimulating B cell proliferation and Ig production, it blocked secretion of Ig and suppressed B cell proliferation in pigs (34). It also did not stimulate T cell proliferation. However, it was capable of blocking inflammatory cytokine production by macrophages of the lung (35), indicating it may still have anti-inflammatory polarizing effects. Instead, porcine IL-13 is emerging as a cytokine that may have similar effects as IL-4 in other species on porcine immune cell types such as monocytes and macrophages. It has been reported that monocytes can be differentiated into DCs more efficiently with IL-13 instead of IL-4, and that IL-13 is more readily detectable in the peripheral lymphoid tissues of common breeds of swine (36).

In addition to their host protective mechanisms, macrophages have also been demonstrated to be a permissive source of entry and pathogenesis for some of the most costly diseases in the swine industry. The porcine reproductive and respiratory syndrome virus (PRRSv) has been demonstrated to infect any fully differentiated macrophage cell type, with the primary target being alveolar macrophages (37). *Salmonella* species exploit the phagocytic nature of macrophages by using them as vehicles for trafficking from the gut to the mesenteric lymph nodes and for intracellular survival (38). The pathogenic
lesions and depletion of lymphoid tissues observed as a result of African swine fever are attributable to the highly activated state of infected monocytes and macrophages and the large amounts of inflammatory cytokines they produce (39). For these reasons and others, macrophage biology continues to be at the forefront of swine disease research.

**Dendritic Cells**

Dendritic cells (DC) are a pivotal cell type in the junction between innate and adaptive immunity with three main functions: survey the body for pathogens to activate innate defenses, efficiently process exogenous antigen to activate adaptive immunity and to regulate both innate and adaptive immune functions (10). They have been classified as the most capable antigen presenting cell (APC) as they can fully prime naïve T lymphocyte responses (40). Given their variety of functionality, these cells are highly plastic in nature depending on tissue type, activation mechanism and origin, and are often divided into two different types. Conventional DCs (cDC) are those that are highly efficient APCs, while plasmacytoid DCs (pDC) are those that secrete large amounts of Type I IFNs. Porcine pDCs are the main DC population that produce IFN-α and TNF-α, and traffic to the lymphoid tissue after activation (40).

In inactivated or immature form, DCs are highly migratory in response to chemokines gradients, express low levels of surface SLA-II and are highly phagocytic (10). They traffic from the bone marrow through the lymphatic system
and peripheral circulation to tissues where they serve as sentinels for capturing invading pathogens. They also can respond to cytokine and chemokines signals by migrating to sites of tissue damage or inflammation. They express PRRs and upon ligation, DC endocytic activity is enhanced in the first 2 h for pathogen uptake and presentation (40). Unlike the phagosomes of neutrophils or macrophages, DC phagosomes do not fuse with lysosomes to achieve a low pH and destroy the pathogen. This results in more efficient preservation of the antigen epitopes for presentation in the context of MHC (10).

Activation in the absence of PRR ligation often leads to the DC adopting a more tolerogenic phenotype. Presentation of these antigenic epitopes to lymphocytes is coupled with the release of cytokines like TGF-β or IL-10, inducing lymphocyte anergy or polarizing T cells to a regulatory phenotype. This is of particular importance in the mucosal tissues, where DCs can mediate self-tolerance and tolerance to harmless antigens. Bimczok, et al. (2006) reported the presence of porcine DCs in the PP and lamina propria of the gut mucosa, and that some DCs directly sample the gut lumen through intraepithelial projections that capture food antigens (41). They also noted the close proximity of PP DCs to M cells, hypothesizing that there is direct antigenic transfer from the M cells to the DCs. Porcine DCs have also been found in the tracheal mucosa, the tonsils and the lungs (41, 42).

Antigen uptake stimulates the DC to adapt a more mature, activated phenotype. Activated DCs express high surface MHC and co-stimulatory
molecules for lymphocyte activation such as CD80/86 and CD40 (7). Surface expression of chemokines receptors is also altered, enabling them to traffic to different tissue types. They also secrete activating cytokines that will differentiate the naïve lymphocyte into an effector phenotype, and this cytokine pattern is dependent upon their mechanism of activation (10). Pathogens that ligate extracellular PRRs often stimulate DCs to secrete cytokines that polarize T cells into a more inflammatory profile, while DCs activated in the presence of factors released from tissue damage will secrete cytokines that polarize T cells to a phenotype involved in wound-healing (40). DCs can also secrete cytokines to induce istotype switching in B cells (40).

Follicular DCs also exist in B cell follicles of lymphoid tissue. These cells present antigen to B cells in stable immune complexes consisting of antigen, antibodies and complement (6). They are long-lived cells, and serve to continually prime proliferating B cells and to secrete survival cytokines in the germinal centers.

Natural Killer Cells

Natural Killer cells (NK) are a cell type that bridges both innate and adaptive immunity. They are of the lymphocyte lineage, but are distinct from both B and T lymphocytes in that they do not undergo receptor rearrangement. They are found in the pig mostly in the spleen and peripheral blood, although a few have been observed in the thymus and lymph nodes (10). They make up 2-
10% of porcine peripheral blood lymphocytes, and decrease in proportion as the animal ages (43). As their name implies, these cells are cytolytic, and their encounters with SLA-I dictate their response. In pigs, the recognition of SLA-I by NK surface receptors known as killer cell immunoglobulin-like receptor (KIR) stimulates an inhibitory pathway within the NK cell. Pig NK cells also recognize SLA-I via surface NKp46, a marker unique to NK cells that recently was described to subdivide porcine NK cells into distinct states of maturation (44). Although cytolytic activity was similar, NKp46+ NK cells produced greater IFN-γ after stimulation. Expression of NKp46 was also induced on NKp46- cells after stimulation with the NK-activating cytokines IL-2, -12, and -18, suggesting that NKp46- cells become NKp46+ upon maturation.

Beyond SLA-I recognition, NK cells can also be activated through ligation of NKG2D, a receptor that recognizes surface proteins expressed by stressed and unhealthy cells such as MICA or MICB (10). Upon ligation, NKG2D signaling overrides the inhibitory signals provided by recognition of SLA-I, permitting NK cells to kill the target cell. Although NKG2D has been identified in porcine NK cells (45), its functionality has yet to be described in pigs. Activation through an antibody-dependent mechanism, known as antibody-dependent cell-mediated cytotoxicity (ADCC) is achieved through ligation of CD16, an FcR (7). Soluble Ig opsonizes or coats target cells, making them recognizable to NK cells by the ligation of CD16. Once activated, porcine NK cells mediate cellular cytotoxicity through perforin and NK-lysin, molecules that create pores in the target cell
membranes and induce cell death. Cytokines such as IL-2, IL-12, IL-18 and IFN-γ alone or in combination have been shown to enhance pig NK cytotoxicity and production of IFN-γ in vitro (43).

Adaptive immunity

Overview

The evolution of the adaptive arm of immunity appeared approximately during the rise of jawless fish with the introduction of lymphocytes (21). Their capability for somatic gene rearrangement to develop diverse antigen receptor genes was the first example of gene alteration throughout the lifetime of an animal to promote survival. The function of adaptive immunity is largely intertwined with and somewhat dependent upon innate immunity, however the unique capability of antigenic memory defines it separately. It is currently recognized that three processes exist to create adaptive memory: somatic gene recombination, somatic hypermutation and allelic exclusion. Somatic gene recombination is the process of recombining gene segments for diversity in antigenic receptors into a productive arrangement, and is mediated by the Recombination Activation Genes (7). This process is also referred to as VDJ recombination, as multiple genes encode for each of these three segments, and only one segment from each is utilized in rearrangement. Somatic hypermutation occurs at a rate of $10^4$ times faster than bacterial mutation, and utilizes activation-induced cytosine deaminase to introduce mutations into the recombined antigenic receptor genes,
repairing them with DNA repair enzymes (46). To ensure only one type of antigen receptor is expressed, allelic exclusion exists to silence the other allele. Through these three mechanisms, T and B lymphocytes are able to provide the antigenic specificity and memory that defines adaptive immunity.

*B cells*

B cells produced from the fetal yolk sac and liver are considered the “first wave,” and in the pig they have nearly 100% productive B cell receptor (BCR) rearrangement on a single allele, with no rearrangement of the second allele. Later in gestation and throughout adulthood, B cells produced from the bone marrow diversify rearrangement. Porcine B lymphocytes can be identified by the expression of IgM, as well as CD79α (Igα chain). The swine genome encodes for the same five isotypes of Ig as found in mice and humans: IgM, IgD, IgG, IgE and IgA. Subclasses of these isotypes continues to expand, and currently there are six classes of IgG (47). There are two forms of IgA (IgAα and IgAβ), and B cells only in secondary lymphoid tissues; i.e. not in the periphery or bone marrow, predominantly express IgD. Transcripts for all five isotypes of Ig have been found in the porcine fetal thymus. The proportion of secretory Ig classes varies with age and tissue location. Newborn piglet serum contains approximately 0.83, 33.9, 2.1mg/ml of IgM, IgG and IgA respectively; and at five weeks of age these concentrations change to 2.2, 7.5 and 0.6 mg/ml. Sow colostrum is extremely high in IgG and contains approximately 9.1, 95, and 21.2
mg/ml of these Ig classes at parturition. Sow milk is then later predominated by IgA.

The light chains of many ungulate species are predominantly \( \lambda \), however swine express near equal \( \lambda \) and \( \kappa \) light chains in their Ig. The genes encoding the heavy chain V segment of Ig are comprised of several subgroups in humans and mice, however all swine IGHV genes come from a single subgroup. Approximately 99% of all VDJ recombinations utilize only seven of 30 IGHV genes, two IGHD genes, and there is only one functional IGHJ gene (22). These numbers limit the combinatorial diversity that can be acquired through gene rearrangement; resulting in 14 possibilities compared to 9,000 in humans (22). Instead, the porcine BCR repertoire achieves diversity after somatic hypermutation is stimulated by exposure to environmental antigens. Studies using isolator-housed piglets indicate that adaptive immunity is dependent upon gut microbiota colonization as evidenced by a lack of Ig changes until a 100-1000 fold increase in serum Ig after gut colonization (48). The term natural antibodies has been used to define the broad specificity and low affinity of IgM antibodies present at birth without antigenic encounter.

Antibody-secreting cells, also referred to as plasma cells, are found in a variety of porcine tissues, including the mucosa, lymph nodes, spleen and even thymus (49). At birth, they produce mostly IgM only, but after isotype switching is induced by environmental stimuli, IgA-secreting cells predominate in the
mesenteric lymph nodes, gut lamina propria and other mucosal tissues. Plasma cells secreting IgG predominate the peripheral lymph nodes and spleen.

In humans and mice, B cells are subdivided into B-1 and B-2 populations based on the expression of CD5. The CD5+ B-1 type develops in the peritoneum, has polyclonally activated BCRs, and secretes highly cross-reactive Ig (7). The CD5- B-2 type expresses more restricted BCRs and their secreted Ig is more specific. These subtypes have not been identified in swine, and the expression of CD5 does not appear to define distinct populations.

T cells

The T cell receptor (TCR) consists of a highly variable (V) region which recognizes antigen, an unchanging constant (C) region, and small transmembrane and cytosolic domains. It is associated with a CD3 complex of five chain types ($\gamma, \delta, \varepsilon, \zeta, \eta$) arranged into three dimers. The expression of $\alpha/\beta$ heterodimer or a $\gamma/\delta$ heterodimer to form the TCR defines it into one of two main types of T cells. As with other livestock species, the proportion of peripheral $\gamma/\delta$ T cells is very high at birth (~40%), and decreases with age (50). Although not definitively described in the pig, it is thought that T cell development follows that which has been described for other species, where thymocyte progenitors undergo sequential TCR rearrangements and testing by resident cells in the thymus (7). Thymocytes capable of self-MHC recognition receive survival signals from resident DCs, macrophages and epithelial cells (positive selection);
while thymocytes that react strongly with self-antigens undergo apoptosis (negative selection). Beyond positive or negative selection, their interaction with SLA-I or SLA-II defines their subsequent phenotype of CD8 or CD4 expression, respectively. As co-stimulatory molecules, CD8 or CD4 associate with the TCR-CD3 complex, and define the function of TCR signaling (6). Once mature, these T cells express either CD4 or CD8αβ heterodimers classifying them by function into T helper (Th) or cytotoxic T lymphocytes (CTL). Pigs have nearly double the number of CD4-CD8+ T cells in peripheral blood than CD4+ T cells, which is the opposite ratio from humans (50). Both α/β Th and CTL are highly mobile, and migrate through the periphery to sites in the spleen and lymph node to continuously scan for antigen presented in the context of MHC on APCs (10). Once they recognize their receptor-specific antigenic epitope, the TCR is strongly engaged, slowing the T cell and activating it to form an immunological synapse. This synapse utilizes co-stimulatory and adhesion molecules to form a strong bond with the APC, fully activating the T cell and polarizing it into one of several effector phenotypes largely based on the cytokine milieu.

The functionality of porcine Th cells in pigs is similar to mice and humans. They interact with SLA-II-presented antigen that stimulates their activation and proliferation, and their main role is to secrete cytokines that stimulate and enhance the functions of surrounding cell types, such as producing IFN-γ to enhance the antibactericidal capacity of macrophages (7). Multiple subtypes of Th cells have been identified based on their differentiation requirements,
expression of transcription factors, and their function in mice and humans, including Th1, Th2, Th3, Th9, Th17, and Th22. The Th1 phenotype is defined by differentiation in the presence of IL-12 and IFN-γ, expression of the transcription factor T-bet, and secretion of IL-2 and IFN-γ (34). Activating microbicidal activity of phagocytes for destruction of intracellular pathogens, stimulating B cell class switching, and stimulating naïve, activated B cells to produce Ig are the main functions of the Th1 cell. The Th2 phenotype is defined by differentiation in the presence of IL-4 and/or IL-13, expression of the GATA-3 transcription factor, and production of IL-4, -5, and -13. Their main role is to provide support for B cell class switching and activation to optimize the humoral response (34). Th17 cells differentiate in the presence of TGF-β and IL-6, are stabilized by IL-23, express RORγT, and produce IL-6, -17, and -21 (7). The full function of these cells is still being elucidated, however the production of these cytokines stimulates surrounding macrophages and epithelial cells to produce chemoattractants of neutrophils and to increase β-defensin expression. Of these phenotypes, only the Th1, Th2 and recently the Th17 phenotypes have been identified in the pig. Kiros, et al. (2011) report the presence of IL-17 secreting T cells in the lungs and peripheral blood of pigs, as well as the ability to differentiate these cells in vitro in the presence of TGFβ and IL-6 or IL-1β.

Regulatory T cells (Tregs) have also been identified in the pig, and similar to other species, they act to downregulate and inhibit the proinflammatory immune response (52). They are identified as CD4+CD25+ T cells that express
the transcription factor FoxP3, and produce IL-10 and TGF-β. Porcine Tregs can be subdivided into two populations based on their expression of CD25 (either high or low), and it appears that only the CD25\textsuperscript{high} subtype is capable of inhibiting activated T cell proliferation. Both types were capable of producing IL-10, and together made up ~9% of the T cell population.

The CTLs of pigs also function similarly to their human counterparts, recognizing SLA-I-presented antigen and responding by killing target cells, proliferating and secreting proinflammatory cytokines such as TNF\textsubscript{α} and IFN\textsubscript{γ}. These cells contain perforin, and can carry out their lytic activity through the release of this pore-forming protein on target cells (53). Upon antigenic stimulation however, pig CD4\textsuperscript{+} T cells can permanently express a CD8\textalpha\textalpha homodimer, resulting in extrathymic CD4\textsuperscript{+}CD8\textsuperscript{+} double-positive T cells (21). Therefore, the expression of CD8 is not unique to CTL, and as a result perforin is used in combination with CD3 for CTL identification. It has also been reported that these double-positive T cells form the CD4\textsuperscript{+} memory population as they can recall antigen, express markers of T cell memory such as CD29, and their numbers positive correlate with animal age (54). Additionally, they upregulate production of IFN-\textalpha, IL-2 and IFN-\textgamma, and the expression of CD4\textsuperscript{+}CD8\textsuperscript{lo} is now commonly used to identify Th memory cells in pigs. Although also described in humans, rodents, monkeys and chickens, the proportion of CD4\textsuperscript{+}CD8\textalpha\textalpha\textsuperscript{+} T cells in swine is the greatest, and at five months of age can comprise a third of all T cells in the secondary lymphoid tissue (55). Activation of CD4\textsuperscript{+} T cells also
leads to their permanent expression of major histocompatibility complex II, although it is unknown if this expression is functional for antigen presentation (50).

Other subtypes of T cells identified in mice, humans and pigs include T follicular helper cells (Tfh) and NKT cells. The expression of CXCR5 on Tfh cells causes them to home to the germinal centers of B cell follicles where B cell proliferation takes place in lymphoid tissues (56). They support B cell activation, proliferation and differentiation in these tissues. NKT cells have been identified in the porcine lung and peripheral blood, and are CD3+perforin+CD16+, making their phenotype similar to both NK and CTL (57). They recognize glycolipid antigens in the presence of CD1d, a molecule similar to SLA in antigen presentation. Porcine NKT are capable of lytic activity through perforin, and also can secrete cytokines similar to Th1 or Th2 when simulated in vitro.

The γ/δ repertoire can also be subdivided based on phenotype and tissue distribution. CD2+CD8αα+ and CD2+CD8- γ/δ T cells are largely located in the spleen where they make up the predominant T cell population, while CD2-CD8- γ/δ T cells are found mostly in circulation (21). Porcine CD2+ γ/δ T cells are not MHC-restricted, and although their mechanism for antigenic recognition is under debate, (55, 58) they will secrete cytokines such as IL-1, IFN-α and CXCL8 in response to stimulation. They can also be cytolytic (59), but their expression of CD3 definitively separates these cells from porcine NK cells (CD3-). It has been demonstrated that CD8- γ/δ T cells may acquire CD8αα upon activation, similar
to $\alpha/\beta$ T cells, as well as SLA-II (58). Takamatsu, et al. (2002) reported this acquisition of SLA-II resulted in the capability of pig $\gamma/\delta$ T cells to present antigen to $\alpha/\beta$ T cells. The repertoire of $\gamma/\delta$ TCRs is highly compartmentalized within organs, and a highly polyclonal repertoire was identified in the spleen. This suggested that antigenic stimulation within tissues selects and maintains the $\gamma/\delta$ TCR repertoire (21).

Resection of the thymus in neonatal pigs greatly reduced the number of T cells, however the reappearance of T cells 3 months later may indicate that extrathymic sites such as lymph nodes or PPs may be sites of T cell production (61).
Agricultural Dust and its Effects on the Respiratory and Immune Systems

Dust-induced respiratory disease

Agricultural dust has been recognized as a source of respiratory complications for centuries (62). It was first documented as a hazard by Ramazzini in 1700 who recognized farmers, grain sifters and horsemen among those who suffered respiratory problems while at work (63). Reports on disease and organic dust continued throughout the nineteenth century (64), and the first direct link to a specific pulmonary disease was identified in 1932 by associating hypersensitivity pneumonitis with exposure to airborne particles from spoiled hay (65). Since that time, exposure to organic dust has also been linked with diseases such as organic toxic dust syndrome, chronic bronchitis, rhinitis, asthma, and chronic obstructive pulmonary disease among others in agriculture workers (66-68). Hyperactivation or repeated activation of the immune system in response to organic dust components is the main mechanism for development of these respiratory diseases. Leukocyte infiltration, alveolar macrophage activation and systemic circulation of pyrogenic cytokines are largely the source for many disease symptoms (69). Thickening of the basement membrane of the airways has also been reported in both symptomatic and asymptomatic agricultural workers (70). Hypersensitivity pneumonitis results from repeated airway inflammation that presents clinical symptoms such as cough, fever and dyspnea in the acute form and granulomatosis and pulmonary fibrosis in the chronic form (71). It comprises a variety of similar diseases that arise from
different dust sources, such as farmer’s lung, grain handler’s disease, suberosis, malt fever, and bird fancier’s lung (72). Organic dust toxic syndrome (ODTS) is an acute airway disease that manifests after initial exposure to organic dust, with symptoms similar to hypersensitivity pneumonitis, but lasting less than 48 hours (73). Repeated or long-term exposure to organic dusts can also cause chronic bronchitis and emphysema, the two forms of chronic obstructive pulmonary disease (COPD) (74). Chronic bronchitis is the result of continued inhalation of irritants causing thickening of the bronchial epithelium and increased mucus production, which in combination obstruct the airway and hinder airflow (75). It has been estimated that approximately 25% of hog barn workers have symptoms characteristic of chronic bronchitis (76). Pathological signs of emphysema include destruction and deformation of alveolar walls as a result of excessive inflammation and the induction of alveoli apoptosis (77). This destruction is irreversible, and leads to an enlargement of the airspaces and a decrease in alveolar wall surface area for gas exchange, resulting in respiratory imbalance (77).

The immune and respiratory response to inhalable dust and its components

Although organic dust inhalation can induce a variety of respiratory diseases, the majority of studies show that most early pulmonary symptoms of these diseases are consistently a result of non-allergic inflammation mediated by neutrophil influx into the airway. This influx is accompanied by both local and
systemic circulation of pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-6, and IL-1β (78, 79). Chemotactic proteins such as CXCL8 have also been detected in the nasal and bronchial lavage fluid, as well as in the periphery in response to dust exposure and its components (80, 81).

Multiple agricultural occupations involve exposure to organic dust, and workers in confinement animal feeding operations (CAFOs) are at the highest risk of dust-induced lung function decline (82). Approximately 60% of all CAFO workers are estimated to develop one or more respiratory disease symptom after six years in their field (83). Their daily environment exposes them to a myriad of organic dust particles that include trace metals, pollen and microbial-associated components among others (84). The source of these particles is mainly feed, dander, fecal waste and microbes. Microbial constituents of organic dust are rich in PAMPs that can ligate PRRs in cells of the airway to activate an inflammatory response. Analysis of organic dust from swine CAFOs demonstrated inhalable dust ranges from 0.16 to 37.2 mg/m³, with mean concentrations of culturable bacteria and fungi of $4.79 \times 10^5$ /m³ and $1.55 \times 10^4$ /m³ respectively (85). Poole, et al. (2008) analyzed dust collected from separate swine barn facilities and demonstrated a predominance of Gram-positive bacteria (98%) compared to Gram-negative bacteria (2%) as identified by colony morphology. Gas chromatography-tandem mass spectrometry indicated high mean concentrations of muramic acid (203.5 ng/mg), a marker of peptidoglycan, and of 3-hydroxy fatty acids (0.0723 nmol/mg), a marker of endotoxin. Mean
endotoxin concentration was approximately 4,800 ng/mL. Further analysis indicated 2-4 mg/mL of total protein, as well as the presence of metals (Mg, Ti, Mn, B, Fe, Co, Ni, Cu, Rb, Mo and Zn).

Lipopolysaccharide (LPS) is found in the endotoxin component of nearly all Gram-negative bacterial cell walls, and its ligation of the PRR toll-like receptor 4 (TLR4) is known to have potent pro-inflammatory effects (67). In the pig, the TLR4 receptor complex (TLR4, CD14, MD-2) is expressed on the surface of many cell types, including blood neutrophils, macrophages, dendritic cells and epithelial cells (10). Mononuclear phagocytes are among the first to respond to inhaled particles in the airways, and are rapidly activated by the components found in inhaled organic dust. Through their secretion of inflammatory mediators, stimulation of these cells by endotoxin has been shown to illicit both a systemic and a focal inflammatory response in the lung (67, 87-89). Upon instilling endotoxin into a lung segment, an early (2-6 h) and a late (24-48 h) phase inflammatory response was detected in BALF (87). The early phase response was characterized by a neutrophil influx, with elevated TNFA, IL-1, IL-6, CXCL8, and macrophage inflammatory protein (MIP)-1. The later phase showed a return to basal levels for these proteins, however there were increased numbers of macrophages, monocytes and lymphocytes as well as neutrophils. Although endotoxin induces a potent inflammatory response, this response varies. Inhalation of LPS resulted in a 20% decrease in forced expiratory volume in one second (FEV₁) from human subjects and this was correlated with ex vivo
production of IL-6 and CXCL8 from peripheral blood monocytes and alveolar macrophages (88). However, LPS sensitivity was varied among subjects and different dosages of LPS were required to elicit the same level of reduction in FEV\textsubscript{1}. Similar variation has been found after inhalation of organic dust from a swine CAFO. Endotoxin exposure in swine barns varies, with reports of “low” levels designated as 452.3±65.8 EU/m\textsuperscript{3} and “high” levels designated as 3,983.5-498.3 EU/m\textsuperscript{3} (89). Burch, et al. (2009) reported that inhaled levels for 93% of agricultural workers studied exceeded the proposed endotoxin exposure limit of 50 EU/m\textsuperscript{3} (67). Reported exposure in swine barns often go beyond the American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV) for organic dust (10 mg/m\textsuperscript{3}) and for endotoxin (10 ng/m\textsuperscript{3}) (90). Studies have correlated total dust concentrations in swine barns with decreased FEV\textsubscript{1}, indicating that concentrations $\geq 2.8$ mg/m\textsuperscript{3} are predictive of a $\geq 10\%$ reduction in FEV\textsubscript{1} (91). Dosman, et al. (2006) measured FEV\textsubscript{1} in naïve individuals working in a high dust level swine barn for 5 h (89). Subjects who had a 10\% or greater decrease in FEV\textsubscript{1} had significantly higher blood lymphocytes, total nasal lavage cells, circulating IL-6, and nasal CXCL8 than those who had less than 10\% decrease in FEV\textsubscript{1} (89). The authors concluded that although variable among subjects, the FEV\textsubscript{1} response to a high dust environment can be predictive of the inflammatory response. Murine mutant studies highlight both the importance and the variation of the inflammatory response to dust. Mice deficient in TLR4 placed in a swine barn for one day showed significantly
decreased leukocyte infiltration and cytokine expression in the lungs than wild-type controls (92). However, TLR4-deficiency had no effect on the induction of airway hyperresponsiveness or cytokine release in response to swine barn air. Endotoxin was present at substantial levels ($2357.8 \pm 2525.16$ EU/m$^3$) and the authors suggest that exposure components other than endotoxin may be the cause of the observed divergent response.

Peptidoglycan is largely a component of Gram-positive bacterial cell walls, and another major constituent of organic dust. Peptidoglycan and its subcomponents diaminopimelic acid and muramyl dipeptide are recognized as a PAMP by TLR2, nucleotide-binding oligomerization domain containing (NOD) 1 and NOD2 respectively, and mediate a potent inflammatory response (93). TLR2 dimerizes with TLR1 or TLR6 to recognize the triacyl or diacyl lipoprotein components of peptidoglycan (93). High concentrations of muramic acid have been found in bioaerosols from swine barns, and are associated with inducing cytokine secretion from both epithelial cells and alveolar macrophages (94). Human bronchial epithelial cells rapidly secrete IL-6 and CXCL8 when treated with an organic dust extract obtained from filter-sterilization of solution containing dust collected from a swine barn (95). Bailey, et al. (2008) demonstrated TLR2 gene and protein expression is increased dose-dependently in airway epithelial cells and in lung tissue after exposure to occupational levels of a similar swine barn dust extract (96). This expression was dependent on IL-6 regulation, as blocking IL-6 attenuated TLR2 expression. In a separate study,
similar airway inflammation was observed in mice after intranasal challenge with either aerosolized peptidoglycan or aerosolized swine barn dust (97). TLR2-deficient mice had dampened neutrophil recruitment and cytokine release into the BALF after exposure to a similar swine barn dust extract (97). Lung pathology was consistent with this finding and demonstrated bronchiolar inflammation was reduced in the TLR2-deficient mice when compared to wild-type controls.

**Negative immune regulation and the adaptation response to dust**

Although exposure to swine barn dust induces a largely proinflammatory profile, evidence exists for a regulatory mechanism to balance this response. Another PRR ligated by the components of peptidoglycan is NOD2, and its expression is regulated by the transcription factor nuclear factor kappa B (NFκB). Murine mononuclear phagocytes rapidly translocated NFκB to the nucleus after treatment with hog barn dust, and this translocation correlated with NOD2 gene and protein expression (98). However, knockdown of NOD2 expression in human monocytes by small interfering RNA enhanced the production of IL-6 and CXCL8. This increase in cytokine production was also demonstrated *ex vivo* in murine alveolar macrophages from NOD2 knockout mice when examining IL-6 and CXCL1 and CXCL2, the murine functional homologs of human CXCL8. Further *in vivo* experiments showed NOD2 knockout mice had increased neutrophils and CXCL1 concentrations in BALF in
response to dust extract. Together, these results indicated a NOD2 may be a negative regulator of inflammation in response to hog barn dust, however the mechanism has yet to be defined. It has been hypothesized that NOD2 activation restricts nuclear translocation of the RelA subunit of NFκB, as RelA activation is increased in the absence of NOD2 (99). Researchers demonstrated NOD2 activation results in the intracellular accumulation of TLR2 suggested a different regulation mechanism where NOD2 is activated to control the internalization of TLR2, limiting the effects of PAMP signaling (100). Further research is required however to fully define this mechanism.

Another mechanism of inflammatory regulation may be a tolerization acquired by agricultural workers exposed to organic dust environments. Hog farmers have a lower sensitization to allergens despite evidence of lower respiratory tract inflammation (90). Although elevated from non-barn workers, numbers of neutrophils, macrophages and lymphocytes were also lower in the BALF of swine confinement workers when compared to naïve subjects after 3-5h of work in a hog barn (101). After a similar work period, non-naïve barn workers had elevated IL-6 in the BALF and serum, however TNF-α was reduced from baseline and remained decreased for 2 weeks after exposure (102). Together, these and other studies are suggestive of an adaptation response (90). However, chronic exposure continues to result in lung function decline and human respiratory disease.
Other *in vitro* techniques have further described the adaptation response to a swine barn dust extract as a model for human exposure. Poole, et al. (2007) found single exposure of human mononuclear cells induced TNF-α, IL-6, CXCL8 and IL-10. After a second exposure however, TNF-α and IL-6, but not CXCL8 or IL-10 production, was attenuated. In addition, basal IL-10 levels remained persistently elevated after a single exposure. Previous studies report that IL-10 reduces airway inflammation in response to agricultural dust, and the authors speculate that IL-10 induction provides an anti-inflammatory balance to a dust-induced response. Only a TNF-α response was retained after treatment with an endotoxin-depleted extract, suggesting different components of the dust extract may elicit different responses. Protein kinase C isoform activation was dampened upon repeat dust extract exposure, potentially identifying a mechanism for the attenuated response.

More recently, the same group has demonstrated that repetitive exposure to dust extract alters the maturation and differentiation of innate immune cells. Monocyte-derived macrophages differentiated in the presence of organic dust extract had an altered immunophenotype, with dampened expression of HLA-DR, CD80 and CD86 after being re-stimulated with the same extract (86). These cells also had diminished phagocytic and anti-bacterial capabilities. Dendritic cell maturation *in vitro* is also dampened in the presence of dust extract (104). As highly-efficient APCs, DCs are an essential link between innate and adaptive immune responses (105). An imbalance in DC maturity and aberrant DC
function have been implicated as a basis for recurrent respiratory infections in COPD patients (106), identifying a potential mechanism for the same issues in swine barn workers who develop COPD. Furthermore, ex vivo stimulation of whole blood from COPD subjects resulted in increased inflammatory cytokine release when compared to healthy subjects (107).

The influence of organic dust exposure on cells of the adaptive immune system has been less well described. Human and murine studies have demonstrated inhalation of organic dust induces CD3+ T cell lung infiltrates, (108, 109) and workers in swine CAFOs have higher basal IL-13 and IL-4–producing Th2 cells in circulation when compared with non-barn workers (78). Production of IL-17 from BALF lymphocytes was enhanced after subjects inhaled organic dust and correlated with an increased influx of neutrophils (110). Along with CXCL8, IL-17 produced by T cells may also promote the neutrophil recruitment into the airways that largely contributes to disease pathology. To further identify the T-cell response to inhaled dust, mice were intranasally challenged with occupational levels of organic dust extract (111). The dust extract stimulated a cytokine profile in the lungs that would favor Th1 or Th17 polarization, yielding production of IL-6, IL-1β, IL-17, and IL-22 (Th17); as well as IFN-γ, TNF-α and IL-12 (Th1). This finding was corroborated by an increased number of CD4+ T cells in the lung predominantly producing IL-17. Utilizing a TCR α/β knockout mouse strain, it was further identified that α/β T cells are required for the aggregation of mononuclear infiltrates in the lung.
Porcine response to swine barn dust

The majority of studies to date on dust-induced inflammatory responses have focused on understanding and modeling the human risk of respiratory disease. There are, however, several studies that have begun to link livestock respiratory disease risk and their constant exposure to dust inside the barns where they are housed. Swine CAFOs often have the highest levels of dust when compared to housing for other species (82), and multiple studies have described similar respiratory inflammation in pigs exposed to dust as in humans and mice (112-114). Controlled chamber studies have shown that combining common levels of dust with ammonia, but neither alone, result in respiratory tract lesions and cilia loss (112, 115). Feed dust at low inhalable concentrations (4.4 mg/m$^3$) induced an increase in alveolar macrophages and lymphocytes in the BALF of pigs after six days, and a positive correlation was found between inhalable dust and total cell numbers in the BALF (114). The amount of LPS in the dust was not correlated with this inflammatory response, and peptidoglycan was not measured. Inhalation of endotoxin alone has been shown to elicit some bronchial inflammation in pigs, but not at the same levels as inhaled dust. This supports the hypothesis in human research that the effects of dust on respiratory tract inflammation are not attributable to endotoxin alone.

Jolie, et al. (1999) studied pigs placed in a continuous flow chamber for 15 weeks to elucidate the effects of known levels of airborne contaminants for
on the porcine respiratory tract (113). Pigs were exposed for 8h per day for 75 days to a fine corn/soybean meal feed dust with or without added LPS. Pigs exposed to dust regardless of LPS treatment had an increase in neutrophils in the BALF and in the lung tissue, indicating an inflammatory response independent of LPS. They found the feed dust to have high levels of peptidoglycan, similar to other hog barn dust analyses, and hypothesized that this may be an alternative source of the dust-induced inflammation.

Currently, the immune mechanisms behind these responses in pigs have yet to be described. Because of the complexity of organic dust and the farm environment, few studies have additionally been able to conclusively define a relationship between swine production and dust levels in the barns. Only dust at very high concentrations has been shown to negatively affect average daily gain and feed efficiency directly (116). Correlations have been found however between dust and/or microbial counts and swine health problems such as pneumonia and pleuritis (117). Research evaluating the dustiness of feed has demonstrated an abrupt reduction in acute respiratory disease when ventilation was altered to reduce dust (118). The negative impact the immune response to antigenic stimulation can have on growth has been well characterized, particularly the metabolic effects of IL-1β, IL-6 and TNF-α (119, 120). Metabolic priorities are altered, and energy is re-partitioned away from growth and towards the increased requirement by the immune response. To meet these enhanced requirements, muscle protein synthesis is limited while hepatic production of
acute phase proteins and muscle protein degradation are both increased (121). The pyrexia induced by these cytokines is also metabolically costly. It has been estimated that a 13% increase in basal metabolism is required to achieve a $1^\circ$C increase body temperature (122). This rise in maintenance energy requirements decreases the efficiency of feed utilization (123), a negative effect that is exacerbated by decreased feed intake as a result of proinflammatory cytokine induction of anorexia (124). It has been demonstrated that production of these cytokines is induced in response to dust inhalation in humans and mice (78, 79, 109), and together these findings may indicate a role for costly growth inhibition mediated by pro-inflammatory cytokines induced by dust inhalation. Further research is needed to understand the mechanisms underlying the porcine immune response to swine barn dust, the capability of dust to enhance susceptibility to respiratory disease, and its potential inhibitory effects on growth.
The Porcine Immune Response to *Salmonella*

**Economic impact**

In 2010, cases of foodborne illness caused by *Salmonella* bacteria reached 80.3 million worldwide (125), making it the leading cause of foodborne hospitalization and death (126). Pork is the mostly highly consumed meat in the world (127), and estimates of human illness attributable to pork can vary from 1-25% (128-130). In the United States, the National Animal Health Monitoring System reported 52.6% of hog farms tested in 17 states were positive for *Salmonella* spp., and listed swine salmonellosis as one of the top ten most common diseases in weaning age and grower/finisher pigs (131). Transmission of bacteria from an infected animal to meat at slaughter presents an interface for foodborne illness and disease. Berends, et al. (1997) reported that pigs infected with *Salmonella* spp. are 3-4 times as likely to result in contaminated meat at slaughter, and they estimated that 5-30% of all carcasses produced are contaminated. Limiting disease transmission is also an economic concern, with annual economic costs of human salmonellosis estimated to be $2.7 billion (133) in the U.S. and nearing €3 billion in Europe (134). In 2005, estimation models predicted 99,430 cases of human salmonellosis and $81.53 million in social costs result from humans consuming contaminated pork (135).

In addition to the economic losses caused by human salmonellosis, the swine industry faces costly losses in animal production. Swine salmonellosis has been estimated to cost pork producers $100 million each year (136). Infected
pigs have a decreased growth rate and average daily gain as a result of pyrexia, anorexia and diarrhea (137). Infected pigs can exhibit both clinical and subclinical symptoms, making it difficult to diagnose (138). As a result, disease spread is frequently not confined to the farm. Transmission of *Salmonella* in swine occurs by fecal-oral route, and the comingling that occurs in transport and at meat processing plants increases the risk of infection (139). Studies examining the spread of *Salmonella* from infected pigs after transport and lairage report an increase in *Salmonella* shedding after transportation of infected pigs (140), and a positive correlation between the length of time held in lairage and *Salmonella* isolation from carcasses (141). Although separating infected from non-infected pigs has been demonstrated to be beneficial in reducing *Salmonella* prevalence, it also increases management costs (142). Consequently, research investigating minimizing initial infection and limiting farm cases of salmonellosis has increased.

**Risk factors**

Identifying the source of *Salmonella* contamination is incredibly difficult, as it is impacted by a variety of risk factors that can impact multiple stages of swine production. The incidence of *Salmonella* infected pigs in swine herds is often much greater than those exhibiting clinical symptoms, increasing the risk of disease spread. Infected pigs can remain asymptomatic carriers for months, intermittently shedding the bacteria and harboring it in tissues such as the
intestinal tract and in the mesenteric lymph nodes (143). Feed contamination has been highlighted as a potential source of *Salmonella* bacteria, and feed consistency has been linked to differences in *Salmonella* prevalence. Hotes, et al. (2010) reported liquid feeding decreased stomach pH, favoring competing microbiota populations that colonize the gastrointestinal tract and limit *Salmonella* colonization. Coarse feed also increased stomach pH compared to pelleted feed and decreased ingested *Salmonella* survival (145). The inclusion of antibiotics in swine feed has resulted in debate on the potential for *Salmonella* to develop antibiotic resistance. Higher *Salmonella* prevalence has been reported both in antibiotic-free herds and in conventional herds, (146) as well as the presence of multi-drug resistant *Salmonella* in antibiotic-free herds (147). Although present in antibiotic-free herds, a greater number of resistant *Salmonella* were isolated from conventionally raised herds (147), indicating a complexity in evaluating antibiotic resistance.

Management practices have also been demonstrated to play a role in *Salmonella* prevalence on farm, largely originating in the fecal-oral method of transmission. Non-slatted flooring retains contaminated feces for longer periods, exposing penmates (144). Bowl-drinking and other methods of feeding, watering and pen design that facilitate nose-to-nose contact have also been implicated (148). Stress has also been demonstrated to have a major impact on livestock homeostasis. Situations such as movement to unfamiliar territory, new penmates to re-establish the social group, human interaction, and transport are all sources
of stress for swine (149). Stress-induced production of glucocorticoids and catecholamines have been demonstrated to directly depress the immune system, having decreased cell proliferation, reduced antibody production, impaired natural killer cell activity and lead to a recrudescence of infection (150). In addition, *Salmonella* Typhimurium exhibits increased growth and enhanced motility in the presence of the catecholamine norepinephrine (151). Experimentally inoculated piglets also had increased fecal shedding and tissue invasiveness of *S*. Typhimurium after periods of social stress (152). Together, these studies indicate the complexity of stress-induced infection, demonstrating effects on both the host and the pathogen.

**Salmonella enterica: mechanisms of pathogenesis**

*Salmonella enterica* is a Gram-negative, facultative anaerobe that can be divided into more than 50 serogroups and greater than 2,400 different serovars by expression of variation in the O (somatic) or H (flagellar) antigens (153). Host-specificity and disease pathogenesis are different among serovars. *S*. Typhi is a human host-adapted serovar that can persist in a long-term chronic carrier state, while *S*. Typhimurium has a broad range of hosts including cattle, pigs and humans, and usually results in a self-limiting gastroenteritis (154). Both the pathogen and the host system play a role in host-specific disease manifestation. *S*. Typhimurium, *S*. Enteritidis, *S*. Agona and *S*. Heidelberg are serovars common to the top ten most-isolated serovars from humans and from
pigs. To achieve colonization, *Salmonella* must survive low stomach pH, compete with the resident microbiota for proximity to the intestinal mucosa, invade intestinal epithelia, and both combat and utilize the host immune system (136). To advance beyond enteritis and cause systemic disease, *Salmonella* must be internalized by host cells, primarily macrophages, and use them to obtain nutrients, as a replication reservoir and for systemic transport (154).

The rooting behavior of swine enhances fecal-oral disease transmission. Upon ingestion and sensing a decreased pH in the stomach, *Salmonella* induces an acid-tolerance response by transcription of genes that encode acid-shock proteins. These genes include *phoP*, *fur*, and *rpoS*, and encode proteins for antimicrobial peptide defense, uptake of the bacterial nutrient iron, and the stress response, respectively. Surviving the swine stomach is dependent upon these genes, as demonstrated by mutant studies (155). *Salmonella* enters the gastrointestinal tract by two hours post inoculation (156), and utilizes quorum sensing to identify the presence of other bacteria and the host environment. Quorum sensing by *Salmonella* in the gut occurs using a two-component system: QseC that receives a signal from the sampled environment, and QseB that responds to that signal by modulation of gene expression (157). Outer membrane proteins such as iroN, FEPA, and CirA also sense the environment (158). Norepinephrine is a host-produced catecholamine that is present in the swine gut. Although a host-protein, it can be beneficial to *Salmonella* by altering iron, making it an available bacterial nutrient (159). It also can be sensed by the
QseBC system to increase transcription of genes involved in the flagella and chemotaxis regulons to enhance bacterial motility when nutrients are available (151). Conversely, the QseBC system can also detect low levels of norepinephrine, and activation of the QseB component will repress this transcription.

As motility and growth increase, *Salmonella* gains closer access to gut epithelial cells by successfully competing with the resident microbiota for proximity, and by traversing through the mucus layer (136). Direct injection of *S. Typhimurium* into ligated ileal loops indicated that the bacteria can adhere to M cells within five minutes, and invade the membrane of these cells within another five minutes (160). M cells can also facilitate internalization of *Salmonella* by macropinocytosis as they directly sample the gut lumen for antigenic priming. *Salmonella* adheres to enterocytes, M cells and goblet cells by utilizing Type I and long polar fimbriae projections from its cell wall (158). Fimbriae attachment triggers transcription of the *Salmonella* pathogenicity island 1 (SPI-1) that encodes the Type III secretion system 1 (T3SS-1) required for invasion into the host cell. A needle complex is assembled from the inner bacterial membrane outward, and a translocation complex is inserted into the host enterocyte membrane. This insertion forms a pore through which chaperone proteins bring effector proteins to the needle complex. An ATPase system then exports effectors through the complex into the host cell. Effector proteins are also encoded by genes within SPI-1, and serve to promote bacterium uptake by the
host cell. SopE, and SopE2 are guanine nucleotide exchange factors that can activate host Rho GTPases by facilitating the release of GDP to allow binding of GTP (158). This action stimulates signal transduction pathways that result in the recruitment of actin reorganization complexes. SipA acts to increase the stability of actin filaments, while SipC bundles actin. Together, these actions result in membrane ruffling of the host cell, and Salmonella uptake.

Simultaneously, inflammatory responses are induced, either directly or indirectly, by bacterial invasion. Salmonella LPS ligates TLR4, resulting in the induction of inflammation. Activation of Rho GTPases can lead to the disruption of tight junctions between intestinal epithelial cells, resulting in increased epithelial barrier permeability. Their activation also leads to MAPK-signaling and nuclear translocation of transcription factors, such as NFκB and activator protein-1 (AP-1). The activation of these pathways results in production of proinflammatory mediators such as TNF-α and CXCL8, which mediate the pyrexia (TNF-α) and neutrophil recruitment (CXCL8) that are considered hallmarks of Salmonella infection (161). Intracellular PRRs such as the NLR family CARD domain-containing protein 4 (NLRC4) can also sense the flagella of internalized bacteria, and form an inflammasome complex of proteins that cleave the inactive, proform of caspase-1 (162). Upon cleavage, caspase-1 is activated, and it in turn cleaves the proforms of IL-1β and IL-18. These cytokines are secreted basolaterally from the epithelial cells and participate in facilitating pyrexia, appetite depression and myosin phosphorylation that opens
tight junctions (120). Combined with the influx of neutrophils, the opening of tight junctions facilitates an increase in fluid entering the bowel, resulting in the characteristic diarrhea of porcine salmonellosis. To combat this, *Salmonella* effector proteins are capable of dampening and negating inflammatory signals within a host cell. SptP can act as a GTPase activating proteins, enhancing the hydrolysis of GTP to GDP and inhibiting the signaling pathways that lead to production of proinflammatory mediators. SspH1 and AvrA can also act directly on the NFκB pathway to inhibit gene expression and activity (158).

In addition to neutrophils, other leukocytes migrate to the gut in response to chemokines gradients and join the resident populations located there. Macrophages and DCs phagocytose the bacteria, encapsulating it into a phagosome (163). These APCs attempt to acidify the phagosome by forming a phagolysosome to break down the pathogen into antigenic epitopes. The phagolysosome then fuses with an SLA-II-containing endosome for SLA-II loading, and the SLA-II-antigen complex is then trafficked to the cell surface for antigen presentation. The APC migrates to the mesenteric lymph node and secretes chemokines such as CCL19 and CCL21 to attract naïve CCR7+ T cells to the parafollicular zones of the lymph nodes (7). Here, the APC can prime the T cells and stimulate at Th1-mediated response.

*Salmonella* has been demonstrated subvert the host immune response by inhibition of phagosome maturation and surviving through lysosome fusion (164), resulting in a compartment termed a *Salmonella*-containing vacuole.
(SCV). The survival of the acidic phagolysosome is largely depending on resistance to antimicrobial peptides, nitric oxide and oxidative killing, as *Salmonella* mutants have attenuated virulence. Systems such as the PhoQ sensor respond to the acidic environment by upregulating survival genes that inhibit nitric oxide formation, degrade antimicrobial peptides and inhibit cellular apoptosis (165). The bacterium undergoes extensive surface remodeling to alter the presence of PAMPs that would continue to trigger an inflammatory response. PhoQ facilitates this by decreasing O-antigen length and altering the lipid A portion of LPS (166). Genes of the *Salmonella* pathogenicity island (SPI-2) are transcribed, and the second T3SS complex is formed and effectors are produced. These effectors are translocated outside the vacuole where SifA and PipB2 mediate filament formation along microtubules for vesicular trafficking (158). SseF and SseG further facilitate this trafficking, and bundle actin to move adjacent to the SCV and direct amino acids and lipids toward it for use in replication. In enterocytes, the SCV also utilizes these microtubules to move toward the basement membrane, where it will fuse with the membrane and enter the lamina propria of the gut (167). Resident macrophages and DCs can phagocytose the bacterium here, similar to after M cell macropinocytosis.

The porcine response to *Salmonella* Typhimurium

Studies to examine the porcine response to *Salmonella* have resulted in further understanding of the pathogenesis of *Salmonella*. Acute phases studies
demonstrated that oral inoculation of five-week-old barrows with $3 \times 10^9$ colony forming units (CFU) of S. Typhimurium increased rectal temperatures by 12 hours post inoculation (h.p.i.), and these temps remained elevated for 5 days (168). Feed intake was depressed by 48h and continued to remain low, and decreased body weights reflected this for 2 weeks post inoculation (p.i.). Peripheral blood concentrations of insulin-like growth factor I (IGF-I) were also depressed, while cortisol concentrations were increased.

Rostagno, et al. (2011) demonstrated that finishing pigs inoculated with S. Typhimurium began shedding the bacteria within 2 h.p.i. Tissue specific inflammatory responses were observed, as TNF-$\alpha$ was increased by 24 h.p.i in the ileum, by 48 h.p.i. in the mesenteric lymph node and remained elevated at 3 weeks p.i. in the ileum, spleen and cecum. These pigs continued to shed S. Typhimurium for 4 weeks p.i., but levels dropped dramatically by 5 weeks p.i.

*In vitro* studies have also demonstrated the proinflammatory effects of S. Typhimurium. Inoculation of porcine jejunal epithelial IPEC-J2 cells seeded onto permeable membrane supports showed both an apical and basolateral secretion of CXCL8 in response to S. Typhimurium inoculation (169). The genes encoded by SPI-1 have profound effects on porcine alveolar macrophage polarization, as demonstrated by SPI-1 mutant *in vitro* studies (170). Alveolar macrophages were polarized toward an alternatively activated, or M2, phenotype in the presence of S. Typhimurium as assessed by M2-characteristic transcriptional patterns. This polarization was lost in SPI-1 mutants, indicating that S.
Typhimurium promotes the less microbicidal M2 phenotype through effector proteins encoded in SPI-1.

Transcriptional studies in swine have further enhanced the knowledge of the porcine immune response to *Salmonella*, both locally and peripherally. The transcriptional response of mesenteric lymph nodes was examined in pigs experimentally inoculated *S. Typhimurium*, and indicated distinct gene expression patterns (171). Seven-week-old pigs intranasally challenged with $1 \times 10^9$ CFU of *S. Typhimurium* had 100 differentially expressed genes as early as 8 h.p.i. when compared to non-inoculated control animals. Differences in expression continued at 24 h.p.i., 48 h.p.i., and at 21 d.p.i., resulting in 848 genes showing differential expression when comparing across timepoints or when comparing to controls. Annotation of these genes indicated a shift over time, with genes involved in pathways for apoptosis and antigen presentation/DC function downregulated at 8 h.p.i., and an upregulation of the Th1 response, innate/inflammatory response, and antigen-processing at 24 and 48 h.p.i. NFκB-target genes were also induced in a time-course fashion, with induction at 24 h.p.i. and suppression by 48 h.p.i. The authors hypothesized that this NFκB suppression may be a mechanism by which *S. Typhimurium* is able to colonize long-term and suppress the immune response in the lymph node.

Similar studies examining the host transcriptional response have confirmed a difference in host response to *S. Typhimurium* and *S. Choleraesuis*. Physiologic studies indicate a divergent response after inoculation in pyrexia,
growth and circulating hormones (172), and gene expression studies of the mesenteric lymph node indicate different kinetics of gene expression between the two serovars, with Choleraesuis-inoculated pigs having a more robust response in inflammatory genes at later times points (48 h-21 d) (173).

It is well established that pigs within a herd will shed *Salmonella* at different levels, with some pigs clearing infection quickly, and others developing a carrier status. Uthe, et al. (2009) demonstrated a positive correlation between *S. Typhimurium* shedding and circulating IFN-γ concentrations at 2 and 7 d.p.i. Circulating neutrophils were also positively correlated with peripheral IFN-γ, indicating a systemic response to infection. Within the same study, a single nucleotide polymorphism (SNP) in the CCT7 gene was associated with *S. Typhimurium* shedding, potentially indicating a genetic mechanism behind the divergent responses. Further identification of SNPs associated with *Salmonella* shedding was conducted on 377 pigs from 3 independent populations (175). Thirteen SNPs were associated with *Salmonella* shedding or tissue colonization, including those found in genes associated with the immune response.

Further characterization of the divergence in *Salmonella* shedding and the porcine immune response has indicated distinct differences in gene pathways and biomarkers (176). Seven-week-old pigs were inoculated with *S. Typhimurium* and fecal shedding of the bacteria was measured over a 20-day period. The extremes of the population were classified into two phenotypes, low shedding (LS) or persistently shedding (PS), based upon their cumulative
Salmonella shedding over time. Global transcriptional differences were identified in the peripheral blood between the two phenotypes and in response to infection (d0 vs. d2). Within the first pig population, the PS pigs had a more robust transcriptional response to infection, with 2,647 differentially expressed genes compared to 545 in the LS pigs. At 2 d.p.i, there was also a large difference in expression between LS and PS pigs (1,071 differentially expressed genes), indicated a divergent response linked to differences in shedding. In both sets of pigs, genes involved in responding to IFN-γ were upregulated by 2 d.p.i., including IL18, TNFA, CD14 and IRF1. Pathway analysis of differentially expressed genes indicated TLR and IFN-γ signaling pathways were prominent in the PS animals, demonstrated by an increase in expression of NF-κB subunits, TLR4 and MD2, among others. Two regulatory factors that control hematopoiesis and some cytokine production, SPI1 and CEBPB respectively, were also upregulated in response to infection, although CEBPB was only identified in the PS pigs. A miRNA known to inhibit these regulators, mir-155, was low in PS pigs, providing further support for pathway integration and the physiologic effects these pathways can exhibit in Salmonella shedding. Taken together, the authors concluded that porcine whole blood can be utilized as a source for identifying biomarkers and pathways that are regulated divergently in pigs that shed different levels of Salmonella Typhimurium after inoculation.

A similar utilization of whole blood transcriptomics in humans has led to identification of disease biomarkers, therapeutic targets and divergent responses
to PAMPs and pathogen challenges (177). Ex vivo experiments stimulated human whole blood from 102 subjects with LPS and measured gene expression to identify the variability in inflammatory responses (178). High and low responders were classified at the extremes of the measured population by the amount of proinflammatory cytokines they produced. Measurements of IL-1β, IL-6, CXCL8, IL-10, and TNF-α all indicated differences in high versus low responders. Gene expression analysis of the whole blood also demonstrated 80 differentially expressed genes between the two groups after LPS stimulation. Moreover, 21 genes were differentially expressed prior to LPS stimulation, indicating an inherent difference between the two groups. The authors concluded that analysis of differential expression in whole blood maybe be a practical approach to identifying distinct populations with variation in response to LPS.

The use of whole blood transcriptomics to profile the systemic immune response has also been utilized as an indicator of disease severity. An 86-transcript signature was identified to discern active tuberculosis infection from other diseases. Moreover, a 393-transcript signature was developed to further classify an intermediate to high burden of infection, as this signature was highly correlated with radiological extent of disease (179). Upon pathway analysis, it was determined that this signature largely consisted of neutrophil-driven, interferon-inducible genes. These results highlighted the capabilities of whole blood transcriptomics for understanding the systemic immune response to a
specific disease, as well as a potential diagnostic and prognostic tool. As a practical means for repeated sampling of the same animal, whole blood transcriptomics in swine is continuing to be developed to further understand the systemic immune response to pathogen challenge, as well as to monitor disease severity and response to treatment.
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CHAPTER 2

SWINE BARN DUST EXPOSURE IMPAIRS PORCINE MACROPHAGE FUNCTION: IMPLICATIONS FOR RESPIRATORY HEALTH

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Abstract

Respiratory diseases are responsible for a significant amount of animal morbidity and mortality in the swine industry, including the majority of nursery and grower/finisher deaths. Innate immunity, including the maintenance of lung macrophage health and function, is an important defense mechanism against respiratory pathogens and their associated losses. Chronic exposure of swine industry workers to airborne barn dust results in significant predisposition to airway diseases and impairment of alveolar macrophage (AMφ) function. Because of their importance in maintaining normal respiratory function, this study was designed to evaluate the impact of barn dust on swine macrophages. As measures of macrophage function, we evaluated the activation of NF-κB, cytokine production, cell surface marker expression and the phagocytic and antibacterial capabilities of porcine macrophages after in vitro exposure to an organic swine barn dust extract (ODE). ODE treatment induced AMφ secretion of both pro- and anti-inflammatory cytokines, suggesting a complex activation profile. Additionally, ODE induced expression of genes (TLR2, NOD2) involved in sensing Gram-positive bacteria, a major component of barn dust. ODE exposure also enhanced the expression of several cell surface markers of activation, including a receptor for the porcine reproductive and respiratory syndrome virus. Moreover, two key functions of AMφ, phagocytosis and bacterial killing, were impaired after exposure to ODE. Treatment with ODE for the first 72 h of differentiation also inhibited the ability of monocyte-derived macrophages to
translocate NF-κB to the nucleus following endotoxin stimulation. Taken
together, these results demonstrate, for the first time, that organic dust extract
exposure negatively affects pig macrophage activation and function, potentially
enhancing host susceptibility to a variety of respiratory infections.

Keywords
Dust; Pig; Macrophage; Airway inflammation; Respiratory disease

1. Introduction

In the swine industry, respiratory diseases account for the highest
percentage of all nursery deaths, cause the majority of grower/finisher deaths
(NAHMS, 2006) and contribute to costly production losses by decreasing feed
intake and average daily gain (Jericho and Harries, 1975; van Reeth and
Nauwynck, 2000). The etiology of respiratory disease is complex, and
susceptibility to infection may be complicated by a variety of environmental and
pathogenic factors, including exposure to swine barn dust. Indeed, conditions
known to contain high levels of swine barn dust impair human lung function, and
swine confinement operation employees are significantly predisposed to airway
diseases, including rhinitis, bronchitis and chronic obstructive pulmonary disease
(Von Essen and Romberger, 2003).

Swine barn dust is composed of a myriad of components derived from
feed, dander, fecal waste, microbial particles and other sources capable of
stimulating immune responses. Alveolar macrophages (AMφ) are among the first immune cells to respond to these inhaled particles (Poole and Romberger, 2012). Microbial constituents of organic dust are rich in highly conserved pathogen-associated molecular patterns (PAMPs) recognized by host pattern recognition receptors (PRRs) present on AMφ and other antigen presenting cells. Signaling cascades initiated via these PRRs ultimately activate cellular inflammatory responses (Barton and Medzhitov, 2002; Poole and Romberger, 2012). The non-allergic inflammation elicited by inhaled dust is accompanied by local and systemic production of inflammatory cytokines, such as TNF-α, IL-1β and the chemoattractant CXCL8, resulting in pyrexia, enhanced mucus production and neutrophil influx into the airways (Larsson et al., 1997; Sahlander et al., 2012; Wang et al., 1998). Exposure to swine barn organic dust extract (ODE) in vitro impaired human and murine macrophage function (Poole et al., 2008) and altered human dendritic cell maturation (Poole et al., 2009a). In vivo, dust inhalation has been directly linked to increased airway inflammation and lung pathology in mice, humans and pigs (Donham et al., 1995; Poole et al., 2009b; Urbain et al., 1999).

To date, few studies have examined the impact of chronic barn dust exposure on the swine immune system, and none have attempted to directly test whether dust impairs porcine macrophage phenotype or function. We sought to define the functional alterations in cytokine production, cell surface marker expression and phagocytosis of pig AMφ exposed to ODE obtained from swine
barns. ODE exposure induced both pro- and anti-inflammatory cytokine production, enhanced surface expression of activation markers and enhanced the expression of genes involved in sensing Gram-positive bacteria. Both phagocytosis and bacterial killing were diminished following ODE treatment. Moreover, ODE exposure during the early differentiation of monocyte-derived macrophages (MDMs) reduced translocation of nuclear factor kappa B (NF-κB) to the nucleus following endotoxin stimulation. Together, these data demonstrate that swine barn ODE suppresses macrophage function. Considering that respiratory immunity must be optimal for ensuring disease resistance and efficient growth in today’s modern swine production facilities, barn dust exposure may be an underappreciated underlying cause of porcine respiratory disease outbreaks.

2. Materials and methods

2.1. Organic dust extract (ODE)

ODE was a kind gift from J. A. Poole, University of Nebraska Medical Center; it was collected, prepared and analyzed for composition as previously described (Poole et al., 2012; Poole et al., 2007; Romberger et al., 2002). Briefly, settled dust was collected three feet above the floor from a swine confinement facility of 500-700 animals. Dust samples were solubilized, vortexed and centrifuged. The supernatant was filter-sterilized (0.22 μM) and frozen (-20°C) until use. The collected dust was analyzed via gas chromatography-
tandem mass spectrometry; results were consistent with previous reports (Poole et al., 2007). Analysis revealed high muramic acid (424.0 pmol/mg ± 17.7 pmol/mg), a component of peptidoglycan, high 3-hydroxy fatty acid (3109.8 ng/mg ± 152.6 ng/mg), a component of endotoxin, and low ergosterol (9.3 pmol/mg ± 0.4 pmol/mg), a component of fungi. The aqueous dust extract was diluted to 12.5% (vol/vol) in sterile PBS for analysis of protein and endotoxin concentrations, and independent batches of ODE were prepared and tested. The range of diluted extract contained 2.91-3.88 mg/mL of total protein and 22.1-91.1 EU/mL of endotoxin as measured by the limulus amebocyte lysate assay (Sigma, St. Louis, MO). ODE concentrations of either 0.1% or 1% were employed to conservatively model low swine barn dust exposure conditions (Poole et al., 2008; Poole et al., 2009a).

2.2. Animals, lavage and macrophage culture

Ten pigs between 8-12 weeks of age of either sex were euthanized with an overdose of sodium pentobarbital given intravenously according to Iowa State University Laboratory Animal Resources experimental guidelines. The IACUC at Iowa State University approved all protocols involving animals. Lungs were removed and lavaged twice with cold PBS. Lavage fluid was centrifuged at 500 x g for 15 min; cell pellets were pooled and washed once in cold PBS. Erythrocytes were lysed in ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Cells were washed again in cold PBS, resuspended in complete
tissue culture media (CTCM; RPMI 1640, 5% heat-inactivated normal swine serum (Sigma-Aldrich, St. Louis, MO), 5 mM HEPES, 1 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 15 µg/mL gentamicin sulfate), plated in 150 x 15 mm tissue culture-treated dishes and allowed to adhere for 2 h at 37°C with 5% CO₂. After 2 h, non-adherent cells were removed and discarded. Adherent cells were harvested by scraping and then washed and counted via trypan-blue exclusion. AMϕ were plated in duplicate wells within pig and treatment at a density of 5 x 10⁵ per well in 24-well plates. Cells were cultured in the presence of 10 µg/mL Salmonella enterica serovar Typhimurium χ4232 endotoxin ODE as a percentage of total culture volume as indicated or a medium only equivalent. No evidence of cell death was observed following incubation with any of the treatments.

Monocytes were isolated from whole blood via density gradient centrifugation to obtain porcine monocyte-derived macrophages (MDMs). Briefly, peripheral blood was diluted 1:2 (v:v) in sterile PBS, overlayed onto Lymphocyte Separation Media (LSM; Mediatech, Manassas, VA) and centrifuged at 500 x g for 30 min. Peripheral blood mononuclear cells (PBMC) were obtained from the LSM/plasma interface and washed twice with sterile PBS. Monocytes were further enriched to > 95% purity by positive magnetic bead selection as previously described by Bimczok et al. (2007) with some modifications. Briefly, PBMC were labeled with a primary antibody against CD172a (SWC3a; clone 74-22-15A; BD Biosciences, San Jose, CA) at a final concentration of 0.5 µg/µL for
30 min at 4°C with gentle agitation. After two washes in PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA), PBMC were labeled with anti-mouse IgG MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's recommendations and sorted with an AutoMACS automated magnetic cell sorter (Miltenyi Biotec). Monocytes were plated at a density of $5 \times 10^5$ per well of a 24-well plate in Dulbecco's modified eagle's medium containing 4.5 mg/mL glucose, 10% heat-inactivated normal swine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 25 mM HEPES and 0.05 µM 2-mercaptoethanol). Medium was supplemented with 30% conditioned media from confluent cultures of L929 fibroblasts to serve as a source of Colony Stimulating Factor (CSF) to induce monocyte differentiation into macrophages. Microscopic identification and flow cytometry were used to assess macrophage differentiation.

2.3 RNA isolation and reverse transcription-PCR

$\text{AM}\Phi$ were harvested after 5 h in culture with treatments as indicated. Total RNA was isolated and purified using an RNeasy Mini kit and DNase I kit (Qiagen, Valencia, CA), and the quantity and quality of RNA was measured using Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Resulting RNA was reverse transcribed into cDNA using 200 ng of total RNA with a SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). Primer designs were obtained from the Porcine Immunology and Nutrition (PIN) database.
(Dawson et al., 2005). Real-time PCR was performed using a SYBR Green PCR kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instructions. Assays were performed in triplicate. Average CT values of duplicate wells were compared to a standard curve for each gene to determine relative expression normalized to expression of RPL32 and ACTB genes.

2.4. Flow cytometry

Macrophages were washed in PBS containing 0.1% NaN3 and 0.1% BSA, blocked using 10 µL normal swine serum and stained for 15 min on ice with monoclonal antibodies against swine workshop cluster 9 (SWC9; CD203a; clone PM 18-7; AbD Serotec, Oxford, UK), monocyte differentiation antigen CD14 (CD14; clone CAM36A; VMRD, Pullman, WA), scavenger receptor cysteine-rich type 1 protein M130 (CD163; clone 2A100/11; AbD Serotec) and swine leukocyte Ag II (SLA-II; clone 2E9/13, AbD Serotec). Irrelevant isotype controls for IgG1 (clone 14-4714, eBioscience, San Diego, CA) and IgG2a (clone 14-4724, eBioscience) were also used to account for non-specific binding. Cells were then washed and stained with either phycoerythrin-conjugated anti-IgG1 or FITC-conjugated anti-IgG2 fluorescent secondary antibodies for 15 min on ice before fixing in BD Stabilizing Fixative (BD Biosciences) as per the manufacturer’s instructions. Cells were acquired on a FACSCanto flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (TreeStar, Ashland, OR). Surface marker expression was reported as mean
fluorescence intensity (MFI) minus isotype MFI to account for non-specific binding. To measure macrophage phagocytosis, 0.2 µM FITC-loaded FluoSpheres (1:1,000 dilution; Invitrogen) were added to macrophage cultures and incubated for 1 h at 37°C. Cells were then placed on ice, harvested via scraping, washed to remove any extracellular particles and fixed in 1% paraformaldehyde prior to analysis via flow cytometry.

2.5. Cytokine analysis

Cell-free culture supernatants were collected and pooled from duplicate wells prior to analysis via a multiplex cytokine assay as described in Lawson et al. (2010) with some modifications. Briefly, magnetic microspheres (Luminex Corporation, Austin, TX) were covalently coupled to capture antibodies against porcine IL-1β (clone DY681 DuoSet, R&D Systems, Minneapolis, MN), CXCL8 (clone 8M6, AbD Serotec), IL-10 (clone 945A4C437B1, Invitrogen), IL-12p40 (clone MCA2414Z, AbD Serotec), IFN-γ (clone A151D5B8, Invitrogen) or TNF-α (clone CSC1753 Detection Kit, Invitrogen). Coupled microspheres for each cytokine were added at a density of 2.5 x 10^3 per well of a 96-well plate and washed twice in wash buffer (PBS, 0.7% Tween-20) on a Bio-Plex Pro Washing Station (Bio-Rad Laboratories, Hercules, CA). Cell-free supernatants were diluted 1:2 in assay buffer (PBS, 1% BSA, 0.05% NaN₃) and 50 µL was added to each well prior to incubation for 2 h. All incubations were performed in the dark at room temperature on a plate shaker at a rotation speed of 3,000 rpm. The
plate was washed three times, and biotinylated detection antibodies against each cytokine were added (IL-1β, 2 µg/mL, clone DY681 DuoSet, Invitrogen; CXCL8, 0.5 µg/mL, clone 105105, Invitrogen; IL-10, 0.5 µg/mL, clone 945A1A926C2, Invitrogen; IL-12p40, 1 µg/mL, clone BAM9122, R&D Systems; IFN-γ, 0.5 µg/mL, clone A151D13C5, Invitrogen; TNF-α, clone CSC1753b, Invitrogen). Following a 1 h-incubation, the plate was washed three times and 50 µL of 10 µg/mL streptavidin-PE conjugate (eBioscience) was added. Following a 30-min incubation, the plate was washed three times, 125 µL of assay buffer was added to each well, and incubated for 3 min with shaking to allow for microsphere resuspension. Cytokine concentrations were then measured using a Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, CA).

2.6. Bacterial culture, phagocytosis and killing assays

A field isolate of Salmonella enterica serovar Cholerasuis cultured from the respiratory tract of an infected pig was a kind gift from R. W. Griffith, Iowa State University. Bacteria were grown on either 5% blood agar plates or cultured in brain heart infusion (BHI) broth.

Phagocytosis assays were performed as previously described (Poole et al., 2009a) with some modification. Bacterial cultures were opsonized with heat-inactivated normal swine serum for 30 min. Macrophages were plated in CTCM without antibiotics, inoculated at a multiplicity of infection of 100:1 (bacteria:macrophages) and incubated for 30 min at 37°C with 5% CO₂. Cultures
were placed on ice, medium removed, and 500 µL of complete culture medium added. Macrophages were harvested via scraping and divided into two aliquots designated as t0 and t120. Aliquots for t0 were lysed immediately by incubating in 1% saponin on ice for 15 min, and the lysate plated in duplicate in serial dilutions on 5% blood agar plates. Aliquots for t120 were incubated with occasional agitation for 120 min at 37°C with 5% CO₂, after which they were lysed and plated as described for t0 aliquots. Phagocytosis was measured by recording the CFU from t0 aliquots, and percent killing was calculated as 

\[
\left( \frac{\text{cfu at t0} - \text{cfu at t120}}{\text{cfu at t0}} \right) \times 100
\]

2.7. Nuclear translocation assay

Monocytes (see section 2.2) were cultured either in the presence or absence of 1% ODE during the first 72 h of differentiation. After a total of 6 d in culture, MDMs were stimulated with 10 µg/mL of \textit{Salmonella} endotoxin or an equal volume of medium alone (No Stim) for 1 h at 37°C to induce NF-κB translocation. Cells were then fixed and labeled with an anti-NF-κB p65 polyclonal antibody (sc-372, Santa Cruz Biotechnology, Inc. Dallas, TX) for 20 min followed by a secondary goat anti-rabbit IgG (A-21422, Invitrogen) antibody conjugated with Alexa Fluor 555. Immediately prior to data acquisition, DRAQ5 (BioStatus, Leicestershire, UK) was added to label the nucleus. Nuclear translocation of NF-κB was visualized by multi-spectral imaging flow cytometry (Amnis ImageStreamX, Seattle, WA). Similarity Scores were calculated by
extrapolation of the log transformed Pearson’s Correlation Coefficient of image pixel intensity for NF-κB and the nucleus (Ideas software, Amnis). NF-κB was considered to have translocated to the nucleus in images with a calculated Similarity Score $\geq 1$. Percent NF-κB translocation for the population was determined by identifying the number of cells with a Similarity Score $\geq 1$ then dividing by the total number of cells in the acquired population.

2.8. Statistics

Data were analyzed using the GLIMMIX procedure of SAS (Version 9.2, SAS Institute, Cary, NC) with treatment as a fixed effect and pig as the subject of repeated measures. For nuclear translocation data analyses, fixed effects were treatment and replicate. Gaussian distribution of response variables was assumed. Least square means were calculated and treatments were compared using the SLICE and SLICEDIFF procedures. An adjusted $p$-value was calculated by using Tukey corrections for multiple comparisons among treatments. Differences were considered to be significant if adjusted $p$-values < 0.05.

3. Results

3.1. Organic dust extract matured and activated AM$.phi.$

Analysis of surface marker expression can be used to characterize the phenotype of a cellular population and/or identify alterations in cellular activation.
For example, human cells have been shown to adopt a more activated phenotype (e.g., increased expression of CD25, CD69 and HLA-DR) after initial exposure to ODE (Bailey et al., 2008; Muller-Suur et al., 1997). After 24 h in culture with medium alone, endotoxin, 0.1% ODE or 1% ODE, cell surface marker expression of AMφ was assessed via flow cytometry. Compared to all other treatments, exposure to 1% ODE significantly increased AMφ expression of SWC9 ($p < 0.05$), a pyrophosphatase widely used as a marker of macrophage maturity (Ezquerra et al., 2009) (Fig. 1A). Similarly, treatment with 1% ODE significantly enhanced surface expression of SLA-II ($p < 0.05$), the porcine homolog of MHCII (Fig. 1B). Both the 0.1% and 1% ODE treatments increased CD163 expression when compared to either medium only ($p < 0.001$) or endotoxin ($p < 0.01$) (Fig. 1C). This is a significant finding, as CD163 has been identified as a receptor facilitating porcine reproductive and respiratory syndrome virus (PRRSv) entry into macrophages, and its expression level is positively correlated with PRRSv replication (Patton et al., 2009). Exposure to ODE did not significantly effect surface expression of CD14 (Fig. 1D). These results indicate that ODE exposure enhances the maturation and activation of AMφ and concomitantly increases CD163 expression, potentially making them more susceptible to PRRSv infection.
**Figure 1. Organic dust extract enhanced maturation and activation of AMφ.**

AMφ were cultured in medium only, 10 μg/mL endotoxin, 0.1% ODE or 1% ODE for 24 h, labeled with antibodies against (A) SWC9, (B) SLA-II, (C) CD163 or (D) CD14 and analyzed via flow cytometry. Data are reported as the mean fluorescence intensity (MFI) minus isotype MFI and presented as the mean ± the SEM of two independent experiments with five pigs per experiment. Treatments with different letters are significantly different from one another at $p < 0.05$.

Representative histograms of treatments and isotype control for each surface marker are shown.

3.2. ODE exposure induced AMφ production of both pro- and anti-inflammatory...
Cytokine production by AM\(\Phi\) exposed to ODE for 24 h was measured via a multi-plex array to assess induction of an inflammatory response. Exposure to ODE induced a dose-dependent secretion of both IL-1\(\beta\) and TNF-\(\alpha\) \((p < 0.05, p < 0.0001;\) Figs. 2A & B). AM\(\Phi\) cultured with 1% ODE produced equivalent levels of IL-1\(\beta\) and significantly more TNF-\(\alpha\) as compared to AM\(\Phi\) stimulated with endotoxin \((p < 0.05)\). Treatment with 1% ODE significantly enhanced CXCL8 (IL-8) secretion \((p < 0.05)\), a chemokine mediating neutrophil recruitment to sites of inflammation (van Reeth and Nauwynck, 2000) (Fig. 2C). Culture of AM\(\Phi\) with either 0.1% or 1% ODE increased production of the pro-inflammatory mediator IFN-\(\gamma\) \((p < 0.05, p < 0.01;\) Fig. 2D). Of interest, AM\(\Phi\) exposed to 1% ODE also markedly increased secretion of IL-10 \((p < 0.01)\), an anti-inflammatory cytokine (Fig. 2E). Endotoxin treatment of AM\(\Phi\) induced production of IL-1\(\beta\), TNF-\(\alpha\) and IFN-\(\gamma\) when compared to medium alone \((p < 0.05)\). IL-12p40 was undetectable for all treatments (data not shown).
Figure 2. ODE exposure induced AMφ production of both pro- and anti-inflammatory cytokines. Analysis of cytokine concentrations in cell-free supernatants via multi-plex fluorescent bead assay for porcine (A) IL-1β, (B) TNF-α, (C) CXCL8, (D) IFN-γ and (E) IL-10. Data are presented as the mean ± the SEM of two independent experiments with five pigs per experiment. Treatments with different letters are significantly different from one another at \( p < 0.05 \).

Expression of IL1B, TNFA, IL8, IFNG, and IL10 RNA was assessed at 5 h post-treatment to support cytokine secretion data. All observed cytokine production responses to ODE and endotoxin were confirmed at the gene expression level (Figs. 3A-E) with the exception of IFNG RNA, which did not
increase following ODE exposure (Fig. 3F). It is possible that macrophage gene expression of IFNG was enhanced in response to dust at a point before or after the cells were harvested at 5 h to yield the secreted IFN-γ measured at 24 h. Others have demonstrated differences in the kinetics of IFNG expression and IFN-γ production (Ye et al., 1995), as well as negative regulation of IFNG RNA by microRNA (Rossi et al., 2011); however, it is unclear why these differences were observed for this cytokine only. Taken together, these results show AMφ produce both pro- and anti-inflammatory cytokines in response to ODE, demonstrating a complex activation profile.

**Figure 3. Gene expression analysis supports cytokine secretion profiles.**

Gene expression of (A) IL1B, (B) TNFA, (C) IL8, (D) IFNG and (E) IL10 was assessed at 5 h post-treatment with medium alone, endotoxin, 0.1% ODE or 1% ODE. Average CT values of duplicate wells were compared to a standard curve.
for each gene to determine relative expression, and expression was normalized to RPL32 and ACTB genes. Data are presented as the mean ± the SEM of two independent experiments with five pigs per experiment. Treatments with different letters are significantly different from one another at \( p < 0.05 \).

3.3. ODE induced expression of genes involved in sensing Gram-positive bacteria.

To identify potential signaling mechanisms involved in the AMφ response to ODE, we evaluated TLR2, TLR4, NOD2 and CXLC2 expression. After 5 h in culture, cells were harvested, total RNA was extracted and reverse transcribed, and the resulting cDNA was used for quantitative PCR. CXCL2 expression was increased in response to both endotoxin and 1% ODE (\( p < 0.05 \); Fig. 4A). Expression of TLR2 was significantly increased after exposure to 0.1% ODE (\( p < 0.05 \); Fig. 4B); however, there were no significant differences in TLR4 expression following treatment with ODE (Fig. 4C). NOD2 expression was significantly enhanced in response to 1% ODE (\( p < 0.01 \); Fig. 4D). These results indicate ODE treatment increased gene expression of PRRs ligated by components of Gram-positive bacteria as well as the neutrophil chemoattractant CXCL2.
Figure 4. ODE induced expression of genes involved in sensing Gram-positive bacteria and neutrophil chemotaxis. Gene expression of (A) CXCL2, (B) TLR2, (C) TLR4 and (D) NOD2 in response to medium, endotoxin, 0.1% ODE or 1% ODE after 5 h. Average CT values of duplicate wells were compared to a standard curve for each gene to determine relative expression, and expression was normalized to RPL32 and ACTB genes. Data are expressed as the mean ± the SEM of two independent experiments with five pigs per experiment. Treatments with different letters are significantly different from one another at $p < 0.05$.

3.4. Exposure to organic dust diminished AMφ phagocytic ability and bacterial
killing capacity.

Because they are key aspects of macrophage function, the phagocytic and microbicidal activities of porcine AMφ were examined following ODE treatment. Phagocytic ability was measured by uptake of 0.2 mm microspheres for 30 min. Exposure to either 0.1% or 1% ODE significantly diminished AMφ phagocytosis as compared to endotoxin stimulated or unstimulated cells ($p < 0.05$, $p < 0.01$; Fig. 5A). A portion of the AMφ population treated with ODE appeared to not phagocytose any microspheres, as the left-most portion of the histogram trace for those cultures was identical to that of AMφ cultured in the absence of particles (Fig. 5B).

To assess bacterial killing capacity, AMφ were treated with medium alone, endotoxin or ODE for 24 h and then cultured with a respiratory isolate of a common swine pathogen, *S. enterica* serovar Cholerasuis. After a 30 min incubation to allow for phagocytosis, AMφ either lysed immediately or after an additional 120 min to evaluate killing of intracellular bacteria. Treatment with 1% ODE significantly impaired bacterial killing when compared to media alone (87.61% vs. 99.11%, $p < 0.05$) (Fig. 5C). Together, these data indicate that exposure to ODE impairs phagocytosis and bacterial killing, two key functions of AMφ in protecting the lung from pathogens and inhaled particles.
Figure 5. Exposure to organic dust diminished phagocytic ability and bacterial killing capacity. (A and B) Flow cytometric analysis of AM\(\phi\) phagocytic activity after treatment with medium, endotoxin, 0.1% ODE or 1% ODE for 24 h. (A) Phagocytic activity reported as the mean fluorescence intensity (MFI). (B) Representative histogram of AM\(\phi\) fluorescence intensity; brighter intensities represent higher levels of phagocytosis. (C) Analysis of intracellular bacterial killing following pretreatment with either medium only, endotoxin, 0.1% ODE or 1% ODE for 24 h. AM\(\phi\) were inoculated with Salmonella enterica serovar Choleraesuis for 30 min. AM\(\phi\) were treated with antibiotics and lysed either immediately (t0) or after 120 min (t120) to assess killing. The number of intracellular bacteria was quantified via plate counts. Percent killing was calculated as \[\frac{(\text{cfu at t0} - \text{cfu at t120})}{(\text{cfu at t0})} \times 100\]. For panels B and C, data
are expressed as the mean ± the SEM of two independent experiments with five pigs per experiment. Treatments with different letters are significantly different from one another at $p < 0.05$.

3.5. Differentiation in the presence of organic dust impaired nuclear translocation of NF-κB.

We next assessed the effect of early ODE exposure on NF-κB translocation. NF-κB activation is critical for mediating inflammatory responses and its inhibition often promotes attenuation. After differentiation in the presence of either ODE or medium only, MDMs were stimulated for 1 h with endotoxin or medium alone (No Stim) induce NF-κB translocation. Treatment with 1% ODE inhibited the ability of MDMs to translocate NF-κB to the nucleus after endotoxin stimulation when compared to differentiation in medium alone (39.25% versus 66.49%, $p < 0.05$) (Fig. 6A). Representative single-cell images of each treatment are shown (Fig. 6B). These results demonstrate that ODE exposure during the first 72 h of macrophage differentiation impairs NF-κB activation in response to stimulation.
Figure 6. Exposure to ODE during differentiation impaired NF-κB activation in response to stimulation. MDM were differentiated in the presence or absence of ODE and then stimulated for 1 h with endotoxin or medium alone (No Stim) induce NF-κB translocation. Cells were labeled with an anti-NF-κB antibody (green) and nuclei stained with DRAQ5 (red). Nuclear translocation of NF-κB (yellow) was assessed via multi-spectral imaging flow cytometry. (A) Representative images for all treatments. (B) Percentage of cells positive for nuclear translocation of NF-κB. Data are presented as the mean ± the SEM of
three independent experiments. Asterisk (*) indicates statistically significant
difference from cells differentiated in medium and stimulated with endotoxin at $p < 0.05$.

4. Discussion

Inhalation of organic swine barn dust induces an inflammatory response
capable of promoting respiratory diseases in humans. Approximately 60% of all
individuals who work in confined animal feeding operations develop at least one
respiratory disease within six years of employment (Donham et al., 1989). The
pigs housed in these barns are constantly exposed to the same organic dust that
elicits these responses in humans; however, the effects of dust on swine
respiratory health are less understood. Given the critical role of AM\(\phi\) in
maintaining respiratory health, we examined the \textit{in vitro} effects of swine barn
dust extract on porcine macrophages and demonstrate adverse effects on their
phenotype, activation and function.

In the present study, ODE enhanced surface expression of SWC9, SLA-II
and CD163. The increase in expression of SWC9 (also known as CD203a) may
be indicative of transient maturation of AM\(\phi\) upon ODE stimulation. Previous
studies indicated that although macrophages are CD203a\(^+\), the presence of
CD203a\(^-\) macrophage-like cells recruited to sites of early inflammation in the
lung may actually be monocytes in the process of differentiation (Ondrackova et
al., 2010). Upon isolation of alveolar macrophages, others have demonstrated
slight variation in degrees of SWC9 expression, although all cells in the population were SWC9⁺ (McCullough et al., 1999). Similarly, the alveolar macrophage population we characterized was SWC9⁺ prior to treatment. The increase in surface expression of SLA-II in response to dust demonstrates a potentially activated phenotype, as porcine PBMCs have similar increases in expression after *in vitro* stimulation with some PAMPs (Van der Stede et al., 2005). The lack of induction of SLA-II surface expression by endotoxin treatment was not surprising, as others have demonstrated repression of the SLA II gene in PBMCs after *in vitro* stimulation with LPS alone (Gao et al., 2010). However, the expression of human HLA-II was also increased on the surface of cells recovered in bronchoalveolar lavage fluid (BALF) after naïve subjects worked in a hog barn for 3 h (Muller-Suur et al., 1997). It is therefore probable that components in the ODE other than endotoxin may be responsible for inducing surface expression of SLA-II.

PRRSv, the causative agent of the most economically important disease in the swine industry, infects pig macrophages via binding to CD163 (Neumann et al., 2005). Non-permissive cells can be made susceptible to PRRSv infection via transfection with CD163 cDNA (Welch and Calvert, 2010). CD163 is a haptoglobin-hemoglobin complex scavenger receptor, and it is hypothesized that CD163 removes these complexes to assist in resolving inflammation (Schaer et al., 2002). We observed an increase in surface expression of CD163 in response to ODE exposure. The mechanism by which ODE enhances CD163
expression likely involves the ligation of TLR2 on pig macrophages, as TLR2 and TLR5 ligand stimulation enhance surface expression of CD163 on human monocytes (Weaver et al., 2007). Chemical analysis of ODE revealed a high concentration of muramic acid, a component of the TLR2 ligand peptidoglycan. Human macrophage surface expression of TLR2 increases in response to ODE (Bailey et al., 2008; Poole et al., 2011b), and we observed increased gene expression of TLR2 in porcine AMΦ following ODE treatment. Although we were not able to quantify peptidoglycan in the ODE, the high concentration of muramic acid indicates that it may be present. It is likely that peptidoglycan in the ODE ligates TLR2, and, in turn, enhances surface expression of CD163. In addition, IL-10 treatment increases CD163 surface expression, with a corresponding increase in PRRSV infection (Patton et al., 2009; Sulahian et al., 2000). Because porcine AMΦ increase IL-10 secretion in response to ODE, there may be an association among TLR2 ligation, IL-10 production and CD163 surface expression in response to swine barn dust.

Consistent with a pro-inflammatory response, ODE exposure increased AMΦ production of IL-1β, TNF-α, CXCL8 and IFN-γ. IL-1β and TNF-α are well-characterized mediators during the early innate immune response, and induce mediators of inflammatory respiratory disease in the porcine lung, including bronchoconstriction and enhanced mucus secretion (van Reeth and Nauwynck, 2000). Endotoxin treatment also induced production of both IL-1β and TNF-α from alveolar macrophages, further supporting its role in induction of
inflammation. In support of the increased production of CXCL8, increased gene expression of CXCL2 in response to ODE was observed. Both chemokines are powerful neutrophil chemoattractants, and our findings are consistent with the observed neutrophil influx in the airways of swine barn workers (Larsson et al., 1997) and pigs following exposure to airborne dust contaminants (Jolie et al., 1999).

Porcine AMφ also enhanced production of IFN-γ in response to ODE. It has been postulated that AMφs produce IFN-γ to enhance their bactericidal capabilities via autocrine or paracrine signaling (Fenton et al., 1997). However, IFN-γ, together with other pro-inflammatory cytokines, has been widely noted to induce lung inflammation and pathology, which often exacerbate porcine respiratory disease (van Reeth and Nauwynck, 2000). The increase we observed in IL-10 production along with pro-inflammatory cytokine secretion at the 1% ODE dose is consistent with other reports. Indeed, AMφ have been shown to produce both IFN-γ and IL-10 simultaneously in disease states as a mechanism to regulate the immune response (Oltmanns et al., 2003). Although AMφ activation is designed to resolve infection, chronic activation induced by long-term inhalation of barn dust can lead to increased lung inflammation and tissue damage (Poole et al., 2009b). Finally, exposing porcine AMφ to ODE diminished phagocytosis and restricted killing of S. enterica serovar Choleraesuis. Together, these findings support the hypothesis that swine barn dust exposure impairs several aspects of macrophage functionality and may
contribute to the increased susceptibility of pigs to inhaled pathogens.

Although dust exposure elicits inflammation, evidence also exists that it induces an adaptation response. Studies examining repeated dust exposure have demonstrated that the inflammatory response to ODE is less robust upon a second exposure (Poole et al., 2009b). After 3 to 5 h of work in a hog barn, leukocyte numbers in the BALF of naïve subjects was greater than those found in the BALF of long-time employees working in swine confinement facilities, although both were elevated above basal levels (Larsson et al., 1994). While it has been previously shown that human monocytes secreted TNF-α, IL-6, IL-10 and CXCL8 in response to an initial treatment with ODE, only the IL-10 and CXCL8 response remained consistently elevated after a second exposure (Poole et al., 2007). A potential mechanism behind this reduction in the inflammatory response may be the negative regulation demonstrated by the constitutively expressed PRR NOD2. NOD2 is ligated by muramyl dipeptide, a constituent of Gram-positive bacterial cell walls, and induces NF-κB translocation for the synthesis of inflammatory mediators, antimicrobial peptides and additional NOD2 molecules (Franchi et al., 2009; Fritz et al., 2006). We demonstrated that a single exposure of ODE induces NOD2 RNA expression in porcine macrophages, a phenomenon also observed in human cell lines that was dependent upon the NF-κB pathway (Poole et al., 2011a). NOD2 knockout mice demonstrate increases in cytokine expression, enhanced airway inflammation and increased TLR2-mediated activation of NF-κB when compared
Researchers demonstrating that NOD2 activation results in the intracellular accumulation of TLR2 have suggested that NOD2 is activated to control TLR2 internalization and limit the effects of PAMP signaling (Muller et al., 2010). Although the mechanism has yet to be defined, NOD2 may play a role in the ODE adaptation response observed in humans.

Nuclear translocation of NF-κB was less pronounced in porcine macrophages differentiated in the presence of ODE, and our assessment of NF-κB translocation on a single-cell basis further supports a potential adaptation response to ODE. Previous studies have reported that differentiation of human monocytes in the presence of ODE yielded an immature and less functional macrophage phenotype (Poole et al., 2008; Poole et al., 2009a). It is possible that monocyte maturity is correlated with NF-κB responsiveness; however, others demonstrated that undifferentiated monocytes are quite capable of rapidly activating the NF-κB pathway (Poole et al., 2007). Moreover, monocytes treated with ODE for only 18 h showed diminished cytokine production upon re-stimulation, indicating that undifferentiated monocytes are susceptible to an adaptation response. It is, therefore, more likely that ODE inhibits macrophage NF-κB activation and/or translocation via a mechanism other than or in addition to restricting maturation. It is possible that cytosolic NF-κB levels were affected by initial ODE treatment during the differentiation process, resulting in less nuclear translocation. Alternatively, NOD2 activation may restrict nuclear
translocation of the RelA subunit of NF-κB, as RelA activation is increased in the absence of NOD2 (Strober et al., 2006). This later scenario may represent a mechanism by which NOD2 participates in an adaptation or attenuation response and negatively regulates the NF-κB activation pathway.

5. Conclusion

In conclusion, our results demonstrate that ODE exposure stimulated AMφ to secrete cytokines linked to increased lung inflammation and tissue damage. Moreover, ODE treatment significantly increased surface expression of CD163, a receptor highly correlated with enhanced intracellular PRRSv replication. Exposure to ODE also negatively affected three important components of macrophage function: phagocytosis, intracellular bacterial killing and NF-κB translocation. The in vitro results described herein indicate that swine barn dust exposure negatively affects macrophages. Maintaining macrophage health is a critical innate defense mechanism, and the inhibition of macrophage function may potentially increase porcine susceptibility to respiratory disease and subsequently decrease health and production efficiency.

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**Conflict of Interest Statement**

None of the authors have any potential conflict of interest regarding or related to this work.
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CHAPTER 3

SALMONELLA ENTERICA SEROVAR TYPHIMURIUM-INFECTED PIGS WITH DIFFERENT SHEDDING LEVELS EXHIBIT DISTINCT CLINICAL, PERIPHERAL CYTOKINE AND TRANSCRIPTOMIC IMMUNE RESPONSE PHENOTYPES

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ABSTRACT

Foodborne salmonellosis costs the U.S. $2.7 billion each year, including $100 million in annual losses to pork producers. Pigs colonized with *Salmonella* are usually asymptomatic with varied severity and duration of fecal shedding. Thus, understanding responses that result in less shedding and transmission may provide a mechanism for early control. Fifty-four crossbred pigs were inoculated with *Salmonella enterica* serovar Typhimurium (ST) and clinical signs, fecal ST shedding, growth performance, peripheral cytokines and whole blood gene expression were measured to characterize the global immune response. The results demonstrated that persistently shedding (PS) pigs had longer pyrexia and elevated serum IL-1β, TNF-α, IFN-γ, and IL-12p40 compared to controls, while low shedding pigs (LS) had brief pyrexia, less shedding that decreased more rapidly, and greater serum CXCL8. The PS pigs up-regulated genes involved in the STAT1, IFNB1 and IFNG networks on day 2, while up-regulation of genes involved in negative immune regulation were seen only in LS pigs. This is the first study to examine these responses to ST infection at a clinical, performance, cytokine, and global transcriptomic level. Results indicate that pigs with different shedding outcomes have distinct immune responses within the first two days of ST infection and elucidate alternative immune mechanisms that can now be targeted to reduce *Salmonella* shedding and spread.
INTRODUCTION

Bacterial contamination of meat from an infected animal during processing presents an interface for animal disease and foodborne illness. In 2010, 80.3 million cases of human foodborne illness were attributed to *Salmonella* worldwide,\(^1\) and salmonellosis was the leading cause of foodborne-associated hospitalizations and deaths in the United States in 2011.\(^2\) Estimated annual economic costs of foodborne salmonellosis are $2.7 billion in the U.S.\(^3\) and nearing €3 billion in Europe.\(^4\) Consumption of contaminated pork has been estimated to result in an average of 99,430 cases of human salmonellosis, costing approximately $82 million.\(^5\) In addition to causing human disease, salmonellosis in swine is one of the top ten most common diseases in weaning and grower/finisher pigs,\(^6\) costing pork producers an estimated $100 million annually.\(^7\) Swine salmonellosis can be both symptomatic and asymptomatic, making it difficult to diagnose,\(^8\) and infected pigs exhibit costly decreases in growth performance.\(^9\) Swine rooting behavior enhances fecal-oral transmission, and as a result, disease is often not confined to the farm. Transport, comingling and holding prior to slaughter increase the risk of infection spread.\(^10\) The increased management costs associated with segregating infected pigs,\(^11\) in addition to the difficulty in diagnosis, makes minimizing initial infection and disease transmission potential targets of intervention strategies to reduce *Salmonella* contamination of pork products. As pork is the most highly
consumed meat in the world, limiting swine salmonellosis would result in improvements in both livestock production and human food safety.

*Salmonella enterica* serovar Typhimurium (ST) is one of the ten most frequently isolated serovars from both pigs and humans, and mediates a self-limiting gastroenteritis in both species. The porcine immune response to ST is largely characterized by the local production of pro-inflammatory mediators that result in the pyrexia and neutrophil influx considered hallmarks of ST infection. Sensing of bacterial components by pathogen recognition receptors (PRRs) also leads to cytokine production and stimulates disruption of tight junctions between intestinal epithelial cells, resulting in a loss of epithelial barrier function. Enhanced intestinal permeability, in combination with neutrophil influx, mediates increased fluid entering the gut lumen resulting in diarrhea characteristic of porcine salmonellosis. However, the severity, duration of disease and even appearance of clinical signs vary greatly from pig to pig, as does the amount of ST shed during the course of infection. Previous work from our laboratory correlated increased circulating IFN-γ levels with increased ST shedding in pigs following challenge, and, using transcriptomic analysis, demonstrated an up-regulation of IFN-γ response pathways in blood of pigs that shed greater amounts of ST. The present study was designed to further elucidate the differential responses to ST challenge by utilizing a larger pig population and combining clinical data, growth performance data, multiple peripheral cytokine measurements, transcriptomics, and bioinformatics. We hypothesized that LS
and PS pigs that differ in their ST shedding characteristics will present with
different early immune responses as determined by cytokines secretion and
transcriptomic analysis. We demonstrate differences between LS and PS pigs in
ST shedding and fever by 2 days post-inoculation (DPI) and distinct cytokine
profiles in response to inoculation for LS and PS pigs. Further, both groups of
pigs up-regulated pro-inflammatory genes in response to ST challenge, but LS
pigs alone up-regulated genes involved in negative regulation of inflammation.
By identifying these responses on multiple levels, the porcine immune response
to ST and the variability in shedding can be further characterized. This valuable
information will, in turn, provide the potential to assist in diagnostic development,
reducing swine disease and limiting food safety risk.

MATERIALS AND METHODS

Animals

Six sows (crossbred or Yorkshire) were bred to boars of different breeds and
treated with antibiotics three times prior to farrowing in isolation facilities at the
USDA-ARS-National Animal Disease Center (NADC) in Ames, IA. All sows
tested fecal-negative for Salmonella twice prior to induced farrowing, and all
piglets tested fecal-negative for Salmonella at 3 and 6 weeks of age. Piglets
were also raised in climate-controlled, fully enclosed isolation facilities. Fifty-four
piglets were divided into two groups, and at 7 to 8 weeks of age were
intranasally inoculated with $1 \times 10^9$ CFU S. enterica serovar Typhimurium
χ4232, and six piglets (one from each sow) were inoculated with PBS alone as previously described.\textsuperscript{17} Data were collected at 0, 1, 2, 7, 14 and 21 DPI. Bacteriology for qualitative and quantitative analysis of \textit{Salmonella} from rectal swabs was performed as previously described.\textsuperscript{16} Peripheral blood was collected on 0, 2 and 21 DPI via jugular venipuncture into serum tubes for cytokine analysis, EDTA tubes for complete blood count (CBC) analysis and PAXgene Blood RNA tubes for RNA extraction according to the manufacturer’s protocol (Qiagen, Cat. No. 762164). A standard CBC analysis was done for blood from each pig at 0 and 2 DPI (including lymphocyte, monocyte, neutrophil, eosinophil, and basophil counts). Body weights were collected and fecal scores were assigned based on the following scale: 1 = dry, 2 = solid but moist, 3 = very moist, 4 = very fluid/watery with particles, 5 = fluid/watery. All animal procedures were approved by the USDA-ARS-NADC Animal Care and Use Committee.

Pigs were selected for subsequent analysis based on the extremes of total ST fecal shedding for the population throughout the study period as previously described.\textsuperscript{17} Briefly, CFU/g feces from each collection time point was logarithmically normalized and plotted to calculate the cumulative area under the plotted log curve (AULC) to determine total ST shedding for each pig for the course of the study. Based on total ST shedding, eight pigs were identified at each of the extremes of the population as low shedders (LS) or persistent shedders (PS).
RNA preparation, microarray hybridization and quantitative PCR

Total RNA was extracted via the PAXgene Blood RNA kit (Qiagen, Cat. no. 762164) from approximately 4.5 mL solution (blood and kit stabilizing solution) as per the manufacturer’s protocol. Samples from 0 DPI and 2 DPI were utilized for analysis, and RNA was purified by DNase I digestion and RNeasy mini elute cleanup kit as recommended by QIAGEN. RNA samples were confirmed DNA-free by lack of genomic DNA PCR amplification. RNA quantity and quality was assessed using Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA), and samples with a RIN number less than 7 were excluded from the experiment. The porcine SNOWBALL Genechip was used as previously described. In brief, microarrays were purchased from Affymetrix (Santa Clara, CA) and RNA labeling, chip hybridization, washing, and signal detection were performed at the GeneChip Facility, Iowa State University, Ames, IA.

Quantitative PCR (qPCR) analysis was used to assess differential expression of genes selected from cytokine and microarray results. Probes and primers were obtained from the Porcine Immunology and Nutrition Database. Synthesis of cDNA was performed with SuperScript Reverse Transcriptase II (Invitrogen) and oligo-dT qPCR amplification was implemented using the Brilliant Kit (Agilent Technologies, Inc., CA) with 25 ng of RNA equivalent of cDNA in an ABI Prism 7500 Sequence Detector System (Life Technologies). Assays were performed in duplicate. The amplification conditions are described in Royaee et al. Ct values were obtained from each individual amplification curve using a
standardized baseline value for each gene and averaged for each gene to
determine the Ct values. Average Ct for RPL32 (housekeeping gene) in each
sample was subtracted from each corresponding average target gene Ct,
producing ΔCt values.

Serum cytokine analysis
Blood collected into serum tubes was centrifuged at 2000 x g and resulting
serum was frozen at -80°C until further analysis. Samples were then thawed
only once and analyzed via a multiplex magnetic bead assay as described
previously (Knetter, et al., manuscript submitted). Briefly, magnetic
microspheres of a unique spectral address were covalently coupled with capture
monoclonal antibodies (mAbs) against porcine IL-1β, CXCL8, IL-10, IL-12p40,
IFN-γ or TNF-α. Microspheres were added to each well of a 96-well magnetic
plate (Bio-Rad Laboratories, Hercules, CA) and washed twice in wash buffer
(PBS, 0.7% Tween-20) on a Bio-Plex Pro Washing Station (Bio-Rad
Laboratories). Serum samples were diluted 1:2 in assay buffer (PBS, 1% BSA,
0.05% NaN3) and 50 µL of diluted samples were analyzed. Plates were
incubated at room temperature in the dark for 2 h on a plate shaker at a rotation
speed of 3,000 rpm, following by washing three times. Pooled biotinylated
mouse anti-porcine cytokine detection mAbs were then added for 1 h with the
same incubation conditions and washed three times, followed by incubation with
a streptavidin phycoerythrin conjugate for 30 min. Plates were washed three
times and microspheres resuspended in 125\(\mu\)L of assay buffer. The mean fluorescence intensity (MFI) for 100 microspheres was measured in conjunction with a Bio-Plex 200 array system (Bio-Rad Laboratories) and analyzed with the Bio-Plex Manager software, version 6.0. A standard curve was generated and cytokine concentrations calculated. For those values below the lower limit of quantitation, a value was assigned for each cytokine based on (lowest detectable value / 2) (IL-1\(\beta\), 1.175 pg/mL; CXCL8, 2.175 pg/mL; IL-10, 3.35 pg/mL; IL-12p40, 1.56 pg/mL; IFN-\(\gamma\), 25.46 pg/mL; TNF-\(\alpha\), 5.90 pg/mL).

**Annotation of microarray and gene function**

An NCBI RefSeq ID was assigned to SNOWBALL probesets using the Affymetrix Genechip annotation as described.\(^{18}\) RNA transcript fold change for 2 DPI over 0 DPI was chosen to indicate up-regulation (\(\geq 1.5\)) or down-regulation (\(\leq 0.667\)) and only those comparisons with a \(q\)-value of \(\leq 0.05\) were included. The Functional Annotation Tool of the DAVID Bioinformatics Database 6.7 was used to assign gene ontology (GO) terms and functional clusters. Only those terms with a false discovery rate (FDR) \(\leq 0.1\) and a \(p\)-value \(\leq 0.05\) were accepted as over-represented. Functional clusters were identified based on the similarity of the function of their biological process with the stringency set to high. Enrichment scores were calculated by the geometric mean of the \(p\)-values for GO terms in a corresponding annotation cluster. Only those with an enrichment score > 1.3 were considered to have biological significance and
included. Enriched clusters were assigned a functional name, based on the collaborative function of each term within the cluster. Pathway Studio 9.0 (Ariadne Genomics, Rockville, MD) was used to find relationships between differentially expressed (DE) genes and to elucidate intersections between response pathways. The text-mining tool MedScan Reader was employed by the Pathway Studio software to scan manuscripts from multiple biomedical web resources to establish known relationships. The datasets were interrogated using Sub-Network Enrichment Analysis (SNEA) to find statistically significant entities connecting the genes in each uploaded list (up- and down-regulated in both LS and PS pigs).

Statistical analysis

The Affymetrix GeneChip data were normalized using the Robust Multi-array Average (RMA) method. Linear model analysis as implemented in the limma R package\textsuperscript{22} was conducted to identify significant expression level changes from day 0 to day 2 within LS and PS pigs. For each gene, the change in log-scale expression within each pig from day 0 to day 2 was used as a response variable, and the mean response was allowed to depend on pig status (LS or PS). Each LS or PS mean was tested for a difference from 0 to obtain two \( p \)-values for each gene. Both sets of \( p \)-values (LS and PS) were separately converted to \( q \)-values\textsuperscript{23} using the approach of Nettleton et al. (2006)\textsuperscript{24} to estimate the number of true null hypotheses. These \( q \)-values were used to identify LS or PS
expression changes from day 0 to day 2 while controlling FDR at approximately the 5% level by considering results with \( q \)-value \( \leq 0.05 \) to be statistically significant. Additional linear model analyses were conducted to test for interactions between shedding phenotype and day, and for differential expression between LS and PS within day for each gene. There was no significance for relevant genes when controlling FDR at approximately the 5% level, and no significance for any gene when controlling FDR at approximately the 10% level. For clarity, differentially expressed SNOWBALL probesets will be referred to as differentially expressed genes from here forward.

For each CBC value, the natural log of the response was modeled using a linear mixed-effects model with random pig effects and fixed effects for groups, shedding statuses (LS v. PS), days (0 v. 2), and status-by-day interaction. Because all interactions were non-significant at \( p < 0.05 \), our analysis focused on the main effects of statuses and days. We estimated the difference between status main effects and the difference between day main effects and tested whether each of these estimates were significantly different from zero using the Kenward and Roger (1997)\textsuperscript{25} method for approximating degrees of freedom. Because we analyzed responses on the log scale, these are estimates of the log fold change across the levels of each factor. The inverse logarithm of estimates and associated 95% confidence interval endpoints were calculated to obtain point and interval estimates of fold changes across the levels of each factor.

The change in log CBC value from day 0 to 2 was tested for correlation
with the change in log expression from day 0 to 2 for each combination of CBC variable and gene. Initial analyses were based on a linear model with the change in log expression as the response and shedding status, change in log CBC value, and the interaction between shedding status and change in log CBC value as explanatory variables. Such a model allows for a separate linear relationship between change in log expression and change in log CBC value for persistent and low shedders. Because there was no evidence that separate linear relationships fit significantly better than a single, common relationship for both LS and PS even at a relaxed false discovery rate (FDR) level of 15%, the analysis was repeated by removing shedding status and the interaction involving shedding status from each model to obtain the simple linear regression of change in log expression on change in log CBC value.

A total of 13 data points were available for fitting each simple linear regression model because only 13 pigs had CBC measurements on both day 0 and day 2 due to blood clotting after collection. For each CBC value, the significance of the simple linear regression slope coefficient was tested for each gene. Using the resulting 47,880 \( p \)-values for each CBC value, the approach of Nettleton (2006)\(^\text{23}\) was used to estimate the number of genes with true null hypotheses among all genes tested, and this estimate was used to convert the \( p \)-values to \( q \)-values\(^\text{24}\). FDR was approximately controlled at 15% by comparing each \( q \)-value to 0.15. Even when allowing a relatively high FDR of 15%, only two genes (SNOWBALL_035461, no gene name; SNOWBALL_023935, CYBRD1)
had log expression changes that were significantly associated with log
lymphocyte levels. Upon examination of these two genes, they were found to be
either not significantly differentially expressed, or had little functional relevance,
and so they were excluded from subsequent analysis. No other combinations of
CBC value and expression were statistically significant at FDR 15%.

Average daily gain correlations were calculated by Pearson’s correlation
coefficient (r) and statistical significance were determined using GraphPad Prism
version 6.0 (GraphPad Software, San Diego, CA). Clinical, cytokine and qPCR
data were analyzed by the GLIMMIX procedure of SAS (Version 9.2, SAS
Institute, Cary, NC) with fixed effects of shedding phenotype and day, and pig as
the subject of repeated measures, assuming Gaussian distribution. For cytokine
analysis, random effect of assay plate was added. Least square means were
calculated and compared using the SLICE and SLICEDIFF procedures, and a
Tukey correction was used to adjust for multiple comparisons among shedding
phenotypes and time points. Differences were considered significant at $p \leq 0.05$.

**RESULTS**

*Identification of LS and PS phenotypes within the Salmonella challenge population*

All challenged pigs were quantitatively positive for *Salmonella* shedding in fecal
swabs at 1 DPI, with the exception of one, which was qualitatively positive. All
control pigs remained negative for ST throughout the course of the study. As
described by others\textsuperscript{16,17} there was a wide range in CFU of ST shed across the
challenge population: CFU/g of feces ranged from qualitatively and quantitatively
undetectable (one LS pig, 21 DPI) to 1 x 10\textsuperscript{7} (one PS pig, 2 DPI). The log
transformed fecal counts were plotted for 0, 1, 2, 7, 14, and 21 DPI and pigs
were ranked based on AULC as described in the Methods section to determine
total ST shedding for the course of the experiment. Eight pigs were assigned to
each phenotype. The calculated total ST shedding averaged 84.43 ± 3.16 for the
LS group and 158.8 ± 6.16 for the PS group (Fig. 1A). Variation in shedding
patterns was observed across the entire population, with some pigs reaching
peak shedding levels on day 1 while others peaked at day 2 (Supplementary
Table 1). The highest level of shedding in LS pigs occurred on day 1, although
this peak was not significantly elevated when compared to other timepoints.
Peak shedding for the PS pigs occurred at 2 DPI and was significantly greater
than all other days ($p \leq 0.0001$) (Fig. 1B). Statistically significant differences in
shedding levels were observed between the selected LS and PS populations on
2 DPI only ($p \leq 0.05$).

\textit{Clinical differences were observed between LS and PS pigs}
At 1 DPI, the mean rectal temperatures of both LS and PS pigs peaked and
were significantly elevated from controls and 0 DPI (Fig. 1C). By day 2 however,
LS pig temperatures significantly dropped ($p \leq 0.001$) compared to 1 DPI and
were not statistically different from controls, or from temperatures taken prior to
inoculation. Conversely, PS temperatures at 2 DPI remained elevated above controls \((p \leq 0.0001)\) and were not statistically different from 1 DPI. At day 7, the rectal temperatures of all challenged pigs were not elevated above non-inoculated controls; however, the PS pigs had a higher rectal temperature when compared to the PS rectal temperature on day 0 \((p \leq 0.05)\). By 14 and 21 DPI, no significant difference in rectal temperature was observed for any pig group.

Complete blood counts (CBCs) were measured for all pigs on day 0 and day 2 (Supplementary Table 2). Blood neutrophils and eosinophils were increased \((p < 0.0001 \text{ and } p < 0.05)\), and blood lymphocytes were decreased \((p < 0.0001)\) in response to infection. The number of circulating monocytes was higher in LS pigs when compared to PS pigs \((p < 0.05)\), and no other differences were significant between LS and PS pigs.

Fecal scores were also assigned to describe the relative condition of diarrhea in all pigs as noted in the Methods section. Scores were assigned on 1, 2 and 7 DPI and ranged from 1.0 (Control pig, 7 DPI) to 4.5 (PS pig, 2 DPI). Both LS and PS pigs had scores significantly higher than controls on day 2, but only PS scores remained higher than controls by day 7 \((p \leq 0.05)\) (Fig. 1D).

Body weight was also measured and average daily gain (ADG) calculated for the 21-day study period as a measure of growth performance (Supplementary Table 3). There was no significant difference in body weights between LS, PS or control pigs prior to inoculation on day 0. There was a significant negative correlation between ADG and total ST shedding \((r = -\)
0.2760, \( p \leq 0.05 \)) for all pigs in the study population. The ADG for PS pigs was also significantly lower than that of control pigs \( (p \leq 0.05) \) at the end of the 21-day study period.

Together, these data indicate that clinical differences observed as early as day 2 after inoculation were predictive of a significant difference in ST shedding over time. Moreover, PS pigs maintain clinical differences from LS pigs for longer periods of time and exhibit decreased ADG as a measure of growth performance.
Figure 1. *Salmonella* fecal shedding and clinical data for LS and PS pigs.

(A) Total ST shedding as calculated by area under the log curve (AULC) for LS and PS pigs, displayed as means ± SEM; hash marks (#) indicate statistically significant difference from the unmarked group at $p \leq 0.05$. (B) Fecal shedding CFU/g for LS and PS pigs. All pigs were qualitatively negative for ST prior to inoculation, and all non-inoculated control pigs remained so throughout the course of the study. Means are plotted ± SEM; hash marks (#) indicate statistically significant difference from unmarked groups at $p \leq 0.05$ for comparisons of shedding type within day; asterisks (*) indicate statistically significant differences from unmarked days at $p \leq 0.05$ for comparisons across time points within shedding type. (C) Rectal temperatures for LS, PS and non-inoculated control pigs. The same data are represented as a line graph (plotted means) and in the bar graph inlay (means ± SEM); hash marks (#) indicate statistically significant difference from unmarked groups at $p \leq 0.05$ for comparisons of shedding type within day; asterisks (*) indicate statistically significant differences from 0 DPI at $p \leq 0.05$ for comparisons across time points within shedding type. (D) Fecal scores to assess diarrhea for LS, PS and non-inoculated control pigs assigned on a scale of 1 – 5, with 5 being the most viscous. Means are represented ± SEM; Means are plotted ± SEM; hash marks (#) indicate statistically significant difference from indicated groups at $p \leq 0.05$ for comparisons of shedding type within day; asterisks (*) indicate statistically significant differences from unmarked days at $p \leq 0.05$ for comparisons across time points within shedding type.
Peripheral cytokine profiles identified for LS and PS pigs

We measured serum concentrations of IL-1β, TNF-α, IL-12p40, IFN-γ, IL-10 and CXCL8 (IL-8) as biomarkers of inflammation to identify potential immune response differences between LS and PS pigs following ST challenge. For the purposes of comparison, peripheral blood samples were collected prior to challenge and at 2 DPI in order to assess differences in circulating cytokines as they related to differences in clinical signs of disease at 2 DPI. Prior to challenge, serum samples from both LS and PS pigs presented with similar concentrations of these six cytokines when compared to control pigs (Fig. 2). However, at 2 DPI, distinct differences emerged between LS and PS pigs when compared to each other and to control pigs. Sera collected on 2 DPI from PS pigs had elevated IL-1β and TNF-α concentrations when compared to all other days, as well as compared to serum samples from LS and control pigs ($p \leq 0.05$). The PS pigs also had increased concentrations of IL-12p40 when compared to control pigs at 2 DPI ($p \leq 0.05$) and had greater IFN-γ levels than both the LS pigs and controls at 2 DPI ($p \leq 0.05$). When compared to concentrations before inoculation, IFN-γ concentrations in PS pigs were increased at 2 DPI, and remained elevated at 21 DPI ($p \leq 0.01$). At 2 DPI, both PS and LS pigs had significantly enhanced levels of IL-10, a classic anti-inflammatory cytokine, when compared to controls ($p \leq 0.05$) on day 2 or to their respective pre-inoculation levels ($p \leq 0.01$). At 2 DPI, the cytokine response of LS pigs was further characterized by a significant increase in CXCL8 ($p \leq 0.05$).
that was elevated above control levels on the same day. Together, these results indicate that while the PS pigs had a cytokine response at 2 DPI dominated by pro- and anti-inflammatory cytokines, the LS pigs induced only inflammatory biomarker CXCL8 and anti-inflammatory IL-10 production.

Figure 2. Peripheral blood cytokine concentrations in LS, PS and non-inoculated control pigs. Serum cytokine levels were measured prior to challenge and at 2 and 21 DPI using a multiplex bead assay. Means are plotted ± SEM; hash marks (#) indicate statistically significant difference from indicated groups at \( p \leq 0.05 \) for comparisons of shedding type within day; asterisks (*) indicate statistically significant difference from unmarked days at \( p \leq 0.05 \) for comparisons across time points within shedding type.
Expression of immune genes are enhanced in response to ST challenge

We have previously determined that there is little difference in gene expression in peripheral blood sampled at 20 DPI for LS and PS pigs. Therefore, we chose to compare gene expression between only samples collected at 0 and 2 DPI. Gene expression was measured in the whole blood by qPCR analysis for CASP1, IL1B, TNFA, IFNG, IFNAR1, IL8, IL10, CCR1, IL12B, and CCR5. The expression of CASP1 was elevated at day 2 compared to before challenge for both LS and PS pigs ($p \leq 0.0001$) as demonstrated by a lower $\Delta$Ct. At 2 DPI, the PS pigs had significantly higher levels of CASP1 expression than the LS pigs ($p \leq 0.05$) (Fig. 3). Levels of IL1B RNA were also increased in response to inoculation for both groups ($p \leq 0.05$), although they were not different between LS and PS on day 2. Similarly, RNA levels of TNFA, IL10, IFNAR1, and CCR1 were also increased on day 2 for both LS and PS groups ($p \leq 0.01$). The expression of IFNG and IL8 was not significantly different for the peripheral blood samples collected on day 0 vs 2 DPI for either LS or PS pigs. Levels of CCR5 and IL12B RNA were undetectable on day 0, and expression was not significantly different for either cytokine on day 2 compared to day 0. We also utilized qPCR results from the cytokine genes to confirm similar expression patterns as a method of microarray chip validation. Five of the six genes that were significantly different by microarray displayed the same significant expression patterns via qPCR analysis (IL1B, IL10, TNFA, CCR1, IFNAR1).
Figure 3. Peripheral blood RNA gene expression in response to ST inoculation. Gene expression was measured as Ct values and ∆Ct values calculated by subtracting the average Ct for the housekeeping gene RPL32 from the average Ct each gene for each pig. Mean ∆Cts are plotted ± SEM; hash marks (#) indicate statistically significant difference from unmarked groups at $p \leq 0.05$ for comparisons of shedding type within day; asterisks (*) indicate statistically significant difference from unmarked days at $p \leq 0.05$ for comparisons across time points within shedding type.

PS pigs elicited a more extensive transcriptional response

To further characterize the response following ST infection, we conducted microarray analysis of gene expression in the whole blood. Comparison of
transcript expression before and after ST inoculation revealed that LS pigs had 4,153 differentially expressed (DE) genes, while PS pigs had 8,952, indicating a more extensive response to infection. Statistical analysis revealed there was not a significant interaction of shedding phenotype and time for any gene, indicating that individual gene expression responses were not different between LS and PS pigs.

Upon functional annotation of these DE genes, several functional gene ontology (GO) terms were over-represented in both the PS and LS responses to ST challenge, including “immune response,” “cell adhesion molecules,” and “STAT transcription factor” (Table 1). The DE genes in the LS pigs mapped to terms such as “STAT transcription factor,” “cytokine binding,” and “Toll-like receptor signaling pathway.” The over-represented GO terms for DE genes in the PS pigs included “natural killer cell mediated cytotoxicity,” “transcription,” and “antigen processing and presentation.”

As differential expression can be indicative of gene up- or down-regulation, fold change of \( \geq 1.5 \) or \( \leq 0.667 \), and \( q \leq 0.05 \) was used to declare up- or down-regulated transcripts, respectively, in both the PS and LS pigs. The numbers of DE genes for these criteria, as well as the number of overlapping genes among these four classes, are shown in Figure 4. A comparison of the numbers of up- or down-regulated genes in each of the classes demonstrated that LS pigs had similar numbers of genes that were up- and down-regulated (1,337 v. 1,902), while PS pigs had more down-regulated genes (2,425 v.
4,044). The up-regulated DE genes in both shedding classes mapped to GO terms typical of a bacterial infection, such as “immune response” and “response to lipopolysaccharide (LPS)” (Table 2). Down-regulated GO terms in both the LS and PS pigs included terms characteristic of cell maintenance, such as “cell cycle.” The gene LOC100525629 of unknown function was the only gene that was regulated in an opposite direction between LS and PS pigs (Fig. 4).

Although there was no significant interaction between shedding phenotype and day for any genes, we interrogated the gene lists for up-regulated or down-regulated genes in LS pigs only or PS pigs only. From these lists, there were no significantly overrepresented GO terms or functional clusters. All of the top ten genes with the highest fold change in LS pigs had more than double the increase in expression in PS pigs, with some gene expression being 3x as high. Both the number of DE genes, as well as the level to which they were expressed, indicate that PS pigs responded to ST infection more extensively than LS pigs.
Figure 4. Differentially expressed genes (day 2 versus day 0) in LS and PS pigs. RNA was extracted from the whole blood and hybridized to Affymetrix Genechips for microarray data collection. Transcripts were categorized as up-regulated on day 2 by a fold change in expression of $\geq 1.5$ (UP) or down-regulated on day 2 by a fold change in expression of $\leq 0.667$ (DN). False discovery rate limit was set at a $q$-value $\leq 0.05$.

There were also distinct differences in function of the up- or down-regulated genes between the shedding phenotypes, as identified by overrepresented GO terms. In addition to terms that are related to inflammation, such as “cytokine binding,” the 1,337 up-regulated LS genes also mapped to regulatory terms such as “regulation of inflammatory response” and “regulation of cellular/leukocyte/lymphocyte activation.” Alternatively, the 2,425 up-regulated
PS genes mapped to GO terms such as “chemokine signaling,” and “NK cell mediated cytotoxicity,” indicating a more inflammatory transcriptional response. There were 1,190 up-regulated genes in the PS pigs that were not represented in the LS pigs, and these also included genes with additional inflammatory annotations, such as “MHC class I,” “immunoglobulin binding,” and “leukocyte transendothelial migration.” Grouping these terms into biological process clusters further demonstrated differences between the LS and PS transcriptional response, as LS clusters demonstrated an up-regulated response to both immune stimuli, such as “Response to Bacteria,” and negative regulation, such as “Negative Regulation of Leukocyte Activation” (Fig. 5). “Regulation of leukocyte/mononuclear cell/lymphocyte proliferation” was also an over-represented term in the PS response; however, there were no enriched terms to indicate regulation of cellular activation.
Figure 5. Biological process clustering characterizing functional annotation of the transcriptional response in LS and PS pigs. The DAVID Functional Annotation Clustering tool (http://david.abcc.ncifcrf.gov) was used to cluster terms based on overlapping genes and functional similarity and to assign an enrichment score for calculating biological significance. Only those clusters with a significant enrichment score of > 1.3 are shown. (A) Functional clusters for genes with a fold change in expression of ≥ 1.5 (up-regulated) for LS pigs or (B) for PS pigs. (C) Functional clusters for genes with a fold change in expression of ≤ 0.667 (down-regulated) for LS pigs or (D) for PS pigs.
The down-regulated genes for both LS and PS pigs mapped to more broad terms, such as “nucleus” for the 1,900 genes in LS pigs, and “nuclear lumen” and “acetylation” for the 4,044 genes in PS pigs. Taken together, these results indicate that the PS pigs expressed RNAs with inflammatory functions at much greater levels than LS pigs at 2 DPI. In contrast, LS pigs at this same time point, are expressing RNAs with more regulatory functions as a component of their response.

To visualize potential regulators and networks involved in the gene expression responses of LS and PS pigs, a sub-network enrichment analysis was used to generate overrepresented regulation networks for up-regulated genes in both classes. Previous research has demonstrated an increase in serum IFN-γ in response to ST that it is correlated with greater ST shedding. Further, of the cytokines detected in the serum that were greater in the PS pigs than the LS pigs, only the IFNG network was significantly overrepresented in the PS pigs. For this dataset, the IFNG response pathway and the STAT1 and IFNB1 regulation networks were evaluated, as these were the most significantly overrepresented networks for this class of genes. Many of the genes in all three regulation networks overlapped with each other and were present in multiple GO terms identified for up-regulated genes in PS pigs. Analysis revealed 66 genes in the STAT1 network, 67 genes in the IFNB1 network and 226 genes in the IFNG network. For clarity, a subset of these genes was selected for representation in the pathways based on functional relevance (Fig. 6). We
demonstrate that although qPCR analysis showed IFNG was not significantly changed in the PS pigs at 2 DPI, many of the genes known to respond to IFNG signaling were up-regulated. Moreover, IFN-\(\gamma\) levels were elevated in the serum of PS pigs. The overlapping nodes of the IFNG, IFNB1, and STAT1 networks illustrate a complex pro-inflammatory profile, indicating potential mechanisms for the enhanced inflammatory response seen in PS pigs.

**Figure 6.** Up-regulated genes in the inflammatory response of PS pigs in the peripheral blood on day 2. Sub-network enrichment analysis was conducted on the up-regulated genes in the inflammatory response of PS pigs. The up-regulated genes present in the IFNG, IFNB1, and STAT1 (yellow) response networks are represented. Genes that were present in both pathways, present in functional annotation GO terms, or those with functional relevance are shown. Green lines indicate promoter binding, lavender lines indicate
expression, gold lines indicate protein modification, gray solid lines indicate direct regulation, and gray dotted lines indicate regulation. Symbols indicate protein classification as shown in the legend.

Overrepresented GO terms related to negative regulation were unique to the up-regulated genes in LS pigs. As such, we chose to analyze the response pathway for 18 genes that were present in the GO terms “regulation of inflammatory response,” “negative regulation of leukocyte activation,” or “negative regulation of lymphocyte activation” (Fig 7). The cytokines, cytokine receptors, and transcription factors present in the pathway regulate activation, as well as potentially mediate alternative activation of cells in the periphery at 2 DPI.
Figure 7. Up-regulated genes in regulatory annotation terms unique to LS pigs. The up-regulated genes presented in the functional annotation GO terms “regulation of inflammatory response,” “negative regulation of leukocyte activation,” and “negative regulation of lymphocyte activation” are shown. Green lines indicate promoter binding, lavender lines indicate expression, gold lines indicate protein modification, gray solid lines indicate direct regulation, and gray dotted lines indicate regulation. Symbols indicate protein classification as shown in the legend.

DISCUSSION

Porcine salmonellosis impacts animal health, food safety, and human health; therefore, understanding host responses to ST infection is vital. It has been estimated that 5 to 30% of all pork carcasses may be contaminated with Salmonella, and infected pigs are three to four times as likely to produce contaminated meat.26 Farm-to-fork estimation models predict consumption of contaminated pork results in an average of 99,430 cases of human salmonellosis, costing approximately $82 million.5 Swine salmonellosis exacerbates the economic impact by costing pork producers approximately $100 million annually.7 The variation in shedding levels, clinical signs, and the asymptomatic nature of both infection and shedding recrudescence make porcine salmonellosis difficult to diagnose, resulting in unrecognized disease transmission and spread.8 As such, characterizing the differences in the
response to ST infection in those animals that shed fewer *Salmonella* bacteria may provide insight to limit disease spread early, minimizing both production losses and food safety risks. To our knowledge, this is the first study to combine analysis of clinical and growth performance data, multiple peripheral cytokine concentrations and whole blood transcriptomic data to identify differences in the response of pigs with differing ST shedding levels.

The fecal measurements of ST for the study population support previous reports that shedding amounts and duration are quite varied. While some pigs reached the minimum of quantitative detection by day 7, others maintained shedding at measurable amounts to the end of the study. Peak shedding levels varied between day 1 and day 2 post-inoculation, and some pigs exhibited a recrudescence in shedding levels as the study progressed. This variability in shedding is characteristic of swine salmonellosis, and supports the need to identify the response that correlates with a reduction in both shedding levels and reoccurrence of shedding. Comparisons of ST CFU shed between LS and PS pigs revealed that these two groups differ distinctly as early as 2 DPI. While shedding counts for the LS pigs were highest at 1 DPI and continued to drop over time, the ST CFU for PS pigs were significantly higher at 2 DPI and remained markedly higher than those for the LS pigs throughout the study. These results indicate that shedding outcomes can be defined as early as day 2. Moreover, the enhanced severity of diarrhea seen in the PS pigs is likely a contributing factor to the decreased ADG observed in this shedding group.
Taken together, these results show LS pigs have lower total ST shedding after challenge, have less severe diarrhea, and reduce these shedding levels more quickly than the PS pigs.

Pyrexia induced by ST challenge was evident as early as day 1, where it peaked for both LS and PS pigs. Others have indicated that body temperatures can be elevated as early as 12 to 24 hours post-inoculation and pigs can begin shedding bacteria at 2 hours post-inoculation. The drop in LS temperatures by day 2 may indicate that LS pigs are limiting the inflammatory response more quickly, as fever is indicative of ongoing inflammation. Combined with reduced shedding at this time point, a reduction in the inflammatory response may also be indicative of more efficient control of ST infection. This may suggest that control of ST infection is mediated through other mechanisms apart from or in addition to inflammation.

Evaluation of gene expression by qPCR at 2 DPI revealed that LS and PS pigs had similar changes in expression for the selected inflammatory genes. Expression of CASP1, IL1B, TNFA, IL10, IFNAR1 and CCR1 was increased on day 2 in response to ST challenge. However, PS pigs had significantly greater CASP1 expression than the LS pigs. This gene encodes the pro-form of caspase-1, an enzyme that is intracellularly stored in its inactive form. Salmonella flagellin activates a cytosolic PRR known as IL-1β converting enzyme-protease activating factor (IPAF), eliciting the formation of a protein complex known as the inflammasome. Pro-caspase-1 is recruited as part of
the inflammasome, where it is cleaved by IPAF into its active, protease form. Simultaneous signals such as TNF-α or the ligation of other PRRs yield the synthesis of inactive pro-IL-1β and pro-IL-18,32 which are proteolytically cleaved by caspase-1 for their activation and secretion. It is, therefore, not surprising that CASP1, IL1B, and TNFA expression would increase in response to bacterial infection. Synthesis of additional pro-forms occurs to replenish the inactive proteins after they are cleaved. Greater expression of CASP1 in the PS pigs could be indicative of an enhanced pro-inflammatory response, either as a measure of replenishing intracellular caspase-1, or as a mechanism for continued signaling. Because the PS pigs did not control ST shedding as quickly as LS pigs, the enhanced systemic inflammatory response observed may have been necessary to maintain the health of the animal.

To further elucidate the global differences between the LS and PS inflammatory responses, we measured serum concentrations of six cytokines as biomarkers of inflammation. Both IL-1β and TNF-α were elevated at 2 DPI in the PS pigs, and concentrations were significantly greater than controls and LS pigs at this time point. This finding is consistent with the observed increases in rectal temperature for PS pigs at 2 DPI, as these two cytokines are acute-phase proteins that can elicit a febrile response. Others have investigated the peripheral TNF-α response to ST infection, with varied results in levels detected and the kinetics of the response.28, 33, 34 We hypothesize that this variation may
be a result of differing experimental factors, such as differences in virulence of the ST strains, differences in host genetics, and/or assay sensitivity.

Serum levels of CXCL8 were increased in LS pigs on day 2. Others have reported increased serum CXCL8 in response to porcine infection with ST,\textsuperscript{34} as well as production by porcine epithelial cells infected \textit{in vitro}.\textsuperscript{35} Recruitment of neutrophils to the gut and their efficient killing of ST has been demonstrated as a primary mechanism for ST clearance in pigs,\textsuperscript{36} and the neutrophil and fluid influx into the gut lumen is a hallmark of diarrhea seen in ST infection. However, high levels of CXCL8 in the periphery and less severe diarrhea were both observed in the LS pigs. Others have demonstrated serum CXCL8 is highest at 1 DPI in response to ST challenge.\textsuperscript{34} Further, IL-1 and TNF-\textalpha not only mediate pyrexia, but also induce production of other inflammatory cytokines, including CXCL8, by blood leukocytes and endothelial cells. As chemokines establish a gradient to attract target cells to sites of inflammation, it is possible that high levels in the blood are secondary to a greater response that has been induced previously by inflammation in the gut. If so, it is likely that the circulation of the inflammatory cytokines that mediated the LS pig pyrexia observed on day 1 also induced subsequent production of CXCL8 measured on day 2, as the half-life of CXCL8 is relatively short (< 4 h). In addition, systemic functions of CXCL8 include neutrophil mobilization from the bone marrow to replenish those in circulation, as well as to delay neutrophil apoptosis.\textsuperscript{37, 38} We hypothesize that neutrophil
recruitment to the gut was rapid and efficient in LS pigs, and the circulating levels observed in the blood on day 2 are a remnant of that response.

Both LS and PS pigs had greater circulating IL-10 concentrations than controls at day 2 or at any other time point. As a negative regulator of the inflammatory response, IL-10 is produced from multiple cell types simultaneously with pro-inflammatory cytokines and may function to balance the cytokine milieu when secreted later in inflammation. It is likely that induction of IL-10 in response to ST is a mechanism for restraining the inflammation elicited by infection.

Circulating levels of IFN-γ at 2 DPI were elevated in the PS pigs compared to pre-inoculation levels and remained elevated at 21 DPI. Caspase-1 activation has been demonstrated to induce IFN-γ rapidly through the activation of IL-18 in the mucosa in response to Salmonella infection, and it is possible that circulating cells in the periphery produce IFN-γ in response to caspase-1-induced IL-18 as well. Indeed, we have previously reported a correlation between IFN-γ levels and ST shedding. The PS pigs also had elevated IL-12p40, a subunit of the heterodimer IL-12, when compared to controls on day 2. Observing similar differences in IL-12p40 and IFN-γ is not surprising, as peripheral blood leukocytes produce IL-12p40 in response to pathogen sensing to induce the Th1 differentiation and induction of lymphocyte cytotoxicity and IFN-γ production. Production of IFN-γ by CD4+ T cells to activate the antimicrobial capacity of macrophages characterizes the adaptive Th1 response
to *Salmonella* infection. The different cytokine profiles observed in the LS and PS pigs may indicate either a difference in the time course of the response, or a difference in the type of response generated between the two shedding phenotypes. As the LS pigs had reduced fever and decreased shedding earlier than the PS pigs, as well as a peripheral cytokine response dominated by IL-10 and CXCL8, it may be reasonable to postulate that their response limited ST invasion and attenuated the immune response by day 2. Conversely, the PS pigs appear to have more sustained inflammation and febrile response than the LS pigs, as well as elevated IFN-γ on day 21. It is possible that the PS pigs responded less quickly, leading to an extended inflammatory response. This biphasic response has been reported in the mesenteric lymph node; expression of genes involved in the Th1 response was not up-regulated until 24 and 48 h.p.i. Moreover, studies that have identified genetic elements of resistance to *Salmonella* indicate that resistance is a result of limiting *Salmonella* replication in macrophages early, and animals lacking resistance are less capable of controlling replication and resulting infection.

To explore the mechanism for this potential divergence in response, microarray experiments were used to compare the global gene expression patterns between LS and PS pigs. Our previous work indicated that PS pigs had a greater transcriptional response to ST challenge than LS pigs. Similarly, the PS pigs identified in this study had a much more extensive transcriptional response to infection, as evidenced by differentially expressing more than
double the number of genes as did the LS pigs. The importance of the IFN-γ response in ST infection continues to be highlighted by our work in this study and by others.\textsuperscript{16, 17, 46, 47} We identified that the IFNG regulatory network was significantly overrepresented by up-regulated genes in the PS pigs, and the STAT1 and IFNB1 networks were the most highly significant overrepresented networks in this group. Interferon signaling pathways vary depending on the stimulus, however they largely result in the binding of transcription factor complexes to the interferon-stimulated response elements in the promoters of interferon stimulated genes.\textsuperscript{48} The induction of these genes by IFN-β stimulation is often mediated by dimerization of STAT1 and STAT2 transcription factors or by TLR signaling to phosphorylate IFN regulatory factor 3 (IRF3).\textsuperscript{49} Ligation of TLR4, a PRR recognizing the lipopolysaccharide component of Gram-negative bacteria, following \textit{Salmonella} infection has been shown to stimulate IFN-β production, which then acted in both a paracrine or autocrine manner to activate dendritic cells and up-regulate co-stimulatory molecules.\textsuperscript{50} The positive regulation of IFN-β on the inflammasome to induce IL-1β production, as well as it’s induction of IFN-γ, have also been reported in response to bacterial infections.\textsuperscript{48} Upon phosphorylation due to IFN-γ signaling, the STAT1 transcription factor forms either a homodimer or a STAT3 heterodimer to stimulate transcription of interferon stimulated genes. Genes represented in these three networks included CASP1, TNFA and IL10, further indicating a relationship between gene transcription and cytokine concentrations we found to
be elevated in the blood of PS pigs. The interactions of IFNβ1, TLR4, STAT1, STAT2, IRF3 and IFNγ in the overrepresented networks of up-regulated genes in the PS pigs on day 2 are complex as indicated by the data. Additionally, four of the five regulatory networks we reported previously in PS pigs in the 40-pig population (CEBPβ, SPI1, TLR4 and IFNγ)\textsuperscript{17} were also significantly overrepresented in the PS group of this study population.

Functional annotation revealed that although both groups were expressing genes involved in the inflammatory response as expected, functions involved in regulation were up-regulated in and unique to LS pigs. Sub-network enrichment analysis of the genes involved in these regulatory functions demonstrated that LS pigs are negatively regulating the immune response on multiple levels and may be inducing alternative activation, an activation phenotype involved in wound healing and inflammation control. The gene products of BCL6, SBNO2, and STAT5B have been shown to repress transcription,\textsuperscript{51-53} and IL-10 and IL-27 are both cytokine suppressors of inflammation.\textsuperscript{54} For example, IL-10 activates the STAT3 pathway, which inactivates NF-κB and induces transcriptional repression by SBNO2.\textsuperscript{52} The protein encoded by the CD274 gene, also known as PD-L1, responds to IL-10 and IL-27 to induce negative regulation in multiple cell types,\textsuperscript{55,56} and the activation of the adenosine A3 receptor encoded by the ADORA3 gene inhibits neutrophil degranulation.\textsuperscript{57} Ligation of IL-4R and IL-13RA by their cognate cytokines induces alternative activation of macrophages and a Th2 phenotype,\textsuperscript{58}
and IL-4 has been demonstrated to have anti-inflammatory activity in pigs and other species.\textsuperscript{59-61} Additionally, the gene products of both THY1 and LST1 can prevent cellular activation, proliferation and promote negative regulation after stimulation.\textsuperscript{62-65} Together, the expression of these genes and their involvement in regulatory pathways provides further support for a negative regulation of the immune response in LS pigs by day 2 post-inoculation.

We hypothesize the differences observed in the febrile response, peripheral cytokine levels, shedding amounts and gene expression are connected by a differential immune response to ST infection, thereby establishing the LS and PS phenotypes. It is also possible that additional mechanisms, such as those mediated by the gastrointestinal microbiota, are responsible for the differential responses. Variation in microbial communities in the gut may limit ST invasion, altering the requirement for an inflammatory response and limiting shedding. Our recent comparison of the gastrointestinal microbiota of a smaller subpopulation of the extreme \textit{Salmonella} shedding pigs identified distinct differences in the microbiota before and after challenge with ST between the pig groups; significant differences were identified at the family and genera classification levels for microorganisms that play a role in gastrointestinal inflammation.\textsuperscript{66} Investigations of the relationship between the porcine transcriptional response and the gastrointestinal microbiota of the extreme \textit{Salmonella} shedding pigs are currently underway. Additionally, measurements taken from the whole blood may not be as indicative of the gastroenteritis
induced by ST in swine compared to sampling the mesenteric lymph node or the gut lumen. Whole blood measurements, however, provide us with a practical, repeatable sampling measure in pigs, as well as a more global view of the response. Whole blood transcriptomics have been highlighted as a useful measure of biomarkers for the immune response to disease in humans, and their capabilities in livestock are beginning to be elucidated.

In conclusion, we characterized two phenotypic populations of pigs that differed in their clinical and shedding responses to ST challenge. These two populations exhibited different circulating cytokine profiles, gene expression functional patterns and regulation networks. Together, these data suggest that distinct, alternative immune responses to ST infection could result in different shedding outcomes in swine. Understanding these differing response mechanisms to *Salmonella* infection is critical to maximizing livestock production, food safety and protecting human health.
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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest for the work described.
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Table 1. Functional annotation of differentially expressed genes in response to inoculation by comparing 0 and 2 DPI for LS and PS phenotypes.

<table>
<thead>
<tr>
<th>Group</th>
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<th>p-value</th>
<th>FDR</th>
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</thead>
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<td>9.7 x 10^-3</td>
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<td>4.4 x 10^{-7}</td>
<td></td>
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Only those genes with differential expression at \( q \leq 0.05 \) were used to generate functional annotation lists.

Terms with \( p \leq 0.05 \) and FDR \( \leq 0.1 \) are considered significantly over-represented.

\(^a\)GO Term, gene ontology term; \(^b\)FDR, false discovery rate
Table 2. Functional annotation of up- or down-regulated differentially expressed genes in response to inoculation by comparing 0 and 2 DPI for LS and PS phenotypes.

<table>
<thead>
<tr>
<th>Group – Fold Change Direction</th>
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<th># of Genes</th>
<th>p-value</th>
<th>FDR^b</th>
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<td>receptor</td>
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<td>$3.50 \times 10^{-7}$</td>
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<td>LS-UP</td>
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1,337 DE^+ genes in LS, fold change ≥ 1.5, q-value ≤ 0.05.
| LS-UP (also PS-UP) | defense response | 28 | $2.40 \times 10^{-6}$ | $4.10 \times 10^{-4}$ |
| LS-UP (also PS-UP) | inflammatory response | 19 | $3.30 \times 10^{-6}$ | $5.50 \times 10^{-4}$ |
| LS-UP (also PS-UP) | regulation of cell activation | 14 | $3.60 \times 10^{-6}$ | $6.10 \times 10^{-4}$ |
| LS-UP (also PS-UP) | SH2 motif | 14 | $4.10 \times 10^{-6}$ | $6.30 \times 10^{-4}$ |
| LS-UP (also PS-UP) | SH2 | 14 | $6.30 \times 10^{-6}$ | $7.60 \times 10^{-4}$ |
| LS-UP (also PS-UP) | regulation of lymphocyte proliferation | 10 | $1.10 \times 10^{-5}$ | $1.80 \times 10^{-2}$ |
| LS-UP | negative regulation of leukocyte activation | 13 | $1.40 \times 10^{-5}$ | $2.40 \times 10^{-2}$ |
| LS-UP | response to bacterium | 13 | $2.10 \times 10^{-5}$ | $3.50 \times 10^{-2}$ |
| LS-UP | glycosylation site:N-linked (GlcNAc...) | 90 | $2.20 \times 10^{-5}$ | $3.30 \times 10^{-2}$ |
| LS-UP | topological domain: Cytoplasmic | 77 | $2.30 \times 10^{-5}$ | $3.60 \times 10^{-2}$ |
| LS-UP | transmembrane | 125 | $2.30 \times 10^{-5}$ | $3.10 \times 10^{-2}$ |
| LS-UP | negative regulation of lymphocyte activation | 12 | $3.10 \times 10^{-5}$ | $5.20 \times 10^{-2}$ |
| LS-UP (also PS-UP) | transmembrane region | 104 | $3.50 \times 10^{-5}$ | $5.40 \times 10^{-2}$ |
| LS-UP | anti-apoptosis | 14 | $3.60 \times 10^{-5}$ | $6.00 \times 10^{-2}$ |
| LS-UP | membrane | 148 | $4.00 \times 10^{-5}$ | $5.40 \times 10^{-2}$ |
| LS-UP (also PS-UP) | SH2 domain | 11 | $4.70 \times 10^{-5}$ | $6.40 \times 10^{-2}$ |
| LS-UP (also PS-UP) | Toll-like receptor signaling pathway | 14 | $4.80 \times 10^{-5}$ | $6.20 \times 10^{-2}$ |
| **1,902 DE \( ^e \) genes in LS, fold change ≤ 0.667, \( q \)-value ≤ 0.05**
| LS-DN (also PS-DN) | phosphoprotein | 245 | $3.80 \times 10^{-10}$ | $5.20 \times 10^{-4}$ |
| LS-DN (also PS-DN) | nucleus | 148 | $4.90 \times 10^{-7}$ | $6.70 \times 10^{-4}$ |
| LS-DN (also PS-DN) | Primary immunodeficiency | 9 | $1.00 \times 10^{-8}$ | $1.40 \times 10^{-3}$ |
| LS-DN (also PS-DN) | alternative splicing | 185 | $1.20 \times 10^{-5}$ | $1.60 \times 10^{-2}$ |
| LS-DN (also PS-DN) | cell cycle | 36 | $1.60 \times 10^{-5}$ | $2.70 \times 10^{-2}$ |
| LS-DN | repressor | 28 | $1.90 \times 10^{-8}$ | $2.60 \times 10^{-3}$ |
| LS-DN (also PS-DN) | DNA binding | 74 | $3.70 \times 10^{-6}$ | $5.00 \times 10^{-4}$ |
| LS-DN | Epithelium | 75 | $5.50 \times 10^{-5}$ | $6.80 \times 10^{-2}$ |
| LS-DN (also PS-DN) | transcription regulation | 78 | $5.80 \times 10^{-5}$ | $7.90 \times 10^{-4}$ |
| **2,425 DE \( ^e \) genes in PS, fold change ≥ 1.5, \( q \)-value ≤ 0.05**
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Only those genes with differential expression at $q \leq 0.05$ were used to generate functional annotation lists.

Terms with $p \leq 0.05$ and FDR $\leq 0.1$ are considered significantly over-represented.

$^a$GO Term, gene ontology term; $^b$FDR, false discovery rate; $^c$DE, differentially expressed
Supplementary Table 1. Fecal shedding for all pigs at 1, 2, 7, 14, and 21 DPI.

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*pigs classified as LS

# pigs classified as PS

aDPI, day post-inoculation; bAUC, area under the curve; cAULC, area under the log curve
Supplementary Table 2. Complete blood count (CBC) analysis measurements on blood collected from LS and PS pigs on 0 and 2 DPI.

<table>
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<th>WBC</th>
<th>RBC</th>
<th>Hb</th>
<th>Hct</th>
<th>MCV</th>
<th>MCH</th>
<th>MCHC</th>
<th>P</th>
<th>MPV</th>
<th>N</th>
<th>L</th>
<th>M</th>
<th>E</th>
<th>B</th>
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Mean CBC Value, LS Day 0
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<th>Hct</th>
<th>MCV</th>
<th>MCH</th>
<th>MCHC</th>
<th>P</th>
<th>MPV</th>
<th>N</th>
<th>L</th>
<th>M</th>
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<td>577.63</td>
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<td>0.52</td>
<td>0.18</td>
<td>0.15</td>
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| PS 4  | 0      | 14.63| 8.14| 13.50| 42.50| 52.20| 16.50| 31.70| 510.00| 11.40| 4.35| 9.40 | 0.51 | 0.18 | 0.12 |
| PS 8  | 0      | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot |
| PS 28 | 0      | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot |
| PS 30 | 0      | 10.80| 7.48| 14.00| 43.30| 57.90| 18.70| 32.20| 506.00| 12.00| 3.15| 6.82 | 0.50 | 0.17 | 0.08 |
| PS 67 | 0      | 17.86| 7.04| 13.00| 38.80| 55.10| 18.40| 33.40| 580.00| 11.50| 5.97| 10.66| 0.67 | 0.21 | 0.22 |
| PS 70 | 0      | 15.74| 6.93| 12.80| 38.40| 55.40| 18.50| 33.30| 610.00| 11.10| 5.59| 9.37 | 0.41 | 0.17 | 0.12 |
| PS 81 | 0      | 11.93| 7.96| 13.40| 40.90| 51.50| 16.80| 32.60| 664.00| 12.20| 4.19| 7.13 | 0.23 | 0.21 | 0.11 |
| PS 83 | 0      | 13.96| 7.41| 12.40| 38.10| 51.40| 16.70| 32.60| 659.00| 12.80| 5.79| 7.43 | 0.30 | 0.32 | 0.09 |

Mean CBC Value, PS Day 0
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<th>Hct</th>
<th>MCV</th>
<th>MCH</th>
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<th>P</th>
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<th>L</th>
<th>M</th>
<th>E</th>
<th>B</th>
</tr>
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<tbody>
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<td>13.18</td>
<td>40.33</td>
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<td>588.17</td>
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<td>4.84</td>
<td>8.47</td>
<td>0.44</td>
<td>0.21</td>
<td>0.12</td>
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| LS 3  | 0      | 10.51| 6.87| 12.40| 37.70| 54.80| 18.00| 32.80| 366.00| 10.50| 5.26| 4.34 | 0.43 | 0.21 | 0.08 |
| LS 18 | 0      | 31.34| 7.58| 13.20| 39.50| 52.10| 17.40| 33.40| 677.00| 10.20| 22.80| 6.83 | 1.06 | 0.33 | 0.23 |
| LS 31 | 0      | 21.75| 6.66| 12.00| 36.60| 54.90| 18.00| 32.80| 266.00| 12.80| 15.22| 5.35 | 0.44 | 0.27 | 0.09 |
| LS 39 | 0      | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot |
| LS 68 | 0      | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot | Clot |
| LS 73 | 0      | 32.14| 6.78| 12.60| 37.00| 54.60| 18.50| 33.90| 523.00| 11.00| 19.60| 10.73 | 0.81 | 0.30 | 0.19 |
| LS 78 | 0      | 21.97| 7.48| 11.30| 36.60| 48.90| 15.10| 31.00| 618.00| 12.00| 14.11| 6.57 | 0.67 | 0.25 | 0.12 |
| LS 82 | 0      | 18.67| 6.63| 11.30| 34.20| 51.60| 17.00| 33.00| 552.00| 11.20| 11.04| 6.17 | 0.76 | 0.19 | 0.41 |

Mean CBC Value, PS Day 0
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<th>MCV</th>
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Blood samples that were too clotted for analysis are indicated, and were removed from statistical analysis. \(^a\)WBC, white blood cell; \(^b\)RBC, red blood cell; \(^c\)Hg, hemoglobin; \(^d\)Hct, hematocrit; \(^e\)MCV, mean corpuscular volume; \(^f\)MCH, mean corpuscular hemoglobin; \(^g\)MCHC, mean corpuscular hemoglobin concentration; \(^h\)P, platelet; \(^i\)MPV, mean platelet volume; \(^j\)N, neutrophil; \(^k\)L, lymphocyte; \(^l\)M, monocyte; \(^m\)E, eosinophil; \(^n\)B, basophil
Supplementary Table 3. Body weights of Control, LS and PS pigs throughout the 21-day study period, reported in lbs.

<table>
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<th>D14</th>
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<th>(D21-D14)</th>
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*BW, body weight*
CHAPTER 4

ENDOTOXIN STIMULATION OF WHOLE BLOOD EX VIVO REVEALS AN ATTENUATION OF THE IMMUNE RESPONSE IN PIGS WITH LOWER SHEDDING LEVELS OF SALMONELLA ENTERICA SEROVAR TYPHIMURIUM

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Abstract

Swine salmonellosis poses a threat to food safety and results in costly losses to swine producers. Infected pigs have varied *Salmonella* shedding outcomes and are often asymptomatic carriers, enhancing the risk of disease transmission and pork contamination at slaughter. In human disease, *ex vivo* stimulation of whole blood has elucidated immune response differences to characterize patient clinical outcomes. We inoculated fifty-four pigs with *Salmonella enterica* serovar Typhimurium (ST) and subsequently stimulated whole blood with ST endotoxin (STE) *ex vivo* to identify RNA or cytokine response differences between persistently shedding (PS) and low shedding (LS) pigs both before and after *in vivo* ST challenge. Stimulation with STE elucidated a similar inflammatory profile prior to ST challenge, with some differences in gene expression and cytokine responses. ST challenge exacerbated these differences; STE stimulation of blood from LS pigs increased plasma TNF-α, CXCL8 and IL-10, while blood from PS pigs had increased plasma IL-1β, TNF-α, IFN-γ, CXCL8 and IL-10. Blood from the PS pigs up-regulated genes and networks involved in inflammation. Conversely, challenge with ST appeared to attenuate the response to STE stimulation in blood from LS pigs, evidenced by dampened plasma IL-1β and an up-regulation of only three genes in response to STE on day 2. These results indicate pigs with lower ST fecal shedding attenuate the inflammatory response upon *ex vivo* stimulation after *in vivo* challenge. Taken together, results demonstrate *ex vivo* stimulation of whole
blood as a useful technique to identify differences in pigs with divergent ST shedding outcomes, with potential to reduce the risks of swine production losses and food-borne illness.
Introduction

Human and livestock infection with *Salmonella* continues to be a serious health problem worldwide. Porcine salmonellosis costs the U.S. swine industry $100 million annually [1], and the economic burden of human cases reaches $2.7 billion each year [2]. One of the most commonly isolated serovars from both pigs and humans is *Salmonella enterica* serovar Typhimurium (ST) [3], which causes a self-limiting gastroenteritis in both species. In pigs, clinical signs and *Salmonella* shedding are quite variable, with many animals developing a subclinical carrier state that can lead to unrecognized spread of *Salmonella* throughout the herd [4]. Infected pigs have a decreased growth rate and average daily gain [5] resulting in costly production losses. Moreover, infected pigs are 3-4 times as likely to produce contaminated pork at slaughter, enhancing the risk of human foodborne illness [6]. As pork is the most highly consumed meat in the world, both an economic and food safety need exists to quickly identify pigs infected with ST, as well as those who limit infection and pathogen spread, to decrease disease transmission across a population.

*Ex vivo* tissue or cell stimulation has been used to examine divergent disease etiologies in a variety of tissues and species, through measuring protein secretion, cellular activation and transcriptomic responses [7-9]. In addition to being a practical and repeatable measure, whole blood analysis provides a global picture of the immune response to pathogens or their components, as immune cells utilize the blood for migration, surveillance of systemic factors, and
to enter the lymph nodes for generating antigen-specific immune responses [10]. Stimulation of healthy, human whole blood with lipopolysaccharide (LPS) identified differences in secreted cytokine responses that correlated with distinct gene expression patterns, identifying factors that influence innate immune response variation [11]. In another study, blood from tuberculosis patients demonstrated transcriptional differences that corresponded with disease burden, as well as a separate signature that differentiates tuberculosis from other infectious diseases [12]. Moreover, differing cytokine responses in whole blood stimulated ex vivo has been demonstrated to distinguish pancreatic cancer patient outcomes in response to therapy [13]. In swine, functional transcriptomics demonstrated moderate to high heritabilities of immune parameters in the whole blood in response to vaccination [14]. These studies highlight whole blood transcriptional analysis as method for understanding and potentially predicting the immune response to pathogens, as well as the individual variation in that response.

We have previously demonstrated distinct whole blood transcriptional patterns in response to ST challenge in pigs with divergent shedding levels [15]. Further, we have found distinct cytokine profiles in pigs that shed low levels of ST (LS) or those that persistently shed ST (PS) during the course of infection (Knetter et al., unpublished data). However, the in vitro responses to STE stimulation, the relationship to in vivo responses to challenge and to differences in shedding levels remain unexplored. As such, we sought to characterize the
porcine whole blood response to *Salmonella* endotoxin (STE) to identify potential differences in the immune response of pigs with reduced ST shedding. Such differences could potentially be developed into predictive biomarkers to improve disease resistance without pathogen challenge. We report that *ex vivo* stimulation of blood from pigs with STE prior to *in vivo* challenge resulted in a significant increase in both pro- and anti-inflammatory cytokines, with distinct differences between LS and PS groups. ST challenge exacerbated the *ex vivo* response differences between these shedding groups; blood from LS pigs had a dramatically attenuated response to stimulation as evidenced by a dampened gene expression response and decreased IL-1β production, while the blood from PS pigs maintained a pro-inflammatory response. Together, these results demonstrate that *ex vivo* stimulation of whole blood from pigs can identify response differences between pigs with divergent shedding outcomes, and may serve as a predictive tool to decrease disease transmission and protect food safety.

**Materials and Methods**

**Animals**

The animal study population has been described previously [15]. Briefly, 60 crossbred piglets were farrowed and reared in isolation facilities as the USDA-ARS-National Animal Disease Center (NADC) in Ames, IA. Sows tested fecal-negative for *Salmonella* twice prior to farrowing, and all piglets also tested
fecal-negative for *Salmonella* at 3 and 6 weeks of age. Intranasal inoculation with $1 \times 10^9$ CFU *Salmonella enterica* serovar Typhimurium χ4232 was performed at 7 or 8 weeks of age (n=54) in two groups, and one piglet from each sow (n=6) was mock-inoculated with PBS alone. Fecal swabs were collected at 0, 1, 2, 7, 14 and 21 days post-inoculation (DPI) for qualitative and quantitative evaluation of *Salmonella* shedding, and bacteriology was performed as previously described [16]. Peripheral blood was collected on 0, 2 and 21 DPI by jugular venipuncture into tubes containing 1 mL 100 mM Na citrate, or into EDTA tubes for complete blood count (CBC) analysis. Lymphocyte, monocyte, neutrophil, eosinophil, and basophil counts were measured in a standard CBC analysis of blood from each pig at 0 and 2 DPI. Citrate tubes were placed on ice until *ex vivo* stimulation. At the end of the study period, eight pigs each were selected for classification as low shedding (LS) or persistently shedding (PS) groups based on calculated cumulative shedding as described [15]. The log transformed CFU/g of feces was plotted for each pig on each day, and the area under the log curve (AULC) was used to determine total ST shedding for the course of the study. All animal procedures were approved by the USDA-ARS-NADC Animal Care and Use Committee.

*Ex vivo stimulation and endotoxin testing*

Peripheral blood collected into citrate tubes was pooled for each pig, and 6 ml was placed into each of four tubes. Blood was treated with either 60 µl of 1
mg/ml *Salmonella enterica* serovar Typhimurium χ4232 endotoxin (final concentration of 10 µg/ml) prepared by extraction via butanol-water solvent or equal volume of PBS alone, followed by incubation for either 2 h or 6 h at 37°C with 5% CO2. Incubation conditions were chosen based on previously reported methods for *ex vivo* stimulation of whole blood [11]. At the end of each incubation period, 2.5 ml of blood from each tube was placed into PAXgene Blood RNA tubes for subsequent RNA extraction as per the manufacturer’s instructions (Cat. No. 762164; Qiagen, Valencia, CA). Citrate tubes containing the remaining 3.5 ml blood were centrifuged at 1500 x g to pellet, and the supernatant was collected into a separate tube. Tubes were frozen at -20°C, followed by storage at -80°C until processing. Endotoxin testing was conducted using the Limulus Amebocyte Lysate QCL-1000 assay kit (Lonza, Basel, Switzerland) as per the manufacturer’s instructions.

**RNA extraction, microarray hybridization and qPCR**

Total RNA was extracted according the manufacturer’s protocol and as previously described [15] from whole blood collected at 0 and 2 DPI. Purification of RNA was performed by DNase I digestion and RNeasy mini elute cleanup kit as recommended (Qiagen). An Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to measure RNA quality and quantity, and samples with an RNA Integrity Number (RIN) less than 7 were excluded. Labeling of RNA, hybridization to microarray chips (Affymetrix, Santa Clara, CA), washing and
signal detection were performed at the GeneChip Facility, Iowa State University, Ames, IA.

*Plasma cytokine measurements*

Plasma samples were thawed once and analyzed using a multiplex magnetic bead assay as described (Knetter, unpublished data) [17], with some modification. Each monoclonal capture antibody to detect porcine IL-1β, CXCL8, IL-10, IL-12p40, IFN-γ or TNF-α was covalently coupled to magnetic microspheres of a unique spectral address, and samples were diluted 1:2 (vol:vol) in PBS. Coupled magnetic beads for each cytokine were added to each well and washed on a Bio-Plex Pro Washing Station (Bio-Rad Laboratories, Hercules, CA) with wash buffer (PBS, 0.7% Tween-20), prior to the addition of 50 µl diluted sample for 2 h in the dark on a plate shaker at 3,000 rpm. Wells were then washed twice, followed by incubation with pooled biotinylated detection antibodies for each cytokine for 1 h in the dark. After washing, a streptavidin-phycoerythrin conjugate was added for 30 minutes and plates were washed. The median fluorescence intensity (MFI) for 100 microspheres was measured via Bio-Plex 200 array system (Bio-Rad Laboratories, Hercules, CA) and analyzed with the Bio-Plex Manager software, version 6.0. A standard curve was generated and cytokine concentrations were measured in conjunction with a Bio-Plex 200 System (BioRad, Hercules, CA). For those values below the lower limit of quantitation, a value was assigned for each cytokine based on (lowest
detectable value / 2) (IL-1β, 1.175 pg/mL; CXCL8, 2.175 pg/mL; IL-10, 3.35 pg/mL; IL-12p40, 1.56 pg/mL; IFN-γ, 25.46 pg/mL; TNF-α, 5.90 pg/mL).

**Microarray and functional annotation**

An NCBI RefSeq ID was assigned to SNOWBALL probesets using the Affymetrix Genechip annotation as described [18]. For each expression comparison, a fold change ≥ 1.5 for an RNA transcript was chosen to indicate up-regulation and a fold change ≤ 0.667 was chosen to indicate down-regulation and only those comparisons with q < 0.05 were included. Functional annotation and clustering was conducted as previously described (Knetter, unpublished data). Briefly, the DAVID Bioinformatics Database 6.7 was used to assign gene ontology terms and functional clusters for functional annotation. Those GO terms with a p-value ≤ 0.05 and a false discovery rate ≤ 0.1 were accepted as over-represented. The stringency was set to high for grouping GO terms in DAVID Functional Clustering. The negative log of the geometric mean of the p-values for GO terms in a corresponding annotation cluster was used to calculate an enrichment score, and only clusters with an enrichment score > 1.3 were considered to have significant functional relevance. Each cluster was assigned an ad hoc descriptive name, based on the overall function of each term within the cluster. Regulatory networks were found using Pathway Studio 9.0 (Ariadne Genomics, Rockville, MD) through the text-mining tool MedScan Reader to scan published manuscripts from multiple biomedical web resources to establish
known relationships. The Sub-Network Enrichment Analysis (SNEA) calculated statistically significant entities connected to the probesets in each uploaded list (day 0 and day 2, up- and down-regulated in blood from both LS and PS pigs in response to STE). Only regulation networks with a \( p \)-value < 0.05 were considered significantly overrepresented. Nodes were selected for representation only if they were present in the up-regulated or down-regulated lists.

**Statistical analysis**

The Affymetrix GeneChip data were normalized using the Robust Multi-array Average (RMA) method [19]. Chip lot, shedding phenotype, treatment, interaction between day and treatment, and interaction between treatment and shedding phenotype were considered fixed effects, and pig and chip wash were considered random effects. The PROC MIXED procedure of SAS (SAS Institute, Cary, NC) was used to calculate a linear mixed model for analysis of expression data for each gene using the Kenward and Rogers method [20] for determining denominator degrees of freedom, and \( p \)-values were obtained for each test. \( P \)-values were converted into \( q \)-values to account for false discovery rate estimation in R using the method of Nettleton et al. [21]. Differentially expressed (DE) probesets were considered to be those with a \( q \)-value of \( \leq 0.05 \), unless where indicated to be those with a \( q \)-value of \( \leq 0.1 \).
Cytokine data were analyzed by the GLIMMIX procedure of SAS (Version 9.2, SAS Institute, Cary, NC) with fixed effects of shedding phenotype, day, stimulation, and incubation length; random effects of assay plate, and pig as the subject of repeated measures. Gaussian distribution was assumed for response variables. Least square means were calculated and compared using the SLICE and SLICEDIFF procedures, and a Tukey correction was used to adjust for multiple comparisons among shedding phenotypes and time points. Differences were considered to be significant if \( p \)-values \( \leq 0.05 \).

The statistical analysis of CBC values was conducted as previously described (Knetter, unpublished data). For each CBC value, the natural log of the response measurement was modeled using a linear mixed-effects model with random pig effects and fixed effects for groups, shedding status (LS v. PS), days (0 v. 2), and status-by-day interaction. Because all interactions were non-significant at \( p < 0.05 \), our analysis focused on the main effects of statuses and days. Thus, as part of each linear mixed-effects analysis, we estimated the difference between status main effects and the difference between day main effects and tested whether each of these estimates were significantly different from zero using the Kenward and Roger [20] method for approximating degrees of freedom. Because we analyzed responses on the log scale, these estimates are estimates of the log fold change across the levels of each factor. The inverse logarithm of estimates and associated 95% confidence interval endpoints were
calculated to obtain point and interval estimates of fold changes across the levels of each factor.

The change in log CBC value from day 0 to 2 was tested for correlation with the change in log expression from day 0 to 2 for each combination of CBC variable and probeset. Initial analyses were based on a linear model with the change in log expression as the response and shedding status, change in log CBC value, and the interaction between shedding status and change in log CBC value as explanatory variables. Such a model allows for a separate linear relationship between change in log expression and change in log CBC value for persistent and low shedders. Because there was no evidence that separate linear relationships fit significantly better than a single, common relationship for both LS and PS even at a relaxed false discovery rate (FDR) level of 15%, the analysis was repeated by removing shedding status and the interaction involving shedding status from each model to obtain the simple linear regression of change in log expression on change in log CBC value.

A total of 13 data points were available for fitting each simple linear regression model because only 13 pigs had CBC measurements on both day 0 and day 2 due to blood clotting after collection. For each CBC value, the significance of the simple linear regression slope coefficient was tested for each probeset. Using the resulting 47,880 p-values for each CBC value, the approach of Nettleton (2006) [21] was used to estimate the number of probesets with true null hypotheses among all probesets tested, and this estimate was used to
convert the $p$-values to $q$-values [22]. FDR was approximately controlled at 15% by comparing each $q$-value to 0.15. Even when allowing a relatively high FDR of 15%, only two probesets (SNOWBALL_035461, SNOWBALL_023935) had log expression changes that were significantly associated with log lymphocyte levels. Upon examination of these two probesets, they were found to be either not significantly differentially expressed, or had little functional relevance. As such, they were excluded from subsequent analysis. No other combinations of CBC value and expression were statistically significant at FDR 15%.

**Results**

*Classification of LS and PS groups*

We previously described the challenge population and identification of LS and PS groups (Knetter, unpublished data). Briefly, all control pigs remained fecal negative for *Salmonella* throughout the study, and all inoculated pigs were qualitatively positive for *Salmonella* by 1 DPI, and shedding measurable amounts by 2 DPI. There was variation in shedding levels, in agreement with other reports of ST-challenged pigs [15,23]. Moreover, the pattern of shedding varied among animals, as demonstrated by greater shedding amounts on different days, as well as a recrudescence in shedding levels at later time points for some pigs. For each shedding phenotype, eight pigs were selected from either extreme (LS or PS) for total ST shedding based on their calculated AULC, as described in the Methods section. As previously reported, the PS pigs shed
significantly greater ST at 2 DPI than the LS pigs \((p \leq 0.05)\), and 2 DPI was the highest compared to all other days \((p \leq 0.0001)\). The LS pigs trended to have highest shedding at 1 DPI, although this was not significantly greater than other days. In addition, the two shedding groups exhibited variable clinical signs. The PS pigs appeared to maintain clinical signs (pyrexia, diarrhea) longer than LS pigs, and had decreased average daily gain for the study period (Knetter, unpublished data). Complete blood count (CBC) data revealed that there was a significant increase in neutrophils and eosinophils in the blood in response to ST challenge \((p < 0.0001\) and \(p < 0.05)\), and a decrease in circulating lymphocytes \((p < 0.0001)\). Monocyte numbers were significantly higher in LS pigs than in PS pigs \((p < 0.05)\), and there was no significant interaction for day post-inoculation and shedding group, indicating that LS and PS differences were consistent with respect to day. Further, we measured endotoxin concentrations in plasma samples to quantify the amount of endotoxin present prior to stimulation. There was no statistically significant difference between LS and PS pigs on either day, indicating that differences between these two groups were not altered by different levels of circulating endotoxin prior to stimulation. These amounts were approximately 1,000-fold lower than the concentration of added STE \((10 \mu g/mL)\), and were similar to ranges reported in uninfected, healthy pigs [24].

*Ex vivo stimulation revealed differences between LS and PS pigs prior to in vivo challenge*
We measured plasma concentrations of six cytokines after ex vivo incubation of whole blood with STE to identify the effects of stimulation on cytokine profiles prior to and following in vivo ST challenge. There was no significant difference among control, LS or PS pigs in response to vehicle incubation for any of the cytokines measured on any day when analyzing all comparisons. However, statistical analysis that excluded STE stimulation results and included only vehicle alone treatments reflected cytokine concentrations and significant differences between LS and PS similar to previously observed comparisons (Knetter, unpublished data). There was no significant difference between 2 h or 6 h with vehicle only for any cytokine levels measured, regardless of day sampled. However, differences between the groups of pigs were revealed in response to STE ex vivo stimulation (Fig. 1 A). Acute-phase cytokines IL-1β and TNF-α had similar response patterns after 2 h of STE stimulation of the blood. Stimulation with STE for 2 h increased IL-1β levels in plasma from controls (p < 0.05) and PS pigs (p < 0.01), and had a trend for increased levels in plasma of LS pigs (p <0.1). Similarly, plasma from all three groups of pigs had elevated TNF-α after 2 h blood stimulation (p < 0.001). After 6 h of blood incubation with STE, control, LS and PS had elevated IL-1β when compared to vehicle only stimulation (p < 0.0001), and the PS group had greater plasma IL-1β than the LS group (p < 0.001). The LS pigs did not have elevated plasma IFN-γ after either a 2 or 6 h stimulation of blood, in contrast to elevated levels in PS and control groups at 2 h. Levels of the neutrophil chemoattractant CXCL8 were elevated in
plasma from controls and the PS group after 6 h, but not the LS group, which were significantly lower than control levels \((p < 0.05)\). Concentrations of IL-10, a classically anti-inflammatory cytokine, were elevated in the plasma after blood was stimulated with STE for 6 h for all groups, however the PS group had significantly greater levels than the other groups \((p < 0.01)\). There was no change in plasma IL-12p40 levels in response to blood stimulation for any of the groups evaluated. Taken together, these results demonstrate blood from LS pigs increases only TNF-\(\alpha\) after 2 h with STE, and IL-1\(\beta\) and IL-10 after 6 h with STE. Blood from PS pigs responds more extensively to STE stimulation, increasing TNF-\(\alpha\), IL-1\(\beta\) and IFN-\(\gamma\) after 2 h, and IL-1\(\beta\), CXCL8 and IL-10 after 6 h.

Challenge with ST resulted in LS and PS cytokine response differences to ex vivo stimulation

As LS and PS pigs were clinically very different on day 2 (ST shedding, febrile response), we assessed the whole blood response to STE after the pigs were inoculated with ST to characterize a secondary response. There was a significant interaction of shedding group and day for IL-1\(\beta\), CXCL8, IL-12p40 and TNF-\(\alpha\) after a 2 h incubation, and for CXCL8, IL-10, IL-12p40 and TNF-\(\alpha\) after 6 h. The PS group appeared to have a greater response to STE on 2 DPI, as they had significantly greater plasma IL-1\(\beta\), TNF-\(\alpha\), IFN-\(\gamma\), and CXCL8 than LS at both 2 h and 6 h (Fig. 1 B). Concentrations of plasma IL-1\(\beta\), TNF-\(\alpha\), IL-10 and CXCL8 were also significantly higher in blood from PS than controls after
stimulation for 6 h with STE, and plasma TNF-α and CXCL8 after 2 h. Blood from the LS group was less responsive, and showed an increase only in plasma TNF-α and CXCL8 at 2 h, and IL-10 at 6 h when compared to vehicle alone. The 6 h STE stimulation elicited greater IL-10 levels in plasma from LS than controls at 6 h ($p < 0.0001$), reaching similar concentrations as PS. Conversely, blood from the LS group produced less IL-1β than PS after 2 h of STE stimulation, and produced less than both PS and controls after 6 h ($p < 0.05$). Further, stimulation of blood from LS pigs produced less IL-1β after the LS pigs were inoculated with ST in vivo, compared to pre-inoculation levels elicited by blood stimulation at 0 DPI ($p < 0.05$) (Supplementary Figure 1).

By day 21, the cytokine response to ex vivo stimulation was less prominent for some cytokines, yet blood responses were distinct between LS and PS pigs. Blood from both controls and PS elicited a significant IL-1β response to a 6 h stimulation that was greater than LS, extending the pattern observed on day 2 (Fig. 1 C). As with day 2, blood from all three groups had increased IL-10 after 6 h incubation with STE, but only LS remained higher than controls ($p < 0.0001$, $p < 0.05$). The only differences in plasma IL-12p40 concentrations were observed on day 21, with blood from LS increasing these levels after 2 or 6 h incubation significantly above the other two groups. Unlike day 0 and day 2, blood from the PS group did not respond to stimulation with increased levels of CXCL8, and were lower than controls or LS after 6 h with STE ($p < 0.01$).
Figure 1 A

Day 0

IL-1β (pg/mL)

- Control
- L6
- PS

Day 0

IFN-α (pg/mL)

Day 0

CXCL1 (pg/mL)

Day 0

TNF-α (pg/mL)

Day 0

IL-10 (pg/mL)

L-tyr (ppm)

Day 0
Figure 1 B

[Graphs showing cytokine levels (IL-1, IP-10, CCL2, TNF-α, IL-10) over different time points (2h Vehicle, 2h STE, 6h Vehicle, 6h STE) for Day 2. The graphs compare control, L6, and PS groups with error bars indicating variability.]
Figure 1. Plasma cytokine concentrations in whole blood from LS, PS and non-inoculated control pigs. Plasma cytokine levels were measured prior to inoculation with ST and after blood stimulation with either vehicle alone or with STE using a multiplex bead assay. Means are plotted ± SEM; hash marks (#) indicate statistically significant difference from indicated bars at $p \leq 0.05$ for comparisons of shedding type within day; asterisks (*) indicate statistically significant difference from unmarked bars at $p \leq 0.05$ for comparisons across time.
points within shedding type. (A) Plasma cytokine concentrations prior to inoculation, 
(B) at 2 DPI, and (C) at 21 DPI.

Transcriptional profiles with similar functions are elicited by LS and PS pigs prior to ST challenge

We also examined the gene expression response of whole blood in response to STE stimulation for 6 h, and compared such responses in blood collected from LS and PS pigs both before and two days after inoculation. We found that the numbers of differentially expressed (DE) genes were similar between LS and PS pigs on day 0 in response to stimulation (represented by 431 and 301 probesets, respectively) (Fig. 2).

Figure 2. Numbers of probesets that represent differentially expressed transcripts from the blood of LS and PS pigs in response to 6 h incubation with vehicle alone or STE. RNA was extracted from the whole blood and
hybridized to Affymetrix Genechips for microarray data collection. Comparisons are indicated by arrows, and only those comparisons at $q < 0.05$ were included.

Functional annotation of these DE genes also revealed similarities, as the lists from both LS and PS pigs had genes with only a few overrepresented gene ontology (GO) terms, including “immune response” and “cytokine–cytokine receptor interaction” (Table 1). To examine the direction of such functions, we also divided the DE genes into increased (fold change $\geq 1.5$) or decreased (fold change $\leq 0.667$) prior to GO analysis. Blood from LS and PS pigs prior to inoculation increased expression of a similar number of transcripts in response to STE (represented by 41 and 36 probesets, respectively), with overrepresented functions such as “cytokine activity” and “inflammatory response.” Clustering these GO terms based on similar functions further demonstrated the pro-inflammatory transcriptional profile of blood from both LS and PS in response to stimulation with STE on day 0. Both LS and PS up-regulated transcripts related to genes in the Inflammatory Response cluster on day 0 (Fig. 3 A,B). Blood from the LS pigs had nearly twice the number of down-regulated genes compared to PS (represented by 155 and 81 probesets, respectively), however no functional annotation or clusters for down-regulated transcripts were significantly overrepresented in either group, indicating genes with diverse functions were down-regulated in both LS and PS.
Figure 3. Significantly enriched clusters of functional GO terms for differentially expressed genes in the blood of LS and PS pigs in response to STE. Functional clusters were obtained using the DAVID Functional Annotation Clustering tool (http://david.abcc.ncifcrf.gov) to assign an enrichment score for calculating biological significance. Clusters were determined to be significantly enriched, and thereby having an important role, if their enrichment score > 1.3.

There were no significantly enriched clusters for down-regulated genes in blood from LS or PS pigs on day 0, or for up- or down-regulated genes in blood from LS pigs on day 2. (A) Clusters for up-regulated genes in blood from LS pigs on day 0;
(B) Clusters for up-regulated genes in blood from PS pigs on day 0; (C) Clusters for up-regulated genes in blood from PS pigs on day 2; (D) Clusters for down-regulated genes in PS pigs on day 2.

Although many transcripts were differentially expressed in blood from both LS and PS groups, the lists of genes represented by these probesets were not identical (Fig 4A). Sub-network enrichment analysis (SNEA) of the genes represented by up-regulated probesets confirmed similarities, as regulation networks for IFNG and TNF were overrepresented in blood from both groups of pigs and depicted an inflammatory response profile (Fig. 5 A,B) (Table 4). Only the LS however had a significant overrepresentation of the IL10 regulation network, demonstrating a difference in the responses on day 0 (Fig. 5A). Further, only blood from the LS pigs had genes represented by down-regulated probesets that were significantly overrepresented by any regulation networks (networks of IL17A, SOX9) (Table 4).
Figure 4. Summary of probeset comparisons for blood from LS and PS pigs in response to STE. Probesets were grouped into up-regulated (fold change in transcript expression of $\geq 1.5$) (UP) or down-regulated (fold change in transcript expression of $\leq 0.667$) (DN) in response to 6h incubation of blood with STE. False discovery rate limit was set at a $q \leq 0.05$. (A) Day 0; (B) Day 2.
Figure 5. The overrepresented regulation networks from probesets representing up-regulated genes in the blood in response to STE
**stimulation at 0 DPI.** Sub-network enrichment analysis was used to define statistically overrepresented regulation networks. Only those networks with \( p < 0.05 \) are shown. Green lines indicate promoter binding, lavender lines indicate expression, gold lines indicate protein modification, gray solid lines indicate direct regulation, and gray dotted lines indicate regulation. Symbols indicate protein classification as shown in the legend. (A) The top three most significant networks (IFNG, TNF and IL10) are illustrated in LS; (B) The only significant networks (IFNG and TNF) are illustrated in PS.

*Challenge with ST exacerbates differences between LS and PS gene expression responses to STE stimulation*

At 2 DPI there appeared to be a more dramatic difference between the LS and PS response. While blood from PS pigs inoculated with ST for two days responded to STE stimulation by differentially expressing transcripts represented by 959 probesets, LS only altered the expression of transcripts represented by 14 probesets in response to stimulation. Of these 14, three transcripts were up-regulated: solute carrier family 1 member 1 (SLC1A1); chloride-ion transporter cystic fibrosis transmembrane conductance regulator (CFTR); and the immunomodulatory microRNA (miRNA) mir-155 (MIR155). There were 11 probesets with transcripts that were down-regulated in LS, and this set of genes had no significantly enriched functions. The response in blood from LS pigs had no unique DE probesets when compared to the response of PS blood samples.
As there were so few probesets undergoing any expression changes in the LS pigs on day 2 in response to *ex vivo* stimulation, no functional signature defined by enrichment of clusters or overrepresented networks was detected. The gene expression response of blood from PS pigs on 2 DPI was somewhat similar to the initial response elicited prior to inoculation, although more enhanced. The PS blood response to STE on day 2 was characterized by up-regulated transcripts represented by 162 probesets described by GO terms such as “inflammatory response,” “cytokine activity,” and “chemokine activity” (Table 2). The day 2 PS blood response, although more extensive than the day 0 PS blood response as defined by numbers of transcripts represented by probesets, was quite similar in function. The up-regulated transcripts in blood from PS pigs again demonstrated an enrichment of inflammatory functional clusters, specifically those for Cytokine Activity, Inflammatory Response, Chemokine Signaling and IL-1 Signaling (Fig 3C). The complexity of this inflammatory profile was illustrated by SNEA, and depicted multiple relationships between cytokine genes (*IL1A, IL1B, IL1RN, IL2, IL8, IL10, IL17A, IL-22, IL27*) and immune gene transcription factors (STAT6, RELA) (Fig 6A) (Table 4). As observed for the day 0 response, there was a greater number of down-regulated transcripts in response to STE, with 756 probesets that mapped to functional terms “alternative splicing,” “RNA binding,” and “regulation of translation” (Table 2). These functional terms grouped into enriched clusters for Lysosome, Cellular Metabolism, and TGF-β Signaling (Fig. 3D). Although these probesets mapped
to a variety of functional terms, SNEA revealed overrepresentation of networks containing many genes involved in cellular adhesion, such as integrins and selectins (Fig. 6B).
Figure 6. The overrepresented regulation networks from probesets
representing differentially expressed genes in blood from PS pigs in response to STE at 2 DPI. Sub-network enrichment analysis was used to define statistically overrepresented regulation networks. Only blood from PS pigs had enough DE probesets to map to overrepresented regulation networks, and only those networks with $p < 0.05$ are shown. Green lines indicate promoter binding, lavender lines indicate expression, gold lines indicate protein modification, gray solid lines indicate direct regulation, and gray dotted lines indicate regulation. Symbols indicate protein classification as shown in the legend. (A) Up-regulated probesets identify regulation networks involved in complex relationships of both pro- and anti-inflammatory mediators. Nodes were selected for representation only if they were present in the up-regulated list and represented in the GO terms “cytokine activity,” “immune response,” “interleukin-1,” and “chemokine receptor binding.” (B) Overrepresented regulation networks for down-regulated probesets illustrate blood from PS pigs down-regulated networks involved in alternative activation, as well as transcription factors that mediate inflammatory responses. Nodes were selected for representation only if they were present in the down-regulated list, and only the top 5 regulation networks were selected for representation (SPI1, IL4, CSF3, CEBPE, IL3).

*Infection status reveals differences in the gene expression profiles elicited from LS and PS pigs after STE stimulation*

The gene expression responses to stimulation between day 0 and day 2
for both LS and PS pigs were compared. Blood from PS pigs had DE transcripts represented by nearly 10 times the number of probesets responding to *in vivo* challenge and *ex vivo* stimulation than blood from LS pigs (4,137 vs. 439) (Table 4). A single overrepresented GO term for “immune response” was demonstrated for the DE transcripts in LS, with no significantly enriched functional clusters. Alternatively, PS probesets that mapped to GO terms such as “phosphoprotein” and “alternative splicing,” with significantly enriched clusters involved in transcription were down-regulated. There were no significant GO terms or functional clusters represented by up-regulated probesets in the DE list of transcripts in PS.

**Discussion**

Infection with *Salmonella* continues to be a worldwide health concern, with 80.3 million cases of human foodborne illness attributed to *Salmonella* bacteria in a single year [25]. As the most highly consumed meat in the world, protecting pork from *Salmonella* contamination sits at the forefront of minimizing the risks and costs associated with foodborne salmonellosis. Human illness from *Salmonella*-contaminated pork consumption has been estimated to cost approximately $82 million [26], and this economic burden is exacerbated by $100 million in losses to the swine industry [27]. As swine infection is frequently asymptomatic and fecal shedding levels are variable, determining a mechanism to characterize infection status and potential outcomes for shedding are a
valuable tool to minimize disease transmission and pork contamination. To our knowledge, this is the first study to utilize whole blood *ex vivo* stimulation as a tool for disease identification and outcomes in swine. Results show that pigs with different shedding outcomes have different cytokine and gene expression responses to whole blood stimulation with STE prior to infection. After 2 DPI, the difference in these responses becomes more dramatic; blood from LS pigs has an attenuated transcriptional response, and has dampened cytokine responses as compared to blood from PS pigs. In contrast, the blood from PS pigs enhances the inflammatory response to *ex vivo* STE at 2 DPI, adding to the differences between LS and PS pigs. Using annotation tools to further describe these transcriptomic differences, the use of *ex vivo* whole blood stimulation was indicated as a useful approach in characterizing swine disease outcomes.

Prior to inoculation, STE-responsive probesets were identified in both shedding groups that are known to respond to LPS stimulation of whole blood, such as IL1B and CCL4 [11]. Blood from both LS and PS pigs had DE transcripts represented by genes with inflammatory functions and regulation networks, however blood from LS had a greater number of DE transcripts. The probesets themselves were not the same however; DE transcripts represented by 106 probesets were unique to LS pigs, and 27 probesets were unique to PS pigs. Although IFN-γ concentrations were not elevated by *ex vivo* stimulation of blood from LS pigs, the IFNG regulation network was significantly overrepresented in both groups, potentially indicating a difference in kinetics
between whole blood gene expression responses and circulating cytokine levels. The up-regulated transcripts in blood from LS pigs alone overrepresented the IL10 regulation network, although both groups had increased plasma IL-10 in response to stimulation and PS was significantly higher. Production of IL-10 simultaneously with pro-inflammatory cytokines to balance the cytokine milieu has been described [28], and may indicate the genes of the IL-10 regulatory network in LS are more sensitive to IL-10. While levels of plasma IL-1β and TNF-α were elevated after blood stimulation in both groups, blood from PS pigs also significantly increased IFN-γ and CXCL8 production. Neutrophil influx into the gut is the primary source of the diarrhea that is a hallmark of swine salmonellosis, and CXCL8 is a powerful neutrophil chemoattractant that is elevated in Salmonella infection [29,30]. Many cell types have been demonstrated to produce CXCL8, including monocytes, lymphocytes, neutrophils, fibroblasts, endothelial cells and epithelial cells [31], and its production can be elicited by IL-1β, TNF-α and a component of endotoxin, lipopolysaccharide (LPS) [32]. In circulation, CXCL8 can also mediate leukocyte extravasation and activate phagocytosis [31,33].

Production of IFN-γ in response to endotoxin stimulation has been well described for a variety of cell types including NK cells, T cells, NKT cells, macrophages and dendritic cells [34]. Further, an interaction between activation of caspase-1, the protease responsible for synthesis of active IL-1β, and IFN-γ induction has been demonstrated [35]. We have previously reported the IFN-γ
regulation network was significantly overrepresented in ST-challenged PS pigs in two study populations (Knetter, unpublished data) [15], and demonstrated a correlation between IFN-γ levels and ST shedding [16]. The production of IFN-γ in the blood in response to endotoxin stimulation prior to in vivo challenge may be an early indicator of the difference in the induced inflammatory responses observed between LS and PS pigs. Additionally, the presence of IFN-γ or LPS alone or in combination elicits different phagosomal processing in macrophages [36], highlighting one mechanism by which endotoxin and the early presence of different cytokines may initiate differences in the subsequent immune response of these two shedding phenotypes. Wilkinson, et al. [37] utilized similar transcriptomic techniques to characterize the response of porcine peripheral blood mononuclear cells to in vitro mitogenic stimulation, and identified distinct functional networks that highlighted a T helper 1 (Th1) phenotype development bias. They hypothesized that this immune response trait may correlate with different responses to intracellular pathogen infection in swine. The LS or PS differences from control on day 0 may demonstrate an inherent predisposition to these responses within the shedding phenotypes. This is difficult to determine, however, as controls were never inoculated, and their potential shedding outcome is unknown.

We have previously demonstrated differences in response to ST inoculation between LS and PS pigs in this and a smaller challenge population at 2 DPI. The PS pigs had a greater gene expression response [15], a longer
febrile response and higher peripheral IL-1β, TNF-α and IFN-γ than the LS pigs (Knetter, unpublished data). The LS pigs alone up-regulated probesets annotated to genes involved in negative regulation and alternative activation of the immune response. These results led us to hypothesize that the LS pigs respond to ST challenge more rapidly, resulting in less *Salmonella* shedding, and the PS pigs maintain a longer and perhaps less effective inflammatory response. Similarly, we show here the blood from PS pigs maintains high levels of inflammatory cytokine secretion after STE stimulation on 2 DPI, as well as a gene expression response characterized by probesets involved in cytokine signaling and inflammation. Alternatively, the LS response to STE is attenuated, with DE transcripts represented by only 14 probesets and lower levels of pro-inflammatory cytokines IL-1β, IFN-γ, CXCL8, and TNF-α produced than PS. Both LS and PS pigs had significantly elevated levels of IL-10 in response to 6 h STE stimulation at similar levels.

Blood from the PS pigs down-regulated transcripts after STE stimulation that clustered to terms involved in wound healing and negative regulation, such as “regulation of tissue remodeling” and “TGF-B Signaling”. Regulation networks for IL-4 and IL-13 [38], cytokines linked to alternative activation; and those for SPI1 and CEBPE, transcription factors that mediate inflammatory responses, were both overrepresented in the down-regulated transcripts of PS. The cytokine transforming growth factor-β (TGF-β) has been demonstrated to play an important role in negative immune regulation and establishing tolerance,
inducing the differentiation of regulatory T cells and suppressing inflammatory cytokine secretion [39]. Together, the functional annotation of down-regulated transcripts suggests blood from PS pigs responds to STE with a bias away from negative regulation and alternative activation. Simultaneously, PS blood up-regulated transcripts that clustered into cytokine, chemokine and inflammatory functions, and overrepresented regulation networks for cytokines and chemokines that reflect these pro-inflammatory functions. A complex intersection of overrepresented networks was identified for the genes encoding cytokines IL1A, IL1B, IL2, IL8, IL10, IL17A, IL27; and for chemokines CCL20, CXCL2 and CXCL10. Like IL-1β, IL-1α can activate the inflammasome, mediate inflammation and the febrile response, and induce production of other cytokines and chemokines [40]. Production of IL-17 and IL-22 by Th17 cells functions primarily to activate mucosal barrier functions such as neutrophil recruitment via CCL20 signaling and antimicrobial peptide production in host defense against bacterial pathogens [41]. An abundance of Th17 cells however has been shown to exacerbate gut inflammatory disease, and Salmonella species exploit this inflammation by utilizing increased epithelial permeability for traversing through the gut epithelium [42]. This pro-inflammatory profile is further supported by chemokines CXCL2 and CXCL10, induced by IFN-γ and TNF-α to attract leukocytes to sites of inflammation [43]. The presence of these regulatory networks in the whole blood of challenged PS pigs after STE stimulation may indicate a bias toward activation of Th17-mediated inflammation, leading to
enhanced inflammation and increased fecal shedding of ST. In addition to the pro-inflammatory networks observed, blood from PS pigs also up-regulated networks with anti-inflammatory effects, potentially to balance the inflamed environment. Competitive binding of IL1RN to the IL1 receptor antagonizes the pro-inflammatory effects of IL-1 cytokines, and anti-inflammatory effects have been characterized for IL-2, IL-10 and IL-27 as components of regulatory T cell differentiation, survival and effects [44]. The complexity of these networks, in addition to their alternative roles in regulating inflammation, indicates STE stimulation elicits a range of immune response functions in blood from PS pigs on day 2. These results support our previous conclusions that the immune response of LS and PS pigs is quite different after ST challenge, and that the PS pigs sustain the capacity for an inflammatory response longer than the LS pigs during the first two days after inoculation.

The IL-1β response to STE in blood from LS pigs was unique, in that it was highest on day 0, and was attenuated after exposure to ST in vivo. Blood from LS pigs also produced significantly lower levels of IL-1β than PS after incubation with STE. We hypothesize this reduction in the IL-1β response is an effect of adaptation in the LS pigs, elicited by a primary response to ST and an attenuation to further stimulation with STE. Foster, et al. [45] treated macrophages with LPS, and demonstrated that the expression of many pro-inflammatory genes, including IL1B, were tolerized to subsequent LPS treatment. This tolerization lasted at least 24-48 h, and was a result of chromatin
modifications that occurred in both tolerized and non-tolerized genes, the latter of which included antimicrobial effectors. The authors concluded that these gene-specific modifications primed the non-tolerized genes for subsequent induction, and silenced the tolerized genes. Others have demonstrated that IL1B tolerance induced by repeated LPS treatment is a function of transcriptional inhibitors IkBα and RelB mediating these epigenetic modifications at the IL1B promoter [46]. Although TNF-α can also be tolerized after repeated endotoxin treatment [47], the retention in the TNF-α response observed in blood from LS pigs may be a result of timing or differing epigenetic mechanisms behind the adaptation of a TNF-α or IL-1β response. Further, identification of aspects of tolerance may be more difficult to define in the mixed cellular population found in a whole blood sample.

The expression of MIR155 as one of only three up-regulated genes in blood from LS pigs after stimulation provides further support for a potential regulation of the inflammatory response. The MIR155 gene product, mir-155, binds to target mRNA sequences for degradation, thereby altering post-transcriptional RNA levels [48]. Activity of mir-155 was initially characterized as pro-inflammatory, as its expression is enhanced after TLR ligation and results in TNF-α production [49,50]. Further evidence, however, supports a dual role whereby mir-155 first acts as a pro-inflammatory mediator, and subsequently is a negative regulator of the induced immune response [48]. The TLR signaling pathway has been demonstrated to be under control of mir-155, as it targets
TAB2 and MyD88, two key signaling proteins that mediate induction of NF-κB [51,52]. Further, mir-155 has been shown to target IKKε, a kinase responsible for phosphorylation of the inhibitor of NF-κB, resulting in decreased production of IL-8 (CXCL8) [53]. It has also been reported that mir-155 inhibits IFN-γ production from CD4+ T cells [54]. Moreover, we have previously described a potential interaction for mir-155 and negative regulation of the IFN-γ signaling pathway, as PS pigs in a smaller study population had high IFNG and low MIR155 expression in response to ST challenge [15]. We hypothesize that up-regulation of MIR155 in LS pigs after STE stimulation may be another mechanism to negatively regulate inflammation and results in a damped immune response in the blood from LS pigs.

Plasma IL-12p40 was only statistically elevated in blood from the LS pigs on day 21 after stimulation, however within this group of pigs, there was large variation in the level detected. This subunit can dimerize with IL-23p19 to become IL-23, or with IL-12p35 to become the active IL-12p70. Additionally, it has been demonstrated to have both a homodimeric and monomeric form with both pro- and anti-inflammatory functions [55]. Alone it can competitively bind the IL-12 receptor to inhibit signaling; however it also can be a chemoattractant in bacterial infections, promoting the migration of macrophages and infected dendritic cells [39]. Because our assay can detect both a dimerized and monomeric form, it is difficult to determine what role elevated IL-12p40 may play in the LS pigs.
The *in vitro* stimulation of cells and tissues has become a useful tool to characterize disease etiologies and develop predictive biomarkers of infection outcomes [7-9,11,12]. Moreover, whole blood stimulation is a practical and repeatable sampling measure in pigs, providing us with a systemic view of the immune response. We demonstrate that *ex vivo* stimulation of whole blood with STE can identify distinct differences in cytokine response profiles and gene expression patterns between pigs with different ST fecal shedding outcomes, both before and after inoculation. Further, we show that blood from pigs with lower fecal shedding has a dramatically attenuated response to stimulation at day 2 following *in vivo* challenge, while blood from pigs with greater fecal shedding has an exacerbated inflammatory response to stimulation. Taken together, these results demonstrate *ex vivo* stimulation of whole blood can identify different responses in pigs with difference ST shedding outcomes, with potential to be developed into a predictive tool to reduce the risk of swine salmonellosis, *Salmonella* contamination of pork, and food-borne disease.
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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest for the work described.
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Table 1. Functional annotation of DE probesets in response to STE stimulation on day 0.

<table>
<thead>
<tr>
<th>Group</th>
<th>GO(^a) Term</th>
<th># of Genes</th>
<th>(p)-value</th>
<th>FDR(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS (also PS)</td>
<td>immune response</td>
<td>26</td>
<td>(1.1 \times 10^{-10})</td>
<td>(1.7 \times 10^{-7})</td>
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<tr>
<td>LS</td>
<td>receptor</td>
<td>26</td>
<td>(1.7 \times 10^{-7})</td>
<td>(2.1 \times 10^{-2})</td>
</tr>
<tr>
<td>LS (also PS)</td>
<td>cytokine-cytokine receptor interaction</td>
<td>12</td>
<td>(3.4 \times 10^{-5})</td>
<td>(4.1 \times 10^{-2})</td>
</tr>
<tr>
<td>LS (also PS)</td>
<td>inflammatory response</td>
<td>11</td>
<td>(3.8 \times 10^{-7})</td>
<td>(4.0 \times 10^{-2})</td>
</tr>
<tr>
<td>PS (also LS)</td>
<td>immune response</td>
<td>22</td>
<td>(5.7 \times 10^{-1})</td>
<td>(8.8 \times 10^{-2})</td>
</tr>
<tr>
<td>PS (also LS)</td>
<td>cytokine-cytokine receptor interaction</td>
<td>11</td>
<td>(1.2 \times 10^{-5})</td>
<td>(1.4 \times 10^{-2})</td>
</tr>
<tr>
<td>PS (also LS)</td>
<td>inflammatory response</td>
<td>28</td>
<td>(1.5 \times 10^{-8})</td>
<td>(1.9 \times 10^{-2})</td>
</tr>
</tbody>
</table>

41 Up-regulated DE\(^c\) genes in LS, fold change \(\geq 1.5\), \(q\)-value \(\leq 0.05\)

| LS (also PS)   | cytokine activity                     | 6          | \(8.7 \times 10^{-9}\)  | \(8.5 \times 10^{-9}\)  |
| LS (also PS)   | inflammation                          | 4          | \(3.2 \times 10^{-1}\)  | \(3.1 \times 10^{-8}\)  |
| LS             | cytokine                              | 5          | \(2.4 \times 10^{-9}\)  | \(2.3 \times 10^{-3}\)  |
| LS             | IL1                                   | 3          | \(1.6 \times 10^{-9}\)  | \(7.8 \times 10^{-9}\)  |
| LS             | inflammatory response                 | 4          | \(9.9 \times 10^{-9}\)  | \(9.7 \times 10^{-9}\)  |
| LS             | extracellular region part             | 7          | \(1.2 \times 10^{-9}\)  | \(1.1 \times 10^{-2}\)  |
| LS             | Interleukin-1                         | 3          | \(1.5 \times 10^{-9}\)  | \(1.3 \times 10^{-4}\)  |
| LS             | macrophage                            | 3          | \(2.0 \times 10^{-9}\)  | \(1.9 \times 10^{-2}\)  |
| LS             | lymphokine                            | 3          | \(2.7 \times 10^{-9}\)  | \(2.6 \times 10^{-2}\)  |
| LS (also PS)   | response to wounding                  | 6          | \(1.9 \times 10^{-9}\)  | \(2.8 \times 10^{-2}\)  |

36 Up-regulated DE\(^c\) genes in PS, fold change \(\geq 1.5\), \(q\)-value \(\leq 0.05\)

| PS             | immune response                       | 6          | \(2.0 \times 10^{-9}\)  | \(2.5 \times 10^{-3}\)  |
| PS (also LS)   | cytokine activity                     | 4          | \(1.3 \times 10^{-9}\)  | \(1.0 \times 10^{-2}\)  |
| PS (also LS)   | inflammation                          | 3          | \(3.4 \times 10^{-9}\)  | \(3.0 \times 10^{-2}\)  |
| PS (also LS)   | response to wounding                  | 5          | \(3.3 \times 10^{-9}\)  | \(4.2 \times 10^{-2}\)  |

155 Down-regulated DE\(^c\) genes in LS, fold change \(\leq 0.667\), \(q\)-value \(\leq 0.05\)

No significantly overrepresented functional annotation

81 Down-regulated DE\(^c\) genes in PS, fold change \(\leq 0.667\), \(q\)-value \(\leq 0.05\)

No significantly overrepresented functional annotation
Only probesets with differential expression at $q \leq 0.05$ were used to generate functional annotation lists.

Terms with $p \leq 0.05$ and FDR $\leq 0.1$ are considered significantly over-represented.

\textsuperscript{a}GO Term, gene ontology term; \textsuperscript{b}FDR, false discovery rate; \textsuperscript{c}DE, differentially expressed
Table 2. Functional annotation of DE probesets in response to STE stimulation at 2 DPI with ST.

<table>
<thead>
<tr>
<th>Group</th>
<th>GO^a Term</th>
<th># of Genes</th>
<th>p-value</th>
<th>FDR^b</th>
</tr>
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<tbody>
<tr>
<td>LS</td>
<td>Too few genes for functional annotation</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PS</td>
<td>cytokine activity</td>
<td>6</td>
<td>1.4 x 10^{-9}</td>
<td>1.6 x 10^{-2}</td>
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<tr>
<td>PS</td>
<td>immune response</td>
<td>32</td>
<td>2.1 x 10^{-8}</td>
<td>3.5 x 10^{-3}</td>
</tr>
<tr>
<td>PS</td>
<td>interleukin-1</td>
<td>5</td>
<td>3.3 x 10^{-9}</td>
<td>3.9 x 10^{-2}</td>
</tr>
<tr>
<td>PS</td>
<td>chemokine receptor binding</td>
<td>8</td>
<td>6.2 x 10^{-9}</td>
<td>4.7 x 10^{-2}</td>
</tr>
</tbody>
</table>

162 Up-regulated DE^c genes in PS, fold change ≥ 1.5, q-value ≤ 0.05

| PS    | inflammatory response              | 8          | 2.1 x 10^{-10} | 2.4 x 10^{-3} |
| PS    | extracellular space                | 14         | 4.8 x 10^{-10} | 4.7 x 10^{-3} |
| PS    | cytokine activity                  | 10         | 1.6 x 10^{-9}  | 2.0 x 10^{-6} |
| PS    | immune response                    | 13         | 8.0 x 10^{-9}  | 1.3 x 10^{-4} |
| PS    | response to wounding               | 11         | 5.9 x 10^{-9}  | 9.3 x 10^{-4} |
| PS    | Cytokine-cytokine receptor interaction | 10      | 6.2 x 10^{-7}  | 5.1 x 10^{-4} |
| PS    | Small chemokine, interleukin-8-like | 5        | 2.0 x 10^{-6}  | 2.2 x 10^{-3} |
| PS    | chemokine activity                 | 5          | 6.7 x 10^{-9}  | 8.0 x 10^{-3} |
| PS    | chemokine receptor binding         | 5          | 8.6 x 10^{-9}  | 1.0 x 10^{-4} |
| PS    | chemotaxis                         | 5          | 1.5 x 10^{-9}  | 1.7 x 10^{-2} |
| PS    | inflammation                       | 4          | 1.9 x 10^{-9}  | 2.1 x 10^{-2} |

756 Down-regulated DE^c genes in PS, fold change ≤ 0.667, q-value ≤ 0.05

| PS    | phosphorylation                    | 193        | 2.3 x 10^{-10} | 3.1 x 10^{-3} |
| PS    | alternative splicing               | 185        | 7.3 x 10^{-9}  | 1.0 x 10^{-5} |
| PS    | regulation of translation          | 76         | 6.0 x 10^{-7}  | 4.7 x 10^{-3} |
| PS    | RNA binding                        | 69         | 5.5 x 10^{-7}  | 9.0 x 10^{-4} |
| PS    | lipid binding                      | 25         | 3.1 x 10^{-5}  | 4.7 x 10^{-2} |

Only probesets with differential expression at q ≤ 0.05 were used to generate functional annotation lists.

Terms with p ≤ 0.05 and FDR ≤ 0.1 are considered significantly over-represented.

^aGO Term, gene ontology term; ^bFDR, false discovery rate; ^cDE, differentially expressed
Table 3. Functional annotation of DE probesets between day 0 and day 2 in response to STE stimulation.

<table>
<thead>
<tr>
<th>Group</th>
<th>GO^a Term</th>
<th># of Genes</th>
<th>p-value</th>
<th>FDR^b</th>
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<td>LS</td>
<td>immune response</td>
<td>16</td>
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<td>phosphoprotein</td>
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<td>PS</td>
<td>Hematopoietic cell lineage</td>
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324 Up-regulated DE^c genes in LS, fold change ≥ 1.5, q-value ≤ 0.05

<table>
<thead>
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<tr>
<td>LS</td>
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115 Down-regulated genes DE^c in LS, fold change ≤ 0.667, q-value ≤ 0.05

<table>
<thead>
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1,013 Up-regulated DE^c genes in PS, fold change ≥ 1.5, q-value ≤ 0.05

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<td>Transcription</td>
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<td>1.5 x 10^-9</td>
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<td>7.2 x 10^-5</td>
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2,991 Down-regulated DE^c genes in PS, fold change ≤ 0.667, q-value ≤ 0.05

<table>
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<th>Group</th>
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Only probesets with differential expression at q ≤ 0.05 were used to generate functional annotation lists.

Terms with p ≤ 0.05 and FDR ≤ 0.1 are considered significantly over-represented.

^aGO Term, gene ontology term; ^bFDR, false discovery rate; ^cDE, differentially expressed
Table 4. Sub-network enrichment analysis of probesets representing
differentially expressed genes in blood from LS and PS in response to
STE.

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Only probesets with differential expression at \( q \leq 0.05 \) were used in sub-network enrichment analysis.

Only networks with \( p \leq 0.05 \) are considered significantly enriched.

*FC, fold change: \( \geq 1.5 \) is considered up-regulated (UP), \( \leq 0.667 \) is considered down-regulated (DN)
Supplementary Figure 1. Cytokine response to stimulation over the 21-day study period for LS, PS and non-inoculated control pigs. Whole blood was collected at 0, 2, and 21 DPI, stimulated with STE for 2 or 6 h, and cytokines
were measured from plasma via multiplex assay. Means are plotted ± SEM; asterisks (*) indicate statistically significant difference from indicated or unmarked days at $p \leq 0.05$ for comparisons across time points within shedding type.
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTION

Summary

The swine industry generates $34.5 billion of gross national product to the U.S. economy each year (1). Pork is the most highly consumed meat in the world, and maintaining pork safety and decreasing swine disease will impact animal health and food security (2). Understanding the swine immune response is therefore important in order to define mechanisms that govern disease susceptibility/resistance and transmission. As such, the experiments presented within this dissertation were designed to characterize the porcine immune response to two challenges: swine barn dust and Salmonella infection. These results demonstrate the negative impacts of swine barn dust on porcine macrophages (Chapter 2), identify divergent immune response profiles of pigs with different Salmonella shedding outcomes (Chapter 3), and characterize distinct differences between these Salmonella-shedding phenotypes in their blood response to stimulation with Salmonella endotoxin (Chapter 4).

Pork producers face significant losses due to respiratory disease each year, resulting in the highest percentage of nursery deaths (53.7%) and grower/finisher deaths (60.1%) (3). Human respiratory disease susceptibility has been demonstrated to increase after work in a hog barn, with links to organic
toxic dust syndrome, chronic bronchitis, rhinitis, asthma, and chronic obstructive pulmonary disease (4, 5). Macrophages have important roles in maintaining respiratory health such as clearing debris, pathogen uptake and promoting subsequent immune responses (6). The results in Chapter 2 demonstrated that swine barn organic dust extract (ODE) increased macrophage cell surface marker expression, enhanced production of both pro- and anti-inflammatory cytokines, and inhibited phagocytosis, bacterial killing, and NF-κB nuclear translocation. Analysis of ODE has demonstrated the presence of multiple ligands for pattern recognition receptors that induce pro-inflammatory signaling and macrophage activation (5). The observed increases in cytokine production are likely the result of this signaling, acting to modulate the inflammatory environment and to balance the cytokine milieu. The dampened phagocytosis and bacterial killing capabilities of alveolar macrophages after ODE exposure provide direct evidence for the harmful effects of hog barn dust on cells critical for maintaining respiratory health. Additionally, the reduction in NF-κB nuclear translocation may demonstrate a tolerization to subsequent stimulation that is elicited by initial hog barn dust exposure. Decreased translocation in this experiment could also be a result of inhibition of maturation or differentiation, as we derived the macrophages for stimulation through an in vitro differentiation system. It would be interesting to evaluate this cellular population in more detail both before and after differentiation to determine if the state of maturity or the cytosolic concentration of NF-κB is affected by ODE. Moreover, expanding these
studies both \textit{in vitro} and \textit{in vivo} to examine a potential adaptation response and the effects of repeated and chronic exposure to swine barn dust on the porcine respiratory system would be intriguing. The immediate immune response of the respiratory system must balance the risk of injury with defense against inhaled pathogens and particles (7), and the adaptation response is likely a mechanism of dampening inflammatory responses in favor of maintaining pulmonary function. Despite evidence in human subjects of an adaptation response to repeated stimuli, respiratory disease is often the result of chronic inflammation, with deleterious effects on gas exchange, enhanced mucous production and bronchoconstriction (8). As such, the swine industry stands to benefit from improved air filtration or electrostatic particle ionization that result in barn dust reduction, thereby minimizing the inhalation of particles that may stimulate chronic inflammation.

Treatment with ODE increased surface expression of scavenger receptor CD163, the primary target of porcine reproductive and respiratory syndrome virus (PRRSv) for macrophage infection. The PRRSv is the cause of the most economically important disease in the swine industry, and increased macrophage infectivity has been correlated with CD163 expression (9). Further, IL-10 treatment and TLR2 ligation have both been shown to increase CD163 expression (10), and we demonstrate an increase in IL-10 production and TLR2 gene expression in response to ODE. A large component of ODE is muramyl dipeptide, a constituent of the TLR2 ligand peptidoglycan found in gram-positive
bacterial cell walls. Taken together, these results may imply that IL-10 and TLR2 signaling are potential mechanisms by which swine barn dust enhances CD163 surface expression and susceptibility to respiratory pathogens. In this regard, it is intriguing to hypothesize that swine barn dust increases swine susceptibility to PRRSv infection. Studies to define these signaling mechanisms and to correlate PRRSv infectivity, IL-10 production, TLR2 expression and CD163 expression are needed. If such correlations can be demonstrated, they may provide evidence that barn dust, especially that which contains components that ligate TLR2, are a risk factor for PRRSv infection. Further research in this area may point to reducing dust in the hog barn environment as one method to decrease the incidence of PRRSv infection, as well as other respiratory diseases. Studies to determine the effects of respiratory disease on inflammation in response to dust would also be interesting. Moreover, vaccine development could benefit from studies examining the IL-10 response, TLR2 ligation and upregulation of surface CD163. In vivo studies of barn dust and correlated respiratory disease susceptibility are needed to fully define the impacts of swine barn dust on the immune system, physiology and growth performance of pigs.

Similar to the respiratory tract, the gastrointestinal tract is open to the outside environment and encounters exogenous foreign material, food antigens, and pathogens routinely. *Salmonella spp.* enter the gut, colonize, and cause a self-limiting gastroenteritis characterized by fever, diarrhea, and anorexia in both pigs and humans. Pigs colonized with *Salmonella* have varied levels of fecal
shedding and can develop a long-term carrier state, intermittently shedding *Salmonella* over time and increasing the risk of disease transmission and pork contamination at slaughter (11). We sought to elucidate differences in the immune response of pigs with different *Salmonella enterica* serovar Typhimurium (ST) shedding phenotypes. We confirmed previous results demonstrating that shedding is variable in duration and severity, pyrexia is induced within the first 24 h, and genes up-regulated in whole blood in response to ST challenge are significantly overrepresented within the IFNG, CEBPB, SPI1, and TLR4 regulons. Interestingly, we further demonstrated distinct differences in responsiveness between pigs that were characterized as low shedding (LS) or persistently shedding (PS). The PS pigs had an extended inflammatory response while LS pigs appeared to have a regulatory, anti-inflammatory component to their response. The PS pigs had longer pyrexia, greater serum levels of pro-inflammatory cytokines, and higher gene expression in the blood within the pro-inflammatory networks STAT1, IFNB1, and IFNG by day 2 post-inoculation. The febrile response of LS pigs, however, had resolved by day 2, accompanied by less ST shedding, and up-regulation of genes in the blood associated with immune regulation. We hypothesize that the LS pigs rapidly initiate an efficient inflammatory response following challenge and resolve or limit the infection more quickly than PS pigs. In contrast, PS pigs respond less quickly and/or more extensively, resulting in an enhanced inflammatory response and increased fecal shedding. It is plausible that the
inflammation in the PS pigs is required in the defense against infection. Additionally, other factors such as the commensal microbiota may contribute to the resolution of infection early in the LS pigs, subsequently limiting the requirement for inflammation. Differences in the structure of bacterial communities have been reported in pigs that differ in their *Salmonella* shedding outcomes, demonstrating variation in the abundance of members of the microbiota before and after infection (12).

Genetic studies have identified that limiting *Salmonella* replication early is a mechanism of *Salmonella* resistance, and animals that cannot reduce replication in the early stages are not resistant (13). Because of the more immediate resolution of fever in LS pigs and a cytokine response dominated by CXCL8 and IL-10 circulation in the blood, it is interesting to postulate that they have a more rapid immune response that quickly limits ST invasion and reduces the need for further inflammation or induction of an adaptive immune response. Antibody production at a later stage of infection may point to differences in eliciting an adaptive immune response. Future studies should also measure inflammatory parameters prior to the first 48 h in order to more clearly define these differences and examine these hypotheses. The gene expression responses measured also point to the induction of negative regulation of the immune system by day 2 in LS pigs. Investigation of negative regulators of inflammation, initiators of alternative activation, and their kinetics of induction would be intriguing.
The third project investigated the *ex vivo* whole blood response to endotoxin stimulation and provided further evidence that by 2 days post-inoculation, the LS pigs down-regulate the inflammatory response. Prior to *in vivo* ST challenge, endotoxin stimulation elicited production of acute-phase cytokines and IL-10 in the blood from both LS and PS pigs. However, blood from pigs that were subsequently categorized as PS pigs following ST challenge also responded to endotoxin stimulation with increased CXCL8 and IFN-γ production. Inflammatory genes with similar functions were up-regulated in blood from both LS and PS pigs, however, these genes were not identical. Transcriptomic studies following *in vitro* stimulation of porcine peripheral blood mononuclear cells have highlighted different inflammatory response patterns within a population (14), providing a potential mechanism for linking immune response traits induced *in vitro* with divergent responses to ST infection of swine. Further investigation of the genes unique to LS or PS on day 0 and their relationships with cytokine production may point to development of a predictive transcriptional signature or biomarker that could classify a pig as LS or PS prior to infection.

At 2 days post-inoculation, the differences between the LS and PS blood response were more dramatic. While blood from the PS pigs appeared to have an exacerbated inflammatory response to endotoxin compared to day 0, blood from LS pigs at 2 days post-inoculation greatly reduced the response to endotoxin stimulation compared to pre-inoculation. ST *in vivo* challenge seemed to induce an attenuation to STE stimulation in blood from LS pigs, with only 14
genes differentially expressed after stimulation. Further, blood from the LS pigs had dampened production of IL-1β when compared to day 0 levels and blood from PS pigs, indicating that the IL-1β response is attenuated. Others have demonstrated tolerization of the IL1B gene after repeated LPS stimulation of murine macrophages, and that this tolerization was a result of toll-like receptor-induced chromatin modifications (15). Studies focusing on epigenetic modifications as a mechanism for the reduction in the LS inflammatory response would be interesting. Another potential mechanism for negative regulation in blood from LS pigs may be mir-155, as it was one of only three up-regulated genes in response to endotoxin. The action of mir-155 as an initiator of inflammatory responses has been widely demonstrated, however it’s role in negative regulation is beginning to be defined. Inhibitory functions of mir-155 now include inhibition of the TLR pathway, IFN-γ signaling and NF-κB translocation (16-18) and it would be interesting to investigate mir-155 as a mechanism of the reduced inflammatory response in blood from LS pigs.

Understanding of the porcine immune response to challenges continues to increase as the economic importance of the swine industry rises and the need for experimental models extends beyond the mouse. The respiratory and gastrointestinal tracts are open to the outside environment, and consist of a large relative surface area with many resident immune cells and mucosally-associated immune tissue. This consequently provides a considerable interface for mucosal immunology and the foreign antigens these tracts routinely
encounter. Chronic immune stimulation in these tissues can elicit inflammation leading to disease, and in the case of dust inhalation, can also suppress normal macrophage function. The complexity of inflammatory responses and the timing at which they occur are also critical to disease outcome, as demonstrated in *Salmonella* infection. Enhanced understanding of these responses, identification of risk factors for disease susceptibility, and development of biomarkers that can predict disease outcome are important tools in maintaining swine health.

In conclusion, this dissertation research is the first to characterize the negative impacts of swine barn dust extract on porcine macrophages, and implicates dust as a factor that may increase swine susceptibility to respiratory disease. Further, we show for the first time that pigs with lower levels of fecal *Salmonella* shedding have distinct differences in the cytokine response and gene expression profiles that point toward early, negative regulation of inflammation. Additionally, we demonstrate support for this negative regulation in an *ex vivo* stimulation setting of whole blood with endotoxin, showing an attenuated gene expression response and decreased cytokine production. Taken together, we are able to provide new findings, postulate additional hypotheses and propose research strategies to further characterize and define the porcine immune response to these environmental and pathogenic challenges.
References Cited


APPENDIX

Characterizing Natural Killer Cells and Leukocyte Phagocytosis in Pigs with Severe Combined Immunodeficiency
Susan M. Knetter and Joan E. Cunnick, Emily Waide, Nicholas K. Boddicker, Jack C. M. Dekkers, Christopher K. Tuggle
*Manuscript in preparation, to be submitted 2013*

Modeling the Expression Patterns of IFN-gamma-Responsive Genes to Differentiate Pigs Shedding High and Low Levels of *Salmonella*
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*Manuscript in preparation, to be submitted 2013*

**Abstract**

Investigating the porcine transcriptional response to Salmonella is important for understanding disease resistance and developing predictive tools for disease outcome. Our whole blood transcriptome analysis revealed many differentially expressed (DE) genes between pigs classified as persistent (PS) or low (LS) Salmonella shedders. Pathway analysis of DE genes showed that IFN-γ-responsive genes represented the largest response network in PS pigs. Here,
we used qPCR to confirm in vivo patterns of 15 DE genes annotated as IFN-γ targets. Further investigations verified that differential patterns observed in vivo in PS and LS pigs could be recapitulated in vitro. Whole blood from 3 healthy pigs was stimulated in vitro with various doses of IFN-γ with or without S. Typhimurium endotoxin (STE). The qPCR analysis revealed that the in vitro response to IFN-γ alone for five genes (CXCL10, IL10, MMP8, PSMB9, TMEM176) was dose-dependent. Simultaneous stimulation of whole blood with STE and IFN-γ induced IFN-γ dose-dependent expression for these genes plus CASP4, CYBA, IRF1, JAK2, NCF1, SOD2, and TAP1. Cluster analysis revealed that in vivo gene expression patterns across all genes in PS swine clustered most closely with patterns in whole blood stimulated with the two highest IFN-γ levels plus STE. We conclude that quantitative differences in IFN-γ levels explain the expression of most tested genes, and that the IFN-γ regulon is a source of genes whose expression levels two days post-infection can predict shedding outcomes. Such genes can now be further evaluated as candidates for development of predictive assays for shedding outcome in swine.

Distinct peripheral blood RNA responses to *Salmonella* in pigs differing in *Salmonella* shedding levels: intersection of IFNG, TLR and miRNA pathways.

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**Abstract**

Transcriptomic analysis of the response to bacterial pathogens has been reported for several species, yet few studies have investigated the transcriptional differences in whole blood in subjects that differ in their disease response phenotypes. *Salmonella* species infect many vertebrate species, and pigs colonized with *Salmonella enterica* serovar Typhimurium (ST) are usually asymptomatic, making detection of these _Salmonella_-carrier pigs difficult. The variable fecal shedding of _Salmonella_ is an important cause of foodborne illness and zoonotic disease. To investigate gene pathways and biomarkers associated with the variance in _Salmonella_ shedding following experimental inoculation, we initiated the first analysis of the whole blood transcriptional response induced by _Salmonella_. A population of pigs (n = 40) was inoculated with ST and peripheral blood and fecal _Salmonella_ counts were collected between 2 and 20 days post-inoculation (DPI). Two groups of pigs with either low shedding (LS) or persistent shedding (PS) phenotypes were identified. Global transcriptional changes in response to ST inoculation were identified by Affymetrix Genechip analysis of peripheral blood RNA at day 0 and 2 dpi. ST inoculation triggered substantial gene expression changes in the pigs and there was differential expression of
many genes between LS and PS pigs. Analysis of the differential profiles of gene expression within and between PS and LS phenotypic classes identified distinct regulatory pathways mediated by IFN-γ, TNF, NF-κB, or one of several miRNAs. We confirmed the activation of two regulatory factors, SPI1 and CEBPB, and demonstrated that expression of miR-155 was decreased specifically in the PS animals. These data provide insight into specific pathways associated with extremes in *Salmonella* fecal shedding that can be targeted for further exploration on why some animals develop a carrier state. This knowledge can also be used to develop rational manipulations of genetics, pharmaceuticals, nutrition or husbandry methods to decrease *Salmonella* colonization, shedding.