Maternal influences on neonatal immune development in pigs

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Meggan Marie Bandrick

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Thomas Molitor, Adviser

August, 2010

Acknowledgements

Earning my PhD has been an exciting journey though which I have learned a lot about myself. I could not have accomplished as much without the support, encouragement, and mentorship of my family, friends, and colleagues. Thank you.

Thank you Tom Molitor, my adviser. Not the sage on the stage but the facilitator of learning. You are an encouraging, supportive, mentor and friend. I have learned a lot from you. Thank you for all of the teaching talk and giving me the opportunity to try my own innovative teaching techniques on your immunology class. And, thanks for the time talking about gardening; my vegetables also grow better because of your input.

Thank you Dave Brown, Scott Dee, Sabita Roy, and Mike Murtaugh, members of my thesis committee, for your guidance and support. Thank you for critically reviewing my thesis work and your enthusiasm in doing so. A special thanks to Carlos Pijoan (in memory).

Thank you to the faculty of the University of Minnesota, especially Sandra Godden and Katey Pelican, for allowing me to help you on your experiments. I learned important skills and gained experience with other species. Thank you Deb Wingert and Al Beitz for mentoring me through the Preparing Future Faculty program.

To the graduate and professional students, especially Mike Olin, Claudia Ariza-Nieto, Maria Pieters, and Kara Theis, thank you for helping me out in the lab and on the farm. Kate O'Conor, Dee Koski, and Julia Manor thank you for keeping me focused, happy, and full of coffee.

I could not have accomplished so much without the support and love of my family. Thank you Mom and Dad and Amanda, Sarah and Arthur, and all of your children. Hanna and Adelle! Yup!

Ryan, my husband, you have always supported me even when my time was short and finances were tight and I needed just one more cup of coffee. Fiona you make me work much more efficient. Thank you.

Thank you also to those agencies that have funded this research. Funding agencies: NIH T32 DA07097, National Pork Board, Minnesota Pork Board, Pfizer, and Syngen. Thank you for your generous donations Midwest Research Swine, Immunochemistry Technologies, LLC, and Prototek. Thank you to the swine facilities and staff for allowing these experiments to be conducted there. Swine facilities: Pipestone System, Prairie Land Pork, and the University of Minnesota, Southern Research and Outreach Center. Without your generous support, this research would not have been possible.

Dedication

Dedicated to my family, especially my parents Linda and Art Hendrickson, my sisters Amanda and Sarah, and my brother Arthur, and to my best friend, my husband Ryan Bandrick, and the beautiful Fiona

Abstract

Adaptive immunity in the neonatal animal is primarily maternally-derived, either by immune components passing into the newborn across the placenta or following colostrum ingestion. Due to their epitheliochorial placentation, maternally-derived immunity (MDI) in swine is first transferred to the piglet in the form of colostrum. It is well established that both maternal antibodies and immune cells are transferred in colostrum and that MDI contributes to the immune repertoire of the neonate. Nonetheless, the consequences of MDI on development of neonatal immune responses, specifically cell-mediated immune (CMI) responses, warrant intense investigation, since the neonatal period is such a critical time in the animal's life. Toward this end, the goal of this thesis was to investigate the influence of MDI on neonatal CMI responses in swine. The central hypothesis to be tested was that maternally-derived CMI is transferred to piglets, and participates in the neonatal immune response, thereby affecting neonatal immune development. In order to test the first part of this hypothesis, the cellular contribution to passive immunity and the transfer of such immunity was determined and compared to the antibody portion of colostrum. T-lymphocytes (CD4+, CD8+, and γδ) were detected in colostrum and were selectively transferred to suckling piglets. The selectivity in colostral lymphocyte transfer was in part due to genetic source since, unlike piglets allowed to suckle on their biological dams, cross-fostered piglets did not have detectable sow-derived CMI responses. To test the second part of the hypothesis, *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*)-specific functional responses of lymphocytes transferred from vaccinated dams to their offspring via colostrum were evaluated. Functional activity of

colostral lymphocytes was demonstrated by antigen-specific *in vivo* delayed type hypersensitivity (DTH) responses and *in vitro* lymphoproliferative responses by piglets of *M. hyopneumoniae* vaccinated dams but not by piglets from nonvaccinated dams. The effect of MDI on vaccine-induced antibody-mediated immune (AMI) and CMI responses *M. hyopneumoniae* was assessed in neonatal piglets. The potential for MDI to interfere with AMI does not extend to CMI against *M. hyopneumoniae* since piglets developed primary and secondary *M. hyopneumoniae*-specific CMI responses when vaccinated in the presence of MDI. Thus, this thesis research demonstrates a potential for MDI to affect but not interfere with neonatal CMI responses following antigen exposure. The advanced understanding of MDI gained from the studies described here will enable veterinarians and physicians to design more effective disease prevention and control strategies for neonates.

Table of Contents

List of Tables

List of Figures

Section A: General introduction and literature review

General Introduction

Transfer of maternally-derived immunity (MDI) acquired across the placenta or via breast milk is critically important for the neonate, especially in regards to pathogen challenge in the neonatal period. Maternal immune factors transferred to the neonate include antibodies, immune cells, and non-specific immune modulators. Although a great deal is known about MDI, the cellular characteristics of colostral immunity have not been well defined. Further, it is unclear how MDI affects piglet cell-mediated immune (CMI) responses or if piglets can respond with CMI responses in the face of MDI. Nonetheless, while MDI provides a critical and immediate protection to environmental and vaccine antigens encountered by the mother, colostral immunity is not always beneficial for the neonate. MDI potentially interferes with endogenous immune responses in the recipient, namely antibody-mediated immune (AMI) responses. Appreciating the impact of passively transferred immunity will enable us to elucidate downstream consequences on neonatal immune development. Toward this end, the goal of this research was to investigate the influence of MDI on neonatal immune development in swine.

The central hypothesis for this dissertation is that maternally-derived cellular immunity is transferred to and participates in the neonatal immune response. This hypothesis was tested in swine for two important reasons (1) to benefit the pig and (2) as a model to understand the influences of MDI on neonatal immune development without the confounding factor of MDI transferred to the fetus *in utero* since there is no transfer of MDI across the porcine placenta. In this thesis four important aspects of MDI were evaluated in swine: the cellular contribution to passive immunity, source specificity of passive MDI transfer, transfer of antigen-specific immunity, and the effects of maternally-derived immunity on neonatal

response to vaccination. The advanced understanding of MDI gained from the studies described here will enable veterinarians and physicians to design better disease prevention and control strategies for neonates.

This dissertation begins with a review of the literature pertaining to MDI, neonatal immunity, and the influence of MDI on neonatal immune development. Four research chapters, each pertaining to an important aspect of MDI, follow the literature review. A chapter summarizing the gaps in the literature and the results of the research chapters completes the thesis. The conclusion chapter also includes a discussion of the limitations of the research chapters and directions for future research.

The first research chapter (chapter 2) is a characterization of the cellular contribution to MDI and the sequential transfer of lymphoid cells from sow periphery to colostrum and finally to the neonate. Maternally-derived antibodies are also characterized and compared to the cellular portions of colostrum and blood in chapter 2. Chapter 2 documents that CD4+, CD8+, and γδ T-lymphocyte are found in colostrum and that these cells are transferred to suckling piglets in a selective manner. Selectivity in transfer is evidenced by the fact that the lymphocyte populations in colostrum did not mimic that found in piglet blood after suckling. No selectivity in antibody transfer was observed between sow blood, colostrum, and piglet blood.

Chapter 3 documents that T-cells are transferred to neonates in colostrum via DNA fingerprinting. Further, chapter 3 demonstrated that colostral T-cells are functional in the recipient animal and participate in the immune response in an antigen-specific manner, in this case to *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*). *M. hyopneumoniae*–specific CMI responses were demonstrated in piglets from *M. hyopneumoniae*-vaccinated dams *in vitro*

by antigen-specific proliferative responses and *in vivo* by delayed type hypersensitivity (DTH) responses.

Chapter 4 documents the effect of cross-fostering on transfer of maternally-derived CMI and AMI to piglets. Cross-fostering piglets affects the transfer of colostral T-lymphocytes and antibodies differently. AMI is transferred to piglets regardless of source. In contrast, CMI is transferred to piglets in a source-dependent manner.

Chapter 5 characterizes the effect of MDI on piglet vaccine-induced CMI and AMI responses. Piglets with MDI to *M. hyopneumoniae* did not develop detectable *M. hyopneumoniae*-specific AMI responses when vaccinated at 7 d of age in the face of MDI. Vaccine-induced CMI responses were evident in neonatal pigs vaccinated in the face of maternal immunity. In this system there was no evidence of passive interference of maternally-derived immune components with neonatal CMI responses following vaccination.

The final chapter (chapter 6) of this dissertation is a summary and conclusion. Chapter 6 also presents limitations of the work performed as part of this thesis. Directions for future research are included in chapter 6.

Chapter 1: Literature review

1.1 Summary

Neonates possess the immune repertoire necessary to mount an immune response at birth but they are immunologically naïve, have a propensity for Th2 responses, and require extra stimulation for their immune systems to perform at a level similar to adults. Adaptive immunity in the neonatal animal is primarily derived from its mother, either by immune components passing into the newborn across the placenta or following colostrum ingestion. Due to the epitheliochorial placentation in swine, maternal immunity is first transferred to the piglet in the form of colostrum. It is well established that both maternal antibodies and immune cells are transferred in colostrum and that maternally-derived immunity contributes to the immune repertoire of the neonate. Yet, the cellular makeup of colostrum and the functional ability of transferred cells in the recipient have largely been neglected. Maternally-derived antibodies are known to interfere with antibody-mediated immune (AMI) responses in neonates. The consequences of maternally-derived cell-mediated immunity (CMI) on development of neonatal immune responses warrant further clarification. Alternatively, the ability of neonates to mount CMI responses in the face of maternally-derived immunity needs to be investigated. The goal of this review is to critically examine the literature concerning maternally-derived immunity especially relevant to the pig, and to hypothesize the role of transferred colostral CMI on neonatal immune development.

1.2 Introduction

Colostrum is a complete diet for newborns. In addition to its nutritive components, colostrum is rich in maternal immunoglobulins, immune and non-immune cells, and non-specific immune modulators. Colostrum varies across species in two important ways (1): the immune constituency and (2): its importance as a first-line defense mechanism in the neonate. Variability in the immune constituency of colostrum is largely dependent on placentation. In swine and other species where maternally-derived immunity (MDI) does not cross the placenta, MDI is wholly acquired in colostrum and milk. As a consequence of their placentation, piglets from a healthy pregnancy are born lacking antigen-specific immunity and the successful absorption of colostrum is essential for disease prevention in the first weeks of life. The benefits of colostrum extend beyond its utility as a source of immediate immune protection, e.g. colostrum adds to the immune repertoire of piglets. However, colostrum can affect immune development in the neonate and can have detrimental effects on immune development in the recipient, i.e. passive interference. There is a plethora of information regarding the immunoglobulin constitution of colostrum and the role of maternally-derived immunoglobulin on neonatal immune development. Published literature pertaining to the cellular constitution of colostrum and the role of those cells in neonatal immune development is sparse. In this review we will first highlight the cellular contribution to colostrum. Secondly, the impact of MDI on neonatal immune development, including the role of maternally-derived cell-mediated immunity (CMI) on neonatal immune response development, will be emphasized.

A relatively large number of leukocytes are found in porcine colostrum, about $1x10^7$ cells per ml, and the lymphocyte contribution to porcine colostrum is about 20% of the

total leukocyte population in colostrum. Colostral lymphocytes are functional in the recipient. Piglets receiving maternally-derived CMI have greater proliferative responses to mitogens (Williams, 1993). Importantly, transfer of CMI reactivity to selected specific antigens as evidenced by delayed type hypersensitivity (DTH), has been detected in recipients of maternal colostrum (Bandrick et al., 2008; Schlesinger and Covelli, 1977; Rifkind et al., 1976; Schnulle and Hurley, 2003), suggesting that maternally-derived CMI may confer protection to suckled neonates.

MDI does influence neonatal immune development. Colostrum is an important source of growth factors necessary for gut closure and the development of immune competence. MDI has also been shown to positively influence the activation status of monocytes and peripheral lymphocytes in the recipient (Donovan et al., 2007; Reber et al., 2008; Reber et al., 2005; Reber et al., 2006). Maternal immunoglobulins are classically blamed for interfering with immune responses in neonates. Whether maternally-derived CMI interferes with neonatal immune responses requires investigation. Neonates often do respond to recall antigens with CMI responses when vaccinated in the face of MDI. However, the conditions concerning the neonatal CMI response in the face of MDI warrants investigation, especially as related to neonatal immunization.

1.3 The requirement for MDI within the neonate

1.3.1 Immune status of the newborn pig and the requirement for maternal immunity

The relative importance of obtaining maternal immunity in the form of colostrum and milk is largely based on placentation. The epitheliochorial porcine placenta is six-layered

and prevents any *in utero* transmission of maternal antibodies or immune cells to the developing fetus. As a result, piglets born following healthy pregnancies are agammglobulinemic (Curtis and Borne, 1971; Porter, 1969) and without the benefit of MDI. In contrast to swine, humans have hemochorial placentae, and as a result are born with the benefit of MDI as the hemochorial placenta allows transfer of maternal antibody mediated immunity (AMI; Simister, 2003) and CMI (Shimamura et al., 1994).

Pigs are useful models of immune development because of their "virgin immunologic" status at birth. The gestation length for pigs is 114 days. Lymphopoietic activity can be detected in fetal pigs as early as the 20th day of gestation (Sinkora et al., 2002). CD45+ cells can be detected as early as day of gestation 17 and 45 in the yolk sac and in the bone marrow, respectively (Sinkora et al., 2002; reviewed in Butler et al., 2006). At day 40 of gestation B cells can be found in the spleen and T cells can be found in the thymus. γδ T lymphocytes can be found peripherally at gestation day 45, almost 2 weeks before αβ T lymphocytes. Yet B cells remain the major peripheral lymphocyte until mid-gestation and αβ T lymphocytes outnumber γδ T lymphocytes from mid-gestation until birth. T and B lymphocytes can be found in the circulation at the beginning of the third trimester, day 76 of gestation.

Pig fetuses have the ability to produce IgG (antigen-dependent) *in utero* (Tlaskalova-Hogenova et al., 1994) and immunoglobulins (about 1000x less than that of a post-colostrum or adult pig) have been detected in piglet serum prior to colostrum ingestion (Bianchi et al. 1992; Porter and Hill, 1970). IgM induction by bacterial mitogens can be detected in the liver and spleen at day 44 of gestation (Sinkora et al., 2002). Despite this, pigs born from healthy pregnancies are born without antigen-specific antibodies. Piglets are born with a B cell

repertoire that includes natural antibodies (Chen et al., 1988). These natural antibody-expressing B cells, also sometimes classified as B-1 cells, have non-random, restricted variable regions (Butler et al., 2000; Sun et al., 1988). Natural antibodies form devoid of environmental antigen stimulation, yet recognize bacterial and self-antigens. Natural antibodies are found in sharks and mammals (Marchalonis et al., 1998).

T lymphocytes have been isolated from piglet blood prior to colostrum ingestion, albeit at half the concentration post-colostrum (Bandrick et al., Dev comp immunol *submitted* 2010, chapter 2 of thesis). Also, the T cell profiles of neonates and adults differ (Bandrick et al., Dev comp immunol *submitted* 2010, chapter 2 of thesis). Pigs have a large population of circulating γδ T lymphocytes, unlike humans and mice in which most γδ lymphocytes are found in mucosal tissues. Maturation of αβ T lymphocytes is thought to be similar among swine and humans. Mature αβ T lymphocytes exported from the thymus into the circulation are CD4+CD8- or CD4-CD8+. However, activation and the generation of memory T cells results in dual expression of CD4 and CD8, i.e. double positive cells. Since concurrent expression of CD4 and CD8 on a single lymphocyte is antigen dependent, double positive cells are absent in the newborn pig (Sinkora et al., 1998).

T lymphocytes isolated from newborn pigs are functional. T lymphocytes isolated from neonatal piglets respond to mitogen stimulation, but to a lesser degree than those from adult pigs (Gaskins, 1998). In addition, like AMI, piglets develop CMI responses to antigen stimulation *in utero*. Recall responses to antigens encountered *in utero* have been detected after birth. For example, piglets exposed to pseudorabies virus prior to birth demonstrate secondary responses upon re-exposure (Sinkora et al., 2002).

Fetal and newborn pigs arising from healthy pregnancies (no *in utero* infection) are

immunologically naïve and immunologically immature (Binns, 1967; Redman et al., 1978; Hammerberg et al., 1989; Gaskins, 1998). Despite their naïveté, piglets are born with a complete, albeit immature, immune repertoire. An important question is how MDI influences the immune status and immune development of piglets. It is clear that piglets that are allowed to ingest colostrum are at a distinct advantage to those piglets that are not. Colostrum contains antigen specific and non-specific factors that contribute to the immune repertoire of piglets (Bouma et al., 1998; Wittman and Ohlinger, 1987); colostrum serves as a source of immediate immune protection. Piglets not fed colostrum do develop detectable primary antigen specific immune responses by 7-10 days (Klobasa et al., 1981; Wilson, 1974.). Nevertheless, the mortality rate among colostrum-deprived piglets can be up to 100% if piglets are not fed sterilized diets or milk replacer and reared in sterile conditions (Aumaitre and Seve, 1978). Thus, acquiring MDI is critical for the conventionally reared pig.

Colostrum influences immune development and maturation in the neonate. For example, newborns require greater immune stimulation in order to mount immune responses at the level of an adult (Kelly and Coutts, 2000); colostrum contains factors that provide the piglet's developing immune system the extra stimulation and antigen presentation required to mount more effective immune responses. The regulatory role of MDI, however, is not clearly established. The relative importance of MDI versus endogenous immunity in the piglet requires investigation. The acquisition of passive immunity is critical for piglets, yet there may be aspects of MDI that are detrimental to the piglet. Table 1.1 describes ways in which maternal immunity is both beneficial and detrimental for the neonate.

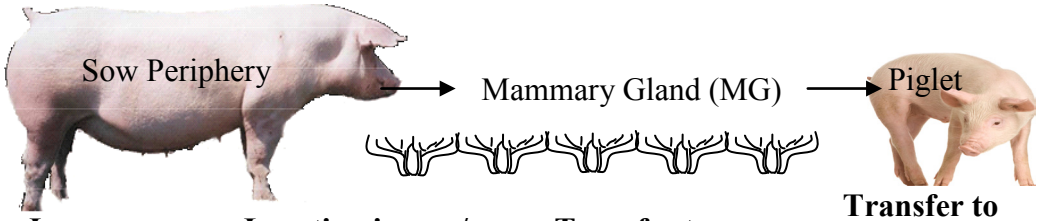
Table 1.1 Pros and cons of maternal immunity in the neonate

Pros of maternal immunity in the neonate	Cons of maternal immunity in the neonate
• Immediate source of immune protection • Contributes to the immune repertoire of the neonate • Antigen neutralization • Limit *in vivo* antigen replication • Opsonize antigen for optimal phagocytosis • Activate neonatal leukocytes • May allow for memory CMI responses in the neonate • May allow for memory AMI responses in the neonate	• Masks B-cell epitopes • Potential interference with antibody-mediated immune priming • Potential interference with cell-mediated immune priming

1.4 Maternally derived immunity: composition and *in vitro* properties

Colostrum is rich in essential nutrients and bioactive substances. These substances are necessary to protect neonates from disease and to foster immune system development, including the induction of tolerance, immune priming, antigen neutralization, and the development of immune memory. Colostrum contains a variety of specific (lymphocytes and antibodies) and non-specific (cytokines, chemokines, growth factors) immune factors (Figure 1.1). These immunomodulating factors contribute to the immune repertoire of piglets. Many good reviews have described the immune and non-immune constituents of colostrum (Wagstrom et al., 2000; Le Jan, 1996; Salmon, 2009). The immune constituency of colostrum, transfer of colostral immunity to neonates, and function of maternal immunity in the neonate will be emphasized here.

Figure 1.1 Integration of immunity: Transfer from sow to piglet



Immune component	Location in sow/ transfer to MG	Transfer to the MG	Transfer to piglet/ location in the piglet
Antibodies (IgG, IgM, IgA): blood & MG	IgG, IgM, IgA from so serum; concentrated via Fc-receptor in MG	IgA, IgG, IgM locally produced in the MG under hormonal control	Transfer via intestinal FcRn; Peripheral or local protection

Bourne & Curtis 1973; Porter 1988; Stokes & Bourne 1989; Schnulle & Hurley, 2003; Watson, 1980; Stirling et al., 2005

Lymphocyte (T & B): mucosa	MadCam-1, CCL25	VCAM-1, CCL28	Unknown transfer mechanism; Mucosa & Lymph node

Tuboly et al., 1988; Harp & Moon, 1988; Kleinman & Walker, 1979; Salmon, 2000; Bourges et a.l, 2008; Wilson & Butcher, 2004

Leukocytes: Macrophages, Neutrophils, Epithelial cells	Peripheral & Local source	Unknown transfer mechanism; Circulation & mucosa

Le Jan, 1996; Wagstrom et al., 1999

Cytokines: Inflammatory: TNF, IL-1, IL-6; Anti-inflammatory: TGF-b, IL-10; Th-1: IFN-g, IL-12; Th-2: TGF-b, IL-4; Growth promotants: Colony stimulating factors; Chemokines: IL-8	Peripheral and local source	Unknown

Ngyuen et al., 2007

Antimicrobial proteins/receptors: Defensins, lactoferrin, transferrin, lysozyme, soluble CD14, serum amyloid A, α-S_1 casein, β-casein, and κ-casein	Local source	Unknown

Shahriar et al., 2006; Labeta et al., 2000; LeBouder, 2003

1.4.1 Non-specific immune components in colostrum and milk:

There are many non-specific immune components in sow colostrum. Enzymes (more than 40), hormones, and other immunomodulatory proteins including cytokines and antimicrobial peptides, have been detected in colostrum. Hormones in colostrum, e.g. cortisol and prolactin, are thought to regulate epithelial cell growth and maturation in the recipient. Factors such as sow colostrum trypsin inhibitor (SCTI) protect transferred immune components from proteolysis (Thode Jensen, 1978; Thode Jensen and Pedersen, 1982).

Cytokines present in sow mammary secretions include pro-inflammatory (TNF, IL-1, IL-6) and anti-inflammatory (TGF-b, IL-10) cytokines as well as growth promotants (colony stimulating factors) and chemokines (IL-8) (Ngyuen et al., 2007). Additionally, Th-1 (IFN-γ, IL-12) and Th-2 (TGF-β, IL-4) response-promoting cytokines are present in mammary secretions. The capacity for colostral cells to produce cytokines had been demonstrated in many species; however cytokine production has only been demonstrated by colostral cells *in vitro*, not within the recipient. Colostral cytokines are absorbed by the piglets and can be detected in piglet blood following colostrum ingestion (Ngyuen et al., 2007). The role of colostral cytokines in the piglet's immune response is unclear.

Antimicrobial peptides, carbohydrates, and lipids have also been detected in colostrum. Antimicrobial peptides found in colostrum include defensins, lactoferrin, transferrin, and lysozyme. Serum amyloid A, α-S_1 casein, β-casein, and κ-casein have been detected in porcine colostrum (Shahriar et al., 2006). Soluble pattern recognition receptors including soluble TLR2 and soluble CD14 have been detected in human (Labeta et al., 2000; LeBouder., 2003; Filipp et al., 2001) and porcine (Shahriar et al., 2006) mammary secretions. Importantly, *in vitro* digestion with pepsin does not inhibit the lipopolysaccharide (LPS)-

binding activity of lactoferrin, α-S_1 casein, and κ-casein (Shahriar et al., 2006). The supposed function of these innate defenses is to bind LPS in the neonatal intestine to prevent bacterial or LPS mediated disease (Shahriar et al., 2006). In support of this supposition, colostrum deprived piglets given lactoferrin orally were protected against endotoxin shock induced by intravenously administered LPS (Lee et al., 1998).

In addition to the passive protection afforded by immunomodulatory proteins, the cellular portion of colostrum also provides an important role in defense. There are over 1-10x10^6 live cells per ml in colostrum and it is estimated that piglets obtain 500-700 million maternal cells daily (Evans et al., 1982; Le Jan, 1994; Magnussen, 1999). Macrophages, neutrophils, epithelial cells, and lymphocytes are all found in colostrum. Colostrum-derived macrophages have been observed migrating from the intestinal lumen to the submucosa (Owen and Heyworth, 1985). Colostrum-derived macrophages have also been found in peripheral lymphoid organs in neonatal mice (Hughes et al., 1988). Colostral polymorphonuclear cells (PMN) (Osterlundh et al., 1998) and macrophages (Weaver et al., 1982) exhibit phagocytic capabilities. Colostral PMN have decreased activity compared to that of PMN found in the blood (Le Jan, 1996). In contrast, colostral macrophages display functions, such as motility (Okaragoz et al., 1988), at a higher degree than blood monocytes. Macrophages found in colostrum express MHC II, which is enhanced following stimulation with IFN-γ (Rivas et al., 1994). Further, calves fed complete colostrum demonstrate greater phagocytosis of *Streptococcus agalactia* than calves fed acellular colostrum (Riedel-Caspari and Schmidt, 1991a). Additionally, calves receiving complete colostrum have higher serum lysozyme levels than calves fed acellular colostrum (Riedel-Caspari and Schmidt, 1991); macrophages are the main source of serum lysozyme in cattle (Gennaro et al., 1978).

Macrophages produce cytokines that direct T cell activity and colostral macrophages have a role in mediating T cell responses in the neonate (Ichikawa et al., 2003).

At 20% of the cellular portion of porcine colostrum, epithelial cells constitute a major portion of the non-antigen specific cells in colostrum. Porcine colostral epithelial cells have characteristics of alveolar epithelial cells, and are thought to act as antigen presenting cells (Le Jan, 1996). Colostral epithelial cells are an important contributor to the neonatal pig's immune repertoire because the neonates' antigen presenting cells require greater stimulation than that of an adult to function at a mature level (Adkins, 2004).

1.4.2 Specific immune components in colostrum and milk

MDI is most often thought about in terms of AMI even though both antibodies and lymphocytes are important specific immune components of colostrum. The immunoglobulin distribution and cellular contribution to mammary secretions varies across lactation stage and species. IgG is the dominant colostral isotype in species where colostral antibodies migrate to the circulation of the recipient (pigs, ruminants, and horses). IgA is the dominant colostral isotype in species where colostral antibodies serve a locally protective role in the intestine of the recipient (humans). Further, lymphocytes are important contributors to colostrum in species where MDI is not acquired across the placenta. The cellular contribution to colostrum has traditionally been overlooked but immune cells are undoubtedly a necessary component of colostrum and warrant characterization.

1.4.2.1 Immunoglobulin in colostrum and milk

IgG, IgA, and IgM are found in swine colostrum, with IgG being the predominant immunoglobin class (Karlsson 1966a). There is approximately 61.8 (SD = 2.44) mg/ml IgG, 9.66 (SD = 0.59) mg/ml IgA, and 3.19 (SD = 0.21) mg/ml IgM in sow colostrum (Courtis and Bourne, 1971). One hundred percent of colostral IgG are derived from serum of sows (Bourne and Curtis, 1973). IgG destined for colostrum moves from sow serum into the mammary gland under hormonal control (Bourne and Curtis, 1973; Porter, 1988; Stokes and Bourne, 1989) and is concentrated in colostrum via Fc-receptors (Fc-r) on glandular epithelial cells (Schnulle and Hurley, 2003). Movement of polymeric IgM and IgA into colostrum is mediated by the poly-Ig receptor within the mammary gland (Kumura et al., 2000). Postpartum hormonal changes stimulate the production of milk and inhibit colostrum production. Concurrent with the change in hormone production and the beginning of milk production, the immunoglobulin origin and predominating immunoglobulin class switches from IgG to IgA.

While IgG constitutes over 80% of the immunologobulin in colostrum, it makes up only about 30% of the immunoglobulin in milk. IgA is the predominant immunoglobulin class in sow milk. There is 3.41 (SD = 1.01) mg/ml IgA, 1.91 (SD = 0.64) mg/ml IgG, and 1.17 (SD = 0.23) mg/ml IgM in sow milk (Courtis and Bourne, 1971). IgA is not concentrated from serum; rather IgA is secreted locally within the mammary gland (Bourne, 1976; Bourne, 1977; Butler, 1998; Stokes and Bourne, 1989; Vaerman et al., 1970). Approximately 30% of the IgG in milk is synthesized locally within the mammary gland (Bourne and Curtis, 1973).

1.4.2.1.1 Acquisition and function of maternally-derived immunoglobulin

One of the first reports to show that IgG, IgM, and IgA make up the immunoglobin repertoire of porcine colostrum also demonstrated that the piglet's agammglobulinemic status at birth is dramatically altered following colostrum ingestion (Karlsson, 1966b; Porter, 1969). The alteration in immunoglobulin status is due to colostral immunoglobulin. Colostral IgG, IgM, and IgA are absorbed across the intestine and are found in piglet serum (Watson, 1980).

Colostral antibodies are nonselectively absorbed via transcytosis into enterocytes (Danielsen et al., 2006) even though Fc-R are present on the apical surface of gut epithelial cells (Stirling et al., 2005). The relative absorption efficiencies of different immunoglobin classes across the neonatal intestinal epithelium requires classification. However, IgA and IgM are found in crypt cells and IgG is found in villus cells after gut closure (Butler et al., 1981), suggesting that immunoglobulin absorption may be isotype specific. Colostral IgA that is first absorbed by enterocytes is then secreted onto the intestinal mucosa; colostral IgG remains in circulation (Stokes and Bourne, 1989).

No source restriction exists for antibody transfer in swine (Klobasa et al., 1981). Colostral immunoglobulins cross the neonatal intestinal epithelium and enter the circulation independent of source, whether it be immunoglobulins from another dam or from another species (Klobasa et al., 1981); however the efficiency of cross species immunoglobulin transfer may be increased in the presence of porcine colostrum (Jensen et al., 2001; reviewed in Sanglid, 2003). Maternally-derived AMI serves an important immune protective role in the neonate. It should be emphasized that the amount of antibodies in colostrum is not directly correlated with protection from disease in the recipient.

Reviews regarding maternal humoral immunity in the pig have been published and will be briefly discussed here. Passive immunity is an immediate source of immune protection for neonates. Passive immunity has been shown to provide at least partial protection when piglets were challenged with porcine circovirus-2 (PCV-2; Opriessnig et al., 2008), *Bordetella pertussis* (*B. pertussis*; Elahi et al., 2006), classical swine fever (CSF; Parchariyanon et al., 1994), transmissible gastroenteritis virus (TGEV; Sestek et al., 1996), pseudorabies virus (Kritas et al., 1997), and other agents. Maternally-derived AMI has been shown to limit antigen replication *in vivo*, neutralize antigen replication, and act as an opsonin to enhance phagocytosis. Shedding of bacteria or viruses and clinical signs of disease following challenge is less when piglets are challenged in the presence of maternally-derived AMI; this is true for *B. pertussis*, (Elahi et al., 2006), *Haemophilus parasuis* (Blanco et al., 2004), PCV2 (Opriessnig et al., 2008), and swine influenza virus (SIV; Loeffen et al., 2003). Maternally derived AMI does allow for neonatal memory B cell generation in certain cases (Kimman et al., 1989) and does not inhibit T cell responses in select systems (Pomorska and Markowsk. 2010; Gans et al., 1998).

On the other hand, maternally-derived AMI has been shown to down regulate endogenous immunoglobulin synthesis (Klobasa et al., 1981; Klobasa et al., 1986). This down regulation of neonatal active immune activation is commonly referred to as "passive interference." Maternal AMI may passively interfere with AMI priming in the recipient. Some degree of passive interference has been documented for maternally immune piglets challenged with many pathogens, including SIV (Kitikoon et al., 2006), CSF (Suradhat and Damrongwatanapokin, 2003) *M. hyopneumoniae* (Haesebrouk et al., 2006), and pseudorabies (Wirrat and Ohlinger, 1987) among others. Interestingly, transferred antibodies inhibit the

neonatal AMI response in a dose-dependent fashion (Siegrist, 2003; Dagan et al., 2000; Albrecht, 1997).

Milk derived immune components provide an alternate means of protection compared to that of colostrum. The switch from colostrum to milk occurs at the same time of gut closure. A consequence of gut closure is that immunoglobulins and immune cells are no longer able to pass across the intestine and enter into the neonatal circulation. Thus, immune components in milk serve to protect the neonate's intestine locally. It has been postulated that the immunoglobulin in milk may be able to cross the neonatal intestine since Fc-R are present on the apical surface of gut epithelial cells (Stirling et al., 2005); however, it is generally accepted that the main function of milk-derived immunoglobulin, namely IgA, is mucosal protection (Stokes and Bourne, 1989).

1.4.2.2 Maternally derived immunity: Cellular immunity

About 20% of the cells found in sow colostrum are lymphocytes and 70-90% of these are T cells (Le Jan, 1996). Contributions by studies of different species have made it clear that colostral lymphocytes are phenotypically memory and activated cells (Parmeley, 1976; Richie et al., 1980; Evans et al., 1982; Bertotto et al., 1990; Jain, 1991; Park et al., 1992; Bertotto et al., 1996). *In vitro* functional activity of colostral lymphocytes has been demonstrated (Figure 1.2). Colostral lymphocytes proliferate following stimulation with mitogens (Evans et al., 1982; Le Jan, 1994) and in response to bacterial and viral antigens (Ogra and Ogra, 1978; Parmely et al., 1976; Park et al., 1992) *in vitro*. Colostral lymphocytes have bactericidal and fungicidal functions (Robinson et al., 1978). Cytokine production by porcine (Le Jan, 1996), human (Kohl et al., 1982; Skansen-Saphir et al., 1993), and bovine

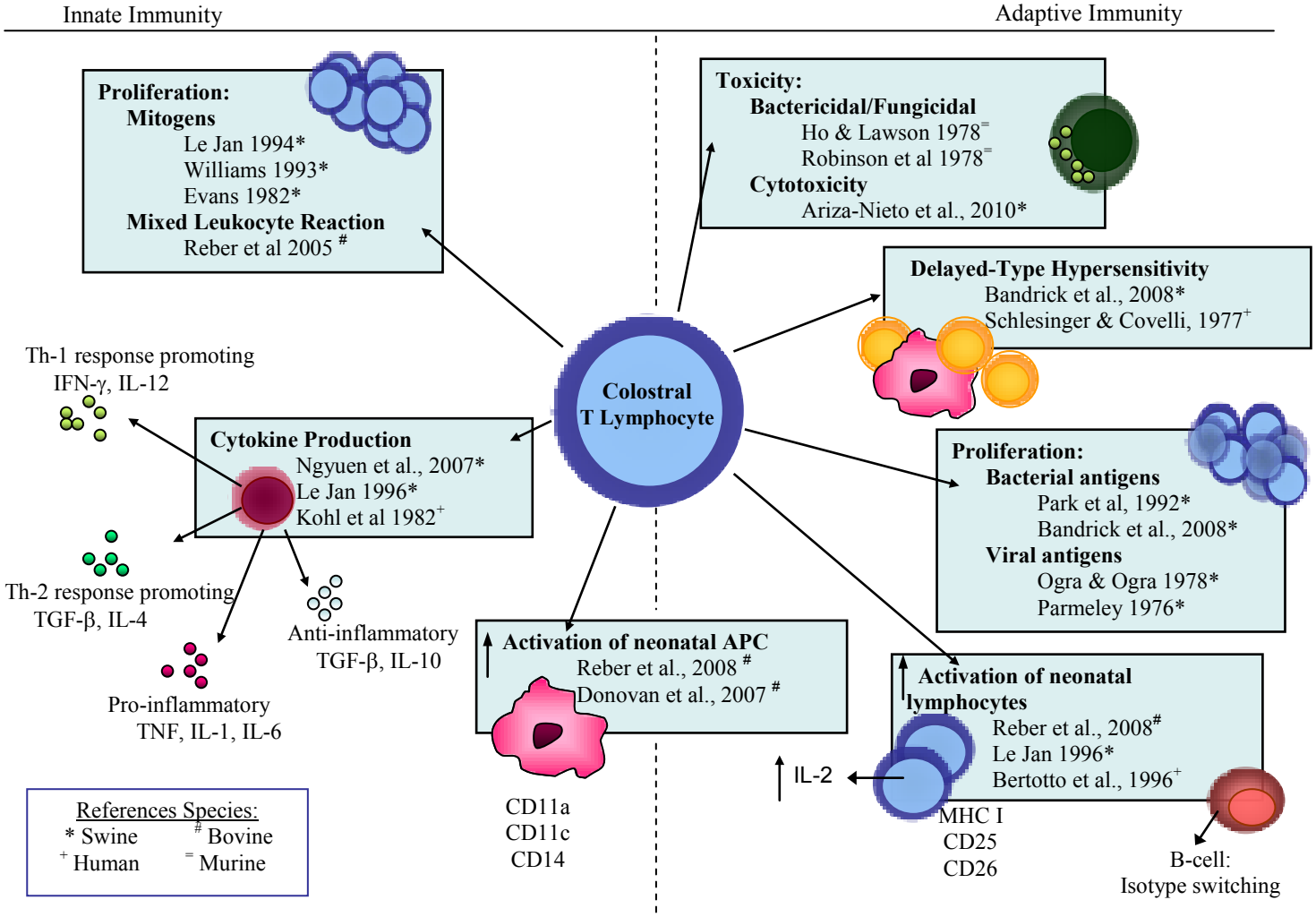
(CD8 cells producing IFN-γ) colostral cells has been demonstrated. Additionally, antibody production by colostral B lymphocytes has been documented (Ahlstedt et al., 2006).

The *in vivo* function of colostral lymphocytes is less well documented compared to that of the *in vitro* functions. Transfer of CMI sensitivity from mom to offspring to selected antigens has been shown in pigs, humans, mice, and cattle. Colostral lymphocytes display proliferative responses when stimulated with recall antigens (Bandrick et al., 2008). The protective capacity of colostral lymphocytes requires further investigation.

Cells found in mammary secretions arise from the common mucosal immune system, which includes the intestinal, respiratory, reproductive, and mammary lymphoid tissues (Harp and Moon, 1988; Kleinman and Walker, 1979; McDermott and Bienenstock, 1979). Using radioactive chromium-labeled lymphocytes that originated from the mesenteric lymph node, Harp and Moon (1988) found that mesenteric lymph node lymphocytes migrated to the mammary gland when intravenously infused into their donor. Experiments demonstrating that cells migrating between mucosal compartments and end in the mammary gland support the entero-mammary immune link (Kleinman and Walker, 1979). Cells normally found within the mammary gland and other organs of the common mucosal immune system prior to lactogenesis serve an immune defensive role locally (Chabaudie et al., 1993; Stokes and Bourne, 1989; Evans et al., 1982; Le Jan, 1994; Magnussen, 1999). However, during lactation many of these cells are found within the lumen of the mammary gland (Chabaudie et al., 1993; Magnussen, 1999; Salmon, 1987; Stokes and Bourne, 1989) and will be transferred to suckling neonates in the form of colostrum or milk.

Homing to the mammary gland may be due to MadCAM-1 and CCL25 recruitment of T cells. MAdCam-1 is the major mucosal vascular addressin and is well know for its role

Figure 1.2 Function of Colostral T lymphocytes



in lymphocyte homing to the Peyer's patch and extravasation into the intestinal lamina propria (Fong et al., 1997). CCL25 is a chemokine that is selectively expressed by small intestinal epithelial cell; CCL25 is attractant for T cells (Salmon, 2000; Bourges et al., 2008). Similarly, homing of lymphoblasts to the mammary gland may be due to VCAM-1 and CCL28. VCAM-1 is an inducible adhesion molecule expressed on activated endothelial cells; CCL28 is expressed by mucosal epithelial cells and is chemoattractant for mucosal homing of T and B lymphocytes (Bourges et al., 2008; Wilson and Butcher, 2004).

1.4.2.2.1 Acquisition of maternally-derived CMI and lymphocyte trafficking in the neonate

Studies in mice (Weiler et al., 1983), primates (Jain et al., 1989), sheep (Schnorr and Pearson, 1984), and swine (Tuboly et al, 1988; Williams, 1993) have demonstrated that cells found within the mammary gland and in lacteal secretions cross the neonatal intestine. Cells are absorbed across the neonatal piglet's intestine via an undescribed selection process. Absorption may take place intercellularly (Tuboly and Bernath, 2002); however, based on research by Tuboly et al., (1988) and Williams (1993), cellular transmigration across the neonatal intestinal epithelium is a receptor mediated event. Using technetium-labeled cells (Tuboly et al., 1988) or fluoroscein-labeled cells (Williams, 1993), it was demonstrated that maternal colostral cells cross the neonatal intestinal epithelium and are found in the neonate's circulation and immune tissues. In contrast, heat-killed cells, blood mononuclear cells (BMC), and cells from sources other than the piglet's own mother do not transverse the intestinal barrier (Tuboly et al., 1988;

Williams, 1993). The receptors involved in cellular transmigration have not been described. MHC class I is likely not involved in piglet recognition of colostral cells since there is very weak expression of SLA 1 in intestinal epithelium and MHC expression does not increase immediately following colostrum ingestion (Le Jan, 1996b). Selectivity in transfer of colostral cells may be related to cellular phenotypic changes incurred while leukocytes are in the colostral environment (Reber et al., 2006).

Following cellular transmigration across the piglet's intestine, colostral cells migrate to secondary lymphoid organs. Technetium labeled colostral cells that were injected directly into the stomach or into the jejunum, or through a naso-esophageal tube into piglets were detected via autoradiography in the mesenteric lymph node (Tuboly and Bernath, 2002; Tuboly et al., 1988; Tuboly et al., 2002). Fluorescein-labeled colostral cells fed to piglets were detected in the neonatal liver, lung, lymph nodes, spleen and gastrointestinal tract (Williams, 1993). Homing to these secondary lymphoid organs is expected since lymphocytes from mucosal compartments express integrins ($\alpha_4\beta_1$ or $\alpha_4\beta7$) or chemokine receptors (CCR9) that bind to mucosal addressins (MAdCAM-1 or CCR25) on mucosal epithelium (Bourges et al., 2008). The colostral environment imparts phenotypic markers to lymphocytes (Reber et al, 2006) and allows for migration within the piglet (Harp and Moon, 1988). Therefore, the colostral environment may activate lymphocytes to express different adhesion molecules.

Unlike immunoglobulin transfer (Klobasa et al., 1981), transfer of colostral cells is dependent on their being maternal in origin and viable (Tuboly et al., 1988; Williams, 1993), indicating an evolutionary importance to colostral cells. A study involving cross-fostering pigs at different times after birth support the hypothesis that only maternal

cells cross the neonatal intestine (Bandrick et al., Vet Record *accepted* 2010). Flow cytometric analysis of the neonatal intestine has revealed that CD3+ lymphocytes are first found when piglets are 2-days-of-age (Herich et al., 2004). Further, incubation of neonatal intestinal explant cultures with maternal colostral cells showed colostral cells infiltrating the submucosa by day 2 of incubation (Williams et al., 1985). Thus, the CD3+ cells found within the 2-day-old pig's intestine are most likely maternal colostral cells populating the intestine. This could be confirmed by feeding piglets labeled maternal colostral cells and later imaging the intestine for labled, CD3+ cells.

1.4.2.2.2 Function of maternally-derived CMI in the neonate

Maternally-derived cells are transferred to neonates where they serve an essential role augmenting the newborns' immune systems (Bourne, 1976; Chabaudie et al., 1993; Le Jan, 1996; Magnussen, 1999; Newby et al., 1982; Porter, 1988; Stokes and Bourne, 1989; Tuboly et al, 1988). MDI to environmental and vaccine antigens are transferred to piglets via colostrum. Vaccination of pregnant dams is often practiced as a way to increase colostrum quality by increasing the antibody titer and specific CMI components found in colostrum. Therefore, colostrum is a medium whereby passive immunity to key pathogens may be conferred to young animals. The action or protective capacity of passively transferred immune components in the neonate is unclear, especially as related to transferred maternal cells.

Studies on the ability of porcine colostral cells to respond in the recipient piglet are restricted to mitogenic responses (Parmeley, 1976; Ogra and Ogra, 1978: Richie

et al., 1980; Evans et al., 1982; Salmon, 1987; Riedel-Caspari and Schmidt, 1991b; Park et al., 1992; Williams, 1993; Le Jan, 1994). A commonality among these studies is that pigs with the benefit of maternal colostral cells demonstrate greater responses to mitogens than to those not allowed to ingest maternal cells. However, none of theses studies investigated colostral cellular immunity directly, rather the activity of BMC between piglets fed maternal colostrum and those not fed maternal colostrum were compared.

Studies focusing on the antigen-specific functional ability of colostral lymphocytes in the recipient are limited. Colostral lymphocytes like colostral macrophages cross the neonatal intestine, suggesting that their influence extends beyond mucosal immunity. Studies in humans and mice suggest that transferred colostral cells have functional ability. Transfer of sensitivity to tuberculin has been shown in breast fed infants (Schlesinger and Covelli, 1977). Further, transfer of sensitivity to nematode and fungal antigens has been demonstrated in mice (Rifkind et al., 1976; Schnulle and Hurley, 2003). Still, humans and mice are exposed to maternal immunity during gestation and the detection of antigen-specific reactivity at birth may be a result of antigen priming while *in utero* (King et al., 1998; Rahman et al., 2010). Therefore direct investigations into the ability of colostral lymphocytes to respond to antigen-specific stimulation in the recipient are necessary.

There are a few examples of maternal antigen specific cellular immunity being functional in the newborn without the confounding issue of transplacentally derived immunity. It has been shown in pigs, sheep, and cattle that transferred maternal cellular immunity confers antigen specific cellular immune responsiveness in the neonate.

Vaccination of ewes against tetanus toxid has shown that antigen specific maternal lymphoid cells are transferred to neonates and that these cells are active in lambs (Tuboly and Bernath, 2002; Tuboly et al., 1995). Also, newborn piglets from *M. hyopneumoniae*-immunized dams demonstrate antigen specific DTH responses and antigen specific lymphoproliferative responses (Bandrick et al., 2008). DTH responses in piglets were due to responding maternal cells since the piglets were not exposed to *M. hyopneumoniae in utero* and timing of immune testing precluded the induction of endogenous anti-*M. hyopneumoniae* immunity (Bandrick et al., 2008). Therefore, viable maternal CMI is transferred in colostrum to lambs and piglets and is functional in the recipients.

1.5 Maternal influence on neonatal immune development

1.5.1 Introduction

Colostral immune components transferred to the neonate serve to aid in protection against pathogen challenge. However, transferred colostral products also play a role in the development of immune competence (Butler and Kehrle, 2004). Colostrum and milk provide factors that regulate or facilitate immune development and responsiveness. For example, porcine reproductive and respiratory syndrome virus (PRRSV) induced immune dysregulation may be less in colostrum fed animals compared to animals not fed colostrum regardless of maternal PRRSV status (Lemke et al., 2004). Further, breast-fed infants have thymuses that are twice the size of that of infants that were not breast-fed (Jeppesen et al., 2004). Thus, colostral components appear to influence immune development in the neonate.

1.5.2 Colostrum stimulates neonatal immune development

1.5.2.1 Colostrum fosters neonatal gut maturation

1.5.2.1.1 Gut Closure

Neonates acquire maternal immunity via absorption of colostrum across the gastrointestinal tract (Butler, 1998; Hammer and Mossmann, 1978; Tuboly et al., 1988; Williams, 1993). Absorption of colostral components occurs for a short period after birth when the neonatal intestinal epithelium lacks tight junctions and epithelial cells are vacuolated (Moon, 1972; Simpson-Morgan and Smeaton, 1972; Werhahn et al., 1981). If suckling is initiated shortly after birth, piglets are able to absorb colostral antibodies and cells for approximately 24 h (Werhahn et al., 1981; Leece, 1973; Moon, 1972). The ingestion of colostral protein and glucose leads to gut closure, the cessation of augmented intact macromolecular uptake (Bourne, 1976; Werhahn et al., 1981). Absorption of colostral components across the neonatal intestinal epithelium decreases over time and the decrease in absorption is positively correlated with the amount of colostrum ingested (Bourne, 1976). Interestingly, the timing of gut closure can be delayed if feeding is delayed (Leece, 1973). Yet, delaying nursing does not result in the normal decrease in colostral whey protein as seen even six hours after nursing has begun (Bourne, 1969). At the same time as gut closure in pigs, colostralgenesis stops and milk production begins in the dam. The immune cells and immunoglobulin in milk are not absorbed; rather they exert their protective effects locally within the intestinal lumen.

Gut closure is also the result of intestinal maturation (Moon, 1972) and human milk has been shown to stimulate enterocyte maturation (Klagsbrun, 1978). The essential ingredient thought to be responsible for enterocyte maturation is epithelia growth

factor (EGF). Porcine colostrum contains about 1500 ng/ml EGF (Jaeger et al., 1987). Maturation of enterocytes involves a decrease in cellular vaculoation and an increase in tight junctions. Thus, colostrum promotes closure and the important barrier function of the intestine.

1.5.2.1.2 Development of immune competence

Intestinal colonization of commensal organisms promotes gut health in pigs and humans alike. In milk from healthy human mothers, there are up to 10^9 microbes/liter and some of these are "pre- and pro-biotics" (Moughan et al., 1992). Exposure to commensal organisms as well as exposure to LPS and other pattern associated molecular patterns (PAMP) alter mucosal T and B cell development. In fact, microbial colonization and exposure to PAMP may be necessary for the development of immune competence in pigs (Butler et al., 2002; Butler et al., 2005). For example, exposure to microbes and PAMP allows immune responsiveness to T-independent and T-dependent antigens (Butler et al., 2002; Butler et al., 2005). Exposure to these microbes/PAMP allows the induction of antibody class switch (Butler et al., 2005; MacPherson et al., 2000). Further, the balance between immune responsiveness and immune tolerance is at least in part directed by exposure to PAMP. Since peripheral tolerance is antigen directed, exposure to PAMP is necessary for tolerance to develop. The mechanism behind development of adaptive immune responsiveness and the requirement for antigen exposure and gut colonization is not clear; however, colostrum clearly influences the development of immune competence via the exposure of PAMP to neonate by way of microbes present in colostrum.

1.5.2.2 Maternal-derived immunity influences the development of immune tolerance

Transferred immunity either across the placenta or via colostrum can lead to the induction of tolerance (Claas et al., 1988). Tolerance to maternal antigens and non-inherited antigens has been documented. The survival of allografts donated by foster mother rats to normally reared neonatal rats is suggestive of tolerance (Head et al., 1977) mediated by MDI components that express maternal alloantigens. The mechanism of neonatal sensitization and tolerance to maternal alloantignes may be a result of maternal cells trafficking across the placenta in humans (Pollack et al., 1982) and mice (Poitrowski and Croy, 1996), or the transfer of soluble maternal immune components (Barneston et al., 1976). In line with neonatal sensitization to maternal antigens, the neonatal MLR is greater to non-inherited antigens compared to alloantigens in cattle, a species in which no MDI is acquired transplacentally (Reber et al., 2005). Others have suggested that the vertical transmission of environmental antigen is a viable mechanism for neonatal sensitization to maternal antigens (Stastny, 1965; Horton and Oppenheim, 1976). The generation of tolerance to maternal alloantigens is established in neonates early on and lasts at least until adulthood, as evidenced by the presence of maternal antigens in 18 year-old humans (Mold et al., 2008).

Tolerance to non-inherited antigens has also been documented. In fact, breastfeeding may be involved in the development of tolerance to oral and airborne allergens. The IgE-mediated allergy response is suppressed in rat pups by milk from immunized dams (Roberts and Turner, 1983); however this response may be due to passive interference and not due to the development of tolerance. Maternal IgA may

block antigen binding in the immature gut and prevent the induction of an allergic response. Tolerance to food protein can be transferred in milk (Korotkova et al., 2004) and this tolerance may be due to TGF-β, which is known to suppress autoimmune responses and may play a role in IgA class switch events (Stavnezer, 1995; Briere et al., 1995). Alternatively, tolerance to food protein may be due to long chain polyunsaturated fatty acids found in milk (reviewed in Palmer and Makrides, 2006). When airborne allergens were transferred to mice pups through milk, tolerance to these allergens and protection against allergic airway disease was observed (Verhasselt et al., 2008). The mechanism behind allergen tolerance is not clear; however, many have postulated that TGF-β and maternal IgA are important mediators.

The transfer of immunity via colostrum leads to the generation of tolerance to maternally-derived cells given their longevity in the recipient, possibly via the induction of a regulatory T cell (T regs) phenotype (Mold et al., 2008). The evaluation of T regs in swine blood or colostrum is problematic and requires more study. There are no CD25 positive cells in sow colostrum (Le Jan, 1994); however, CD25 expression on swine cells is not a valid measure of T cell activation as it is in other species (Bandrick et al., unpublished observations). Accordingly, while CD25 expression on CD4+Foxp3+ T cells dictates a regulatory T cell phenotype in mice and humans, the phenotype of the regulatory T cell may be different in swine than in mice and humans. Protocols for the assessment of Foxp3 expression on swine lymphocytes have recently been characterized (Kaiser et al., 2008; Wongyanina et al., 2010) but have not been applied to cells isolated from neonatal piglets or colostral cells.

1.5.2.3 Effects on neonatal leukocytes

1.5.2.3.1 Antigen Presenting cells

Maternal colostral leukocytes are able to influence the activation status of neonatal leukocytes. In calves that received acellular colostrum, greater numbers of CD11a, CD11c, and CD14 expressing cells were found in blood compared to calves that received whole colostrum (Donovan et al., 2007). This elevation in phenotypic markers is most likely due to the greater number of monocytes in calves receiving acellular colostrum compared to calves that received whole colostrum (Reber et al., 2008). Yet, monocytes and other antigen presenting cells (APC) from whole-cell colostrum fed calves bore higher levels of MHC class I and CD25 expression than those calves that received acellular colostrum (Donovan et al.,2007; Menge et al., 1999). Thus, whole colostrum appears to activate the recipient's monocytes. It is possible that an immune modulator or growth factor in colostrum stimulates APC to express MHC. Also, stimulation of neonatal APC with colostral leukocytes may activate APC to express MHC (Donovan et al., 2007).

The absolute number of APC in blood of whole-cell colostrum fed animals is higher compared to cell-depleted or non-colostrum fed animals (Donovan et al., 2007). Interestingly, calves that were fed whole colostrum had less CD11a positive cells and less density of CD11a per cell during the first 2 weeks of life compared to calves fed acellular colostrum (Reber et al., 2008). Since APC and their effector molecules direct the antigen-specific responses of T lymphocytes, it can be inferred that colostrum directly affects not only innate immune development, but antigen specific immune development as well.

1.5.2.3.2 Effects on T lymphocytes

1.5.2.3.2.1 Colostrum stimulates T lymphocyte maturation

MDI has been shown to nonspecifically modulate T cell maturation and activation. Calves fed acellular colostrum had fewer CD11a positive blood cells compared to those fed whole colostrum (Reber et al., 2005). Further, calves receiving whole colostrum have a higher percentage of lymphocytes expressing the activation markers CD25 and CD26 compared to those given acellular colostrum (Reber et al., 2008). The level of MHC I expression on lymphocytes isolated from calves fed whole colostrum was higher during the first week of life compared to cells from calves receiving cell-free colostrum (Reber et al., 2008). Thus, MDI influences development of neonatal lymphocytes as evidenced by the expression of lymphocyte activation markers. Mice born to dams stimulated with *Leishmania major*, a potent inducer of Th1 responses, had different ontologic T cell and NK cell profiles compared to progeny of non-immune dams (Fagoaga and Nehlsen-Cannarella, 2000), suggesting that the immune status of the dam also has a role in neonatal T cell development.

Neonates are predisposed to Th2 biased immune responses (Adkins and Du, 1998); however, the colostral environment may help regulate the Th1/Th2 balance. Intraepithelial lymphocytes of adult mice orally fed bovine colostrum had a greater propensity for Th1 type responses compared to those not fed bovine colostrum (Yoshika et al., 2005). Th1-response skewing induced by colostrum would be valuable for the neonate in the immune response against many infectious diseases and allergens.

1.5.2.3.2.2 Colostrum stimulates non-specific T lymphocyte reactions

The colostral environment stimulates functional activity of neonatal lymphocytes. Colostrum stimulates IL-2 production by BMC *in vitro* and inhibits natural killer (NK) activity in a dose dependent fashion (Sirota et al., 1995). In addition, colostral leukocytes stimulate neonatal lymphocytes to respond to mixed lymphocyte populations. Reber and colleagues (2005) showed that leukocytes isolated from calves receiving maternal whole-cell colostrum proliferated more and reached the same level of proliferation as that from a mature animal more quickly in response to stimulation with mixed leukocyte populations than those from calves receiving cell-depleted colostrum (Reber et al., 2005). Mixed leukocyte reactions (MLR) are stimulated by differences in MHC between the leukocyte populations. Colostral APC may be responsible for augmenting the neonatal T lymphocyte response to mixed leukocyte populations directly via antigen presentation and costimulatory factor expression.

Cells treated with colostrum or cells isolated from animals fed whole colostrum display greater proliferative responses than those not incubated with colostrum or from animals not fed colostrum or fed acellular colostrum. Incubation with low doses of milk has a stimulatory effect on human cord blood mononuclear cell proliferation; however, high doses of milk suppress proliferation of these same cells (Zizka et al., 2007). Maternal colostral leukocytes influence the neonatal lymphoproliferative response to superantigens and mitogens. Lymphocytes from calves that received whole colostrum demonstrated greater proliferative responses to stimulation with Staphylococcal enterotoxin (SE), a superantigen, compared to calves that received acellular

colostrum (Reber et al., 2005). SE, produced by *Staphylococcus aureus*, is a potent activator of T lymphocytes. The proliferative response to SE is mediated by APC presenting SE along with MHC II to T lymphocytes (Johnson et al., 1991). In the report by Reber and colleagues, 2005, it is not clear whether proliferation induced by SE was due to SE being presented by colostral or neonatal APC (Reber et al., 2005). The origin of APC in this case along with the MLR may be important since it is the neonatal APC that seems to be the limiting factor in appropriate immune responsiveness (Adkins et al., 2004). APC from neonates require greater co-stimulatory factor activation for effector function compared to APC from mature animals (reviewed in Adkins et al., 2004).

Colostral leukocytes also stimulate the neonatal T cell response independently from antigen presentation or the use of maternal APC to stimulate a T cell response. Lymphocytes isolated from piglets receiving whole cell colostrum display greater lymphoproliferative responses to phytohemagglutinin (PHA), a T cell mitogen, compared to piglets not receiving colostral cells (Williams, 1993). Similarly as in the study by Reber et al., (2005), it is not clear if the T lymphocytes responding to superantigen or mitogen stimulation are maternal or neonatal in origin. However, lymphocytes from pigs receiving maternal colostral cells proliferate more than lymphocytes from pre-colostral pigs. Thus, it appears that maternal colostral lymphocytes are contributing to neonatal proliferative responses. The contribution of maternally-derived CMI to the piglet's immune response to mitogens provides evidence that colostral T lymphocytes may also impart antigen specific reactivity and possibly protection to the neonate.

1.6 Passive interference of maternally-derived immunity on neonatal antigen-specific immune responses

1.6.1 Introduction

Maternal vaccination is often practiced in veterinary medicine as an indirect means to protect neonates by providing immediate protection from pathogens in the form of antigen specific AMI and CMI. Transferred MDI undoubtedly affects the development of the newborn's immune repertoire and response to specific antigens. While MDI is necessary for the survival of the neonate, it is not always beneficial for the neonate. Actively acquired immunity decreases disease associated with many infectious agents; however, the immune factors passively acquired by the neonate from its mother could be detrimental since passive immunity may interfere with active immune development following antigen exposure, i.e. passive interference.

Passive interference occurs when passively acquired immunity impedes the immune response in the recipient. Passive interference has been documented for many specices and many antigens. The neonatal immune response to environmental organisms encountered during the suckling period is largely uncharacterized. On the other hand, neonatal responses to vaccination in the face of MDI have been well documented. Neonatal vaccination is not often practiced in veterinary medicine because of the immaturity of the neonatal immune system and the potential for passive interference with MDI. Nonetheless, neonates vaccinated against certain pathogens do respond to vaccination in the face of MDI and are protected upon challenge in select systems (Ridpath et al., 2003).

1.6.2 Mechanisms of passive interference

The role of MDI in the piglet's response to antigen exposure is unclear but may depend on the antigen, the dose and route of exposure, the presence and type of adjuvant employed, piglet age at exposure, and the concentration of MDI, among other factors. It is generally accepted that transferred MDI, specifically maternal antibodies, will interfere with endogenous AMI responses (Reviewed in Siegrist, 1999). In fact, the degree at which maternally-derived antibodies inhibit endogenous antibody production by the piglet is directly related to the amount of antibody at the time of antigen exposure (Siegrist, 2003; Dagan et al., 2000; Van Oirschot, 1987; Albrecht et al., 1977). However, whether endogenous CMI responses are generated following antigen stimulation in the face of MDI is unclear.

Recent studies investigated the impact of high versus low maternal antibody titers on AMI responses in the neonate have found that in contrast to high titers, low titers of maternal antibodies do not suppress the induction of IgA responses (Nguyen et al., 2006a; Nguyen et al., 2006b). Nguyen and colleagues (2006b) suggest that the presence of maternal antibodies may alter effector cell trafficking within the recipient and thus, impact the development of protective AMI. Altered effector cell trafficking would decrease the likelihood of T cell-B cell interactions necessary for T helper functions, in this case, isotype switching.

The mechanism of passive interference is thought to be antigen neutralization by maternal antibodies (reviewed in Siegrist, 1999). Neutralization results in a lack of antigenic determinant binding sites, a lack of inflammation and the resultant Th1 response, and rendering of the antigen unable to bind or infect host cells. The

increased need for co-stimulatory factors for endogenous APC to induce an appropriate lymphocyte response (Adkins et al., 2004) in combination with the propensity for Th2-type of responses (Adkins and Du, 1998) and the low antigenic load due to neutralization, results in the neonate not responding or responding at a low level to antigenic stimulation.

1.6.3 Vaccination in the face of maternally-derived immunity

Passive interference has been a commonly documented event, especially as it pertains to AMI (Loeffen, 2002; Kitikoon et al., 2006; Haesebrouk et al., 2006; Wittman and Ohlinger, 1987; Kono et al., 1994; Klinkenberg et al., 2002; Surdahat and Damrongwatanapokin, 2003). It is commonly accepted dogma that vaccinating a young animal in the presence of MDI will result in no or delayed endogenous immune priming in the neonate because of passive interference. Passive interference is a major cause for concern because it prevents the neonatal immune system from mounting a response as well as from preparing for future antigen challenges. This holds true for many pathogens in many species. In swine, these pathogens include SIV (Loeffen, 2002; Kitikoon et al., 2006), *M. hyopneumoniae* (Haesebrouk et al., 2006), Pseudorabies (Wittman and Ohlinger, 1987), *Bordetella bronchiseptica* (Kono et al., 1994), and CSF (Klinkenberg et al., 2002; Surdahat and Damrongwatanapokin, 2003) (see table 1.2). On the other hand, recent evidence suggests that transferred maternal immunity may not inhibit the neonatal CMI response to certain pathogens. For example, MDI to SIV delays antibody responses in piglets but does not prevent the development of CMI responses (Pertmer et al.,2000; Radu et al., 2001; Loeffen et al., 2002; Kitikoon et al., 2006). Therefore, passive

interference may not be as restrictive as previously thought and may not apply to CMI responses in the recipient of passive immunity.

Table 1.2: Pathogens for which AMI responses do not occur in the presence of MDI in swine

Pathogen	Reference
Swine influenza virus	Loeffen et al., 2003
Pseudorabies	Wittan and Ohlinger, 1987
Mycoplasma hyopneumoniae	Haesebrouk et al., 2006
Bordetella bronchiseptica	Kono et al., 1994
Classical Swine fever	Klinkenberg et al., 2002

Recent studies suggest that passively acquired CMI is not subject to interference and may be useful in overcoming the inhibitive effects associated with maternally-derived antibodies in the newborn (Endsley et al., 2004; Gans et al., 1999; Siegrist et al., 1998; Cherry et al., 1973; Gans et al., 1999; Siegrist, 2001). For example, neonates vaccinated in the face of MDI respond with specific CMI responses to *M. hyopneumoniae* (Bandrick et al., unpublished data), bovine herpesvirus-1 (Endsley et al., 2003), pseudorabies virus (Van Rooj et al., 2006; Bouma et al., 1998, Wittman and Ohlinger, 1987), bovine viral diarrheal virus (BVDV; Endsley et al., 2004) and others (see

table 1.3). Yet, information that does exist on the ability of neonates to mount CMI responses in the face of MDI is largely limited to *in vitro* studies. Furthermore, the literature regarding CMI development in the face of MDI is inconsistent. For example, in one study, piglets vaccinated against SIV in the face of MDI responded with SIV-specific CMI responses (Loeffen et al., 2002), but did not in another investigation (Kitikoon et al., 2006).

Antigen specific lymphocyte proliferation and cytokine production have been demonstrated for maternally immune infants vaccinated against measles virus (Gans et al., 2001; Gans et al., 1998), and neonatal mice vaccinated against measles virus (Capozzo et al., 2006; Siegrist, 1998) or lymphocytic choriomeningitis virus (Seiler et al., 1998) (Table 1.3). Notably, humans and mice are exposed to MDI during gestation, and the detection of antigen specific reactivity in the neonate may be a result of antigen priming *in utero* (Rahman et al., 2010) or the transfer of maternally-derived CMI *in utero* (Pollack et al., 1982). The lack of exposure to MDI across the placenta makes pigs, cattle, and horses excellent models to study the role of colostral immunity in the neonate, yet few reports have investigated activation of CMI following vaccination of maternally immune neonatal piglets, calves, and foals. Bouma et al., (1998) showed that 3-week-old piglets challenged with pseudorabies virus developed virus-specific lymphoproliferative responses regardless of maternal pseudorabies status. Further, vaccinated maternally immune piglets had lower pseudorabies virus antibody titers than their non-maternally immune counterparts (Bouma et al., 1998). Bandrick et al., (*submitted* 2010b; chapter 5) also showed that maternally immune piglets respond to vaccination with CMI responses; however, unlike the previous study, Bandrick et al., (2010b; chapter 5) showed

evidence of *in vivo* CMI stimulation by DTH testing. DTH testing assesses *in vivo* anamnestic CMI responses.

Maternally immune calves vaccinated against BVDV developed CMI responses (Endsley et al., 2004) but did not show evidence of anamnestic AMI responses upon re-exposure to the antigen (Endsley et al., 2004; Ellis et al., 1996). Interestingly, BVDV vaccinated calves are protected from disease even in the absence of a specific antibody response (Ridpath et al., 2003), as are bovine respiratory syncytial virus-vaccinated calves (Mawhinney et al., 2005). Furthermore, Opriessnig and colleagues (2008) showed that piglets vaccinated against porcine circovirus-2 (PCV2) in the presence of MDI had reduced viremia and fewer microscopic lesions associated with PCV2 even without AMI responses as compared to piglets without MDI, suggesting that endogenous CMI responses protects piglets, at least partially, from PCV2 challenge. Therefore, just because maternally immune neonates fail to develop vaccine-induced AMI responses does not signify that there is an inhibition of the immune response. Rather, vaccination may induce protective T cell responses. Collectively these studies show that maternally immune neonates are capable of both generating CMI responses and that passively transferred MDI may not interfere with CMI induction (see Table 1.3).

On the other hand, antigen exposure of maternally immune neonates does not always result in CMI responses. Further, passively transferred MDI may not always be helpful for the piglet. Neonates vaccinated in the face of MDI do not demonstrate CMI responses following vaccination with CSF (Klinkenberg, et al., 2002) or SIV (Kitikoon et al., 2006). Vaccination of maternally immune neonatal piglets against CSF does not result in the induction of CMI and AMI responses when maternal antibody levels in

Table 1.3 Cell-mediated immune responses in the presence of maternally-derived immunity

Pathogens for which CMI responses occur in the presence of MDI			Pathogens for which CMI responses do not occur in the presence of MDI		
Pathogen	**Species**	**Reference**	**Pathogen**	**Species**	**Reference**
Swine influenza virus	Swine	Loeffen et al., 2002	Swine Influenza virus	Swine	Kitikoon et al., 2006
Mycoplasma hyopneumoniae	Swine	Bandrick et al., 2010	Classical Swine Fever	Swine	Klinkenberg et al., 2002
Bovine herpesvirus-1	Cattle	Endsley et al., 2003			
Pseudorabies	Swine	Van Rooij et al., 2006; Bouma et al., 1998; Wittman and Ohlinger, 1987			
Bovine viral diarrheal virus	Cattle	Endsley et al., 2004			
Measles virus	Humans	Bertley et al., 2004; Gans et al., 1998 ; Gans et al., 2001			
Lymphocytic choriomeningitis virus	Mice	Seiler et al., 1998			
Sindbis virus	Mice	Capozzo et al., 2006			
Porcine circovirus-2	Swine	Opriessnig et al., 2008			

the piglet are high (Klinkenberg et al., 2002; Surdahat and Damrongwatanapokin, 2003). Provided the level of maternal antibodies was low, piglets were protected from disease following vaccination and this protection was most likely due to enodogenously primed CMI, given the lack of AMI stimulation (Surdahat and Damrongwatanapokin, 2003).

Piglets with MDI to SIV were partially protected from disease following intra-tracheal (Kitikoon et al., 2006) or intranasal aerosol (Loeffen et al., 2002) challenge. However, pigs with maternal AMI specific for SIV developed weaker challenge induced anti-SIV immunity compared to pigs not receiving these antibodies (Loeffen et al., 2002; Kitikoon et al., 2006). Further, piglets with MDI to SIV shed virus for the same amount of time as maternally-non immune piglets (Kitikoon et al., 2006) or longer than piglets without maternal SIV-specific immunity (Loeffen et al., 2002). The difference in shedding between maternally immune and non-immune piglets following challenge may be due to a delay in nasal IgA production because of interference of maternal antibodies with AMI responses in the piglet (Loeffen et al., 2002). In fact, maternally immune piglets demonstrated no rise in anti-HI or anti-NP titers following a second inoculation (Loeffen et al., 2002). Thus, it would appear that MDI does not influence or negatively influences the neonate's response to SIV challenge. However, antibody titers are not necessarily correlated with protection (Ridpath et al., 2003).

On the other hand, SIV-specific lymphoproliferative responses were demonstrated by BMC isolated from piglets after the first (7 weeks) and second (15 weeks) inoculations. However, piglets from non-SIV immune dams had significantly greater lymphoproliferative responses at 7 days post inoculation than piglets with MDI (Loeffen et al., 2002). Yet the kinetics of responses were comparable between maternally-

immune and non-immune piglets 7 and 14 d post inoculation. Thus, MDI may also interfere with neonatal CMI responses. No memory T cell responses were detected 21 d post inoculation in piglets with SIV-specific MDI. Interestingly, upon challenge with a heterologous SIV strain, piglets with MDI to SIV demonstrated enhanced disease, potentially due to skewing of the T helper response to a Th2-type of response (Kitikoon et al., 2006) or due to original antigenic sin.

1.6.4 What conditions allow for maternally-derived immunity to passively interfere with neonatal CMI responses?

The potential for MDI to interfere with CMI responses in the neonate may be antigen dependent or dependent upon stimulation route, dose, use of an adjuvant, and the concentration of MDI at stimulation, similar criteria as used for interference with AMI priming in the neonate. The capacity for neonates to generate CMI responses following vaccination in the face of MDI may depend on their age and immune status at antigen exposure. Variables such as TH1/Th2 bias, APC maturity, and others contribute to the potential for passive interference in the neonate. Results from studies by Kitikoon et al., (2006) and Klinkenberg et al., (2002) provide evidence of selective responses imparted by the antigen. Since these two mentioned studies employed different vaccine preparations, modified-live (CSF) or inactivated (SIV) virus preparations, the vaccine type does not solely dictate the neonatal response to vaccination. DNA vaccines are an especially alluring neonatal vaccination paradigm since they replicate *in vivo* but do not pose a risk of infection. Some DNA vaccines induce CMI responses in neonates (Capozzo et al., 2006; Hassett et al., 1997; Manickan et al., 1997) while others do

not (Wang et al., 1998; Le Potier et al., 1997; Capozzo et al., 2006). Further, adjuvants that enhance CMI responses or antigen presentation (such as CpG motifs) may help the neonatal immune system overcome the opposing effects of maternally-derived AMI (Salerno-Goncalves and Sztein, 2006; Linghua et al., 2006).

Differences in neonatal responses to vaccination may also be due to the presence of functional maternal lymphoid cells at the time of vaccination. Various studies have shown that transferred maternal cells augment the neonatal response to nonspecific (Williams, 1993; Reber et al., 2005) and specific antigens (Bandrick et al., 2008; Kumar et al., 1989; Schelsinger and Covelli, 1977). We have shown that only piglets born to immune mothers exhibited *M. hyopneumoniae*-specific CMI responses 7 dpv (Bandrick et al., *submitted* 2010b). Therefore, CMI priming and anamnestic responses were not solely dependent upon piglet vaccination. Capozzo et al., (2006) showed that lymphocytes from maternally immune mice produce IFN-γ and proliferate to measles virus after only one vaccination. These studies suggest that maternal lymphoid cells play a role in the vaccine-induced CMI response detected in neonates.

The exact mechanism of CMI interference is not known. Transferred maternal CMI may interfere with endogenous CMI response development in the neonate via regulating inflammation or the activation status of endogenous lymphocytes. For example, in humans and other similarly placented mammals, T cells cross the placenta and develop a tolerant phenotype to survive (Mold et al., 2008). Therefore, transferred maternal cells react to their antigen inasmuch as their tolerant or regulatory phenotype allows them. Alternatively, transferred cells act as memory cells and respond to antigen in the standard way. In both cases passively transferred cells neutralize the antigen

before endogenous cells can respond or induce a regulatory phenotype of responding cells**.** A different mechanism whereby MDI may interfere with active CMI responses in the neonate is that transferred maternal antibodies neutralize the antigen prior to antigen recognition by T lymphocytes.

1.6.5 How can interference be overcome?

In order to overcome the inhibitive effects of passive interference, more information regarding the mechanism of passive interference needs to be gleaned. Only then can we begin to understand the variables of vaccination that lead to interference or immune priming in the maternally immune neonate. It is clear that the vaccination route (mucosal vs parenteral vs enteral), the vaccination type (killed or MLV), and immunogenicity, i.e. use of and type of adjuvant, influence a neonate's response to vaccination. Maternally immune neonates demonstrate cellular immune priming in the absence of AMI priming following a variety of vaccination regimens. For example, vaccination of maternal immune piglets against pseudorabies virus with DNA or killed vaccines induced AMI and CMI responses (Van Rooij et al., 2006) and calves vaccinated with MLV or killed BVDV vaccine respond with anamnestic T cell responses even in the presence of maternal antibody (Endsley et al., 2004). Still, piglets vaccinated against SIV via an inactivated virus preparation do not experience CMI stimulation when vaccinated in the face of maternal immunity (Kitikoon et al., 2006). Maternally-immune piglets vaccinated with an inactivated vaccine *M. hyopneumoniae* vaccine do manifest CMI responses (Bandrick et al., 2010b). Therefore, the vaccine type does not solely dictate the neonatal response to vaccination.

DNA vaccines are an especially alluring neonatal vaccination paradigm since they replicate *in vivo* but do not pose a risk of infection. Some DNA vaccines induce T cell responses in neonates (Capozzo et al., 2006; Hassett et al., 1997; Manickan et al., 1997) while others do not (Wang et al., 1998; Le Potier et al., 1997; Capozzo et al., 2006). Further, adjuvants that enhance CMI or antigen presentation (such as CpG motifs) may help the neonatal immune system overcome the opposing effects of maternal antibodies (Salerno-Goncalves and Sztein, 2006). Other adjuvant systems that should be investigated as inducers of neonatal CMI responses are mycobacterial antigens (Vekemans et al., 2001), Immune Stimulating Complexes (ISCOM), and biodegradable polymer microspheres (Gupta et al., 1998).

1.7 Conclusions

Neonates depend on MDI acquired across the placenta or via breast milk to overcome pathogen challenge while their own immune systems mature. Maternal immune factors transferred to the neonate include antibodies, immune cells, and non-specific immune modulators, yet the cellular characteristics of colostral immunity are not well defined. Colostral immunity is not always beneficial for the neonate. In fact, MDI is a double-edged sword. While MDI provides a critical and immediate protection to environmental antigens, it potentially interferes with endogenous immune responses in the recipient. Further, maternal immune factors with reduced function immunoreactivity due to prenatal opiate use or other immune-altering activity may also be transferred to the neonate in colostrum.

Recent evidence suggests that neonatal CMI responses are not subject to

passive interference. If MDI does not interfere with CMI responses in neonates, neonatal vaccination to selected pathogens could be routinely practiced. Neonatal vaccination would open up many doors for pathogen control during the critical neonatal period. In this case, how the immune response to vaccination would be measured need to be altered. No longer could antibody-based tests be the sole methods for determining if vaccination resulted in immune priming. CMI priming would need to be measured following vaccination since there may be no evidence of AMI responses due to passive interference. In fact, the CMI response to vaccination in the maternally immune neonate may be protective even in the absence of evidence of AMI responses. However, the mechanism of cell transfer, the role of transferred cells in neonatal immune development, and the functional and long-term biological significance of these cells remains requires further study.

An advanced understanding of MDI will enable physicians and veterinarians to design better disease prevention and control strategies for neonates. Appreciating the impact of passively transferred immunity and maternal drug use on colostrum quality and immune transfer will enable us to elucidate downstream consequences on neonatal immune development. Toward this end, the goal of future research should be to investigate the influence of MDI on neonatal immune development.

Section B: Research Chapters

Chapter 2:

Cellular contribution to passive maternal immunity in pigs

Submitted in part to Comparative and Developmental Immunology

2.1 Summary

Maternal immune components bound for colostrum are concentrated from blood in the mammary gland. Upon ingestion immune components are absorbed across the neonatal intestine and can be detected in neonatal blood. Aims of this study were to sequentially characterize the lymphocyte and immunoglobulin profiles of sow blood, colostrum, and piglet blood, and to determine how colostral immunity affects piglet immune responses. Sow blood and colostrum-derived lymphocytes were characterized and compared to piglet blood before (PS) and after (AS) colostrum ingestion. CD4, CD8, and γδ T lymphocytes were found in colostrum and blood; each had a unique T lymphocyte profile. Immunoglobulins were detected in sow blood and colostrum but only in piglets AS. The immunoglobulin pattern detected AS mimicked sow serum. Unlike immunoglobulin, there may be selectivity in lymphocyte concentration into colostrum and lymphocyte transfer into the neonate based on differences in T-lymphocyte profile in colostrum and blood. Assessment of NK-activity and *Mycoplasma hyopneumoniae*-specific lymphoproliferation revealed that colostrum is capable of influencing the piglet response to innate and specific antigens.

2.2. Introduction

Maternal immunity is transferred to offspring *in utero* across the placenta or after birth via the ingestion of mammary secretions. Neonatal piglets first receive maternal immunity in the form of colostrum since their placenta prohibits transfer of immunity *in utero*. Therefore, successful absorption of colostrum is essential for disease prevention and growing healthy pigs. Immunomodulatory and antimicrobial factors including antibodies and a variety of cells are integral parts of colostrum (reviewed in Wagstrom et al., 2003; Salmon et al., 2009). Maternal immunoglobulin are concentrated from the blood into colostrum in the mammary gland via a Fc-receptor dependent mechanism (Hammer and Mossmann, 1978). Lymphocytes derived from the common mucosal immune system migrate to the mammary gland (Salmon, 1987) and can be found in colostrum (Harp and Moon, 1988).

Cell-mediated immunity is undoubtedly a necessary component of disease control but the cellular contribution to colostrum has traditionally been overlooked. Colostrum is rich in cells; there are more than $1x10^6$ cells per ml, and it is estimated that piglets obtain $5.0x10^8$-$7.0x10^8$ maternal cells daily (Evans et al., 1982; Le Jan, 1994; Magnussen, 1999). A relatively high percentage of these cells are lymphocytic (15-25%) (Le Jan, 1994). Using technetium-labeled cells (Tuboly et al., 1988) or fluoroscein-labeled cells (Williams, 1993), it was demonstrated that maternal colostral cells cross the neonatal intestinal epithelium and are found in the neonate's circulation and immune tissues. In contrast, heat-killed cells and cells from a source other than the piglet's own mother do not transverse the intestinal barrier (Tuboly et al., 1988; Williams 1993). Colostral immunoglobulins cross the neonatal intestinal epithelium and enter the circulation

independent of source, whether it be immunoglobulins from another dam or from another species (Klobasa et al., 1981); however the efficiency of cross species immunoglobulin transfer may be increased in the presence of porcine colostrum (Jensen et al., 2001; reviewed in Sanglid, 2003). The mechanism involved in selective lymphoid cell transfer and whether transfer of specific maternal lymphocyte subsets is subject to further selectivity remains to be clarified.

The majority of colostral lymphocytes are of the memory phenotype as indicated by the expression of activation markers (Bertotto et al., 1990; Evans et al., 1982; Jain, 1991; Park et al., 1992; Parmely, 1976; Richie et al., 1980). The presence of activation markers suggests that colostral lymphocytes are functional. The ability of human colostral cells to respond to bacterial and viral antigen stimulation (Ogra and Ogra, 1978; Parmely, 1976) and to produce cytokines has been observed *in vitro* (Kohl et al., 1982; Skansen-Saphir et al., 1993). However, studies on the ability of porcine colostral cells to respond in the piglet are restricted to mitogenic responses (Bertotto et al. 1990; Evans et al., 1982; Jain, 1991; Le Jan, 1994; Ogra and Ogra, 1978; Park et al., 1992; Parmely, 1976; Richie et al., 1980; Riedel-Caspari and Schmidt, 1991; Salmon, 1987; Williams, 1993). The ability of colostral lymphocytes to respond to antigen-specific stimulation in the recipient remains unclear.

The present study was undertaken to assess the lymphocyte contribution to passive immunity in pigs. T lymphocyte subsets (CD4, CD8, γδ) were analyzed and functional activity as determined by natural killer (NK) activity and antigen specific proliferation to a previously exposed antigen was assessed. Cellular and humoral immunity in sow blood and colostrum and piglet blood were assessed and compared

to investigate differences in passive transfer of specific immune components and the role of those immune components in immune development of young pigs.

2.3. Materials and Methods

Animals

Animals (English Belle; GAP Genetics, Winnipeg, Manitoba Canada) were housed at a commercial farm and treated in accordance with the University's Institutional Animal Care and Use Committee regulations. Sows were fed commercial corn-soybean based diets and water was provided ad libitum. Randomly chosen sows (12 of a group of 24) were vaccinated with a commercially available, federally approved *Mycoplasma hyopneumoniae* vaccine (*M. hyopneumoniae*; Respisure, Pfizer Animal Health, Kalamazoo, MI, USA). Vaccination was performed at five and three weeks prior to farrowing according to the vaccine manufacturer's instructions.

All farrowings were monitored and piglets were placed into plastic tubs immediately after birth to prohibit suckling. Piglets were returned to their dams immediately following blood collection. Cross-fostering was not practiced. Sows and their litters were kept in individual crates; farrowing rooms were maintained at 18°C. Heat lamps provided additional heat to newborn piglets for 72 h and subsequent extra heat was provided by heat-pads (Osborne, NE, USA).

Sample collection

Blood was collected from 16 sows prior to farrowing and from their piglets before suckling (PS) and 24 h after suckling (AS) colostrum. Blood for the PS piglet sample

was collected within 1 h of birth. All blood samples were collected from the jugular vein into sterile heparinized Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA). Colostrum was collected manually into sterile 50 ml conical tubes from all functional teats for a total volume of 25-30 ml. Teats were scrubbed with alcohol and gloves were worn to minimize contamination. Colostrum was obtained within 1 h of farrowing.

Lymphocyte purification

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density centrifugation using Lymphocyte Separation Media (Cellgro, Inc, VA, USA; Bautista et al., 1999). Colostral lymphocytes were purified as described (Le Jan, 1994) with modifications. Colostrum was diluted 1:3 in sterile PBS and centrifuged at 800 x g for 10 min. The cell pellet was retained and the supernatant was centrifuged again at the same specifications. The cell pellets were washed 3x and resuspended in PBS, then centrifuged on Lymphocyte Separation Media for 35 min at room temperature at 1000 x g. The lymphocyte enriched layer was retained and washed with PBS. Isolated PBMC and mononuclear colostral cells were resuspended in RPMI medium supplemented with 10% heat-inactivated, irradiated fetal calf serum, 2 mM L-glutamine, 100 U penicillin G per ml, and 100 μg of streptomycin per ml. The serum portions of blood and whey portions of colostrum were retained for IgG and IgA or *M. hyopneumoniae*-specific antibody analysis and stored at -80C until ELISAs were performed.

Lymphocyte phenotyping

Cells were enumerated using a hemocytometer and viability was assessed by trypan blue exclusion. Lymphocytes were phenotyped as described (Yang and Parkhouse, 1996) with modifications. Anti-pig CD8-PE, CD4-PE, and γδ-FITC antibodies (VMRD, Inc Pullman, WA, USA) were added at a concentration of 1 μg/10^6 target cells in 200 μl of PBS. Cells were allowed to incubate for 30 min in the dark at room temperature. Samples were washed and immediately analyzed via flow cytometry. A Facs Caliber flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA) was used and the FL-4 laser was calibrated using the manufacturer's calibration beads. Non-stained cells were used to establish a base line for phenotyping. Event acquisition was set at 10,000 within the regions encompassing PE or FITC depending on subset being analyzed.

Natural killer assay

The NK assay was performed as described (Olin et al. 2005a). The K562 (human tumor) cell line was membrane stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; 10 μM concentration; Immunochemistry Technologies, LLC, Bloomington, MN, USA). CFSE stained K562 cells were added to purified porcine lymphocytes at a ratio of 1:50. Samples were incubated for 4 h at 37°C in 5% CO2. The cell stain 7-amino-actinomycin D (7AAD: Immunochemistry Technologies, LLC, Bloomington, MN, USA) was added and the samples were placed on ice for 15 min prior to analysis via flow cytometry. Non-stained effector (porcine lymphocytes) and target cells (K562) were used to establish a baseline for NK activity. CFSE stained K562 cells in suspension with

non-stained swine lymphocytes were used to verify the separation of effector and target cells. Water-lysed K562 cells stained with 7AAD were used to calibrate acquisition of live and dead K562 cells. Event acquisition was set for 10,000 events in a region encompassing the CFSE positive, 7AAD positive and negative quadrants (upper and lower right quadrants only). Natural killer activity was assessed by flow cytometry and analyzed by CellquestPro (Olin et al., 2005b).

Antigen specific blastogenesis

Mononuclear cells isolated from colostrum and blood were stained with CFSE as stated in section 2.5 and plated in duplicate in v-bottom 96-well-plates at a concentration of $5x10^5$ cells per well. Cells were stimulated with 10 μg/ml purified *M. hyopneumoniae* antigen as described (Thacker et al., 2000). Non-stimulated cultures served as negative controls and concanavalin A (conA) stimulated cultures (5 μg/ml) served as positive controls. Proliferation was assessed by flow cytometry and analyzed by CellQuestPro (Olin et al., 2005b).

IgG and IgA quantitation

ELISAs were performed to quantitate the immunoglobulin concentrations in blood and colostrum. ELISA reagents and antibodies from Bethyl Laboratories (Montgomery, TX, USA) were used for IgA quantitation (Foss and Murtaugh 1999). ELISA reagents from Immunochemistry Technologies, LLC (Bloomington, MN, USA) and antibodies from Immunology Consultants Laboratory, Inc (Newberg, OR, USA) were used for IgG quantitation. Plates were coated with either anti-swine IgG (4

μg/ml) or anti-swine IgA (10 μg/ml) and incubated overnight at 4º C. Plates were washed 4x and blocked for 1 h. Diluted samples were added to the plates and incubated for 1 h before 4x washing and addition of the secondary antibody. The secondary antibody was incubated for 1 h in the dark, the plates were washed 4x and read on a plate reader at absorption 540 nm.

Antigen specific antibody detection

ELISAs were performed on colostral whey and serum collected from sows and from serum collected from piglets, before and after colostrum ingestion. The HerdCheck ELISA for *M. hyopneumoniae* (Idexx Laboratories, Westbrook, Maine, USA) was performed per manufacture's instructions (Erlandson et al., 2005). Positive and negative status was determined based on sample to positive (S:P) ratio: S:P = (sample OD - negative control OD)/(positive control OD - negative control OD). S:P ratios >0.4 were classified as positive; S:P ratios < or = 0.4 were classified as negative. All samples were run in duplicate and sample means were used to determine the S:P ratio.

Data analysis

Flow cytometry data were analyzed using a dot plot separated into four quadrants. Percentages of cells killed by cytotoxic activity were calculated by dividing the (upper right quadrant by the sum of the upper and lower right quadrants) x 100, minus the percentage of spontaneous dead cells obtained from target cell control tube. Lymphocyte subsets, natural killer activity, and immunoglobulin concentrations were analyzed by ANOVA. Significant interactions among variables ($P<0.05$) were analyzed by

Tukey's method. Statistical analysis was performed using commercially available statistical software.

2.4 Results

Immune components in colostrum and sow blood

Cellular immune components in colostrum and sow blood

To assess the lymphocyte contribution to porcine colostrum and compare this to sow blood, isolated lymphocytes were enumerated and their viability was assessed. Viability was determined to be greater than 97% cells/ml for blood and 90% cells/ml for colostral lymphocytes. Significantly more lymphocytes were isolated from peripheral blood than from colostrum (n=16), $1.69x10^7$ (+/-$2.3x10^6$) cells/ml and $4.07x10^6$ (+/-$1.29x10^6$) cells/ml, respectively. CD4, CD8, and γδ T lymphocyte subpopulations were enumerated by flow cytometry (Fig 2.1). All three T lymphocyte subsets were observed in sow peripheral blood and colostrum. CD8 (35% +/- 2%) and γδ (33% +/- 4%) lymphocytes predominated in sow peripheral blood. In colostrum γδ lymphocytes (42% +/- 8%) alone predominated. CD4 and CD8 lymphocytes constituted significantly lower proportions of cells in colostrum compared to sow blood (P=0.001). While the ratio of CD4 to CD8 lymphocytes in blood (0.7) and colostrum (0.6) were similar, CD4 and CD8 lymphocytes combined constituted approximately 60% of the lymphocytes isolated from blood but only 25% of the colostral lymphocytes. The disparity in CD4 and CD8 combined percentage is due to the dramatic increase in γδT lymphocytes from blood to colostrum.

Figure 2.1 T lymphocyte distribution in sow blood and colostrum.

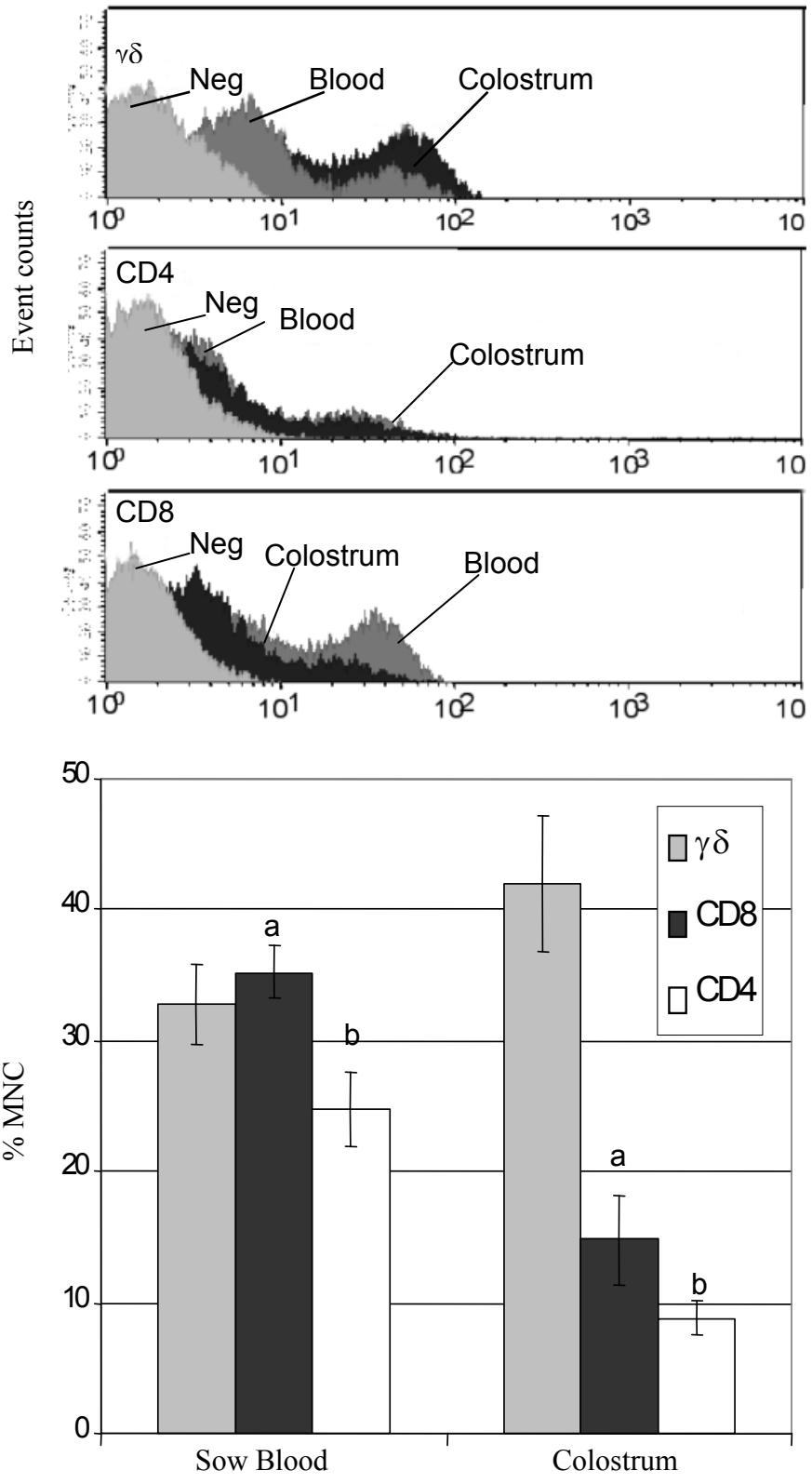


Figure 2.1 T lymphocyte distribution in sow blood and colostrum.

Mononuclear cells (MNC) were isolated from sow blood or colostrum (n=16) and viability was determined to be greater than 97% for blood and 90% for colostral lymphocytes. MNC were phenotyped with swine specific γδ, CD8, or CD4 monoclonal antibodies and analyzed with flow cytometry. (A) Representative flow cytometric analysis of T lymphocyte subpopulations in sow blood and colostrum. Flow cytometric analysis of colostral lymphocytes is in black and flow cytometric analysis of blood lymphocytes is in dark gray; light gray represents unstained control. Unstained control is labeled as "Neg." (B) Percentages of T lymphocyte subsets in sow blood and colostrum (n=16). Variance is given by standard error. Samples with same letters are significantly different; for samples marked "a," p=0.001, for samples marked "b" p=0.0001.

Humoral immune components in colostrum and sow blood

To compare the immunoglobulin concentrations in colostrum to serum, IgG and IgA sandwich ELISAs were performed on colostral whey and sow serum (n=16). Mean immunoglobulin levels in sow colostrum and sow serum are shown in Fig. 2.2. Both IgG and IgA were found in greater concentrations in colostrum compared to serum ($P<0.001$ and $P<0.001$, respectively); approximately 2.5 fold more IgG and 5 fold more IgA was found in colostrum compared to serum. Additionally, the ratio of IgG:IgS immunoreactive material was greater in colostrum (12 IgG: 1 IgA) than in serum (8 IgG: 1 IgA), however this difference was not significant. The concentrations of IgM in colostrum and blood were not determined.

Immune components in neonatal piglet blood

Neonatal peripheral T lymphocytes

Piglets used in this study were offspring of the sows used in this study. PS and AS samples were taken from the same piglets. Peripheral lymphocytes were enumerated and CD4, CD8, and γδ subpopulations were assessed to compare the distribution of circulating T lymphocytes in pre- and post-colostral piglets. The number of PBMC isolated per ml of blood increased by 63% after colostrum ingestion ($7.0x10^6$ +/- $0.71x10^6$ cells/ml) compared to before colostrum ingestion ($4.4\ x10^6$ +/- $0.73x10^6$ cells/ml; $p=0.018$). The distribution of CD4, CD8, and γδ lymphocytes in piglet blood varied depending on colostrum ingestion status (Fig 2.3). Prior to receiving colostrum, CD4 (19% +/-2%) lymphocytes represented the greatest percentage of lymphoid cell subset taken from piglets, whereas after colostrum ingestion, γδ (24% +/-3%) and

Figure 2.2 Immunoglobulin G and A concentrations in sow serum and colostrum.

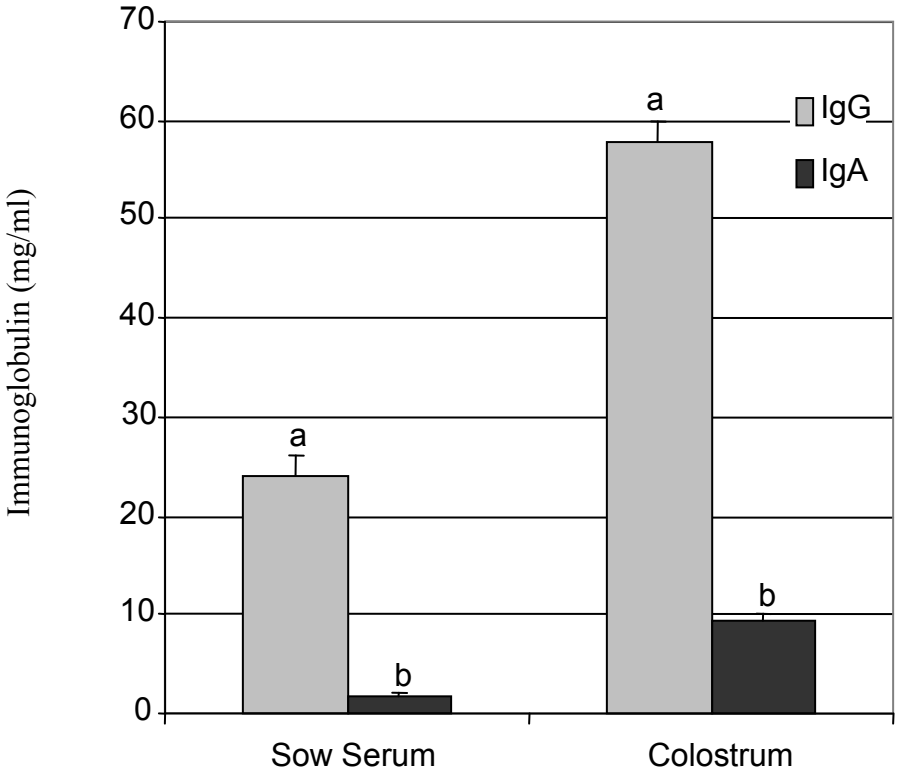


Figure 2.2 Immunoglobulin G and A concentrations in sow serum and colostrum.

IgG and IgA concentrations from sow serum and colostral whey were determined by ELISA (n=16). Variance is given by standard error. Samples with same letters are significantly different, $p<0.001$

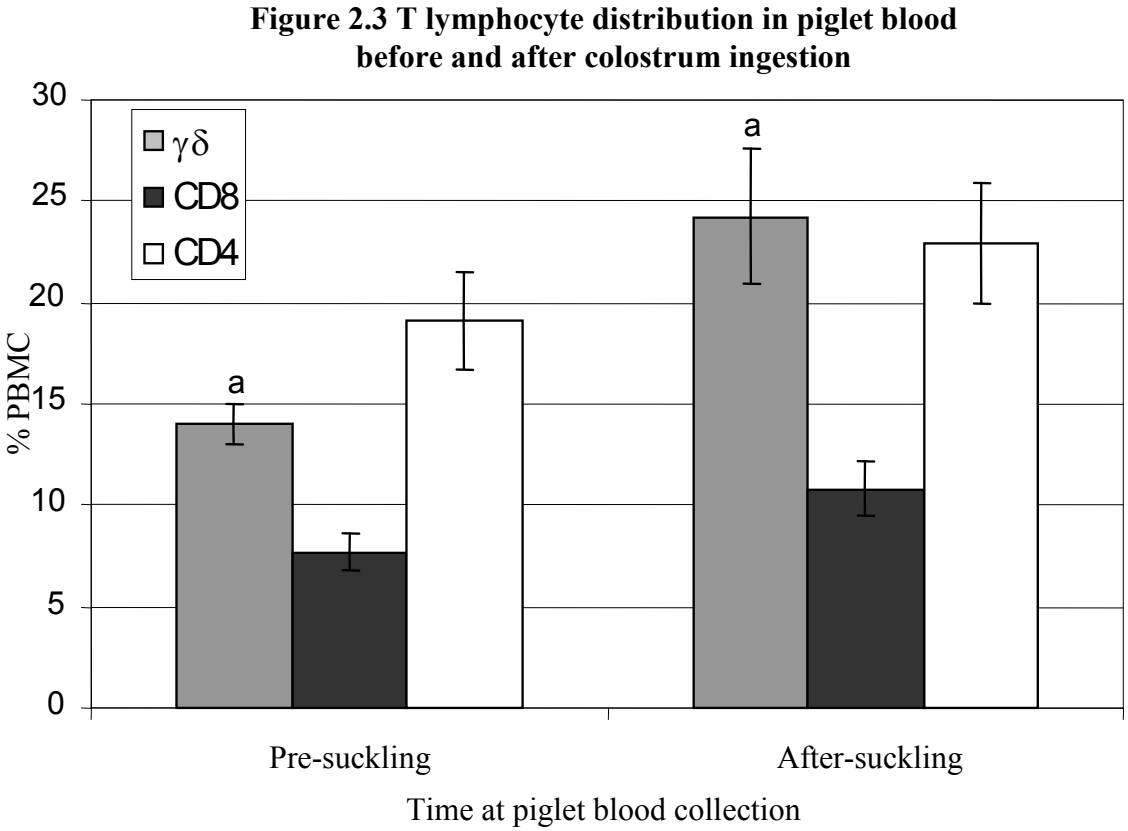


Figure 2.3 T lymphocyte distribution in piglet blood before and after colostrum ingestion.

Blood was collected from piglets prior to colostrum ingestion (pre-suckling) and again at 24 h (after-suckling). PBMC were isolated from piglet blood and phenotyped with swine specific γδ, CD8, or CD4 monoclonal antibodies. T lymphocyte subpopulations were analyzed by flow cytometry. Variance is given by standard error. Samples with same letters are significantly different, $p<0.01$, $n=34$.

CD4 (23% +/- 3%) lymphocytes increased. It is noteworthy that the percentage of γδ cells observed in PBMC of post-colostral piglets was greater than pre-colostral piglets (P=0.009), while the percentage of CD4 and CD8 lymphocytes remained constant. The distribution of γδ and CD8 T lymphocytes found in piglet blood after colostrum ingestion was different from what was found in sow blood (P=0.05 and P<0.001, respectively). The distribution of γδ and CD4 T lymphocytes found in piglet blood after colostrum ingestion was different from what was found in colostrum (P=0.02 and P=0.004, respectively).

Neonatal serum immunoglobulins

To compare the concentrations of IgG and IgA in piglet serum before and after colostrum ingestion, blood samples were collected from pigs immediately following birth and 24 h later. The serum portions of blood were collected and IgG and IgA concentrations were determined by ELISA. Neither IgG nor IgA were detected from pre-colostral piglet serum samples. Approximately 20 (+/- 0.4) mg/ml IgG and 1.6 (+/- 0.04) mg/ml IgA were found in piglet serum after suckling (Fig 2.4). The immunoglobulin pattern detected in the post-colostral piglet was similar to that found in sow serum.

Natural killer activity of colostral and peripheral lymphocytes

Natural killer (NK) activity was used to assess the innate functional ability of colostral lymphocytes and peripheral lymphocytes of sows and piglets. NK activity is a critical defense mechanism for killing intracellular pathogens in an antigen-nonspecific manner. NK activity is accomplished by activated NK cells, γδ T lymphocytes, and select CD8 lymphocytes (Charerntantanakul and Roth, 2007). Colostral lymphocytes

Figure 2.4 Immunoglobulin concentration in piglet serum before and after colostrum ingestion.

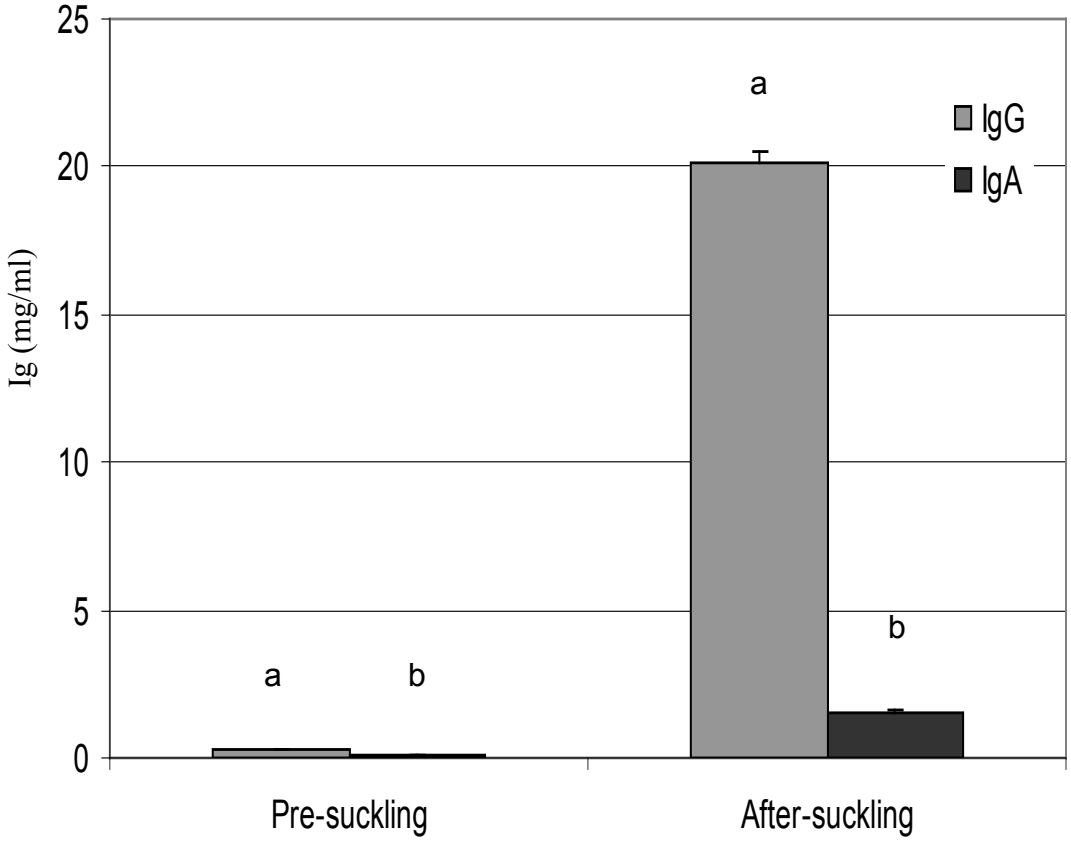


Figure 2.4 Immunoglobulin concentration in piglet serum before and after colostrum ingestion.

IgG and IgA concentrations in piglet serum before colostrum ingestion (pre-suckling) and 24 h later (after-suckling) were determined by ELISA. Neither IgG nor IgA were detectable in pre-suckling samples. Variance is given by standard error. Samples with same letters are significantly different, $p<0.001$, $n=34$.

demonstrated the greatest amount of NK activity (16% +/- 1%) and this was significantly greater NK activity than was observed for sow PBMC (9% +/- 1%; P=0.003) or any piglet sample (Fig 2.5). Sow PMBC demonstrated greater NK activity than PBMC isolated from piglets before suckling (P=0.03) but similar activity to PBMC isolated from piglets after colostrum ingestion (P=0.4).

Antigen specific humoral and cellular immunity in colostrum and blood

M. hyopneumoniae-specific antibodies and *M. hyopneumoniae*-specific lymphocytic proliferation were measured to assess and compare antigen specific humoral and cellular immunity in colostrum. Antibodies specific to *M. hyopneumoniae* were found in serum and colostrum of vaccinated sows (Fig 2.6.). In addition, *M. hyopneumoniae*-antibodies were detected in the serum of piglets from vaccinated dams one day after colostrum ingestion but not from piglets of unvaccinated dams (data not shown). Pre-colostral piglets and unvaccinated animals had *M. hyopneumoniae*-antibody S:P values <0.4 and were classified as negative. Colostral lymphocytes from vaccinated sows proliferated in response to *M. hyopneumoniae* stimulation and this response was mimicked in offspring of vaccinated sows after suckling only (Fig. 2.7). Antigen specific proliferation was not detected from PBMC of pre-colostral piglets and antigen specific proliferation was not detected from colostral lymphocytes of non-vaccinated sows or from PBMC of their offspring (before and after colostrum ingestion).

Figure 2.5 Natural killer activity of colostral lymphocytes and peripheral lymphocytes

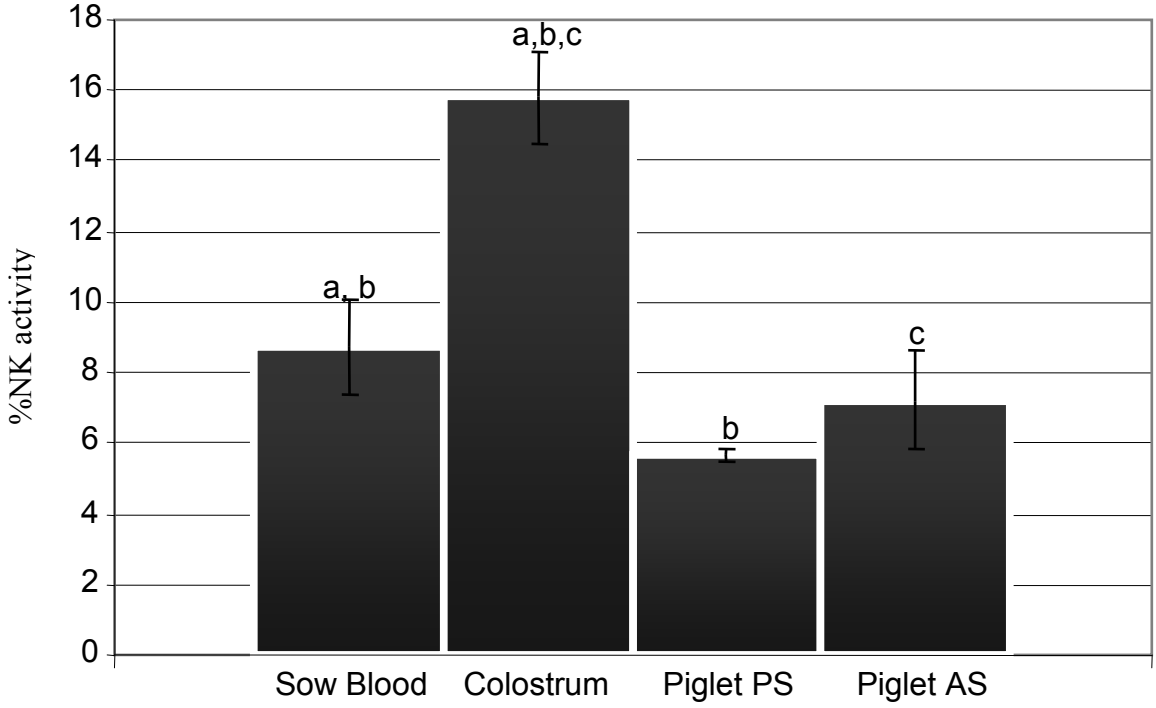


Figure 2.5 Natural killer activity of colostral lymphocytes and peripheral lymphocytes.

Innate natural killer activity was used to assess the functional ability of T lymphocytes from sow colostrum and blood of sows and piglets, before (PS) and after (AS) colostrum ingestion. Variance is given by standard error. Samples with same letters are significantly different, $p<0.05$.

Figure 2.6 *Mycoplasma hyopneumoniae* specific antibodies in serum and colostrum of unvaccinated and vaccinated sows

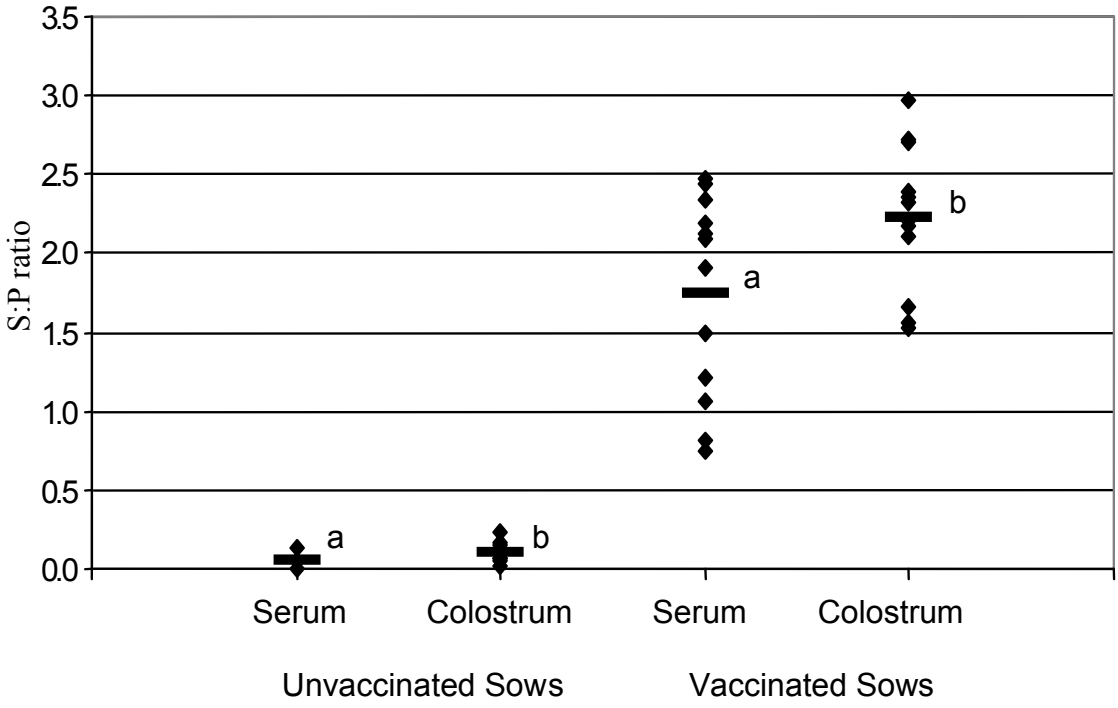


Figure 2.6 *Mycoplasma hyopneumoniae* specific antibodies in serum and colostrum of unvaccinated and vaccinated sows.

Mycoplasma hyopneumoniae specific antibodies were measured in serum and colostrum of unvaccinated (n=12) and vaccinated (n=12) sows using the Idexx HerdCheck ELISA. Black diamonds represent S:P ratios for individual animals. Horizontal lines represent S:P ratio means. Positive and negative antibody status was based on sample to positive (S:P) ratio; S:P= (sample OD-negative control OD) / (positive control-negative control OD). Samples with the same letters are significantly different, $p<0.05$.

Figure 2.7. Antigen specific proliferation by lymphocytes isolated from piglets before and colostrum ingestion and from sow colostrum.

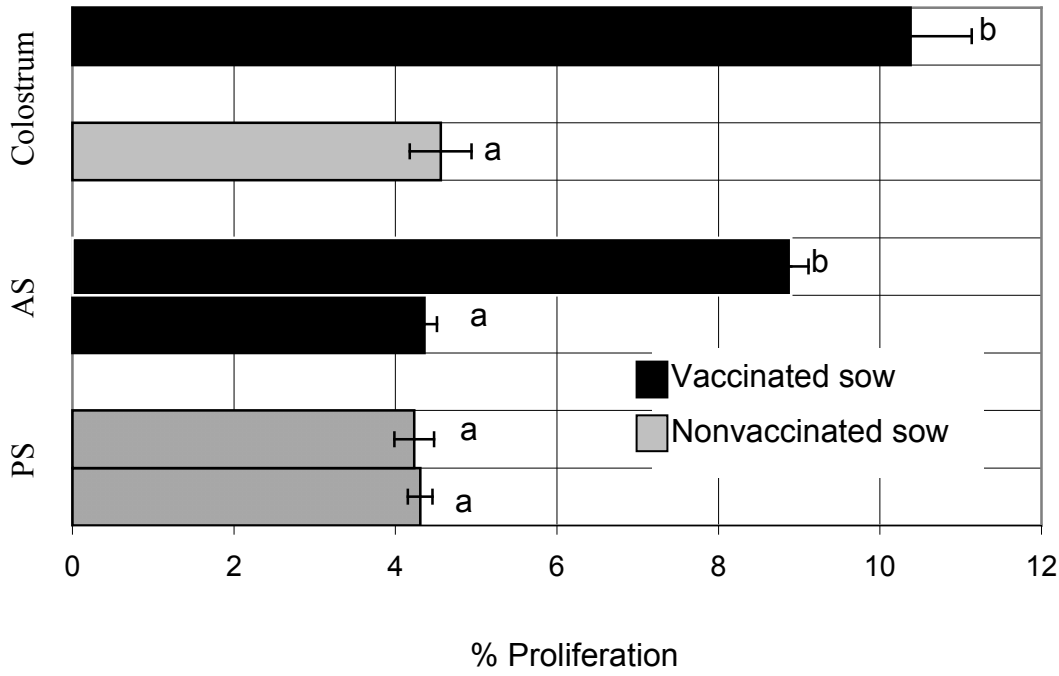


Figure 2.7 Antigen specific proliferation by lymphocytes isolated from piglets before and colostrum ingestion and from sow colostrum.

Lymphocytes from sow colostrum (n=10) and from piglet blood (n=20) before (PS) and after (AS) colostrum ingestion were stimulated with *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) antigen. Proliferation was assessed by flow cytometry. Mycoplasma specific proliferation was used to assess the ability of colostral lymphocytes to respond in an antigen-specific manner. Antigen specific proliferation was compared between colostrum of vaccinated and unvaccinated sows and piglets receiving colostrum from vaccinated and unvaccinated sows. Variation is expressed as standard error; samples with *different* letters indicate significance at $p<0.05$.

2.5. Discussion

The epitheliochorial nature of the porcine placenta prohibits transfer of maternal immune cells and immunoglobulins to the fetus. Therefore, piglets are born agammaglobulinemic and must rely on the successful absorption of colostral components to acquire maternal immunity. Maternal immunity serves to protect young pigs while their own immune systems develop. The amount of maternal immunity in the post-suckling pig depends on the concentration of immune and other bioreactive products in colostrum, the amount of colostrum ingested, and timing of gut closure (Rooke and Bland, 2002). Their unique placentation, large litter size, and precocial nature make swine an excellent model to study the role of passive immunity in the neonate. Immune components in colostrum can be measured and are often manipulated to provide protection in young animals.

Immunoglobulins in colostrum are concentrated from sow serum in the mammary gland and pass across the piglet's intestinal epithelium nonselectively by endocytosis (Burton and Smith, 1977; Clarke and Hardy, 1971). In agreement with published findings (Curtis and Bourne, 1971; Porter, 1969), our results show that IgG and IgA are more concentrated in colostrum than sow serum. Furthermore, the distribution pattern of IgG and IgA across colostrum and sow serum follows what was found by Curtis and Bourne (1971). Levels of IgG and IgA in the post-colostral pig suggest that absorption of colostral immunoglobulins by the neonate was nonselective since the serum IgG and IgA levels of post-colostral piglets mimicked the distribution of IgG and IgA in sow colostrum. As expected, IgG and IgA were not detected in the serum of blood from piglets prior to colostrum ingestion. Since fetuses have the ability to produce IgG

while gestating (Tlaskalova-Hogenova et al., 1994), the lack of IgG in pre-colostral piglet serum indicates that the piglets were not facing antigen challenge *in utero*.

T lymphocyte subpopulations in colostrum did not mimic what was seen in sow blood, indicating that the cellular portion of colostrum is selectively concentrated. While the relative ratio of CD8:CD4 cells remained similar across blood and colostrum, in accordance with Magnussen (1999) and Le Jan (1994), there was a significantly greater percentage of CD8 and CD4 lymphocytes in blood compared to colostrum (P=0.001). The reduction in CD4 and CD8 lymphocytes in colostrum is compensated by a significant increase in γδ T lymphocytes, which constitute almost half of the lymphocytes found in colostrum. The disparity in the T lymphocyte contribution to colostrum and blood indicate that there is selectivity in the lymphocyte contribution to colostrum. While other reports have suggested that γδ T lymphocytes contribute to porcine colostrum (Wagstrom et al., 2000), this is the first report to show γδ T lymphocytes are indeed in porcine colostrum. γδ T lymphocytes have also been shown to be present in human colostrum (Bertotto et al., 1990).

Pigs have a significant population of circulating γδ T lymphocytes. The presence of these lymphocytes in colostrum was anticipated and of interest because of their unique immunologic activities. γδ T lymphocytes respond to antigens differently than αβ T cells. γδ T lymphocytes are MHC unrestricted and have the ability to recognize unprocessed non-protein antigens (Tanaka et al., 1994). In addition, γδ T lymphocytes in murine and bovine species possess pattern recognition receptors (PRR; Mokuno et al., 2000, Hedges, 2003). Whether porcine γδ T lymphocytes possess PRR or Toll-like receptors is unknown (Takamatsu 2006); however scavenger receptors on γδ T lymphocytes have been

demonstrated (Carr et al., 1994). Moreover, γδ T lymphocytes are thought to have NK activity and antigen presentation abilities (Bandes et al., 2005; Sinkora et al., 2005). Having mature antigen presenting cells (APC) would be advantageous to the neonate since neonatal APC are immature and require increased stimulation in order to function at the level of a mature animal (Adkins, 2004). γδ T lymphocytes' ability to "bridge" the innate and adaptive immune responses would be an asset for the developing pig.

The mechanism of maternal lymphocyte transport across the neonatal intestinal epithelium is unknown; however, transfer may be a receptor mediated event due to the observed T lymphocyte distribution in the post-suckling pig. Selectivity in lymphocyte transfer across the neonatal intestinal epithelium has been suggested by other studies (Tuboly et al., 1988; Williams, 1993) and selectivity in transfer may be related to cellular phenotypic changes incurred while in the colostral environment (Reber et al., 2006). While the increase in γδ T lymphocytes in pre- to post-colostral pigs may be due to the high percentage of γδ T lymphocytes found in colostrum, the high number of CD4 T lymphocytes present in post-colostral piglets cannot be explained in the same way since CD4 T lymphocytes constituted the smallest percentage of colostral lymphocytes. While this finding suggests selectivity in uptake, we cannot conclude selectivity because we did not control for endogenous lymphogenesis and did not distinguish maternally-derived cells from neonatally derived cells.

It is important to note that a proportion of swine αβ T lymphocytes co-express CD4 and CD8 and are known as double positive (DP) cells (reviewed in Charerntantanakul and Roth, 2007). In addition, some swine γδ T lymphocytes express CD4 and/or CD8 antigens (Sinkora et al., 2005). A limitation of this study is that all

flow cytometric analysis done was single color and double positive as well as γδ T lymphocytes expressing CD4 and CD8 were not examined. Therefore, it is possible that some of the cells were classified more than once and as more than one cell type. Despite this, most γδ T lymphocytes do not express CD4 and those that do are mainly found within the thymus (Sinkora et al., 2005). Further, γδ T lymphocytes expressing CD8 only constitute about 2% of the peripheral lymphocyte pool (Yang and Parkhouse, 1996). The large proportion of γδ T lymphocytes in colostrum is still an interesting finding, especially given that the number of γδ T lymphocytes in peripheral blood dramatically decreases with age (Yang and Parkhouse, 1997). Conversely, the percentage of DP αβ T lymphocytes increases with age (Zuckermann and Husmann, 1996). DP lymphocytes are effector memory cells and stimulation with their specific antigen induces DP lymphocytes to proliferate (Dillender and Lunney, 1993; Saalmuller et al., 1987, 2002; Zuckermann and Husmann, 1996). Thus, many of the CD4 expressing cells in colostrum likely express CD8 as well. We are focusing future investigations of maternal immunity to include multi-color analysis of colostrum for a more complete characterization of its cellular constitution.

To assess the ability of colostral cells to participate in an innate immune response, natural killer activity was measured. Colostral lymphocytes demonstrated the greatest amount of natural killer activity across all samples. While no clear difference was observed in natural killer activity between PBMC isolated from pigs that had ingested colostrum compared to those that did not, this does not necessarily indicate non-function of colostral cells in the recipient. Neonates have higher numbers of circulating NK cells than adults (Yabuhara et al., 1990), yet reports have indicated that NK activity in

neonates is lower than in adults (Baley and Schacter, 1985; Lin et al., 2004). Consequently, the contribution by colostrum may not be significant enough to enhance measurable NK activity in the post-colostral piglet. Additionally, the presence of an inhibitory factor in colostrum could explain the lack of difference. Corticosteroids found in colostrum have been shown to be immunosuppressive and influence immune activity in the neonate (Borghetti et al., 2006). The potential Th2 bias of newborns and increased apoptosis of Th1 cells may also contribute to the lack in difference of NK activity (Adkins et al., 1996).

Antigen specific proliferation was measured to assess the ability of primed colostral cells that have crossed into the neonate's circulation to respond in an antigen specific manner upon stimulation. Findings shown here indicate that antigen specific cells transfer from colostrum into the neonate's circulation. Additionally, these antigen specific cells are functional, as evidenced by *M. hyopneumoniae* specific proliferation by PBMC isolated from one-day-old colostrum fed piglets. Antigen specific proliferation by PBMC from offspring of vaccinated dams but not from unvaccinated dams indicates that transfer of primed maternal cells may influence the neonate's response to environmental antigens (Donovan et al., 2007; Bandrick et al., 2008).

The short and long-term impact colostral cells have on neonatal immune development is not fully understood at this point. The newborn's immune system is suppressed at birth due to its immaturity, an imbalance in Th1/Th2 type responses, endocrine factors and passive interference (Barrios et al., 1995). Passive interference is a major cause for concern because it disallows the neonatal immune system from mounting a response as well as from preparing for future antigen challenge. Little is known

about interference regarding passively transferred cells or the role these cells may play in active immune development of the newborn. Reports in humans and rodents suggest that maternally derived T lymphocytes do not interfere with active immune development in the newborn and in fact may be useful in overcoming the suppressive effects of passive immunoglobulin (Cherry et al., 1973; Gans et al., 1999; Siegrist, 2001). Animal studies of colostral lymphocytes are limited; therefore, the functional and long-term biological significance of these cells remains to be elucidated. Future studies should emphasize the mechanism of maternal cell transfer, the role of transferred cells in neonatal immune development and passive interference, and the ability of transferred cells to protect young pigs from specific pathogens.

Acknowledgements

The authors would like to thank Dr. Mike Olin for assistance with flow cytometry and Dr. Alejandrina Da Silva for the mycoplasma antibody quantitation. The authors would like to thank Dr. Brian Lee and Immunochemistry Technologies, LLC for assistance with ELISA setup and generous donation of ELISA reagents. The authors gratefully acknowledge the staff of the University of Minnesota, Southern Research and Outreach Center, for their help during sampling sessions. MB was in part supported from NIH T32 DA07097.

Chapter 3:

Passive transfer of maternal *Mycoplasma hyopneumoniae*-specific cellular immunity to piglets

In part published in Clinical and Vaccine Immunology, 2008, 15(3): 540-543

3.1 Summary

Immunity in the neonatal animal is primarily maternally derived, either by lymphocytes that pass into the newborn across the placenta or following colostrum ingestion. However, the effect of this passively transferred cellular maternal immunity on the newborn's immune repertoire is not clearly understood. Various studies have shown that colostral lymphocytes are activated and possess functional abilities; however, no studies have shown the transfer of colostral antigen specific T cell specific responses in a newborn. In this study we examined the transfer of vaccine-induced *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) cellular immunity from immune dams to newborn piglets. Newborn piglets from vaccinated and non-vaccinated dams were assessed in two ways for cellular immune responses specific to *M. hyopneumoniae*: (1) Delayed-Type Hypersensitivity (DTH) testing; and (2) *in vitro* lymphocyte proliferation, assayed on piglet blood lymphocytes and sow colostral lymphocytes. DTH responses to *M. hyopneumoniae* were detected only in offspring of vaccinated sows, whereas DTH responses to the nonspecific mitogen phytohemagglutinin (PHA) were seen in all piglets. *M. hyopneumoniae*-specific proliferation was seen in colostral lymphocytes from vaccinated sows and in blood lymphocytes from neonatal piglets of vaccinated dams, but not in blood lymphocytes from piglets of non-vaccinated sows. Functional antigen-specific T cells were transferred to offspring from vaccinated sows and participated in the neonatal immune response upon stimulation. This data has implications for defining disease intervention strategies.

3.2 Introduction

The immediate postnatal life is a critical time in the development of young animals' immune systems because it involves a major shift from reliance on innate immunity to adaptive immunity. During this transition period neonates are protected by passively acquired maternal immunity. In most species, the fetus acquires passive immunity *in utero* when immune factors cross the placenta. However, some animals first receive maternal immunity at birth through colostrum ingestion, specifically, animals with epitheliochorial (swine and equine) or hematochorial (bovine) placentation. Immunomodulatory factors are integral parts of colostrum and include hormones and cytokines, as well as antibodies and a variety of cells (reviewed in Wagstrom et al., 2000). While an extensive literature exists regarding the immunoglobin composition of porcine mammary secretions, little attention has been given to colostral cells. There are more than $2x10^6$ cells per ml in colostrum, approximately 20% of which are lymphocytes, and an estimated 500 million maternal cells transverse the intestinal epithelium daily (Evans et al., 1982; Le Jan, 1994. Interestingly, the transfer of lymphocytes from colostrum into the circulation of the neonate is ordered, not random, indicating an evolutionary importance of maternal lymphocytes (Tuboly et al., 1988; Wiliiams, 1993). The present study investigated whether the colostrum of vaccinated sows transfers functional, antigen-specific lymphocytes to newborn piglets.

Contributions by studies of different species have made it clear that colostral lymphocytes are phenotypically memory and activated cells and can proliferate following mitogen stimulation *in vitro*. Transferred colostral cells also have functional ability. Transfer of sensitivity to tuberculin has been demonstrated in human infants from

vaccinated mothers by delayed type hypersensitivity (DTH) testing, a T lymphocyte-mediated response (Schlessinger and Covelli, 1977). In addition, transfer of sensitivity to nematode and fungal antigens in neonatal mice born to immunized mothers has been demonstrated (Rifkind et al., 1976; Kumar et al., 1989). Still, humans and mice are exposed to maternal immunity during gestation, and the detection of antigen specific reactivity at birth may be a result of antigen priming while *in utero*. Therefore, the responding cells in these reports may be neonatal cells that were primed during gestation and not maternal cells.

To date, no reports have investigated the ability of colostral cells to respond in an antigen specific manner in the neonate. It is of great interest and practicality to harness the ability of passively transferred antigen-specific cellular immunity because of the potential role maternal cells have in protecting the neonate from disease. We evaluated the ability of passively transferred *M. hyopneumoniae*-specific colostral cells to function in a specific manner in the newborn pig. *M. hyopneumoniae* was chosen because of its clinical importance to the swine industry (reviewed in Ross, 1999) and the importance of cellular immunity associated with this infection (Adegboye, 1978; , Roberts, 1973; Tajima et al., 1983; Thacker et al., 2000). Newborn piglets are naïve to *M. hyopneumoniae,* and there is no evidence of transplacental infection. Newborn piglets from *M. hyopneumoniae* vaccinated and non-vaccinated dams were tested for both specific lymphoproliferative responses at birth (before colostrum ingestion) and 24 h later and delayed type hypersensitivity (DTH) responses at four days of age. DTH responses and specific lymphoproliferation are by definition secondary immune responses. Therefore, a positive DTH or lymphoproliferative response in a neonatal

pig would be due to responding antigen-specific maternal cells. Although the use of sow vaccination for *M. hyopneumoniae* is uncommon, sow vaccination and antibiotic treatment during lactation results in a significantly lower prevalence of *Mycoplasma*-positive piglets at weaning (Ruiz et al., 2003) and subsequent lower prevalence and severity of lung lesions at slaughter (Fano et al., 2005). *M. hyopneumoniae*-specific antibodies have been demonstrated in blood and milk of sows following vaccination, yet direct evidence for the passive transfer of cellular immunity specific for *M. hyopneumoniae* has not been demonstrated. A greater understanding of the role of maternal cellular immunity in protection of neonates and potential interference with active immunity could be used to define more effective disease intervention strategies.

3.3 Materials and Methods

Animals

All animals were treated in accordance with the University of Minnesota's Institutional Animal Care and Use Committee regulations. Sows were housed at a commercial farm in gestation crates until one week prior to farrowing at which time they were moved to farrowing crates. Food and water were provided ad libitum. At the beginning of the study 15 sows were randomly assigned to vaccinated sows (VS) or non-vaccinated sows (NVS) groups. At five and three weeks antepartum VS were vaccinated against *M. hyopneumoniae* (Respisure, Pfizer) according to manufacturer's instructions. Colostrum was collected from all sows at farrowing. Blood was collected from seven piglets per sow at 0 h. To ensure 0 h piglet blood was collected prior to colostrum

ingestion, farrowings were monitored and piglets were placed in tubs immediately after birth. Blood was collected from piglets within 30 min of being placed in the tub and then immediately returned to their dams. Piglets were ear-notched for identification purposes. Based on previous studies, only piglets that had not been cross-fostered (to ensure maternal lymphocyte transfer into circulations) were included in the 24 h blood collection. Blood was collected from two to three piglets per sow at 24 h.

DTH testing

DTH validation was performed prior to experimental use of the DTH. Different concentrations of *M. hyopneumoniae* antigen (300 μg/ml in 0.1 ml, 200 μg/ml in 0.1 ml, 150 μg/ml in 0.1 ml) and Phytohemagglutinin (PHA; 7 μg/ml in 0.1 ml, 12 μg/ml in 0.1 ml, 20 μg/ml in 0.1 ml; Sigma, St. Louis, MO) were intradermally injected into the inguinal area of preweaning pigs. Injection sites were clearly marked with livestock paint prior to injection. Each concentration of antigen or mitogen was evaluated in at least 3 pigs. DTH injection sites were evaluated immediately before injection, 1 h following the injection, and at 12 h, 36 h, and 48 h post injection. The concentrations of PHA and *M. hyopneumoniae* to be employed in experimental sessions were chosen based on lesion response time (to insure proper hypersensitivity was being measured), lesion size (to insure the right concentration of antigen/mitogen was being used), and congruence of pig *M. hyopneumoniae* status and lesion status (to insure experimental specificity). The time at DTH lesion measurement was chosen similarly.

Experimental DTH testing was performed on 20 piglets per group at 4 days of age. Concentrated and purified *M. hyopneumoniae* (300 μg/ml in 0.1 ml) antigen

was injected intradermally into the inguinal area (Roberts, 1973). PHA (20 μg/ml in 0.1 ml) and saline (0.1 ml) were used as positive and negative controls, respectively. Injection sites were clearly marked with livestock paint. Skin fold thickness was measured 30-36 h later with calipers. Final DTH lesions were determined as orthogonal diameter x skin thickness (DxT).

Sample collection

Twenty ml colostrum was manually collected from each functional udder in 50 ml conical tubes following alcohol swabbing of teats. Blood was collected by jugular puncture into sterile heparinized vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ).

Antigen preparation

M. hyopneumoniae was cultured in Friis' media. At passage 15 and when the pH of the culture reached 6 or lower, the organisms were harvested by continuous flow centrifugation at 70,000 x g. The harvested *M. hyopneumoniae* was resuspended in Tris-sodium chloride (TN) buffer (pH 7.2- 7.4) in 1/100 of the original volume of the culture and washed three times by centrifugation, each with the same proportion of TN buffer. The washed *M. hyopneumoniae* was inactivated by one freeze-thaw cycle and then by sonic disruption. *M. hyopneumoniae* was solubilized with 1% NP-40 and the antigen concentration was adjusted to 4 mg/ml.

Lymphocyte stimulation

Colostrum was diluted 1:3 with sterile PBS to decrease viscosity and then washed 3x prior to lymphocyte isolation. Piglet blood was diluted 1:1 in sterile PBS to improve cell recovery yield. Colostral and blood lymphocytes were isolated by Ficoll density centrifugation as described (Bautista et al., 1999; Olin et al., 2007). Cells were stained with the membrane stain CFSE (ICT, Bloomington, MN) to evaluate antigen specific proliferation (Thacker et al., 1998). CFSE was added to cells in PBS and allowed to incubate for 20 min at room temperature in the dark. Cells were washed 2x with RPMI supplemented with 10% FBS, 100 U penicillin G per ml, and 100 μg of streptomycin per ml (from here known as RPMI) and reconstituted in RPMI. Viability was deemed to be greater than 97% by Trypan Blue exclusion. Cells were plated in v-bottom 96 well plates in duplicate at a concentration of $4x10^5$ cells per well. Cells were stimulated with 10 μg/ml purified *M. hyopneumoniae* antigen as described (Thacker et al., 2000). The *M. hyopneumoniae* antigen used *in vitro* was the same as used for *in vivo* testing but at different concentrations. Non-stimulated cultures served as negative controls; concavalin A-stimulated cultures (5 μg/ml; Sigma, St. Louis, MO) served as positive controls. Cells were allowed to incubate for four days. Flow cytometry was used to assess lymphocyte proliferation utilizing a Facs Caliber flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA). The FL-4 laser was calibrated using the manufacturer's calibration beads. Non-stained, non-stimulated cells were used to establish a baseline for the proliferation assay. Event acquisition was set for 10,000 events in a region encompassing the CFSE-positive quadrants (lower quadrants only). Results were analyzed by CellquestPro software.

Detection of maternal DNA in piglet blood

In order to determine if cells were transferred in a source-dependent manner to piglets, sow colostrum and piglet blood, pre and post colostrum ingestion, were subject to DNA testing using a battery of 5 primer sets. DNA isolation was undertaken on blood or colostrum using DNAeasy kits (manufacturer). DNA was quantified via mass spectrophotometry. Approximately 50ng DNA was used in each PCR reaction. Published primers sets were used to amplify sow-derived and piglet derived DNA (Putnova et al., 2003). Amplified PCR products along with a DNA ladder were run on agarose gels. Overlapping PCR products as visualized on the agarose gels were indicative of cells transferred via colostrum to the piglet.

Statistics

Data were analyzed using the Linear Mixed Effect model in SAS program and means were compared using the Least Square Means and HSD comparison. Data were transformed to log(y), but the original means are used in the text and figures.

3.4 Results:

In order to confirm that maternal cells transferred into neonates, maternal DNA isolated from one sow (colostrum) and one of her piglets (both pre and post suckling colostrum samples) were subject to PCR amplification using five different sets of published primers (Putnova et al., 2003) and the amplified products were run on an agarose gel. Maternal DNA was not detected in pre-suckling samples after PCR amplification using any of the 5 primer sets (Fig 3.1). Maternal DNA was detected

Figure 3.1 Detection of maternal DNA in piglet blood

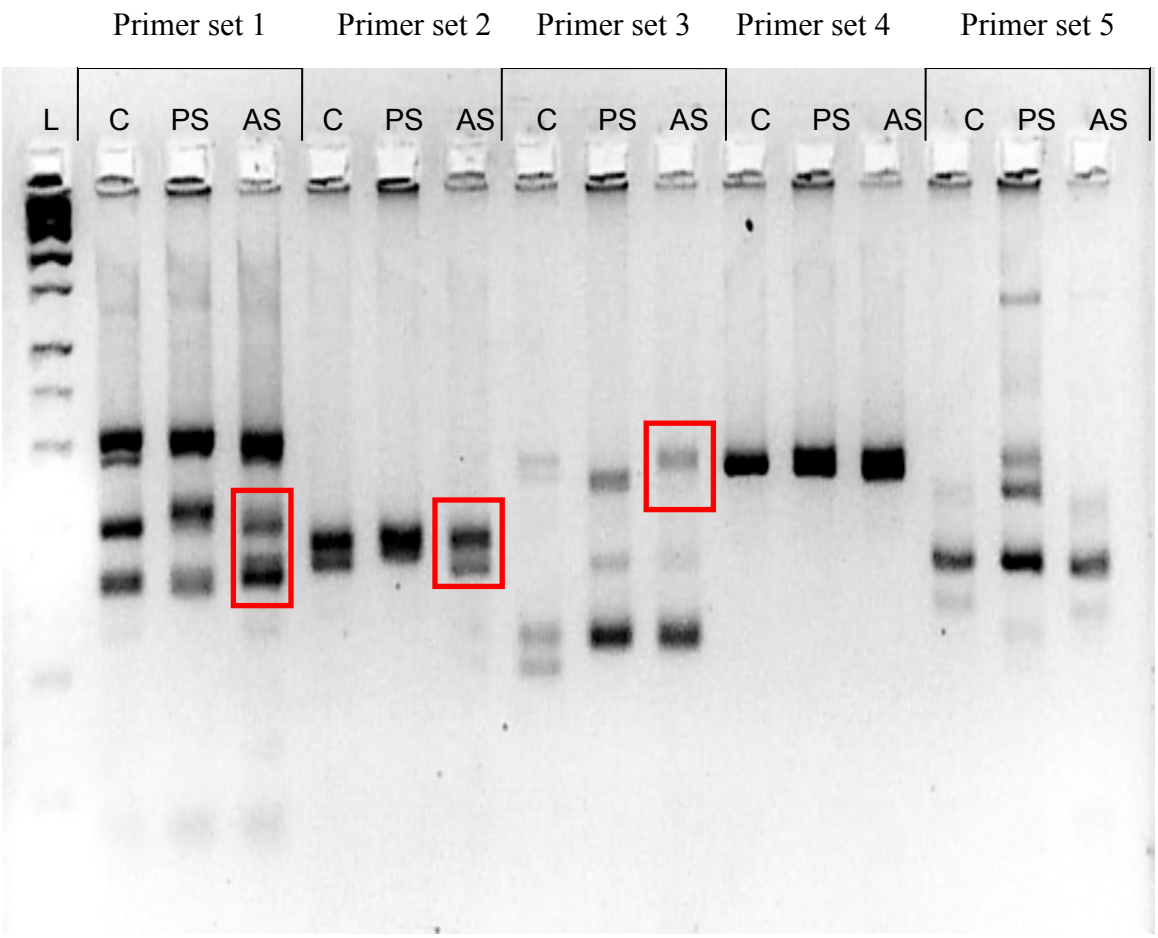


Figure 3.1 Detection of maternal DNA in piglet blood

Sow colostrum and piglet blood, pre and post colostrum ingestion, were subject to DNA isolation and amplification. Published primers sets were used to amplify sow-derived and piglet derived DNA. Brackets are used to highlight the different primer sets. Colostrum (C) from one sow and blood from one of her piglets (PS: pre-suckling; AS: after suckling) are shown in the picture of the agarose gel. The red boxes highlight the colostral (maternal) DNA found in neonate. L: ladder

in post-suckling piglet blood after PCR amplification using 3 of the 5 primer sets.

In order to characterize the *in vivo* cellular immune response induced by the injection of the non-specific mitogen PHA (Fig. 3.2A) and the *M. hyopneumoniae* antigen (Fig. 3.2B), the inflammatory response was measured at 1, 24, 36, and 48 h post-injection. Hypersensitivities were not evident immediately post-injection. Hypersensitivity responses were maximal at 36 h post-injection, and the magnitude of the response as measured by orthogonal diameter and skin fold thickness, declined by 48 h post injection. No pigs demonstrated hypersensitivity responses to saline injection (Fig. 3.2C). All pigs responded to PHA injection with DTH responses (Fig 3.2D). Piglets from *M. hyopneumoniae* vaccinated dams responded with *M. hyopneumoniae*-specific DTH responses (Fig 3.2.E). The timing and characteristics of the inflammation confirmed that the responses were delayed-type hypersensitivity (DTH) responses.

Experimental use of DTH testing in VS and NVS groups of piglets was performed based on results of DTH validation experiments. The concentrations of PHA (20 μg/ml in 0.1 ml) and *M. hyopneumoniae* antigen (300 μg/ml in 0.1 ml), as well as timing at measurement (36 h post-injection) were chosen based on lesion response time, lesion size, and congruence of pig *M. hyopneumoniae* status and lesion status. Newborn piglets from both the VS and NVS groups showed DTH responses to the nonspecific mitogen PHA, thus indicating cellular immune competence. No difference in the magnitude of PHA DTH-response size was observed among piglets across sow vaccination groups (Fig. 3.3). One piglet did not respond to PHA. Inflammation was not observed at the saline injection site in any of the neonatal pigs.

Figure 3.2 Validation of DTH response in young pigs

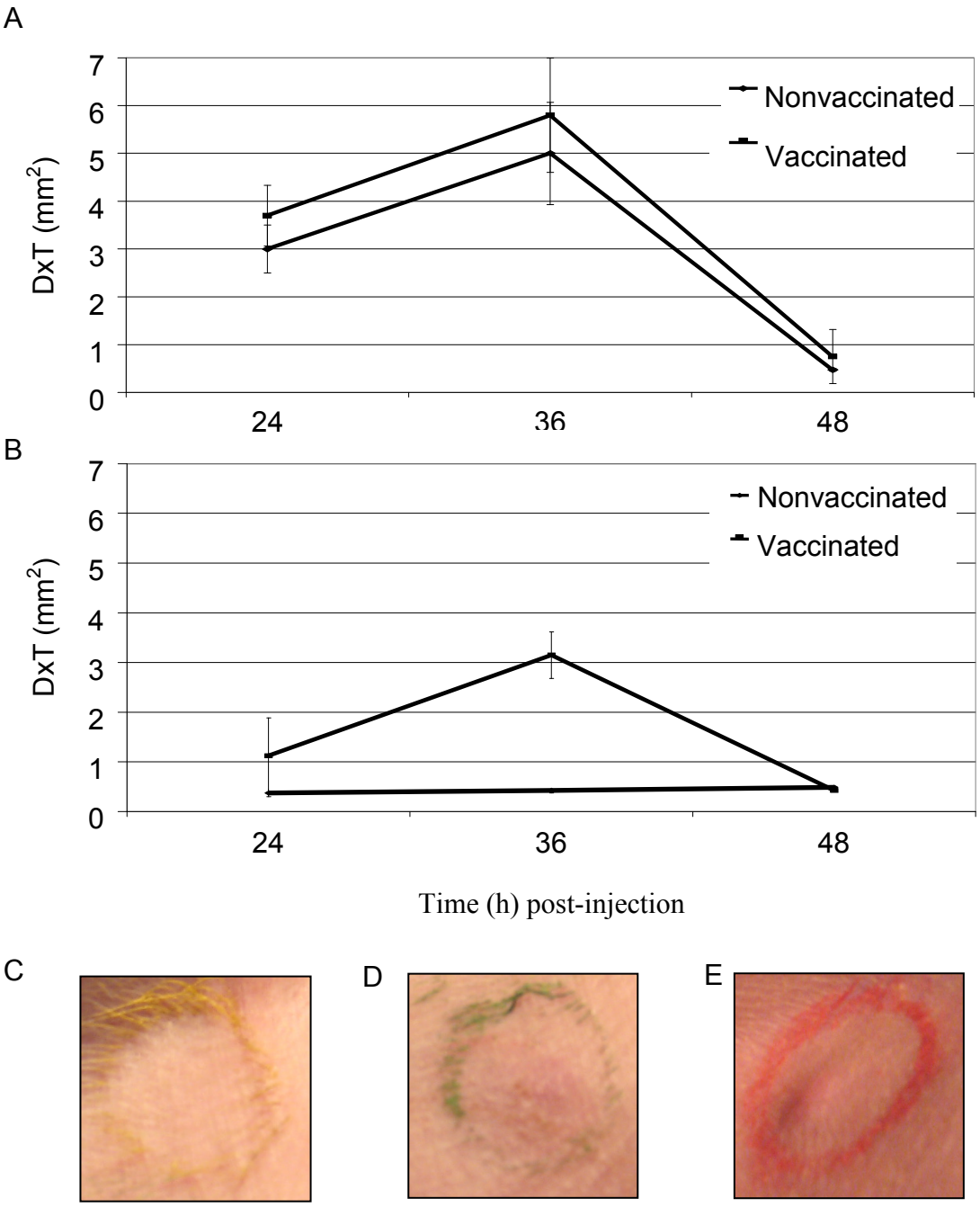


Figure 3.2 Validation of DTH responses in young pigs

M. hyopneumoniae antigen, PHA, and saline were injected intradermally into the inguinal area of preweaning pigs. Skin fold thickness and mean lesion diameter were measured with calipers immediately before (zero h) and 1, 24, 36, and 48 h post-injection to verify that the inflammation was a result of a DTH response. Pig responses from non-vaccinated dams are represented by triangles; pig responses from vaccinated dams are represented by squares. *A*. PHA-induced DTH mean lesion diameters over time. All animals tested responded to PHA whereas none of the piglets responded to saline (data not shown). *B*. *M. hyopneumoniae*-specific DTH responses over time. *C*. Photo of representative saline-injection site 36 h post-injection. *D*. Photo of representative PHA-induced DTH lesion 36 h post-injection. *E*. Photo of representative *M. hyopneumoniae*-induced DTH lesion in a piglet from a vaccinated dam36 h post-injection. Variation is expressed as standard error.

To investigate the *in vivo* cellular immune response to *M. hyopneumoniae*, concentrated *M. hyopneumoniae* antigen was injected intradermally into newborn pigs. Previous studies have demonstrated DTH responses to *M. hyopneumoniae* in infected pigs (Adegboye, 1978; Roberts, 1973), but this is the first to use DTH as a measure to assess passive maternal cellular immune transfer to piglets. Offspring from sows vaccinated with *M. hyopneumoniae* had significantly greater Mycoplasma-specific DTH responses (3.1 +/- 0.1 mm^2) than offspring from non-vaccinated sows (0.8 +/- 0.1 mm^2) ($p > 0.001$) (Fig 3.3). It should be noted that one piglet from a non-vaccinated sow did have a detectable DTH response to *M. hyopneumoniae*.

To assess *in vitro* cellular immune responses, *M. hyopneumoniae*-specific proliferation was measured. Lymphocytes isolated from colostrum of vaccinated sows proliferated significantly more in response to *M. hyopneumoniae* than colostral lymphocytes from non-vaccinated sows ($p<0.05$) (Fig. 3.4). Among lymphocytes isolated from piglet blood, *M. hyopneumoniae*-specific proliferation was observed only from lymphocytes isolated from piglets of vaccinated sows, and only after suckling ($p<0.05$). There was no statistical difference between antigen-specific proliferation of colostral lymphocytes and that of lymphocytes isolated from piglets of vaccinated sows after suckling. Antigen-specific proliferation was not observed from lymphocytes isolated from pre-colostral pigs of any group.

3.5 Discussion

In order to verify that colostral cells are transferred to neonates in a source-dependent fashion, DNA was isolated from colostrum and piglet blood (before and

Figure 3.3 DTH responses in young pigs

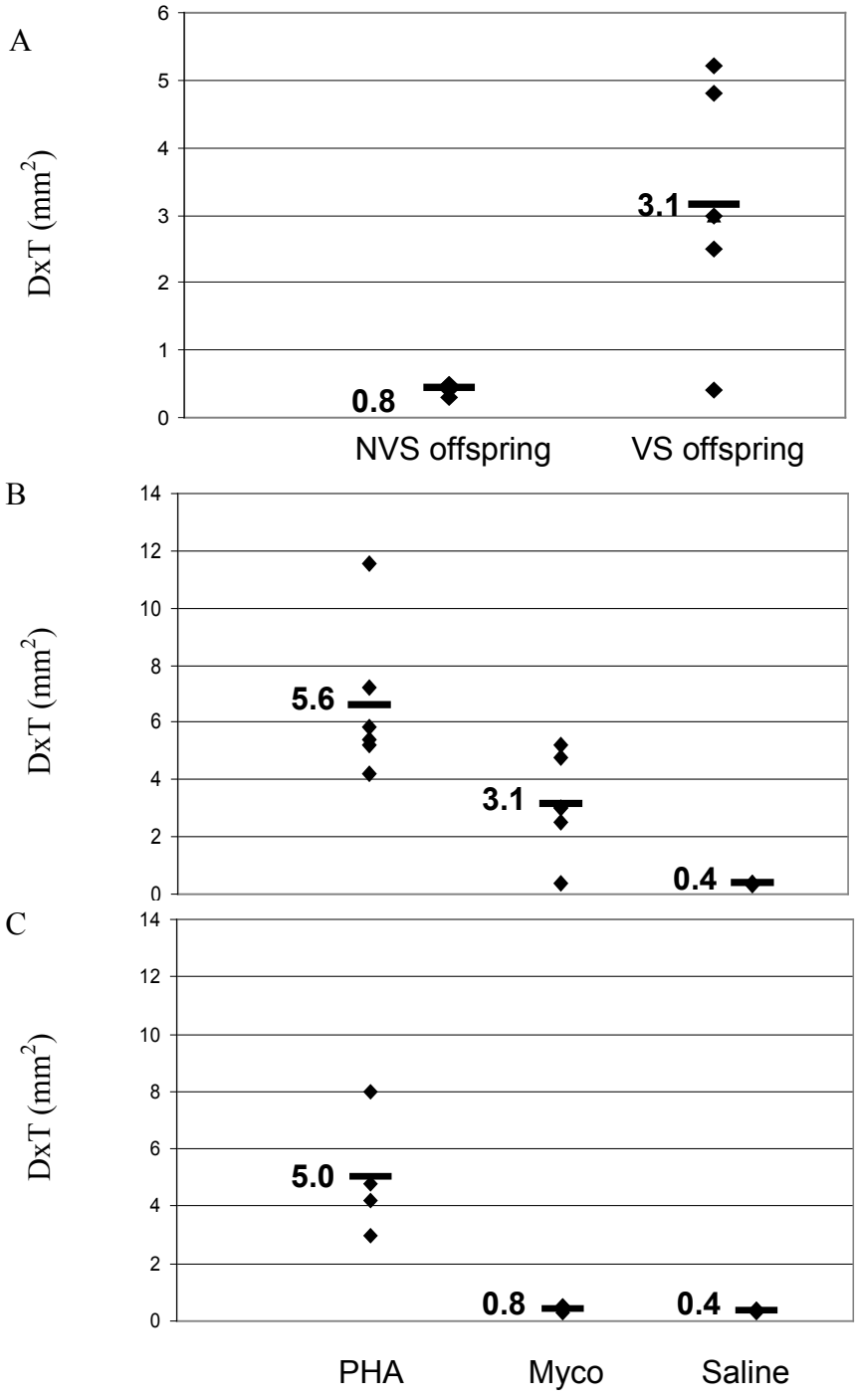


Figure 3.3 DTH responses in young pigs.

M. hyopneumoniae antigen was injected intradermally into the inguinal area. Skin fold thickness and mean lesion diameters were measured 36 h later with calipers. *A*. Mycoplasma-specific DTH responses in offspring of vaccinated (VS) and non-vaccinated (NVS) sows. *B*. DTH responses in offspring of vaccinated sows. *C*. DTH responses in offspring of non-vaccinated sows. *M. hyopneumoniae*-specific DTH responses were evident only in offspring of vaccinated sows. All animals responded to the nonspecific mitogen PHA and none responded to saline. (* indicates significance at $p<0.05$)

Figure 3.4 *Mycoplasma hyopneumoniae*-specific lymphocyte proliferation

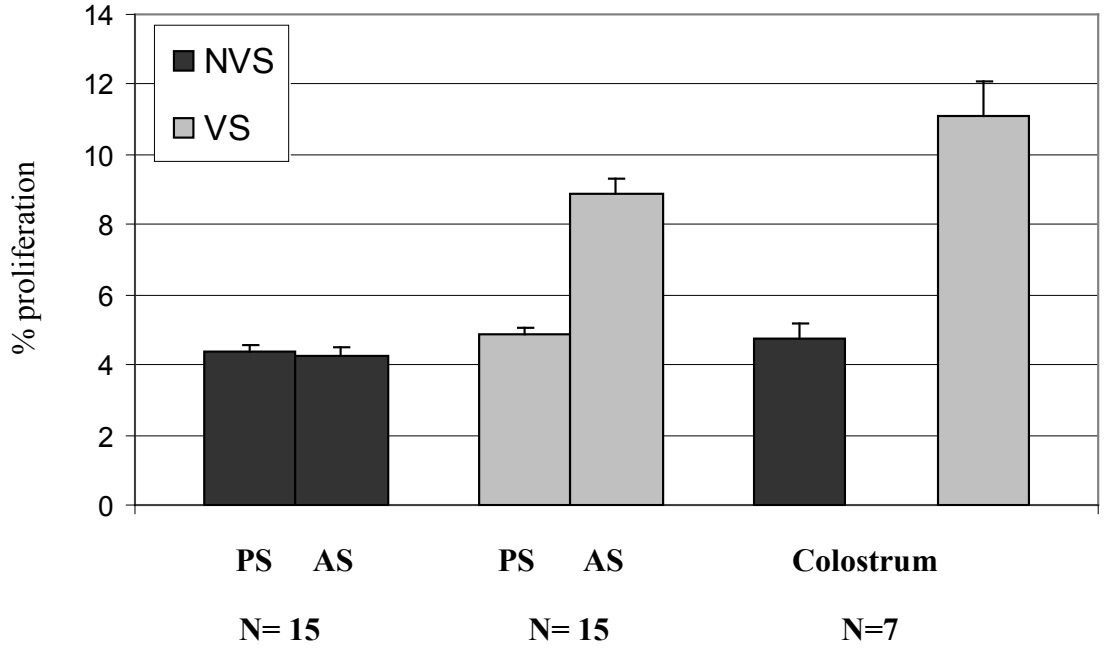


Figure 3.4 Mycoplasma-specific lymphocyte proliferation.

Lymphocytes were isolated from sow colostrum and from piglet blood pre (PS) and 24 h after (AS) colostrum ingestion and stimulated with *M. hyopneumoniae* antigen. *A*. Mean antigen specific proliferation by lymphocytes isolated from PS and AS piglets and from sow colostrum. (Variation is expressed as standard error; * indicates significance at $p<0.05$)

after colostrum ingestion) and amplified via PCR. Maternal DNA was visualized in post-suckling piglet blood when DNA was amplified with three of the five primer sets tested.

Thus, maternal cells do transfer to and are detectable in offspring piglet blood. Future studies should be done to evaluate the source-specificity of colostral cell transfer and the mechanism of cell transfer in pigs.

In this study, we have demonstrated for the first time that lymphocytes that are passively transferred from vaccinated dams to their offspring in colostrum are able proliferate and participate in a functional response to a particular antigen, in this case *M. hyopneumoniae*. Evidence that colostral lymphocytes were transferred into the newborn's circulation and that colostral lymphocytes were functional came from antigen-specific *in vivo* DTH responses and *in vitro* lymphocyte proliferation. *M. hyopneumoniae*-specific DTH lesions were only found in offspring of vaccinated sows. Colostral lymphocytes from vaccinated sows proliferated in response to stimulation with *M. hyopneumoniae* antigen and colostral lymphocytes from non-vaccinated sows did not.

Lymphocytes isolates from piglets before suckling did not respond to *M. hyopneumoniae* stimulation, indicating that piglets are naïve to *M. hyopneumoniae* at birth. Moreover, lymphocytes isolated from the offspring of vaccinated sows after suckling proliferated to the same degree as the colostral lymphocytes they received. Antigen specific DTH responses and lymphoproliferation are by definition secondary immune responses. Since newborn piglets are otherwise naïve to *M. hyopneumoniae*, the DTH responses and lymphoproliferative responses we observed in neonates of vaccinated sows were due to the action of antigen-specific maternal colostral cells. Therefore, maternal colostral cells transferred into the newborn's circulation and participated in

the immune response in an antigen specific manner.

Infection control in newborns often relies on maternal vaccination with the assumption that maternal immunity is transferred to the newborn and that maternal immunity is protective. Maternal vaccination is more widely practiced than newborn vaccination due to complications of the newborn's maturing immune system and of passive interference. Specifically regarding Mycoplasma,.several control studies have documented that when sow vaccination is followed by suckling pig vaccination, many piglets do not seroconvert to the vaccine, suggesting interference (Haesebrouck et al., 2004; Gross Beilage, 2005). Further, in most vaccination regimens timing is most often correlated with antibody titer without regard to cellular immunity. In fact many studies have indicated that cellular immunity is important in the response to *M. hyopneumoniae* (Adegboye, 1978; Roberts, 1973; Tajima et al., 1983; Thacker et al., 2000), and there appears to be no correlation between serum antibody level and protection from bacterial colonization or Mycoplasma disease (Thacker et al., 1998). The studies reported here are the first to demonstrate a functional response of passively transferred colostral cells in the newborn to *M. hyopneumoniae*.

The transfer of colostral cells with the ability to participate in a functional immune response suggests that passively transferred maternal cellular immunity can affect immune development of the newborn piglet. The impact of passively transferred maternal immunity on the development of the newborn's immune repertoire is not clear; however, development of adaptive immunity is undoubtedly affected by components of colostrum, notably maternal antibodies. Transferred antibodies are functional and can serve protective roles in the neonate, yet maternal antibodies can also interfere with

active immune induction. Studies in rodents suggest that passively acquired cellular immunity is not subject to interference and may be helpful in overcoming the inhibitive effects associated with maternal antibodies in the newborn (Siegrist, 2001). In addition, colostral cells have been found to modulate the proliferative response of piglet lymphocytes by significantly increasing blastogenesis to pokeweed mitogen, as compared to piglets not given maternal colostral cells (Wiliiams, 1993). A common practice in commercial swine production is to cross foster newborn piglets soon after birth. The timing of cross fostering practices may limit the number of cells transferred into the\ neonate and may hinder immune development of those animals since colostral cells are transferred into the neonate only if the colostrum is from the piglet's own mother (Tuboly et al., 1988; Williams, 1993). Using technetium-labeled cells (Tuboly et al., 1988) or fluoroscein-labeled cells (Williams, 1993), the two groups demonstrated that heat killed cells and cells from a source other than the piglet's own mother do not transverse the intestinal barrier. Thus, the transfer of T cells is dependent on their being viable and maternal in origin. No source or class restriction exists for antibody transfer (Klobasa and Butler, 1981) or for colostral cell transfer in other species including primates (Jain et al., 1991). Due to the very probable importance of T cells in neonatal immune development, timing of cross fostering practices warrants further investigation.

It should be noted that *M. hyopneumoniae* infection was evident on the farm and a single positive DTH response was observed from a piglet of the non-vaccinated sows group. This response could be a sensitivity issue; however, no animals in the study responded to saline and all responded to PHA. More likely, the piglet with the positive DTH response was the offspring of a sow that was actively infected with *M.*

hyopneumoniae. In an infected sow, the population of *M. hyopneumoniae*-specific transferred cells may be great enough to mount a specific cell-mediated response in the newborn.

Acknowledgements:

The work was performed on a commercial swine farm. The authors wish to thank the Pipestone Veterinary Clinic and staff veterinarians, Pipestone System producers, and Pipestone System employees for the assistance in this study. The authors also thank Juan Antonio Aeson, Seth Baker, Jean Paul Cano, Alejandrina Da Silva, Tug Peterson, Alejandro Pieters, Albert Rovira, and Nathan Schafer for assistance with sample collection and Abdoraghman Alghamdi for assistance with statistical analysis. Special thanks to Shi-Jun Ma and Prototek for assistance in the Mycoplasma antigen used in this study. This work was supported by Pfizer, Syngen, and the National Pork Board.

Chapter 4:

The effect of cross-fostering on transfer of maternal *Mycoplasma hyopneumoniae* immunity to piglets

Veterinary Record accepted

4.1 Summary

In spite of its widespread usage, the implications of cross-fostering on piglet immunity have not been examined. The goal of this study was to assess the effect of cross-fostering on the transfer of maternal *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*)-specific antibody-mediated and cell-mediate immunity. Cross-fostering was based on gilt *M. hyopneumoniae* vaccination status following this scheme: vaccinated gilt x nonvaccinated gilt, nonvaccinated gilt x vaccinated gilt, and vaccinated gilt x vaccinated gilt. Piglets were cross-fostered within the gilt pair at either 0, 6, 12, or 20 h after birth. Two piglets per gilt per time point were cross-fostered, thus 8 piglets per gilt were moved; remaining piglets served as non-cross-fostered controls. Four litters served as non-cross-fostered controls. A maximum of 10 piglets per gilt were sampled. Piglet *M. hyopneumoniae*-specific humoral and cellular immunity were assessed via ELISA and delayed type hypersensitivity, respectively. *M. hyopneumoniae*-specific antibodies transferred to piglets independent of cross-fostering status. Piglets from nonvaccinated mothers cross-fostered onto vaccinated dams had detectable *M. hyopneumoniae*-specific antibodies when moved within 6 h of birth. *M. hyopneumoniae*-specific cellular immunity transferred to piglets in a source dependent manner and was only detected in piglets maintained on their mothers for at least 12 h. Therefore, cross-fostering does impact the transfer of cell-mediated and antibody-mediated immunity differently.To overcome the effects of cross-fostering without foregoing the practice it should be done after 12-20 h to ensure colostral cell transfer.

4.2 Introduction

Despite advances in farrowing house management pig producers struggle to maintain the health of young pigs. The highest risk period for pig mortality is in the preweaning stage; mortality is estimated to be 10.9 +/- 0.4% (USDA 2007). Preweaning mortality is due to infectious and noninfectious causes; however, management of the farrowing house plays the largest role in lowering the risk of mortality in preweaning animals. Studies published in the 1970's and 1980's suggest that variation in birth weight is a major contributor to preweaning mortality and that moving piglets to balance sow burden reduces preweaning mortality (English and Smith 1975, English and Wilkinson 1982, English 1984, Marcatti Neto 1986). As a result of these studies cross-fostering became a widespread farrowing house management strategy with over 98% of farms in the US and Canada exercising cross-fostering (Straw and others 1998). However, it was contended that cross-fostering practices actually decrease piglet survivability and performance (Stewart and Diekman 1989, Neal and Irvin 1991, Straw 1997). More recently, cross-fostering has been used as a way to increase survivability of low birth weight piglets (Cutler and others 1999) since low birth weight piglets consume less colostrum (Cutler and others 1999) and have a higher risk of dying (Milligan and others 2001, Deen and Bilkei 2004) when raised with heavier littermates. Nevertheless, low birth weight piglets gain the same amount of weight as heavier birth weight piglets (Milligan and others 2002) but do not catch-up to the weights of their heavier litter mates, reiterating the idea that cross-fostering does not improve piglet performance. In spite of its widespread usage and the controversy it has endured, the implications of cross-fostering on piglet health and immunity have not been examined.

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) infects pigs of all ages and is associated with respiratory disease and decreased productivity. Mortality caused by *M. hyopneumoniae* infection alone is rare (Piffer and Ross 1984); however *M. hyopneumoniae* infection predisposes the colonization of other pathogens, bacterial as well as viral, along the respiratory tract (Thacker 2006). *M. hyopneumoniae* plays a major role in porcine respiratory disease complex (PRDC) when combined with viral infections, and enzootic pneumonia when combined with other bacteria. While the most important means of *M. hyopneumoniae* transmission is by sow-to-piglet contact during lactation (Calsamiglia and Pijoan 2000, Rautiainen and Wallgren 2001), this close relationship also assures the transfer of immunity from the mother to the baby pig via colostrum.

Due to their epitheliochorial placentation, pigs are born without specific immunity and must rely on maternal immunity in the form of colostrum to overcome antigen challenge within the first weeks of life. Recently it was demonstrated that *M. hyopneumoniae*-specific cellular and humoral immunity is transferred from the immunized sow to her offspring in colostrum (Bandrick and others 2008). Colostral *M. hyopneumoniae* antibodies do not affect protection against *M. hyopneumoniae* (Wallgren and others 1998, Palzer and others 2006); however maternal immunity lowers the antibody response of the piglet (Haesebrouck and others 2004). Given the source specificity of colostral lymphocyte absorption by newborn pigs (Tuboly and others 1988, Williams 1993), *M. hyopneumoniae*-specific cellular immunity may not be transferred to piglets that have been cross-fostered immediately after birth. The objective of the present study was to assess the effect of cross-fostering piglets on the transfer of antigen

specific immune components from mother to piglet. In this study we have shown that humoral and cellular immunity are transferred to piglets via different mechanisms and that cross-fostering affects the transfer of humoral and cellular immunity.

4.3 Materials and Methods

Animal housing and vaccination

All animals were treated in accordance with the University of Minnesota's Institutional Animal Care and Use Committee regulations. Twenty gilts and their litters were housed in an 800-sow farm belonging to the University of Minnesota Southwest Experimental Station, Waseca, MN, USA. The farm was known to be free of *M. hyopneumoniae* and PRRS virus infection for at least 4 years prior to and during the experiment. Gilts were chosen for the study based on breeding dates to ensure synchronized farrowings and to allow cross-fostering of piglets. Gilts were individually identified and randomly allocated into two groups. Twelve gilts were vaccinated (IM), while eight gilts remained unvaccinated. The vaccinated gilts were immunized twice (5 and 3 weeks pre-farrow) with a commercial *M. hyopneumoniae* killed bacterin (RespiSure®, Pfizer Animal Health, Kalamazoo, MI, USA). Gilts were tested for *M. hyopneumoniae* specific antibodies prior to being vaccinated.

Cross-fostering

In order to study the effect of cross-fostering on immune transfer, piglets needed to be cross-fostered between matched gilt pairs at specific times. Gilts were matched into pairs at farrowing and matching was based on gilt vaccination status and time when

farrowing started (appearance of first pig). To ensure piglets did not ingest any colostrum before cross-fostering, all farrowings were closely monitored. At farrowing and prior to suckling piglets were individually identified and placed into plastic tubs. Plastic tubs were placed under heat lamps. Piglets were cross-fostered within the matched gilt pair only when farrowing time differences were less than 3 h.

Piglets were cross-fostered based on gilt vaccination status following this scheme: vaccinated gilt x nonvaccinated gilt (V x N), nonvaccinated gilt x vaccinated gilt (N x V), and vaccinated gilt x vaccinated gilt (V x V) (Fig. 1A). Piglets were cross-fostered within the matched gilt pairs at four time points: 0, 6, 12 and 20 h after birth (Fig. 1B). Two piglets per time point were cross-fostered within the gilt pair, thus 8 piglets per gilt were moved. The remaining piglets stayed with their biological mother and served as non-cross-fostered controls. A maximum of ten piglets per gilt were included in the sampling sessions. At the end of the study six V x V, five V x N, and five N x V gilt pairs were formed. Four gilts served as controls (3 vaccinated and 1 nonvaccinated) and their piglets were not cross-fostered.

Sampling

Colostrum was manually collected from all functional teats within 1 h of farrowing. Gloves were worn and teats were scrubbed with alcohol wipes to minimize sample contamination. Colostrum (25 ml) was collected in sterile 50 ml conical tubes. Piglet blood was collected by jugular vein puncture into sterile EDTA tubes (Becton Dickinson, Vacutainer® Blood Collection Tubes, Franklin Lakes, NJ, USA) using sterile needles (Becton Dickinson Vacutainer® Blood Collection Needles, Franklin Lakes,

NJ, USA). Blood (4 ml) was collected from three piglets per gilt before colostrum ingestion and from all piglets 24 h after birth.

Cell-mediated immunity assessment

Cell-mediated immunity was assessed *in vivo* by DTH testing. DTH testing antigens were injected intradermally into the left inguinal region of 4-day old piglets. Injection sites were clearly marked with livestock paint. Concentrated and purified killed *M. hyopneumoniae* (300 µg/ml in 0.1 ml; generously provided by Shi-Jun Ma, Prototek, Minneapolis, MN, USA) was used to measure antigen specific cellular immunity in piglets (Roberts 1973). The mitogen PHA (20 µg/ml in 0.1 ml; Sigma, St. Louis, MO, USA) and biological saline (0.1 ml) were used as positive and negative controls, respectively. Inflammatory reactions, skin fold thickness and the mean orthogonal diameter of induration, were measured 36 h later with calipers (Bandrick and others 2008).

Detection of Mycoplasma specific antibodies by ELISA

M. hyopneumoniae-specific antibodies were measured in colostral whey and serum collected from gilts and from serum collected from piglets. Antibodies were measured using a commercial ELISA kit (IDEXX Laboratories, Westbrook, Maine, USA) for *M. hyopneumoniae*-specific antibody detection (Erlandson and others 2005). Positive and negative *M. hyopneumoniae* serological status was determined based on sample to positive (S:P) ratio: S:P = (sample OD - negative control OD) / (positive control OD - negative control OD). S:P ratios >0.4 were classified as positive; S:P

ratios <0.4 were classified as negative. All samples were run in duplicate and sample means were used to determine the final S:P ratio.

Statistics

The effect of cross-fostering on transfer of *M. hyopneumoniae*-specific cellular immunity was analyzed with a two-way ANOVA with time at cross-fostering (0, 6, 12, 20 h), DTH lesion size, and their interactions in the model. The effect of cross-fostering on transfer of *M. hyopneumoniae*-specific humoral immunity was analyzed with a two-way ANOVA with time at cross-fostering (0, 6, 12, 20 h), ELISA S:P ratios, and their interactions in the model. Interactions found to be significant by ANOVA ($p<0.05$) were analyzed by Tukey's method. Statistical analysis was performed using GraphPad Prism 5 (Graph Pad Software, Inc, CA, USA). The difference in *M. hyopneumoniae*-specific DTH lesion size among control piglets (non-cross-fostered piglets from vaccinated dams vs. non-cross-fostered piglets from nonvaccinated dams) was analyzed with the Student's t-test. The difference in *M. hyopneumoniae*-specific antibody S:P ratios among control piglets, before and after vaccination in gilt serum, in colostrum of vaccinated or nonvaccinated gilts, and in serum of vaccinated or nonvaccinated gilts was analyzed with the Student's t-test.

4.4 Results

Farrowings were synchronized to allow for cross-fostering of age-matched piglets. The cross-fostering scheme and the experimental design are shown in Fig 4.1. Piglets were only moved when 2 gilts began farrowing within 3 h of each other and

piglets were only moved within the gilt pair. Of the 20 gilts included in the study, 8 pairs of cross-fostered litters were formed. The final cross-fostering scheme was as follows: 6 V x V litters, 5 V x N litters, and 5 N x V litters. Each gilt within the cross-fostered pair had 8 foster piglets and 2 of her own non-cross-fostered piglets (one gilt had 8 foster piglets and one non-cross-fostered piglet because she farrowed 9 piglets). The remaining 4 gilts did not have their litters cross-fostered and served as controls.

Gilt vaccination

At the onset of the study all twenty gilts were negative for *M. hyopneumoniae*-specific antibodies by ELISA (Fig. 4.2). Twelve of these gilts were vaccinated at three and five weeks prefarrowing and all twelve seroconverted following vaccination (Fig. 4.2). Serum and colostrum collected from all vaccinated gilts were positive for *M. hyopneumoniae*-specific antibodies while serum and colostrum collected from nonvaccinated gilts were negative for *M. hyopneumoniae*-specific antibodies. In vaccinated gilts, ELISA S:P ratios measured from colostral samples were significantly greater than S:P ratios measured from serum ($p=0.02$).

M. hyopneumoniae-specific cell-mediated immunity

Transfer of *M. hyopneumoniae*-specific immunity from dam to offspring was assessed by antigen specific DTH responses in young pigs. DTH responses to *M. hyopneumoniae* antigen and PHA were measured as an assessment of the *in vivo* cellular

Figure 4.1 Experimental design

A

Cross-fostering scheme	Number of piglets fostered per time point (hrs after birth) per litter				Not cross-fostered	Total piglets/gilt	Total gilts/ scheme
	0	**6**	**12**	**20**			
V x N	2	2	2	2	2	10	**5**
N x V	2	2	2	2	2	10	**5**
V x V	2	2	2	2	2	10	**6**
N control	0	0	0	0	10	10	**1**
V control	0	0	0	0	10	10	**3**

B

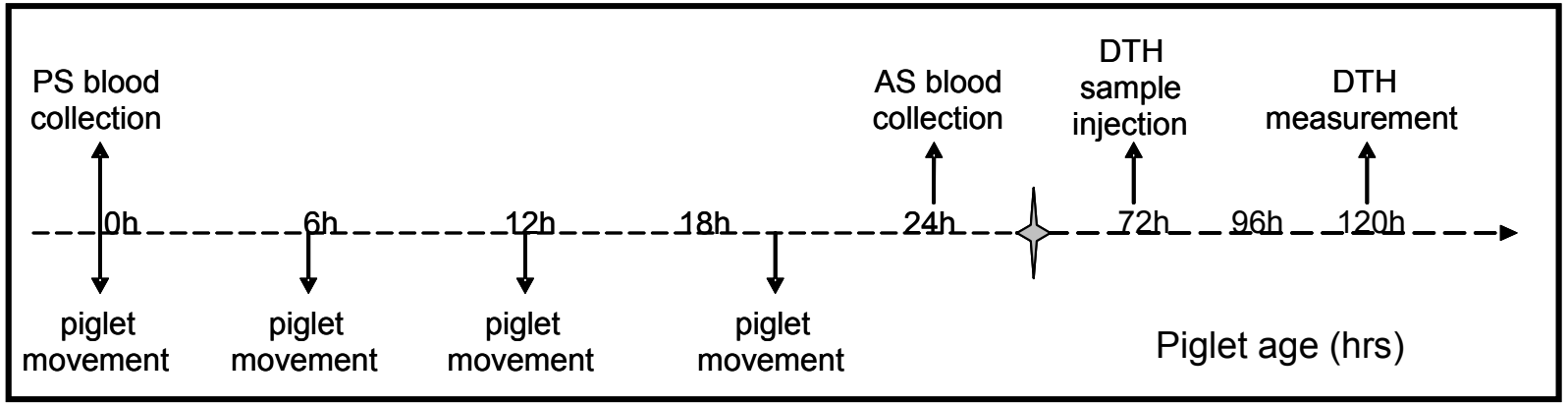
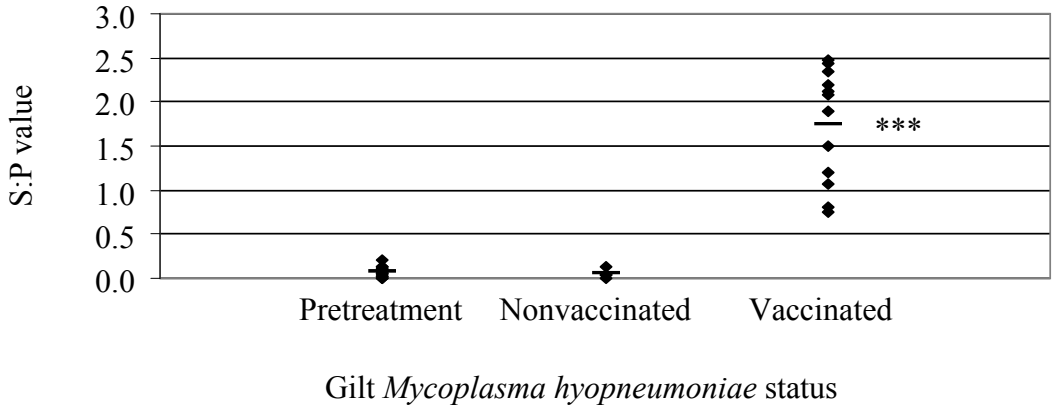


Figure 4.1. Experimental design

Piglets were left on their biological dam or cross-fostered based on gilt vaccination status at either 0, 6, 12, 20 h after birth. Two piglets were moved per time point; thus a total of 8 piglets per litter were moved to foster dams. The remaining piglets stayed on their biological mother as controls. Blood was collected before and 24 h after colostrum ingestion for *M. hyopneumoniae* antibody testing. Antigens were injected intradermally into the inguinal area at 3 days of age and DTH lesion size was measured 36 h later with a calipers. *A*. Cross-fostering scheme. Piglets that were born to vaccinated gilts were cross-fostered onto nonvaccinated gilts or other vaccinated gilts. Piglets born to nonvaccinated gilts were cross-fostered onto vaccinated gilts. Piglets were only moved within the gilt pair. *B*. Experimental time line. Blood was collected from piglets pre-suckling (PS) and after suckling (AS). Piglet movement times correspond to time at cross-fostering. Piglets were subject to delayed type hypersensitivity (DTH) tests at 36 h of age. DTH lesions were measured 36 h later with calipers.

Figure 4.2 *Mycoplasma hyopneumoniae*-specific antibodies in gilts

A. Serum



B. Colostrum

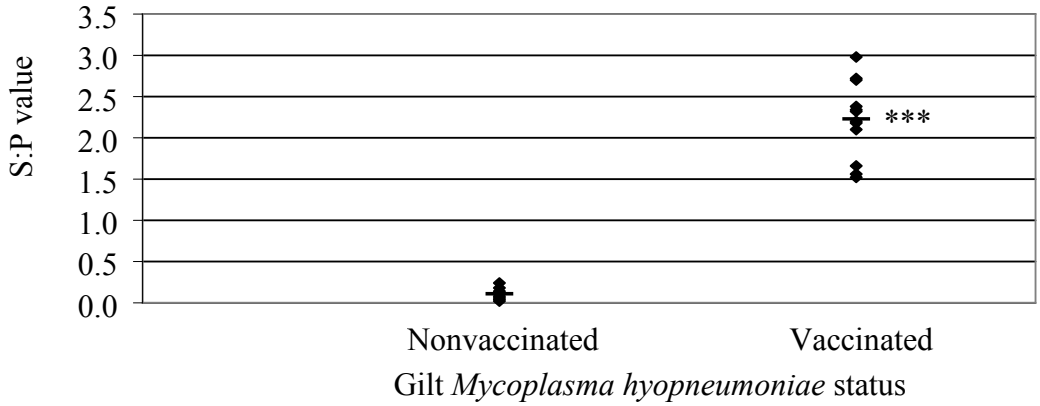


Figure 4.2 *Mycoplasma hyopneumoniae*-specific antibodies in gilts

M. hyopneumoniae specific antibodies were measured in *A*. serum and *B*. colostrum of vaccinated and nonvaccinated gilts by ELISA. Positive and negative antibody status was based on sample to positive (S:P) ratio; S:P= (sample OD-negative control OD) / (positive control-negative control OD). S:P ratios >0.4 were classified as positive; S:P ratios <0.4 were classified as negative. Horizontal bars indicate mean S:P ratios; ***p<0.0001.

immune response. Inflammatory reactions were not observed at the injection site immediately post-injection. Hypersensitivity responses were measured at 36 h post-injection and the magnitude of the response was measured as orthogonal diameter with calipers. Young piglets across control and cross-fostered groups responded to the nonspecific mitogen PHA, indicating cellular immune competence. There was no difference in the size of DTH responses to PHA among piglets despite gilt vaccination status or time at cross-fostering. Inflammation was not observed at the saline injection site in any of the piglets.

Non-cross-fostered piglets were used as a control. Among non-ross-fostered piglets, DTH responses to *M. hyopneumoniae* antigen were only detected in piglets from vaccinated gilts ($p<0.001$, n=21 piglets) (Fig. 4.3) No non-cross-fostered piglets from Nonvaccinated gilts had *M. hyopneumoniae*-DTH lesions.

Piglets that were moved from a nonvaccinated dam to a vaccinated dam did not respond to *M. hyopneumoniae* antigen injection with a DTH reaction at any cross-fostering time. Only piglets that were born from vaccinated gilts and maintained with them for 12 h or more had *M. hyopneumoniae*-specific DTH responses that were on average significantly greater than DTH responses from piglets of different cross-fostering schemes or times ($p=0.01$; Fig. 4.4). Piglets that were moved from a vaccinated dam to a nonvaccinated dam at 12 h and 20 h had significantly greater DTH responses compared to the same group of piglets moved at 0 and 6 h ($p=0.01$). In addition, piglets from vaccinated gilts that were cross-fostered onto vaccinated gilts at 12 h and 20 h had greater DTH responses compared to the same group of piglets moved at 0 and 6 h ($p=0.01$). There was no difference in DTH lesion size among piglets from vaccinated gilts that

Figure 4.3 *Mycoplasma hyopneumoniae*-specific DTH in piglets from vaccinated or nonvaccinated dams

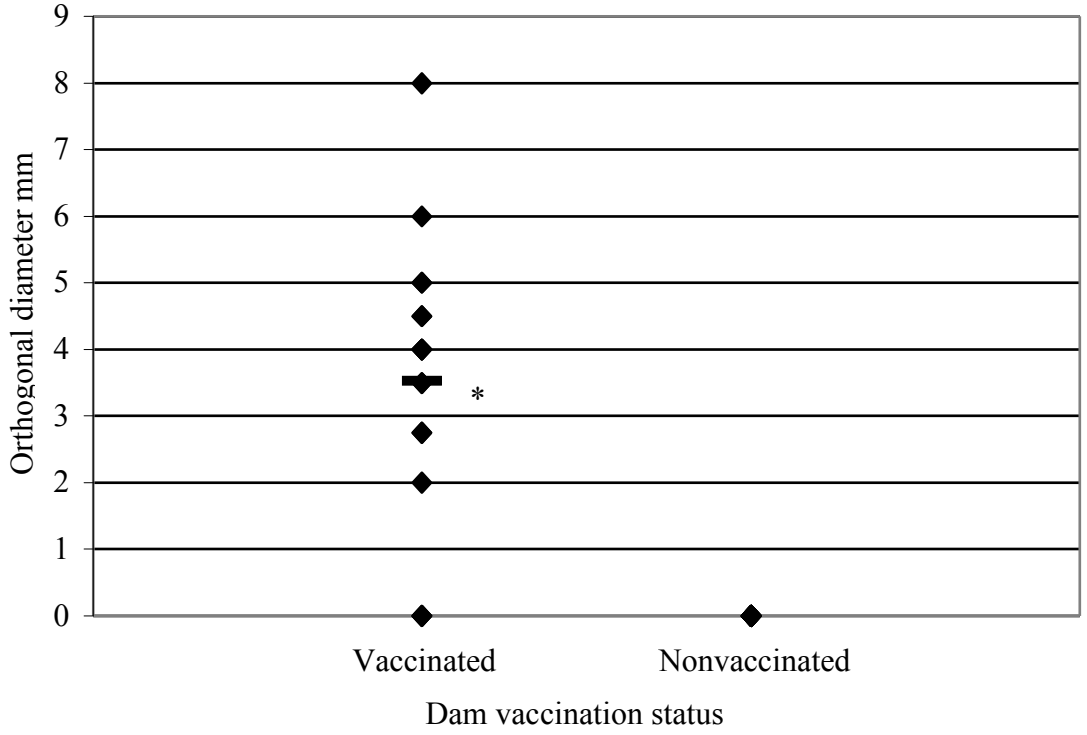


Figure 4.3 *Mycoplasma hyopneumoniae*-specific DTH in piglets from vaccinated or nonvaccinated dams

Purified and concentrated *M. hyopneumoniae* antigen was injected intradermally into the left inguinal area. Mean lesion induration was measured 36 h later with calipers. Mycoplasma-specific DTH responses were measured in piglets of vaccinated (n=21 piglets) and non-vaccinated (n=21 piglets) gilts. Variation is given by standard error; horizontal bars indicate mean lesion size; ** $p<0.01$.

cross-fostered onto vaccinated or nonvaccinated gilts at 12 h or at 20 h (Fig 4.4). It should be noted that two piglets that were cross-fostered onto (different) vaccinated dams at 0 h demonstrated positive DTH responses to *M. hyopneumoniae* antigen. Despite these rare positive reactions, the average DTH lesion size among piglets cross-fostered at 0 h was not different.

M. hyopneumoniae-specific humoral immunity

Serum samples collected from piglets pre-suckling were tested for *M. hyopneumoniae*-specific antibodies by ELISA. All pre-suckling serum samples were negative for *M. hyopneumoniae*-specific antibodies regardless of gilt vaccination status (data not shown). *M. hyopneumoniae*-specific antibody S:P ratios from piglets that were maintained with their mothers are shown in Fig 4.5A. None of the non-cross-fostered piglets from nonvaccinated gilts had detectable *M. hyopneumoniae* antibodies by ELISA. All non-cross-fostered piglets from vaccinated gilts had measurable anti-*M. hyopneumoniae* antibodies (Fig 4.5A) 24 h after birth.

M. hyopneumoniae-specific antibody S:P ratios in cross-fostered piglets were measured in blood samples collected 24 h after birth and are shown in Fig.4. 5. All of the piglets that were born to vaccinated dams and cross-fostered to vaccinated dams had *M. hyopneumoniae* antibodies. Further, there was no difference in antibody S:P ratio relative to time at cross-fostering for piglets born to vaccinated dams and cross-fostered to vaccinated dams (Fig. 4.5C). Piglets born to vaccinated gilts and moved to nonvaccinated gilts were *M. hyopneumoniae*-antibody positive as long as they remained on their vaccinated dam for at least 6 h ($p=0.01$; Fig. 4.5B). *M. hyopneumoniae*-specific

Figure 4.4 *Mycoplasma hyopneumoniae*-specific DTH responses in cross-fostered animals

Figure 4.4 *Mycoplasma hyopneumoniae*-specific DTH response in cross-fostered piglets

Purified and concentrated *M. hyopneumoniae* antigen was injected intradermally into the left inguinal area. Mean lesion induration was measured 36 h later with calipers. *A*. *M. hyopneumoniae* specific DTH responses were measured in piglets that were cross-fostered based on gilt vaccination status (vaccinated: V or nonvaccinated: N) at 0, 6, 12, and 20 h after birth. *B.* Mean *M. hyopneumoniae*-specific DTH lesion size in cross-fostered pigs. Variation is given by standard error. Different superscripts (a or b) represent significantly different mean lesion size; p=0.01.

Figure 4.5 *Mycoplasma hyopneumoniae*-specific antibody S:P ratios in piglets

Figure 4.5A Non-Cross-Fostered

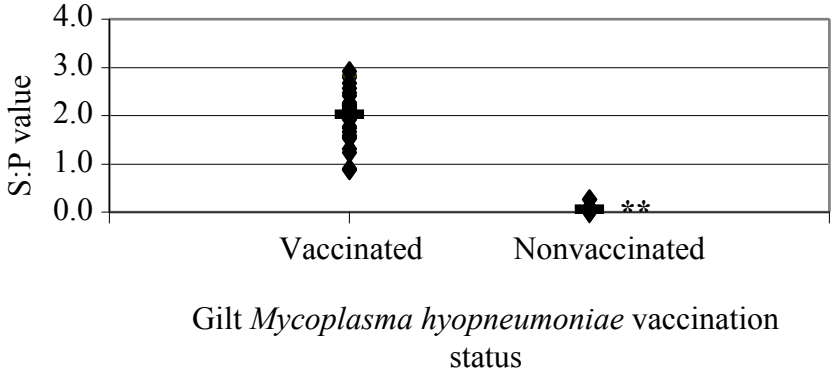


Figure 4.5 B Vaccinated x Nonvaccinated

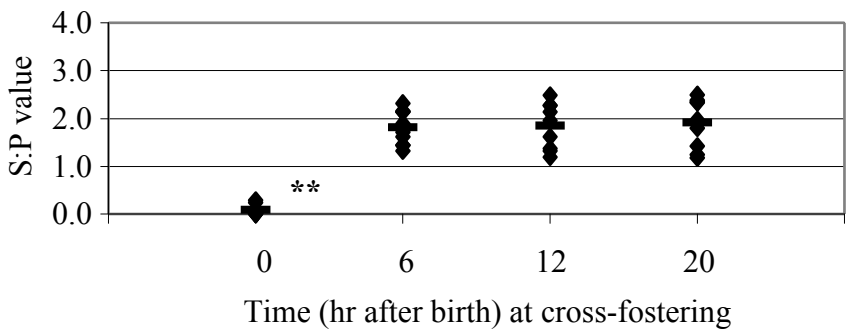


Figure 4.5C Vaccinated x Vaccinated

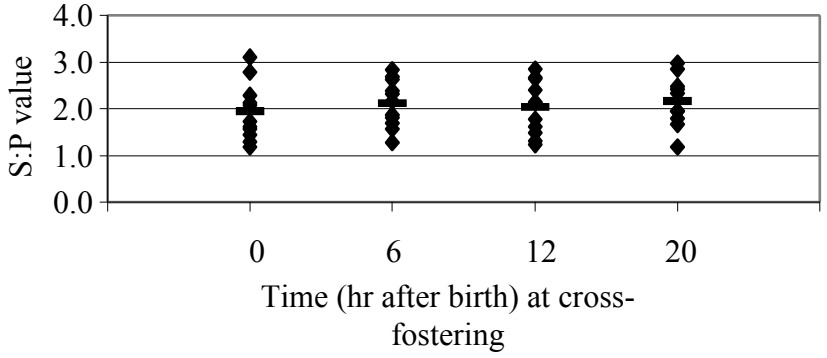


Figure 4.5 D Nonvaccinated x Vaccinated

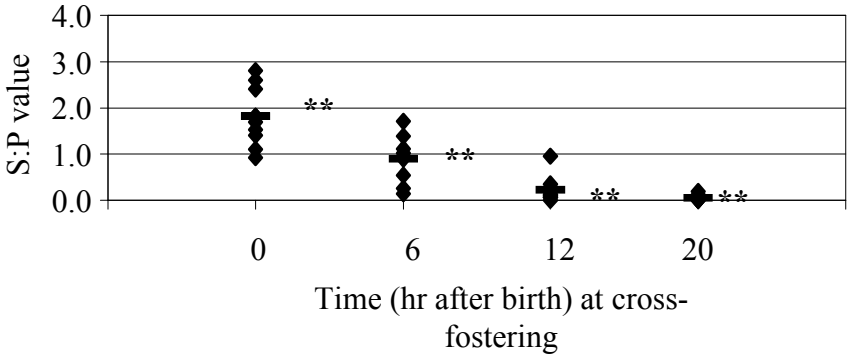


Figure 4.5 *Mycoplasma hyopneumoniae*-specific S:P ratios in piglets

M. hyopneumoniae specific antibodies were measured in serum of control piglets from vaccinated (n=30) and nonvaccinated (n=32) gilts and in serum of piglets that were cross-fostered based on gilt vaccination status (vaccinated: V or nonvaccinated: N) at 0, 6, 12, and 20 h after birth via ELISA. Positive and negative antibody status was based on sample to positive (S:P) ratio; S:P= (sample OD-negative control OD) / (positive control-negative control OD). S:P ratios >0.4 were classified as positive; S:P ratios <0.4 were classified as negative; horizontal bars indicate mean S:P ratios. Different superscripts (a, b, c or d) represent significantly different average lesion size; p=0.01; ***p<0.0001

antibodies were not detected in piglets that were born to vaccinated gilts and moved to nonvaccinated gilts at 0 h. Piglets that were born to and remained with their nonvaccinated dams for more than 6 h were *M. hyopneumoniae*-antibody negative (Fig. 4.5D). Only piglets that were born to nonvaccinated dams and moved immediately or at 6 h to vaccinated dams had detectable *M. hyopneumoniae*-antibodies ($p=0.01$).

4.5 **Discussion**

Piglet survival within the first few days of life is dependent on passive immunity acquired in colostrum because the porcine placenta is largely impermeable to immune proteins and cells. Antibodies and immune cells are transferred to the piglet via colostrum ingestion, yet these two types of immune components are not transferred in the same fashion. Antibodies are absorbed across the neonatal intestinal mucosa for a selected period of time (up to 12-24 h) regardless of maternal source or even donor species (e.g. bovine antibodies) (Payne and Marsch 1962, Klobasa and others 1981). Conversely, in order for lymphocytes to cross the neonatal intestinal mucosa they need to be viable and derived from the piglets' mother (Tuboly and others 1988, Williams 1993). Consequently, piglets fostered to sows other than their biological mother will be deficient in maternally derived cellular immunity. The present study assessed the effect of cross-fostering piglets up to one day of age on the transfer of immune components, cells and antibodies, from mother to offspring.

In order to determine if cross-fostering affects passive immune transfer, gilts were vaccinated against *M. hyopneumoniae*. At all times during the study the only gilts that were positive for *M. hyopneumoniae* antibodies were those that were vaccinated,

indicating that *M. hyopneumoniae* was not endemic in the herd and that vaccination induced an immune response. As expected, no piglets had humoral *M. hyopneumoniae*-specific immunity before colostrum ingestion. Further, piglets that were maintained with their nonvaccinated gilts were negative for *M. hyopneumoniae* antibodies 24 h after birth, and had negative *M. hyopneumoniae*-specific DTH responses. In contrast, all piglets maintained with their vaccinated gilts had *M. hyopneumoniae*-specific antibodies when evaluated 24 h after birth, and a percentage had *M. hyopneumoniae*-specific cellular immune responses. The degree of *M. hyopneumoniae*-specific cells transferred to piglets may have varied in vaccinated gilts since maternal immunity was induced solely by vaccination and not by active infection. Furthermore, colostrum quality and yield of gilts is less than that of higher parity sows. Active infection and the use of sows rather than gilts would have led to more specific cells transferred to piglets and consequently, more piglets responding in the DTH test. However, the amount of specific cells transferred in colostrum was not quantified. Since newborn pigs are naïve to *M. hyopneumoniae* and there was no evidence of on-site exposure, the DTH responses observed in the piglets were due to the action of antigen-specific maternal colostral cells.

This is the first report to show an immediate effect of cross-fostering on passive immune transfer in pigs. In addition, this and other studies indicate that cellular immunity does not transfer into the piglet via a mechanism that is similar to the transfer of humoral immunity (Tuboly and others 1988, Williams 1993, Klobasa and others 1981). In this study, we have shown that piglets receive *M. hyopneumoniae*-specific cell-mediated immunity only from their genetic dams and receive humoral immunity from any dam (provided the dams have *M. hyopneumoniae* immunity), but that transfer is

dependent upon the time at cross-fostering. Immune cells are thought to traverse the intestinal epithelium via a receptor-mediated event since cells cross the neonatal intestine only if the cells are of maternal origin (Tuboly and others 1988, Williams 1993). In this study, piglets that were cross-fostered from their vaccinated dam to any other dam (vaccinated or nonvaccinated) before 12 h did not receive sufficient *M. hyopneumoniae*-specific cellular immunity to elicit significant *M. hyopneumoniae*-specific DTH responses. On the other hand, antibodies cross the intestinal epithelium nonselectively via endocytosis (Clarke and Hardy 1971, Burton and Smith 1977). Here, piglets that were moved from a nonvaccinated dam to a vaccinated foster dam had *M. hyopneumoniae* antibodies but only when moved immediately or within 6 h of birth. Piglets within the same cross-fostering scheme had no *M. hyopneumoniae*-specific antibodies if moved 12 or 20 h after birth. The difference in how cells and antibodies are absorbed across the neonatal intestinal epithelium along with timing of gut closure may explain why cells are maximally transferred after piglets are maintained with their dams for at least 6 h and antibodies are transferred immediately.

The protective immune response from *M. hyopneumoniae* disease needs clarification; nonetheless, antibodies (Messier and others 1990, Okada and others 2000) and cells (Roberts 1973, Adegboye 1978, Tajima and others 1983, Thacker and others 2000) participate in the anti-*M. hyopneumoniae* immune response. Therefore, it appears that both cells and antibodies are necessary to protect against *M. hyopneumoniae* disease and receiving both in colostrum would be beneficial to the young pig. Speculations exist, however, that the immune factors acquired by the piglet from its mother may be detrimental to the piglet since passive antibodies could interfere with endogenous

effector function in the piglet. Maternally derived cellular immunity may be especially important in the newborn since it may not interfere with active immune development, as is often the case for maternally derived humoral immunity (Siegrist 2001), and is the case for *M. hyopneumoniae*–specific humoral immune development (Wallgren and others 1998, Hodgins and others 2004). If cross-fostering practices are timed such that maternal cells are not transferred, endogenous immune effector function may be interfered with by maternal antibodies and piglets will not have developed immune memory to antigens encountered early in life. Therefore, upon repeat exposure to pathogens, piglets without specific immunity due to interference by maternal antibodies and a lack of maternal cellular immunity due to cross-fostering may be at a greater risk for disease once maternal antibody levels have waned.

Cross-fostering is a commonly used management practice on swine farms; however, the consequences of cross-fostering for piglet health have been largely neglected. This study provides striking evidence that cross-fostering impacts transfer of immunity from sow to piglet via colostrum. Therefore, cross-fostering should be done judiciously since adequate time spent on the sow prior to cross-fostering is critical for successful transfer of immune components. Additionally, to overcome the effects of cross-fostering without foregoing the practice it should be done after 12-20 h to ensure colostral cell transfer. Further, sow immune status should be standardized to ensure antibody transfer. A major goal of the swine industry should be to elucidate the long term impacts of cross-fostering on piglet health.

Acknowledgements

M. Bandrick and M. Pieters contributed equally to this study. The authors would like to thank the staff at the University of Minnesota Southwest Experimental Station for their assistance in this study. We also thank Kara Theis, Alejandrina da Silva, Katy Ptak, Chistall Norell, and Susana Mesonero for their assistance with sample collection and laboratory assays. Special thanks to Abdorrhaman Alghamdi for assistance with DNA isolation and running all PCR assays. Special thanks to Shi-Jun Ma and Prototek for the Mycoplasma antigen used in this study. This research study was supported by a grant from the National Pork Board (07-023).

Chapter 5:

Maternal Influences on Neonatal Cell-Mediated Immune Response to Vaccination

Clinical and Vaccine Immunology *Submitted*

5.1 Summary

Passively acquired maternally-derived immunity (MDI) is a double-edged sword. Maternal antibody-mediated immunity (AMI) and cell-mediated immunity (CMI) are critical immediate defenses for the neonate; however, MDI may interfere with the induction of active immunity, i.e. passive interference, in the neonate. The effect of MDI on vaccine-induced AMI and CMI responses to *Mycoplasma hyopneumoniae (M. hyopneumoniae*) was assessed in neonatal piglets. To determine whether CMI and AMI responses could be induced in maternally immune piglets, piglets with and without maternal *M. hyopneumoniae*-specific immunity were vaccinated against *M. hyopneumoniae* at 7 d of age. Piglet *M. hyopneumoniae*-specific antibody, proliferation, CD25 expression, and DTH responses were measured 7 and 14 d post vaccination. This study demonstrated that CD25 expression is not a reliable indicator of *M. hyopneumoniae*-specific CMI responses in pigs. Further, piglets with *M. hyopneumoniae*-specific MDI failed to show vaccine induced AMI responses; there was no rise in *M. hyopneumoniae* S:P ratios following vaccination of maternally immune piglets. In contrast, evidence from both antigen-specific proliferation and delayed-type hypersensitivity (DTH) testing demonstrates that *M. hyopneumoniae*-specific CMI priming and anamnestic responses are induced following vaccination in the presence of *M. hyopneumoniae*-specific MDI. Thus, neonatal *M. hyopneumoniae*-specific CMI is not subject to passive interference by MDI under the conditions of the study. Whether these conclusions can be applied to other pathogens warrants intense investigation, especially considering the potential impact of these studies on improving disease control and prevention strategies in neonates.

5.2 Introduction

Infectious disease is a major contributor to morbidity and mortality among infants and children. Ideally, vaccination should be practiced at an early age to confer protection from infectious disease-related morbidity and mortality experienced later in life. Vaccination is a commonly used disease control measure; however, there are many issues that complicate vaccinating neonates including vaccine safety and vaccine efficacy. Vaccination regimens may be unsuccessful in stimulating protective immunity in neonates due to both ontogenic immune immaturity and passively acquired maternally-derived immunity interfering with active immune responses.

An important contributor to the neonatal immune repertoire is passively acquired maternally-derived immunity. Maternally-derived immunity is transferred to neonates across the placenta in humans and mice (Simister, 2003; Saji et al., 1999; Papadogiannakis, 1997) or via colostrum and milk in pigs (Wagstrom et al., 2000; Salmon et al., 2008), horses (Crisman and Scarratt, 2008), and cattle (Chase et al, 2008). Maternally-derived immunity acts in a beneficial role by preventing or reducing the impact of infectious diseases in the neonate. Maternally-derived immunity in piglets has shown to be protective against transmissible gastroenteritis virus (Sestak et al., 1996), classical swine fever (CSF; Suradhat and Damrongwatanapokin, 2002), *Bordetella pertussis* (Elahi et al., 2006), *Mycoplasma hyopneumoniae* (*M. hyopneumoniae;* Rautianinen and Wallgren, 2001), and other agents. However, immunity acquired by the neonate from its mother can be detrimental since passive immunity may interfere with active antibody-mediated (AMI) and cell-mediated immune (CMI) responses following vaccination, i.e. passive interference. For example, young pigs fail to develop

anamnestic antibody responses to *M. hyopneumoniae* (Haesebrouk et al., 2006), Pseudorabies (Wittman and Ohlinger, 1987), swine influenza virus (SIV; Loeffen et al., 2003), *Bordetella bronchiseptica* (Kono et al., 1994), and CSF (Klinkenberg et al., 2002) when re-stimulated in the face of MDI. Both CMI and AMI mediators are transferred to neonates in colostrum, but maternally-derived antibodies are classically blamed for impeding active AMI responses. Effects of maternally-derived CMI on neonatal immune response development are less clearly understood.

Maternally-derived CMI transferred to neonates across the placenta or in the form of colostrum clearly participate in the neonatal CMI response. Transfer of functional tuberculin-specific immune cells as evidenced by delayed-type hypersensitivity (DTH) has been demonstrated in infants from vaccinated mothers (Schlesinger and Covelli, 1977). Transfer of CMI sensitivity to nematode and fungal antigens, as evidenced by antigen-specific proliferation and nematode load, and DTH, respectively, has been demonstrated in neonatal mice born to vaccinated mothers (Kumar et al, 1989; Rifkind et al, 1976). Further, enhanced CMI responsiveness to bovine viral diarrheal virus (BVDV) has been documented *in vitro* in calves receiving colostral cells from BVDV vaccinated mothers (Donovan et al., 2007). We have shown that maternal colostral cells from *M. hyopneumoniae* vaccinated dams are transferred to piglets and participate in the *in vivo* neonatal CMI response to *M. hyopneumoniae* upon antigen challenge (Bandrick et al., 2008). Still, it is unclear whether maternally-derived immunity participates in immune interference or otherwise affects active CMI response development in the piglet.

CMI is critical in the immune response to *M. hyopneumoniae* (Adegboye, 1978; Roberts, 1973; Tajima et al., 1983; Thacker et al., 2000) and maternal *M.*

hyopneumoniae specific cells participate in the immune response following *M. hyopneumoniae* antigen challenge in piglets (Bandrick et al., 2008). This study was conducted to test the hypothesis that piglets respond to *M. hyopneumoniae* vaccination with CMI but not AMI responses in the face of maternally-derived immunity. Piglets with maternally-dervied immunity failed to show vaccine-induced AMI responses. In contrast, piglets with MDI and subsequently vaccinated developed primary and secondary *M. hyopneumoniae*-specific CMI responses in the face of maternally-derived immunity.

5.3 Materials and Methods

Animals

All animals were cared for and housed under the Institutional Animal Care and Use Committee guidelines. At all times during the study, animals were housed at a commercial facility known to be porcine reproductive and respiratory syndrome virus (PRRSV) negative. According to the facility's protocol, all replacement gilts were vaccinated against a standard battery of vaccines including a commercial *M. hyopneumoniae* vaccine (Myco Silencer, Intervet, Whitehouse Station, NJ, USA) at days 17 and 45 of gilt acclimatization by farm staff; the total acclimatization period was 110 days. Randomly selected piglets were tested for *M. hyopneumoniae* by PCR following nasal swabbing prior to starting the present study. *M. hyopneumoniae*-specific PCR testing was performed by the University of Minnesota Veterinary Diagnostic Laboratory. All swabs were negative for *M. hyopneumoniae* DNA, indicating that *M.*

hyopneumoniae was not circulating on the farm.

Animals were randomly chosen to participate in this study based on breeding date and on gilt and first parity sow status. Gilts and first parity sows (from here known as sows) were randomly stratified into experimentally vaccinated or nonvaccinated groups. Vaccination was against *M. hyopneumoniae* (Respisure®, Pfizer Animal Health, Kalamazoo, MI, USA) at 5 and 3 weeks prior to the anticipated farrowing date. Farrowings were monitored and piglets were ear-tagged at birth. No cross-fostering was practiced among study animals. Eighty piglets were randomly chosen based on experimental sow vaccination status; 3-4 piglets per litter were randomly selected to participate in the study. Twenty piglets from vaccinated sows and 20 piglets from nonvaccinatred sows were immunized with the same vaccine (Respisure®, Pfizer Animal Health, Kalamazoo, MI, USA) as given to the sows. Piglet vaccine was administered at 7 d of age and resulted in four treatment groups of 20 piglets each as follows: (1) sow vaccination, piglet vaccination (V V); (2) sow vaccination, piglet nonvaccination (V N); (3) sow nonvaccination, piglet vaccination (N V); and (4) sow nonvaccination, piglet nonvaccination (N N).

Sampling

Blood collection of sows occurred at 5 weeks antepartum and 3 weeks postpartum. Blood was collected from the jugular vein into EDTA Vacutainer tubes. Colostrum was collected from all sows within 2 h of farrowing. Teats were scrubbed with alcohol wipes and gloves were worn to minimize sample contamination. Colostrum (25 ml total) was collected manually from all functional teats. Blood was collected from

piglets before colostrum ingestion, 24 h after colostrum ingestion, and at 7, 14, and 21 days of age. Blood sampling times are expressed in terms of days post vaccination (dpv).

Mononuclear cell isolation and lymphocyte stimulation

Mononuclear cells were isolated from sow and piglet blood via Ficoll density centrifugation as described (Bautista et al., 1999) with modifications. Piglet blood was diluted 1:2 in sterile PBS prior to layering on lymphocyte separation media to improve cell recovery yield. Mononuclear cells were isolated from colostrum as described (Le Jan, 1996) following 1:3 dilution in PBS to decrease viscosity. Cells were microscopically enumerated and viability was assessed via trypan blue exclusion. Viability of blood mononuclear cells (BMC) was at least 95% and viability of colostral mononuclear cells (CMC) was at least 90%.

A dye-dilution method was used in order to evaluate the proliferative response to *M. hyopneumoniae*. Cells were stained with the membrane stain carboxy fluorescein isothiocyante (CFSE; 5 μM; ICT, Bloomington, MN) and washed with RPMI supplemented with 10% FBS, 100 U penicillin G per ml, and 100 μg of streptomycin per ml. Cells were resuspended in RPMI and plated in duplicate at $5x10^5$ cells/well in 200 μl in round bottom 96-well plates. CMC and BMC were stimulated with 10 μg/ml *M. hyopneumoniae* antigen as described (Thacker et al., 2000). The *M. hyopneumoniae* antigen was prepared as described (Bandrick et al., 2008); *M. hyopneumoniae* at passage 15 was harvested by continuous flow centrifugation at 70,000 x g and resuspended in Tris-sodium chloride buffer. *M. hyopneumoniae* was inactivated by one freeze-thaw cycle and then by sonic disruption. Nonstimulated and ConA (5 μg/ml) stimulated cultures

served as negative and positive controls, respectively. Experimental, negative, and positive controls were setup for each animal. Following 5 d incubation, cells were transferred to sterile FACS tubes and washed. Proliferation was analyzed by flow cytometry using a Facs Caliber flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA). Non-stained, non-stimulated cells and stained, non-stimulated cells were used to establish a baseline for the proliferation assay. Event acquisition was set for 10,000 events in a region encompassing the CFSE-positive quadrant (bottom quadrants only). Results were analyzed by CellquestPro software. Data are described as *M. hyopneumoniae*-stimulated proliferation - nonstimulated proliferation.

CD25 expression was also utilized to assess lymphocyte stimulation (Quade and Roth., 1999). CMC and BMC were plated in 96-well round-bottom plates in duplicate at a concentration of $5x10^5$ cells per well. Cells were stimulated with 10 μg/ml *M. hyopneumoniae* antigen as described (Thacker et al., 2000). Non-stimulated cultures served as negative controls; conA-stimulated cultures (5 μg/ml) served as positive controls. Experimental, negative, and positive controls were setup for each animal. Cells were allowed to incubate for five days. Following incubation cells were transferred to sterile FACS tubes and washed. Mouse anti-swine CD25 antibody (10 μl; clone PGBL25A; IgG1 isotype; VMRD, Pullman, WA, USA) or mouse IgG1 isotype control (SouthernBiotech, Brimingham, AL, USA) was added to the cells and allowed to incubate for 30 min. Cells were washed three times before adding goat anti-mouse IgG1 conjugated to PE (10μl; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA). Cells were again incubated for 30 min in the dark and washed 3x before

analysis. Flow cytometry was also used to assess CD25 expression. A FACS Caliber flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) was utilized. Non-stained, non-stimulated cells were used to establish baseline CD25 expression. Event acquisition was set for 10,000 events in a region encompassing the PE-positive quadrants. Results were analyzed by CellquestPro software. Data are described as *M. hyopneumoniae*-stimulated CD25 expression-negative control CD25 expression.

DTH testing

DTH was used as an *in vivo* measure of the CMI response. The DTH assay for *M. hyopneumoniae* was performed as originally applied (Roberts et al 1973) with modifications. The *M. hyopneumoniae* antigen used in the DTH assay was the same antigen as used in the *in vitro* cell proliferation assay. *M. hyopneumoniae* antigen (300 μg/ml in 0.1 ml physiological saline) was injected intradermally in 10 piglets per group at 14 d of age and a second set of 10 piglets per group at 21 d of age. Phytohemagglutinin (PHA) (20 μg/ml in 0.1 ml physiological saline; Sigma, St. Louis, MO, USA) and physiological saline (0.1 ml) were used as positive and negative controls, respectively. Injections were performed in the inguinal region. Injection sites were clearly marked with livestock paint. The DTH injection sites were assessed immediately and 36 h post injection. The diameter of induration was measured 36 h post injection with calipers. DTH data are shown as mean orthogonal diameter of induration.

Antibody measurement

M. hyopneumoniae-specific antibodies were measured in all blood and colostrum samples employing Idexx ELISA kits (Idexx Laboratories, Westbrook, Maine, USA) as described (Erlandson et al., 2005). Positive and negative *M. hyopneumoniae* serological status was determined based on optical density (OD) of sample to positive (S:P) ratio; S:P = (sample OD - negative control OD) / (positive control OD - negative control OD). S:P ratios ≥0.4 were classified as positive; S:P ratios <0.4 were classified as negative. All samples were run in duplicate and sample averages were used to determine the final S:P ratio.

Data and statistical analysis

The difference in *M. hyopneumoniae*-specific antibody S:P ratio in sow sera before and after vaccination, the difference between *M. hyopneumoniae*-specific antibody S:P ratios in sera and colostrum of vaccinated or nonvaccinated sows, and the difference between *M. hyopneumoniae*-specific proliferation by CMC isolated from vaccinated or nonvaccinated sows were analyzed by the student t-test. The effect of piglet vaccination on *M. hyopneumoniae*-specific AMI and CMI (piglet *M. hyopneumoniae*-specific antibody S:P ratios, *M. hyopneumoniae*-specific proliferation, *M. hyopneumoniae*-specific CD25 expression, and *M. hyopneumoniae*-specific DTH induration diameter) was analyzed via standard unpaired two-way ANOVA. Interactions found to be significant by ANOVA ($p<0.05$) were analyzed by Tukey's HSD method. Student t-test was used to analyze the difference in mean *M. hyopneumoniae*-specific DTH induration diameter, *M. hyopneumoniae*-specific CD25 expression, and *M. hyopneumoniae*-

specific antibody S:P values between 7 and 14 dpv among piglets of the same group. Statistical analysis was performed using GraphPad Prism 5 (Graph Pad Software, Inc, CA, USA).

The correlation between *M. hyopneumoniae*-specific CD25 expression and *M. hyopneumoniae*-specific proliferation, *M. hyopneumoniae*-specific CD25 expression and *M. hyopneumoniae*-specific DTH responses, and between *M. hyopneumoniae*-specific proliferation and *M. hyopneumoniae*-specific DTH responses was determined by drawing best fit curves for the variables and determining the R^2 values. Correlation analysis was performed using Microsoft® Excel 2002.

5.4 Results

Sow response to vaccination

Prior to experimental vaccination, sows did display *M. hyopneumoniae*-specific antibodies (Fig. 5.1A) due to prior on-farm vaccination. *M. hyopneumoniae* experimental vaccination of sows resulted in *M. hyopneumoniae*-specific AMI responses in blood and colostrum (Fig. 5.1A) and *M. hyopneumoniae*-specific CMC proliferative responses in colostrum (Fig. 5.1B). Sows vaccinated as part of this study had significantly higher *M. hyopneumoniae*-specific serum antibody S:P values post-vaccinated compared to before vaccination ($p<0.0001$) and compared to nonvaccinated sows ($p=0.002$; Fig. 5.1A). Similarly, experimentally vaccinated sows had significantly greater *M. hyopneumoniae*-specific antibody S:P values in colostrum compared to nonvaccinated sows ($p=0.03$; Fig. 5.1A). CMC from experimentally vaccinated sows demonstrated significantly more

M. hyopneumoniae-specific proliferation compared to CMC from nonvaccinated sows ($p=0.04$; Fig. 5.1B).

Passive transfer of M. hyopneumoniae-specific immunity to piglets

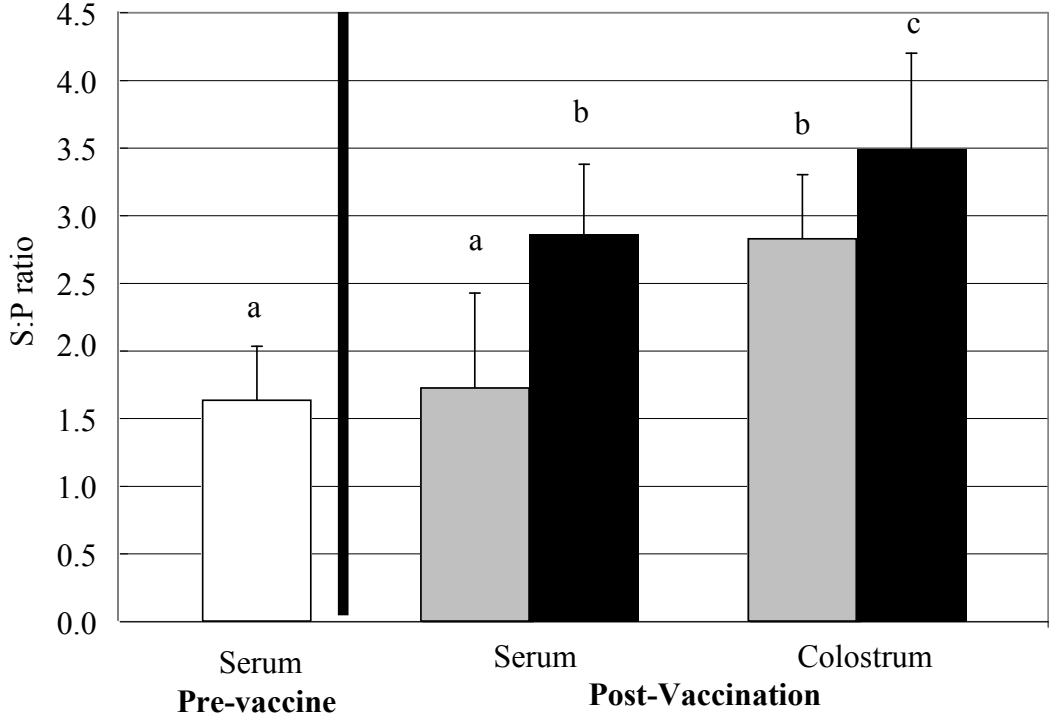
Newborn piglets are naïve to *M. hyopneumoniae* (the swine placenta prohibits transfer of immune components *in utero*), and there is no evidence of transplacental infection with *M. hyopneumoniae*. Prior to colostrum ingestion piglets did not demonstrate *M. hyopneumoniae*-specific immunity, either AMI (Fig. 5.2A) or CMI (Fig. 5.2B). Following colostrum ingestion, piglets from experimentally vaccinated sows had significantly greater *M. hyopneumoniae* antibody S:P values compared to piglets from nonvaccinated sows ($p<0.0001$; Fig. 5.2A). Mononuclear cells isolated from the blood of piglets of experimentally vaccinated sows 24 h after suckling proliferated significantly more in response to stimulation with *M. hyopneumoniae* antigen compared to BMC isolated from piglets of nonvaccinated sows ($p=0.04$; Fig. 5.2B). CD25 expression on BMC isolated from piglets of experimentally vaccinated sows 24 h after suckling tended to be greater than CD25 expression compared to PBMC isolated from piglets of nonvaccinated sows ($p=0.08$; data not shown).

Piglet response to vaccination

M. hyopneumoniae vaccine was administered when piglets were 7 d of age. Piglets were vaccinated at 7 d of age in an attempt to stimulate endogenous immunity in

Figure 5.1 Sow response *to Mycoplasma hyopneumoniae* vaccination

A: *M. hyopneumoniae* specific antibodies in sow blood and colostrum



B: *M. hyopneumoniae* specific colostral cell proliferation

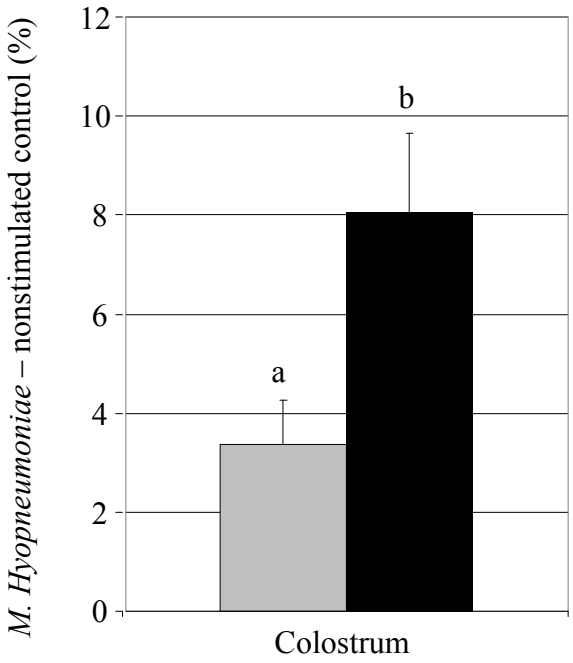


Figure 5.1 Sow response *to Mycoplasma hyopneumoniae* vaccination

M. hyopneumoniae specific humoral (panel A) and cellular immunity (panel B) were measured in sow blood and colostrum. *A. M. hyopneumoniae* specific antibodies were measured in serum and colostrum of vaccinated and nonvaccinated sows using the Idexx HerdCheck™ ELISA. *B. M. hyopneumoniae* specific cellular immunity was measured in sow colostrum via specific lymphoproliferation. Antigen specific lymphoproliferation was determined by subtracting unstimulated lymphoproliferation from *M. hyopneumoniae* specific lymphoproliferation. Black bars represent experimentally vaccinated sows; gray bars represent nonvaccinated sows; white bars represent sows pre-vaccination. Error bars are SEM; different subscripts represent significance at $p<0.05$.

Figure 5.2 *M. hyopneumoniae* specific immunity is transferred to piglets

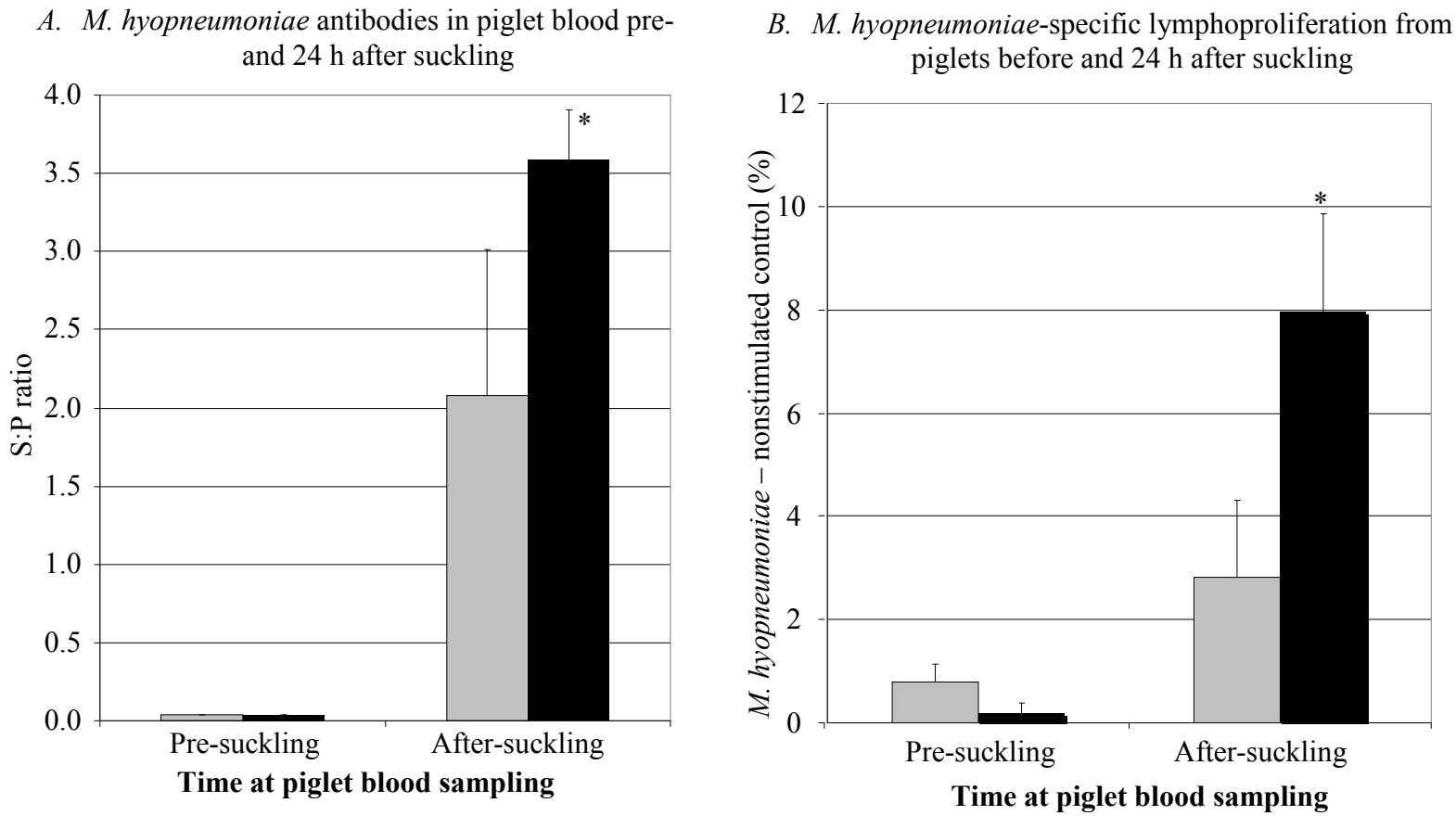


Figure 5.2 *M. hyopneumoniae* specific immunity is transferred to piglets

M. hyopneumoniae specific humoral (panel A) and cellular immunity (panel B) were measured in piglet blood before and 24 h after colostrum ingestion. *A. M. hyopneumoniae* specific antibodies were measured in serum of piglets pre-suckling and 24 h after suckling using the Idexx HerdCheck^{TM} ELISA. *B. M. hyopneumoniae* specific cellular immunity was measured in piglet peripheral blood via specific lymphoproliferation. Antigen specific proliferation was determined by subtracting unstimulated proliferation from *M. hyopneumoniae*-specific proliferation. Black bars represent piglets from experimentally vaccinated sows; gray bars represent piglets from nonvaccinated sows. Error bars are SEM; *: $p<0.05$.

the presence of maternally-derived CMI. *M. hyopneumoniae*-specific antibody levels did not differ among piglets from experimentally nonvaccinated sows relative to vaccination status 7 dpv or 14 dpv (Fig. 5.3A). Further, there was no change in the *M. hyopneumoniae*-specific antibody S:P values among piglets from experimentally nonvaccinated sows relative to piglet vaccination from 7 dpv to 14 dpv. *M. hyopneumoniae*-specific antibody S:P values among piglets from experimentally vaccinated sows was not different 7 dpv relative to piglet vaccination status (Fig. 5.3A). There was no change in *M. hyopneumoniae*-specific antibody S:P value among V N piglets from 7 dpv to 14 dpv; however, V V piglets had lower *M. hyopneumoniae*-specific antibody S:P values at 14 dpv compared to the same group of piglets 7 dpv ($p=0.05$). V N piglets had greater *M. hyopneumoniae* antibody S:P values compared to V V piglets 14 dpv ($p=0.002$; Fig. 5.3B).

The BMC isolated from V V piglets proliferated significantly more in response to *M. hyopneumoniae* stimulation compared to all other groups of piglets 7 dpv ($p<0.05$; Fig. 5.4A). Further, BMC isolated 14 dpv from V V piglets proliferated more in response to stimulation with *M. hyopneumoniae* than BMC isolated from the same group of piglets at 7 dpv ($p=0.02$). Similarly, *M. hyopneumoniae*-specific proliferative responses from N V piglets 14 dpv were significantly greater than those from the same group of piglets at 7 dpv ($p<0.001$). Interestingly, BMC isolated from vaccinated piglets proliferated significantly more in response to stimulation with *M. hyopneumoniae* compared to BMC isolated from nonvaccinated piglets regardless of sow vaccination status 14 dpv ($p<0.01$; Fig. 5.4B). There was no difference in proliferation by BMC isolated from N V compared to V V piglets at 14 dpv.

Figure 5.3 *M. hyopneumoniae* antibodies in piglet blood

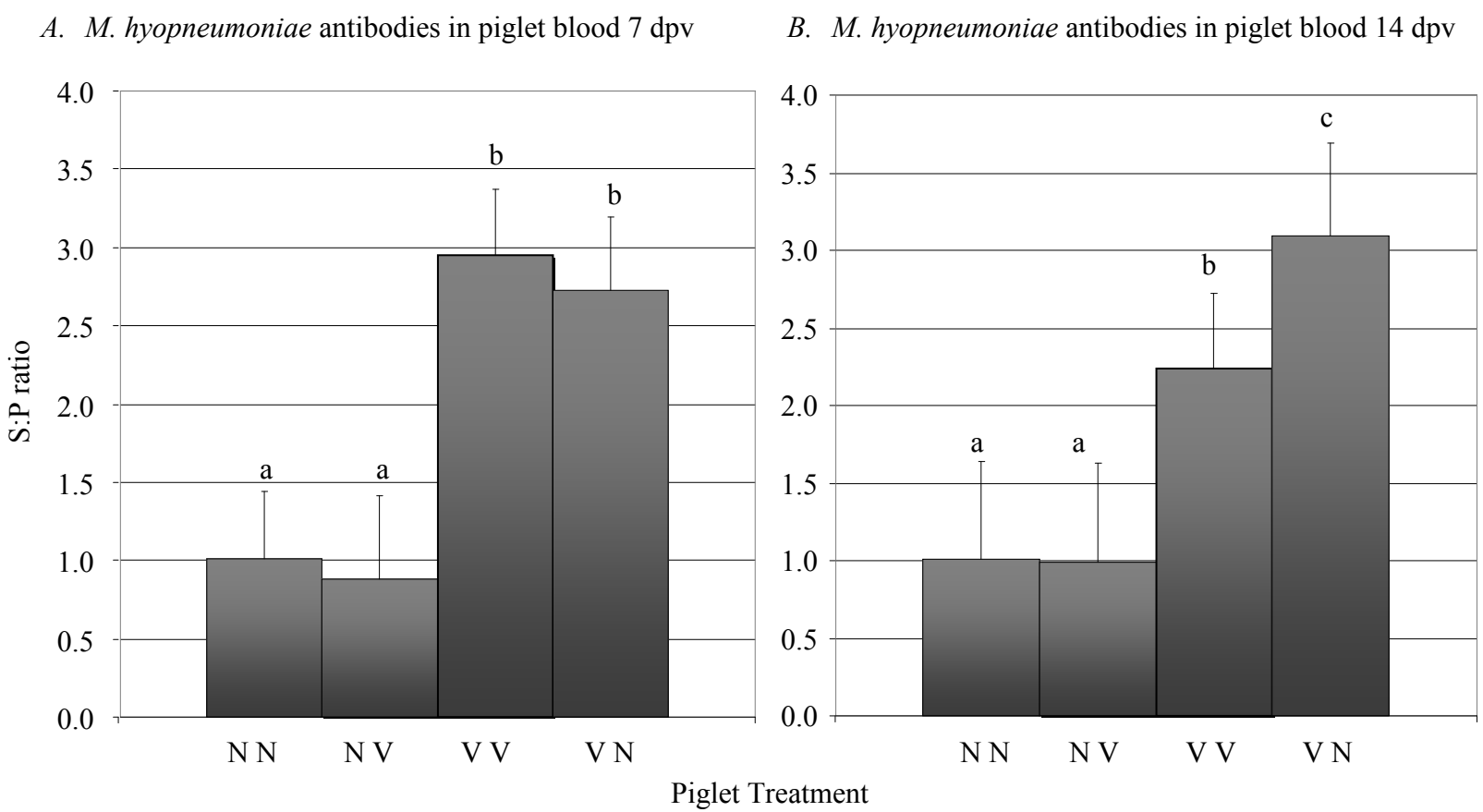


Figure 5.3 *M. hyopneumoniae* antibodies in piglet blood

M. hyopneumoniae specific humoral immunity was measured in piglet blood 7 days post vaccination (dpv) (panel A) and 14 dpv (panel B) using the Idexx HerdCheck ™ ELISA. Treatment groups are as follows: N N: nonvaccinated sow, nonvaccinated piglet; N V: nonvaccinated sow, vaccinated piglet; V V: vaccinated sow, vaccinated piglet; V N: vaccinated sow, nonvaccinated piglet. Error bars are SEM; different subscripts represent significance at $p<0.05$.

Figure 5.4 *M. hyopneumoniae*-specific proliferation

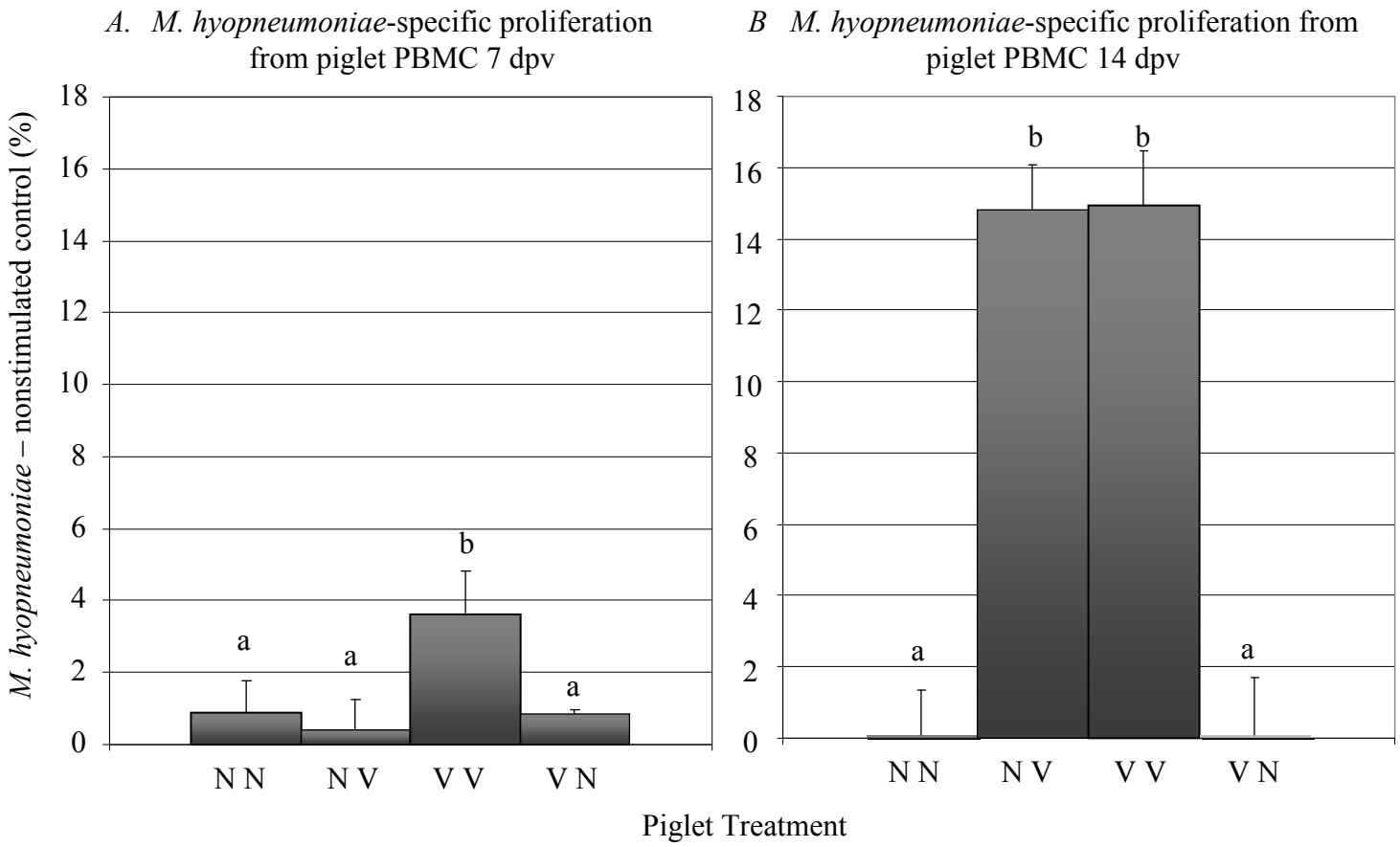


Figure 5.4 *M. hyopneumoniae*-specific proliferation

M. hyopneumoniae specific proliferation from piglet BMC was measured 7 days post vaccination (dpv) (panel A) and 14 dpv (panel B). Antigen specific proliferation was determined by subtracting unstimulated proliferation from *M. hyopneumoniae* specific proliferation. Treatment groups are as follows: N N: nonvaccinated sow, nonvaccinated piglet; N V: nonvaccinated sow, vaccinated piglet; V V: vaccinated sow, vaccinated piglet; V N: vaccinated sow, nonvaccinated piglet. Error bars are SEM; different subscripts represent significance at $p<0.05$.

Figure 5.5 *M. hyopneumoniae*-specific CD25 expression

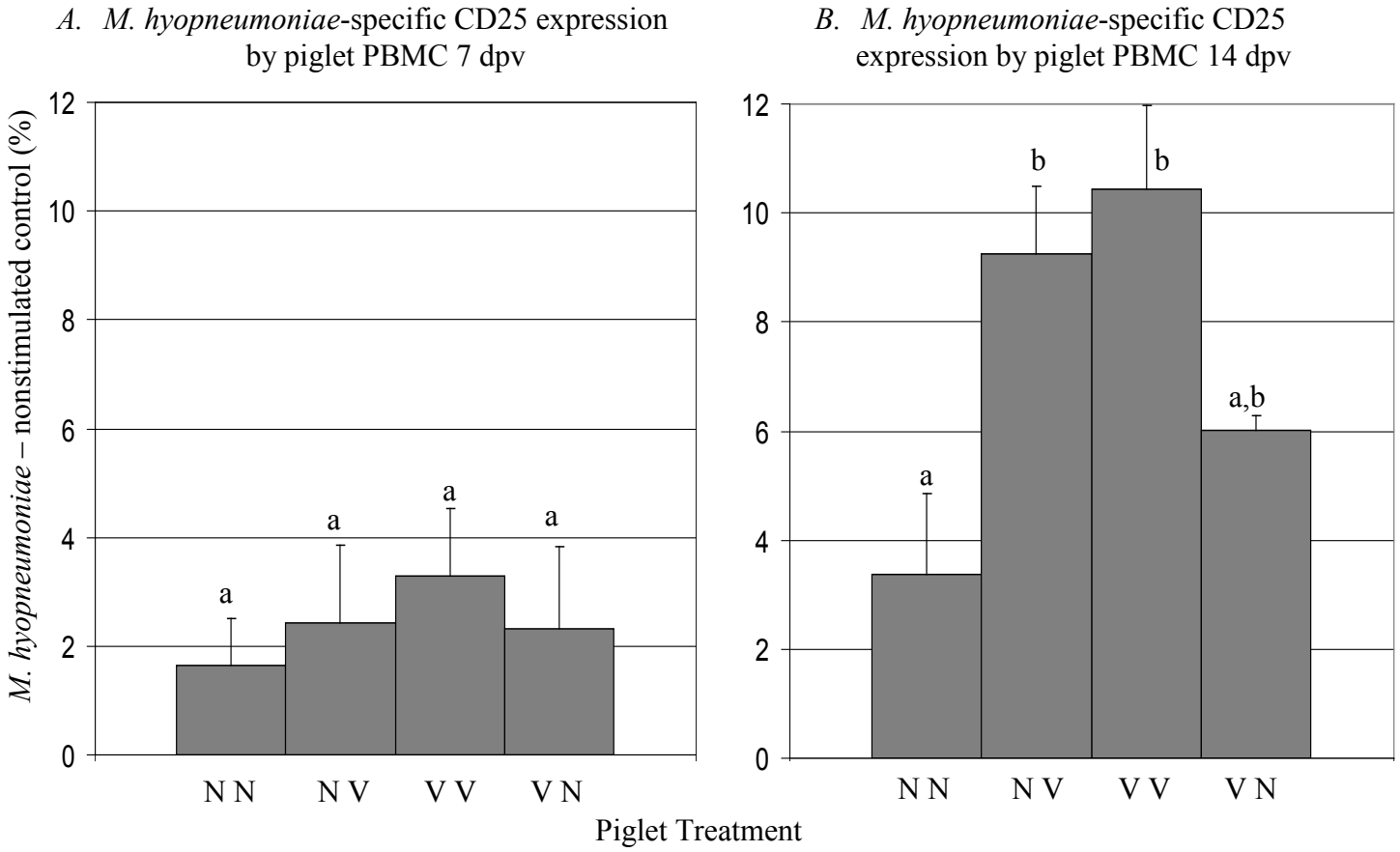


Figure 5.5 *M. hyopneumoniae*-specific CD25 expression

M. hyopneumoniae specific CD25 expression from piglet BMC was measured 7 days post vaccination (dpv) (panel A) and 14 dpv (panel B). Antigen specific CD25 expression was determined by subtracting unstimulated CD25 expression from *M. hyopneumoniae* specific CD25 expression. Treatment groups are as follows: N N: nonvaccinated sow, nonvaccinated piglet; N V: nonvaccinated sow, vaccinated piglet; V V: vaccinated sow, vaccinated piglet; V N: vaccinated sow, nonvaccinated piglet. Error bars are SEM; different subscripts represent significance at $p<0.05$.

CD25 expression was used as an experimental indicator of antigen-specific lymphocyte function as has been demonstrated in bovine (Endsley et al, 2002; Platt et al 2006; Quade and Roth, 1999). There was no difference in CD25 expression by BMC isolated from piglets at 7 dpv (Fig. 5.5A). At 14 dpv, PBMC isolated from N N and V N piglets had the lowest levels of CD25 expression (Fig. 5.5B). CD25 expression on BMC isolated from N N and V N piglets was not different 14 dpv; however, CD25 expression on BMC isolated from N N piglets was less than that of N V and V V piglets while CD25 expression on BMC isolated from V N piglets was the same as that of N V and V V piglets. Interestingly, CD25 expression on BMC derived from N V and V V piglets was significantly greater 7 dpv compared to 14 dpv. The level of BMC-CD25 expression 14 dpv compared to 21 dpv was the same among N N and V N piglets.

To detect piglet *M. hyopneumoniae*-specific CMI *in vivo*, DTH testing was performed. DTH responses to PHA were detected in piglets across all treatment groups at both injection times (7 dpv and 14 dpv). No piglets developed DTH lesions in response to saline injection at either time point. *M. hyopneumoniae*-specific DTH lesions were detected in some piglets across all treatment groups at both time points (Fig. 5.6). V V piglets had significantly larger *M. hyopneumoniae*-specific DTH othagonal induration diameters compared to all other treatment groups at 7 dpv ($p<0.01$; Fig. 5.6A). *M. hyopneumoniae*-specific DTH lesions among nonvaccinated piglets did not differ in othagonal induration diameter 14 dpv in terms of sow vaccination status; however, the orthogonal induration diameter of *M. hyopneumoniae*-specific lesions of nonvaccinated piglets was smaller than that of N V piglets ($p=0.05$) and V V piglets

Figure 5.6 *M. hyopneumoniae*-specific DTH responses

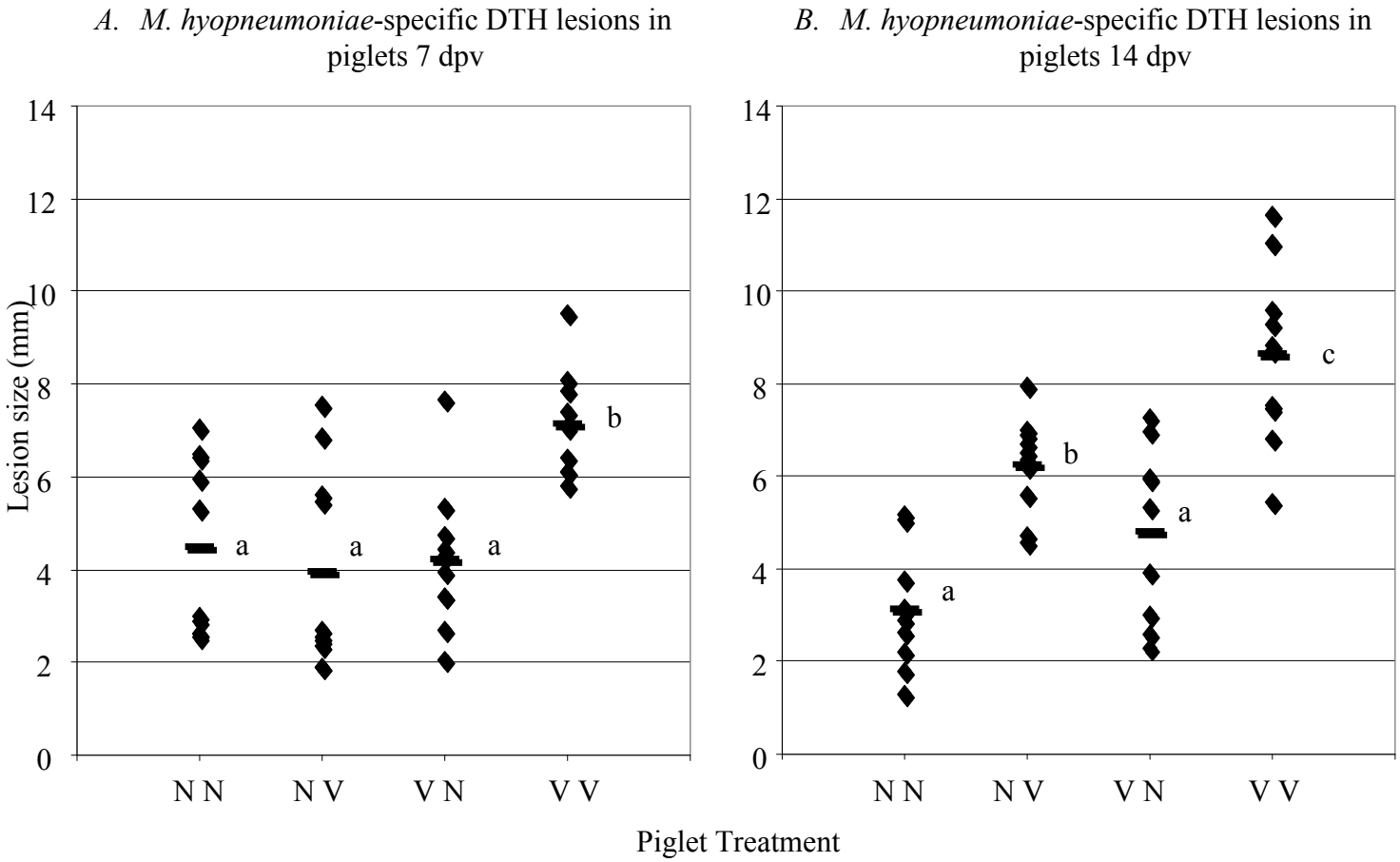


Figure 5.6. *M. hyopneumoniae*-specific DTH responses

Mean orthoganol diameter of *M. hyopneumoniae*-specific delayed type hypersensitivity (DTH) induration was measured 7 days post vaccination (dpv) (panel A) and 14 dpv (panel B) in piglets. Each diamond represents an individual animal. Horizontal bars represent mean orthoganol diameter of DTH lesion induration. Treatment groups are as follows: N N: nonvaccinated sow, nonvaccinated piglet; N V: nonvaccinated sow, vaccinated piglet; V V: vaccinated sow, vaccinated piglet; V N: vaccinated sow, nonvaccinated piglet. Different subscripts represent significance at $p<0.05$.

(p=0.01) (Fig. 5.6B). In contrast to proliferative responses, *M. hyopneumoniae*-specific DTH lesions of V V piglets were significantly larger than that of N V piglets (p=0.01) 14 dpv. The mean *M. hyopneumoniae*-specific DTH induration diameter was not different within piglet treatment groups from 7 dpv to 14 dpv, except for N V piglets. The mean orthogonal diameter of *M. hyopneumoniae*-specific DTH lesion induration from N V piglets was significantly larger at 14 dpv compared to 7 dpv (p=0.01).

5.5 Discussion

Maternally-derived lymphocytes participate in the neonatal CMI response upon antigen challenge; however, it is unclear how maternally-derived CMI affects neonatal CMI development. This study was conducted to determine whether neonatal piglets respond with AMI and/or CMI responses to *M. hyopneumoniae* when vaccinated in the face of maternally-derived AMI and CMI. To determine whether CMI and AMI responses could be induced in maternally immune piglets, piglets with and without maternal *M. hyopneumoniae*-specific immunity were vaccinated against *M. hyopneumoniae* at 7 d of age. Piglet *M. hyopneumoniae*-specific antibody, proliferation, CD25 expression, and DTH responses were measured 7 dpv and 14 dpv. Vaccination of piglets from vaccinated sows did not induce AMI responses in those piglets; there was no rise in *M. hyopneumoniae* S:P ratios following vaccination of maternally immune piglets. In contrast, evidence from both antigen-specific proliferation and DTH testing demonstrates that *M. hyopneumoniae*-specific CMI priming and anamnestic responses are

induced following vaccination of maternally immune piglets.

Maternally-derived immunity acquired across the placenta or postnatally in the form of colostrum or milk is a requirement for all mammals. While maternally-derived immunity provides an immediate source of immune protection for neonates, maternal immunity may interfere with immune priming and the generation of memory responses in the neonate. A consequence of passive interference is that neonates often fail to develop AMI responses to antigens upon re-stimulation. On the other hand, little attention has been given to whether neonatal CMI is subject to passive interference, or alternatively if neonatal CMI can be stimulated in the face of maternally-derived immunity. This is the first study to demonstrate that piglets vaccinated against *M. hyopneumoniae* develop primary and secondary CMI responses in the face of maternally-derived immunity.

In an effort to confer protection from specific pathogens during the period in which the neonatal immune system matures and maternal antibodies wane, neonatal vaccination has been practiced across a variety of antigens. There is a plethora of data demonstrating passive interference by maternal immunity with AMI stimulation in neonates following vaccination; however, evidence for activation of CMI following neonatal vaccination is limited. Not only is there a paucity of information regarding the neonatal CMI response to vaccination, the information that does exist is largely restricted to *in vitro* studies. Antigen specific BMC proliferation and cytokine production have been demonstrated for maternally immune infants vaccinated against measles virus (Gans et al, 2001; Gans et al., 1998), and neonatal mice vaccinated against measles virus (Capozzo et al., 2006; Siegrist, 1998) and lymphocytic choriomeningitis virus (Seiler et

al., 1998). Notably, humans and mice are exposed to maternally-derived immunity during gestation, and the detection of antigen specific reactivity in the neonate may be a result of antigen priming while *in utero* (Rahman et al., PlosOne). The lack of exposure to maternally-derived immunity across the placenta makes pigs, cattle, and horses excellent models to study the role of colostral immunity in the neonate, yet few reports have investigated activation of CMI following vaccination of maternally immune neonatal piglets, calves, and foals. Bouma et al., (1998) showed that 3-week-old piglets challenged with pseudorabies virus developed virus-specific proliferative responses regardless of maternal-pseudorabies status. Further, vaccinated maternally immune piglets had lower pseudorabies virus antibody titers than their non-maternally immune counterparts (Bouma et al., 1998). The current study also shows that maternally immune piglets respond to vaccination with CMI responses; however, unlike the previous studies, we show evidence of *in vivo* CMI stimulation by DTH testing.

Similar to the present study, calves vaccinated against BVDV in the face of maternally-derived immunity developed CMI responses (Endsley et al., 2004) but did not show evidence of anamnestic AMI responses upon re-exposure to the antigen (Endsley et al., 2004; Ellis et al., 1996). Interestingly, calves vaccinated against BVDV are protected from disease even in the absence of specific AMI responses (Ridpath et al. 2003). Therefore, just because neonates fail to develop detectable anamnestic AMI responses following vaccination in the face of maternally-derived immunity does not signify that there is an inhibition of the immune response. Rather, vaccination may induce CMI responses that are protective. Collectively these studies show that neonates are capable of

generating CMI responses in the face of maternal immunity and that passively transferred maternally-derived immunity may not interfere with CMI induction.

Neonatal vaccination in the face of passive immunity does not always result in CMI responses. The capacity for neonates with maternally-derived immunity to generate CMI responses following vaccination may depend on their age and immune maturation at vaccination. Further, the concentration of maternally-derived AMI within the neonate may play a role in the neonatal immune response since the level of interference with AMI stimulation is dependent on the initial level of maternally-derived AMI in the neonate (Siegrist, 2003; Dagan et al., 2000; Van Oirschot, 1987; Albrecht et al., 1977). Further, the capacity for neonates to generate specific CMI responses in the face of MDI seems to be antigen specific or may be reliant on the type or immunogenicty of the vaccine and administration method. For example, piglets vaccinated against SIV (Kitikoon et al., 2006) or CSF (Suradhat and Damrongwatanapokin, 2003) do not experience CMI stimulation when vaccinated in the face of maternally-derived immunity. Results from these studies provide evidence of selective responses imparted by the antigen. Since these two previously mentioned studies and the current study employed different vaccine preparations, modified-live (CSF) or inactivated (SIV) virus preparations, the vaccine type does not solely dictate the neonatal response to vaccination. An inactivated vaccine was used in the present study and resulted in neonatal CMI responses. DNA vaccines are an especially alluring neonatal vaccination paradigm since they replicate *in vivo* but do not pose a risk of infection. Some DNA vaccines induce CMI responses in neonates (Capozzo et al., 2006; Hassett et al., 1997; Manickan et al., 1997) while others do not

(Wang et al., 1998; Le Potier et al., 1997; Capozzo et al., 2006). Further, adjuvants that enhance CMI responses or antigen presentation (such as CpG motifs) may help the neonatal immune system overcome the opposing effects of maternally-derived AMI (Salerno-Goncalves and Sztein, 2006; Linghua et al., 2006).

Differences in neonatal responses to vaccination may also be due to the presence of functional maternal lymphoid cells at the time of vaccination. Various studies have shown that transferred maternal cells augment the neonatal response to nonspecific (Williams, 1993; Reber et al., 2005) and specific antigens (Bandrick et al., 2008; Kumar et al., 1989; Schelsinger and Covelli, 1977). In the current study, only piglets born to immune mothers exhibited *M. hyopneumoniae*-specific CMI responses 7 dpv. Therefore, CMI priming and anamnestic responses were not solely dependent upon piglet vaccination. Capozzo et al (2006) showed that lymphocytes from maternally immune mice produce IFN-γ and proliferate to measles virus after only one vaccination. These studies suggest that maternal lymphoid cells play a role in the vaccine-induced CMI response detected in neonates. Capozzo et al (2006) suggested that the CMI responses observed in mice of immune dams following one vaccination was due to neonatal T cells that were stimulated by endogenous dendritic cells, which had engulfed maternal antibodies bound to measles antigens. We suggest that the neonatal CMI responses observed are due to functional maternal lymphocytes transferred via colostrum. Maternal cells primed during sow vaccination were transferred to piglets via colostrum and were detected in piglet blood (proliferation results) and in the periphery (DTH tests). These functional cells demonstrated a booster response upon re-exposure to the *M.*

hyopneumoniae antigen. Importantly, multiple exposures of the sows to the vaccine antigen were necessary for the transfer of CMI responses from sows to piglets; only BMC of piglets born to experimentally vaccinated sows demonstrated significant *M. hyopneumoniae*-specific proliferation. Further, while piglets across all groups demonstrated antigen-specific DTH responses 7 dpv, the mean induration diameter of DTH lesions was largest in V V piglets.

Recent reports in cattle suggest that CD25 expression can be used as a reliable indicator of specific CMI responses (Endsley et al, 2002; Platt et al 2006; Quade and Roth, 1999). This is the first reported use of CD25 expression in swine that attempts to use CD25 expression as a marker of specific CMI responses. Interestingly, *M. hyopneumoniae*-specific proliferative and DTH responses were positively correlated (R^2=0.5377), but *M. hyopneumoniae*-induced CD25 expression was not correlated to *M.* hyopneumoniae-specific proliferation (R^2=0.0029) or DTH (R^2=0.02) results. Based on the conditions of this study, CD25 is not a reliable indicator of *M. hyopneumoniae*-specific CMI responses in swine.

There was no evidence that piglets developed AMI responses within 14 dpv. These results are in agreement with Hodgins et al. (2004) who showed no rise in serum *M. hyopneumoniae* antibody titer in piglets with maternally-derived immunity 9 weeks following vaccination, which occurred when piglets were 14 d-old. The lack of AMI induction in maternally immune vaccinated piglets is most likely due to passive interference. It is unclear why *M. hyopneumoniae* S:P values were greater in V N piglets compared to V V piglets at 14 dpv. This difference is likely due to interanimal variation

or due to maternally-derived antibodies binding vaccine antigen in the piglet.

In the present study, we showed that maternal AMI and CMI are transferred to and detectable in piglets. Further, passively transferred maternal cells are functional and participate in the antigen-specific immune response in piglets. We also demonstrated that vaccination was sufficient to prime a 7-day-old pig's immune system. Piglets did develop CMI responses in the face of maternal immunity. In conclusion, vaccination of neonatal pigs against *M. hyopneumoniae* in the face of maternal immunity results in CMI priming and anamnestic CMI responses following subsequent exposure to *M. hyopneumoniae* antigen.

Acknowledgements

The authors wish to thank Don Johnson for help arranging the farm Prairie Land Pork for this study. The authors also wish to thank Prairie Land Pork for help during sampling sessions. Thanks to Deepti Joshi for laboratory assistance. Thank you to Midwest Research Swine for the generous donation of supplies and Prototek for the generous donation of the *M. hyopneumoniae* antigen. MB was supported from NIH T32 DA07097.

Section C: Summary and Conclusions

Chapter 6:

Summary and conclusions

6.1 *Statement of the problem*

Neonates depend on maternally-derived immunity (MDI) acquired across the placenta or via breast milk to overcome pathogen challenge while their own immune systems mature. Maternal immune factors transferred to the neonate include antibodies, immune cells, and non-specific immune modulators, yet the cellular characteristics of colostral immunity have not been well defined. It is unclear how MDI affects piglet cell-mediated immune (CMI) responses or if piglets can respond with CMI responses in the face of MDI. While MDI provides a critical and immediate protection to environmental and vaccine antigens encountered by the mother, colostral immunity is not always beneficial for the neonate. MDI potentially interferes with endogenous immune responses in the recipient, namely antibody-mediated immune (AMI) responses. An advanced understanding of MDI will enable physicians and veterinarians to design better disease prevention and control strategies for neonates. Appreciating the impact of passively transferred immunity will enable us to elucidate downstream consequences on neonatal immune development. Toward this end, the goal of this research was to investigate the influence of MDI on neonatal immune development.

6.2 *Results Summary and limitations/pitfalls organized by chapter*

Chapter 2: Cellular contribution to maternal immunity in pigs

Immune cells, antibodies, and soluble immune factors have been found in colostrum (reviewed in Le Jan, 1996; Wagstrom et at., 2000). Further, contributions by colostrum-derived immune cells and antibodies to the immune repertoire of piglets is clear, yet the cellular contribution to colostrum was unclear and the focus of chapter 2. Chapter 2 presents a characterization of the IgG, IgA, and T lymphocyte profiles within sow blood, colostrum, and piglet blood, before and 24 h after colostrum ingestion. IgG and IgA were found in sow colostrum and blood, and in blood of piglets post-suckling only. CD4+, CD8+, and γδ T lymphocytes were also observed in sow colostrum and blood, and in blood of piglets pre and post suckling. Interestingly, the profile of immune cells found in colostrum is different than that of sow blood and of piglet blood post-suckling, suggesting that there is selectivity in transfer to these compartments. The mechanism for selectivity in cell transfer needs to be elucidated but may be related to the trafficking ability of different cell types. Conversely, the antibody profiles of sow blood, colostrum, and piglet blood post-suckling are similar and there is no indication of selectivity in transfer. The mechanisms of cell and antibody transfer into these compartments is unclear, however the mechanism of transfer was not the focus of the presented research.

Importantly, differences in placentation and normal lymphocyte pool/location of immune cells, translates into these results being not directly applicable to other species.

For example species in which immunity is transferred across the placenta will have different colostrum characteristics and colostrum will differ in its importance as a first line defense for the neonate. Swine have inverted lymph nodes and so blood sampling is a reliable source of blood-borne and lymph node-lymphocytes; this is not true in other species. Additionally, swine are known to have CD4+CD8+ double positive lymphocytes and γδ T cells that express CD4 or CD8; this may also not be true in other species. A major limitation of chapter 2 is that only single staining of lymphocytes was done, limiting the knowledge gained from that study. Future studies should use triple or more color staining in order to more fully characterize the lymphocyte contribution to sow colostrum.

Chapter 3: Passive transfer of maternal Mycoplasma hyopneumoniae-specific immunity in pigs

As shown in chapter 2, maternally-derived lymphocytes are transferred to piglets via colostrum. The goal of the experiment presented in chapter 3 was to determine if maternally-derived cells are functional in the neonate. Chapter 3 documents that suckling maternal colostrum results in the transfer of functional maternally-derived immune cells. The transfer of colostral antigen-specific CMI responses was evidenced by antigen-specific CMI reactivity in immunologically naïve piglets. Functional maternally-derived vaccine-induced *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*)-specific immune cells were detected in blood of neonatal piglets (lymphoproliferation) and in the periphery (delayed type hypersensitivity (DTH) responses) in 3-4 d old pigs whereas

these antigen-specific immune responses were not detected in piglets from *M. hyopneumoniae*-naïve sows. Therefore, not only were transferred maternally-derived cells functional, they responded in an anamnestic manner to their specific antigen when in the neonate.

Notably, functional responses by maternally-derived CMI were observed not only *in vitro*, but also *in vivo*. The functional ability of transferred colostral cells, as evidenced by *in vitro* mitogen-induced proliferation, has been observed previously (Williams, 1993; Le Jan 1994, Evans 1982). This is the first report to demonstrate *in vivo* antigen-specific activity of maternally-derived colostral immune cells in pigs. The immune responses detected in piglets were performed by transferred maternal cells since DTH are secondary immune responses. Endogenous production of antigen-specific immunity was not accounted for in this experiment; however, it is highly unlikely that healthy piglets would develop *M. hyopneumoniae* specific immune responses by three days of age, especially since there is no evidence for transplacental infection with *M. hyopneumoniae*. Further, DTH responses were due to colostral cells and not maternally-derived cells acquired prior to birth since there is no transfer of maternal immune components across the placenta in pigs.

In order to directly confirm that DTH and proliferative responses were due to transferred maternal cells, maternal colostral cells would need to be labeled prior to ingestion by the neonate. Labeled cells could then be isolated from DTH lesions or from the pool of proliferating cells. Labeling of colostral cells was not done in this experiment because of the logistics associated with withholding colostrum from piglets due to the

potential for gut closure to occur and the time involved in colostral cell isolation and labeling. Maternally-derived blood monoculear cells (BMC) were not obtained for the purpose of labeling and feeding to piglets since BMC do not migrate across the neonatal intestine (Williams, 1993; Tuboly, 1988).

The experiment presented in chapter 3 was performed on a commercial swine farm where *M. hyopneumoniae* infection was evident. Based on the data, sow *M. hyopneumoniae* priming by vaccination in addition to live bacterial exposure may be necessary for the transfer of sufficient maternally-mediated CMI response to induce DTH responses in offspring. A single positive DTH response was observed from a piglet of the NVS group. This response may be a sensitivity issue, however, no animals in the study responded to saline and all responded to PHA. Therefore the piglet with the positive DTH response was most likely the offspring of an actively infected and non-vaccinated sow. In a *M. hyopneumoniae* infected sow, the population of *M. hyopneumoniae*-specific transferred cells may be great enough to mount a specific DTH response in the newborn.

Chapter 4: The effect of cross-fostering on the transfer of maternally-derived immunity in pigs

The research presented in chapter 2 documents that maternally-derived AMI and CMI are transferred into the neonate differently based on differences in the lymphocyte profile of colostrum and piglet blood. Previous studies have suggested that unlike transfer of antibodies in pigs (Klobasa et al., 1981) transfer of colostral cells is dependent on their being maternal in origin and viable (Tuboly et al., 1988; Williams, 1993). A common

practice in commercial swine production is to cross-foster newborn piglets soon after birth. The timing of cross fostering practices may limit the number of cells transferred into the neonate and may hinder immune development of those animals. Chapter 4 demonstrates that cross-fostering affects the transfer of maternally-derived antigen-specific CMI and AMI differently. Where AMI is transferred to piglets regardless of source, CMI is transferred to the recipient only if it is from the mother. The source restriction of passively transferred CMI is evidenced by the lack of DTH response in piglets that were cross-fostered onto dams other than their biological mother within 12 h of birth.

The number of animals chosen per experiment in this thesis was most often determined based on a power analysis. However, no preliminary data were available for the cross-fostering experiments so a power analysis was not feasible. A minimum number of piglets was chosen for this experiment based on estimated standard deviations and means, yet significant differences in DTH lesion sizes and antibody S:P values were detected.

While swine are an excellent model to study transfer of MDI due to their placentation, large litter size, and precosial nature, there are also negative aspects to using swine. Swine are outbred animals; thus, much inter- and intra-animal variation is obtained and expected. Animal differences explain the variability in mean DTH lesion sizes in this experiment and those obtained in chapter 3. Further, the facts that the sows used in the cross-foster experiments were naïve to *M hyopneumoniae* other than vaccination and that piglet DTH lesions were smaller than those obtained from piglets in

chapter 3, lends credence to the idea that multiple exposures to the antigen or possibly infection are required for the transfer of maternally-derived CMI detectable by DTH. Studies controlling for the amount of sow antigen exposure or the robustness or the ensuing immune response are needed to clarify the role of antigen exposure in the transfer of maternally-derived CMI detectable by DTH.

Chapter 5: Effect of maternally-derived immunity on vaccine-induced immune responses in pigs

Maternally-derived lymphocytes participate in the neonatal CMI response upon antigen challenge as demonstrated in chapters 3 and 4; however, these studies do not clarify how maternally-derived CMI affects neonatal CMI development. The study in chapter 5 was conducted to determine the effect of vaccination in the face of maternally-derived AMI and CMI on development of neonatal vaccine-induced AMI and CMI responses. To determine whether CMI and AMI responses could be induced in maternally immune piglets, piglets with and without maternal *M. hyopneumoniae*-specific immunity were vaccinated against *M. hyopneumoniae* at 7 d of age. Piglet *M. hyopneumoniae*-specific antibody, proliferation, CD25 expression, and DTH responses were measured 7 days post vaccination (dpv) and 14 dpv.

Vaccination of piglets from vaccinated sows did not induce AMI responses in those piglets; there was no rise in *M. hyopneumoniae* S:P ratios following vaccination of piglets with MDI. While evidence of AMI responses would most likely be detectable by 14 dpv, the piglet AMI response was not assessed later than 14 dpv. The fact that AMI

responses were not measured in piglets more than 14 dpv is a limitation since the AMI response to *M. hyopneumoniae* may be delayed compared to other antigens.

Evidence from both antigen-specific proliferation and DTH testing demonstrates that *M. hyopneumoniae*-specific CMI priming and anamnestic responses are induced following vaccination of piglets in the face of MDI. There is no evidence that neonatal *M. hyopneumoniae*-specific CMI responses are interfered with by MDI. However, the conclusions drawn from the data presented in this chapter may not be extendable to other antigens. Neonates vaccinated in the face of MDI respond with specific CMI responses to bovine herpesvirus-1 (Endsley et al., 2003), pseudorabies virus (Van Rooj et al., 2006, Bouma et al., 1998, Wittman and Ohlinger 1987), bovine viral diarrheal virus (BVDV; Endsley et al., 2004) and others. Yet, piglets vaccinated against swine influenza virus (Kitikoon et al., 2006) or classical swine fever (Klinkenberg et al., 2002) in the face of MDI do not respond with antigen-specific CMI responses. The role of MDI in the piglet's response to antigen exposure is unclear but may depend on the antigen, the dose and route of exposure, the presence and type of adjuvant employed, or the piglet age at exposure. It is generally accepted that transferred MDI, specifically maternal antibodies, will interfere with endogenous AMI responses (Reviewed in Siegrist, 2003). In fact, the degree at which maternal antibodies inhibit endogenous AMI responses is directly related to the amount of antibody at the time of antigen exposure (Siegrist, 2003; Dagan et al., 2000; Van Oirschot, 1987; Albrecht et al., 1977). Thus, before the mechanisms of passive interference with CMI responses is elucidated, the conclusions from this study should not be freely applied to other pathogens.

6.3 *Contributions to the field and future investigations.*

The studies presented in this thesis contribute to the field of immunology. Chapter 2 is the first description of γδ T cells in porcine colostrum. The presence of γδ T lymphocytes in colostrum is anticipated and of interest because of their unique immunologic activities. Maternally-derived γδ T lymphocytes are beneficial to the neonate because of the γδ T lymphocytes' unique mode of antigen recognition (Tanaka et al. 1994), possession of pattern recognition receptors (Mokuno et al. 2000, Hedges 2003; Carr et al., 1994), and their abilities to present antigen (Bandes et al., 2005; Sinkora et al., 2005) and act as NK cells. The ability of maternally-derived γδ T lymphocytes to "bridge" the innate and adaptive immune responses would be an asset for the developing pig and warrants further investigation. The goal of future investigations should be to characterize the *in vivo* function of maternally-derived γδ T lymphocytes.

Maternally-derived CMI contributes to passive immunity in pigs. In fact, this thesis provides the first evidence for the transfer of functional antigen-specific CMI *in vivo* in pigs. The goal of future research should be the development of more sensitive methods of evaluating the CMI response, especially if the CMI response elicited in neonates by maternal cells is only detected when the transferred CMI is robust. Having more sensitive tests of CMI will allow for the detection of a fewer number of antigen-specific immune cells in the recipient. The fact that functional *M. hyopneumoniae*-specific maternally-derived CMI is transferred to and detected in the pig is an indication that maternally-derived CMI may protect the pig against mycoplasmal disease. Studies

evaluating the protective capacity of maternally-derived CMI in neonates are needed.

Data presented in this thesis clearly show that neonatal *M. hyopneumoniae*-specific CMI is not subject to passive interference by MDI under the conditions of the study. If MDI does not interfere with neonatal responses as is the case for AMI, neonatal vaccination to selected pathogens could be routinely practiced. Neonatal vaccination would open up many doors for pathogen control during the critical neonatal period. Whether neonatal CMI responses develop under other conditions (different antigen, different antigen exposure route or age at exposure….) in the face of MDI warrants intense investigation.

The idea that piglets are immunocompetent at 7 days of age is enticing as concerning neonatal vaccination strategies. This thesis demonstrated that piglets vaccinated at 7 days of age in the face of MDI respond with antigen-specific CMI responses. Thus, even if vaccination in the face of MDI results in a lack of detectable AMI does not indicate a lack of immune response. In this case, how the immune response to vaccination is measured (antibody-based testing is routinely practiced) need to be altered. No longer could antibody-based tests be the sole methods for determining if vaccination resulted in an immune response. CMI responses would need to be measured following vaccination since there may be no evidence of AMI responses due to passive interference. In fact, the CMI response to vaccination in the maternally immune neonate may be protective even in the absence of evidence of AMI responses; however, the protective capacity of neonatal CMI induced in the presence of MDI needs investigation.

It is clear that maternally-derived CMI and AMI are transferred into piglets

differently, yet the mechanism of cell transfer is unclear. Future studies should include elucidating the mechanism of cell transfer across the neonatal intestine. The using chamber may be a viable tool to verify the source restriction associated with cellular migration across the intestine. Future studies are also needed to evaluate whether selectivity in cell transfer remains in neonatal disease states or alternatively, in immune-altering states in the dam, e.g. maternal drug abuse.

Cross-fostering is a common farrowing house management tool, yet data presented here is the first to document the effects of cross-fostering on the transfer of MDI. Based on the results of the cross-fostering studies, maternally-derived CMI and AMI are transferred to piglets differently. Adequate time spent on the biological sow is required to ensure transfer of CMI and AMI into piglets. If cross-fostering will be practiced, it should be practiced after the piglets are at least 12 hours old.

Studies evaluating the persistence of transferred maternally-derived cells need to be performed. Regulatory T cells likely influence the acceptance and maintenance of colostral cells within the neonatal pig, but the reagents required to study regulatory T cells in swine are currently lacking. This thesis demonstrates the presence of functional maternally-derived cells in 21-d-old piglets as evidenced by *M. hyopneumoniae*-specific DTH responses in piglets otherwise naïve to *M. hyopneumoniae*. Future studies should evaluate the longevity of viable maternally-derived cells and whether the longevity of these cells is dependent on antigen stimulation.

The functional and long-term biological significance of transferred maternally-derived cells requires further study. The mechanism of interference with neonatal CMI

responses needs to be elucidated Studies evaluating the protective capacity of maternally-derived cells in the neonate need to be performed. Understanding the mechanisms of passive interference will better allow us to overcome passive interference and generate protective immune responses in the face of MDI.

Section D: References

References

1. **Adegboye, D.S.** 1978. Attempts to demonstrate cell-mediated immune responses during *Mycoplasma suipneumoniae* infection of pigs. Res in Vet Sci **25**:323-330.
2. **Adkins, B. and R. Du.** 1998. Newborn mice develop balanced Th1/Th2 primary effector responses in vivo but are biased to Th2 secondary responses. J Immunol. **160**:4217-4224.
3. **Adkins, B., C. Leclerc, and S. Marshall-Clarke**. 2004. Neonatal adaptive immunity comes of age. Nat Rev Immunol. **4:**553-564.
4. **Adkins, B., K. Chun, K. Hamilton, and M. Nassiri**. 1996. Naive murine neonatal T cells undergo apoptosis in response to primary stimulation. J Immunol. **157**:1343-1349.
5. **Ahlstedt, S., B. Carlsson, A. Hanson, and R. M. Goldblum**. 2006. Antibody production by human colostral cells I. Immunoglobulin Class, Specificity, and Quantity. Scand J Immunol. **4**:535-539.
6. **Albrecht, P., F. Ennis, E. Saltzman, and S. Krugman.** 1977. Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. J Pediatr. **91**:715–718.
7. **Baley, J. E., and B. Z. Schacter**. 1985. Mechanisms of diminished natural killer cell activity in pregnant women and neonates. J Immunol. **134**:3042-3048.
8. **Bandes, M., K. Willimann, and B. Moser.** 2005. Professional antigen-presentation function by human γδ T cells. Science. **309**:264-268.
9. **Bandrick, M., Pieters, M., Pijoan, C. and T. W. Molitor.** 2008. Passive transfer of maternal *Mycoplasma hyopneumoniae*-specific cellular immunity to piglets. Clini Vaccine Immunol. **15:**540-543.
10. **Barneston, R., S. T. C., G. Bjune, and M. E. Duncan.** 1976. Evidence for a soluble lymphocyte factor in the transplacental transmission of T-lymphocyte responses to *Mycobacterium leprae*. Nature. **260**:150-151.
11. **Barrios C., P. Brawand, M. Berney, C. Brandt, P. H. Lambert and C. A. Siegrist.** 1996. Neonatal and early life immune responses to various forms of

vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. Eur. J. Immunol. **26**:1489-1496.

12. **Bautista, A. E., M. P. Suarez and T. W. Molitor.** 1999. Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Arch Virol. **144**:117-134**.**
13. **Bertotto, A., G. Castellucci, M. Radicioni, M. Bartolucci, and R. Vaccaro.** 1996. CD40 ligand expression on the surface of colostral T cells. Arc Dis Child Fet Neonat Ed. **74**:F135-136.
14. **Binns, R. M.** 1967. Bone marrow and lymphoid cell injection of the pig foetus resulting in transplantation tolerance or immunity, and immunoglobulin production. Nature. **214**:179–80.
15. **Blanco, I., L. Galina-Pantoja, S. Oliveira, C. Pijoan, C. Sánchez, and A. Canals.** 2004. Comparison between *Haemophilus parasuis* infection in colostrums-deprived and sow-reared piglets. Vet Microbiol. **103**:21-27.
16. **Borghetti, P., E. De Angelis, R. Saleri, V. Cavalli, A. Cacchioli, A. Corradi, E. Mocchegiani, and P. Martelli.** 2006. Peripheral T lymphocyte changes in neonatal piglets: Relationship with growth hormone (GH), prolactin (PRL) and cortisol changes. Vet Immunol and Immunolpath. **110:**17-25.
17. **Bouma, A., M. D. M. De Jong, and T. G. Kimman.** 1998. The influence of maternal immunity on the development of the in vitro lymphocyte proliferation response against pseudorabies virus in pigs. Res Vet Sci. **64**:167-171.
18. **Bourges, D., F. Meurens, M. Berri, C. Chevaleyre, G. Zanello, B. Levast, M. Sandrine, G. Volker, and H. Salmon.** 2008. New insights into the dual recruitment of IgA(+) B cells in the developing mammary gland. Mol Immunol. **45:**3354–62.
19. **Bourne, F. J. 1969.** Studies on colostral and milk whey proteins in the sow. 1. The transition of mammary secretion from colostrum to milk with natural suckling. Anita. Prod. **11**:337-334.

20. **Bourne, F. J. and J. Curtis.** 1973. The transfer of immunoglobins IgG, IgA and IgM from serum to colostrum and milk in the sow. Immunology. **24**:157–162.
21. **Bourne, F.J.** 1976. Humoral immunity in the pig. Vet Rec. **25**:499–501.
22. **Bourne, F.J.** 1977. The mammary gland and neonatal immunity. Vet. Sci. Commun. **1**:141–151.
23. **Briere, F., T. Defrance, B. Vanbervliet, J. M. Bridon, I. Durand, F. Rousset, and J. Banchereau.** 1995. Transforming growth factor beta (TGF beta) directs IgA1 and IgA2 switching in human naive B cells. Adv Exp Med Biol. **371A**:21-26.
24. **Burton, K.A., and M. W. Smith.** 1977. Endocytosis and immunoglobulin transport across the small intestine of the new-born pig. J. Physiol. **270**:473–488.
25. **Butler, J. E.** 1999. Immunoglobulins and immunocytes in animal milks. In: Mucosal Immunity. New York: Academic Press, pp 1531-1554.
26. **Butler, J. E., and M. E. Kehrle.** 2004. Immunocytes and immunoglobulins in milk, in: Ogra P.L., Mestecky J., Lamm M.E., Strober W., McGhee J.R., Bienenstock J. (Eds.), Mucosal Immunology, 3rd ed., Academic Press, New York, pp.1763–1793.
27. **Butler, J. E., D. Francis, J. Freeling, P. Weber, and A. M. Krieg.** 2005. Antibody repertoire development in fetal and neonatal piglets. IX. Three PAMPs act synergistically to allow germfree piglets to respond to TI-2 and TD antigens, J. Immunol. **175**:6772–6785.
28. **Butler, J. E., F. Klobasa, and E. Werhahn.** 1981. The differential localization of IgA. IgM and IgG in the gut of suckled neonatal piglets. Vet Immunol Immunopathol. **2**:53–65.
29. **Butler, J. E., P. Weber, M. Sinkora, D. Baker, A. Schoenherr, B. Mayer, and D. Francis.** 2002. Antibody repertoire development in fetal and neonatal piglets. VIII. Colonization is required for newborn piglets to make serum antibodies to T-dependent and type 2 T-independent antigens, J. Immunol. **169**:6822–6830.
30. **Butler, J. E., P. Weber, M. Sinkora, J. Sun, S. J. Ford, and R. Christenson.**

2000. Antibody repertoire development in fetal and neonatal piglets. II. Characterization of heavy chain CDR3 diversity in the developing fetus. J. Immunol. **165**:6999–7011.

31. **Butler, J.E. 1998.** Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals. Rev Sci Tech. **17**:43–70.
32. **Calsamiglia, M. and C. Pijoan.** 2000. Colonization state and colostral immunity to *Mycoplasma hyopneumoniae* of different parity sows. Vet Rec. **146**:530-532.
33. **Capozzo, A.V., K. Ramírez, J. M. Polo, J. Ulmer, E. M. Barry, M. M. Levine, and M. F. Pasetti.** 2006. Neonatal immunization with a Sindbis virus-DNA measles vaccine induces adult-like neutralizing antibodies and cell-mediated immunity in the presence of maternal antibodies. J Immunol. **176**:5671-5681.
34. **Carr, M. M., C. J. Howard, P. Sopp, J. M. Manser, and K. R. Parsons.** 1994. Expression on porcine gamma delta lymphocytes of a phylogenetically conserved surface antigen previously restricted in expression to ruminant gamma delta T lymphocytes. Immunol. **81**:36–40.
35. **Chabaudie, N., C. Le Jan, M. Olivier, and H. Salmon.** 1993. Lymphocyte subsets in the mammary gland of sows. Res. Vet. Sci. **55**:351–355.
36. **Charerntantanakul, W., and J. A. Roth.** 2007. Biology of porcine T lymphocytes. An Hlth Res Rev. **7**:81–96.
37. **Chase, C., D. Hurley, and A. Reber. 2008. Neonatal immune development in the calf and its impact on vaccine response. Vet Clin N Am: Food An Prac 24**:87–104.
38. **Chen, P.P., M.-F Liu, S. Sinha, and D. A. Carson.** 1988. A 16/6 idiotype positive anti-DNA antibody is encoded by a conserved VH gene with no somatic mutation. Arthr Rheum. **31**:1429–1431.
39. **Cherry, J. D., R. D. Feigin, P. G. Shackelford, D. R. Hinthorn, and R. R. Schmidt.** 1973. A clinical and serological study of 103 children with measles vaccine failure. J Pediatr. **82**:802-808.
40. **Claas F. H., Y. Gijebls, J. van-der-Velden-de-Munck, and J. J. van-Rood.**

1988. Induction of B cell unresponsiveness to noninherited maternal HLA antigens during fetal life. Science. **241**:1815-1817.

41. **Clarke, R. M., and R. N. Hardy.** 1971. Histological changes in the small intestine of the young pig and their relationship to macromolecular uptake. J. Anat. **108**:63–77.
42. **Crisman, M. V., and W. K. Scarratt.** 2008. Immunodeficiency disorders in horses. Vet Clin N Am Eq Pract. **24**:299-310.
43. **Curtis, J. and F. J. Bourne.** 1971. Immunoglobulin quantitation in sow serum, colostrum and milk and the serum of young pigs. Biochim Biophys Acta. **236**:319-332.
44. **Cutler, R. S., V. A. Fahi, E. M. Spicer, and G. M. Cronin.** 1999. Preweaning mortality. IN: Diseases of swine. 8th edn. Eds. Straw, B. E., S. D. D'Allaire, W. L. Mengeling and D. J. Taylor. Ames, IA, Iowa State University Press. pp. 985–1002.
45. **Dagan, R., J. Amir, A. Mijalovsky, I. Kalmanovitch, A. Bar-Yochai, S. Thoelen, A. Safary, and S. Ashkenazi S.** 2000. Immunization against hepatitis A in the first year of life: priming despite the presence of maternal antibody. Pediatr Infect Dis J. **19**:1045–1055.
46. **Danielsen, M., T. Thymann, B. B. Jensen, O. N Jensen, P. T. Sangild, and E. Bendixen.** 2006. Proteome profiles of mucosal immunoglobulin uptake in inflamed porcine gut. Proteomics. **6**:6588–6596.
47. **Deen, M. G. H., and G. Bilkei.** 2004. Cross fostering of low-birthweight piglets. Livestock prod sci. **90**:279-284.
48. **Dillender, M.J., and J. K. Lunney.** 1993. Characteristics of T lymphocyte cell lines established from NIH minipigs challenge inoculated with Trichinella spiralis. Vet Immunol Immunopathol. **35**:301–319.
49. **Donovan, D. C., A. J. Reber, J. D. Gabbard, M. Aceves-Avila, K. L. Galland, K. A. Holbert, L. O. Ely, and D. J. Hurley.** 2007. Effect of maternal cells transferred with colostrum on cellular responses to pathogen antigens in neonatal

calves. Am J Vet Res. **68**:778-782.

50. **Elahi, S., R. M. Buchanan, L. A. Babiuk, and V. Gerdts.** 2006. Maternal immunity provides protection against pertussis in newborn piglets. Infect Immun. **74**:2619-2627.
51. **Ellis, J. A., L. E. Hassard, V. S. Cortese, and P. S. Morley.** 1996. Effects of perinatal vaccination on humoral and cellular immune response in cows and young calves. J Am Vet Med Assoc. **208**:393–400.
52. **Ellis, J., S. Gow, K. West, C. Waldner, C. Rhodes, G. Mutwiri, and H. Rosenberg. 2007**. Response of calves to challenge exposure with virulent bovine respiratory syncytial virus following intranasal administration of vaccines formulated for parenteral administration. J Am Vet Med Assoc. **230**:233–243.
53. **Endsley, J. J., J. A. Roth, J. Ridpath, and J, Neill.** 2004. Maternal antibody blocks humoral but not T cell responses to BVDV. Biologicals. **31**:123–125.
54. **Endsley, J. J., J. F. Ridpath, J. D. Neill, M. R. Sandbulte, and J. A. Roth.** 2004. Induction of T lymphocytes specific for bovine viral diarrhea virus in calves with maternal antibody. Viral Immunol. **17**:13-23.
55. **English, P. R. and W. J Smith.** 1975. Some causes of death in neonatal pigs. Vet Ann. **15**:95-104.
56. **English, P. R. and V. Wilkinson.** 1982. Management of the sow and litter in late pregnancy and lactation in relation to piglet survival and growth. IN Control of pig reproduction. Eds. D. J. A. Cole and G. R. Foxcroft. London. Butterworths. pp, 479-506.
57. **English, P.R. 1984.** Fostering of piglets: Principles and practices. Publication no. 114. Scottish Agricultural college, Edinburgh.
58. **Erlandson, K. R., R. B. Evans, B. J. Thacker, M. W. Wegner, and E. L. Thacker.** 2005. Evaluation of three serum antibody enzyme-linked immunosorbent assays for *Mycoplasma hyopneumoniae*. J Swine Health and Production. **13**:198-203.
59. **Evans, P.A., T.J. Newby, C.R. Stokes, and F.J. Bourne.** 1982. A Study of cells

in the mammary sectetions of sows. Vet. Immunol. Immunopathol. **3**:515-527.

60. **Fagoaga, O. R., S. L. and Nehlsen-Cannarella.** 2000. Maturation of lymphocyte immunophenotypes and momory T helper cell differentiation during development in mice. J Dev Immunol. **8:**47-60.
61. **Fano, E., C. Pijoan, and S. Dee.** 2005. Mycoplasma hyopneumoniae prevalence at weaning as a predictor of the group's subsequent Mycoplasma status. Allen D. Leman Swine Conference. **32**:109-113.
62. **Filipp, D., K. Alizadeh-Khiavi, C. Richardson, A. Palma, N. Paredes, O. Takeuch. S. Akira, and M. Julius**. 2001. Soluble CD14 enriched in colostrum and milk induces B cell growth and differentiation. Proc Nat Acad Sci. **98**:603-608.
63. **Fong, S., S. Jones, M. E. Renz, H. H. Chiu, A. M. Ryan, L. G. Presta, and D. Jackson.** 1997. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Its binding motif for alpha 4 beta 7 and role in experimental colitis. Immunol. Res. **16**:299-311.
64. **Foss, D. L., and M. P. Murtaugh.** 1999. Mucosal immunogenicity and adjuvanticity of cholera toxin in swine. Vaccine. **17**:788–801.
65. **Gans, H.A., Y. Maldonado, L. L. Yasukawa, J. Beeler, S. Audet, M. M. Rinki, R. DeHovitz, and A. M. Arvin. 1999**. IL-12, IFN-γ, and T cell proliferation to measles in immunized infants. J Immunol. **162**:5569-5575.
66. **Gaskins, H. R.** 1998. Immunological development and mucosal defence in the pig intestine. IN Progress in Pig Science (Eds J. Wiseman, M. A. Varley & J. P. Chadwick), pp. 81–102. Nottingham, UK: Nottingham University Press.
67. **Gennaro, R., C. Schneider, G. De Nicola, F. Cian, and D. Romeo.** 1978, Biochemical properties of bovine granulocytes. Proc Zoo Exp. Biol. Med. **157**:342-347.
68. **Gross Beilage, E., and A. Schreiber.** 2005. Vaccination of sows against *Mycoplasma hyopneumoniae* with Hyoresp. Dtsch Tierarztl Wochenschr. **112**:256-261.

69. **Haesebrouck, F., F. Pasmans, K. Chiers, D. Maes, R. Ducatelle, and A. Decostere A.** 2004. Efficacy of vaccines against bacterial diseases in swine: what can we expect? Vet Microbiol. **100**:255-268.
70. **Hammer, D. K., and H. Mossmann.** 1978. The importance of membrane receptors in the transfer of immunoglobulins from plasma to the colostrum. Ann. Recherches Vet. **9:**229–234.
71. **Hammerberg, C., G. G. Schurig, and D. L. Ochs.** 1989. Immunodeficiency in young pigs. Am J Vet Res. **50**:868–874.
72. **Harp, J. A., and H. W. Moon.** 1988. Lymphocyte localization in lymph nodes of swine: changes induced by lactation. Vet Immunol Immunopathol. **18**:219–227.
73. **Hassett, D. E., J. Zhang, J. L. Witton.** 1997. Immune responses following neonatal DNA vaccination are long-lived, abundant, and qualitatively similar to those induced by convention immunization. J Virol **74**:2620-2627.
74. **Hassett, D., J. Zhang, and L. Whitton** 1997. Neonatal DNA immunization with a plasmid encoding an internal viral protein is effective in the presence of maternal antibodies and protect against subsequent viral challenge. J Virol. **71**:7881-7888.
75. **Head, J. R., A. E.Beer, and R. E. Billingham.** 1977. Significance of the cellular component of the maternal immunologic endowment in milk. Transplant. Proc. **9**:1465–1471.
76. **Hedges, J.F., J. C. Graff, and M. A., Jutila.** 2003. Transcriptional profiling of γδ T cells, J. Immunol. **171**:4959–4964.
77. **Herich, R., M. Levkut, A. Bomba, S. Gancarcikova, and R. Nemcova**. 2004. Differences in the development of the small intestine between gnotobiotic and conventionally bred piglets. BerlinMunch Tierarztl Wochenschr. **117**:46-51.
78. **Hodgins, D.C., P. E. Shewen, and C. E. Dewey.** 2004. Influence of age and maternal antibodies on antibody responses of neonatal piglets vaccinated against *Mycoplasma hyopneumoniae*. J Swine Health Prod. **2**:10-16.
79. **Horton, J. E., and J. J. Oppenheim.** 1976. Relationship of transformation of

newborn human lymphocytes by dental plaque antigen to the degree of maternal periodontal disease. Cell Immunol. **21**:153-160.

80. **Hughes, A., J. H. Brock, D. M. Parrott, and F. Cockburn.** 1988. The interaction of infant formula with macrophages: effect on phagocytic activity, relationship to expression of class II MHC antigen and survival of orally administered macrophages in the neonatal gut. Immunology. **64**:213-218.
81. **Ichikawa, M., M. Sugita, M. Takahashi, M. Satomi, T. Takeshita, T. Araki T, and H. Takahashi.** 2003. Breast milk macrophages spontaneously produce granulocyte-macrophage colony-stimulating factor and differentiate into dendritic cells in the presence of exogenous interleukin-4 alone. Immunology. **108**:189-195.
82. **Jaeger, L. A., C. H. Lamar, G. D. Bottoms, and T. R. Cline.** 1987. Growth-stimulating substances in porcine milk. Am J Vet Res. **48**:1531–1533.
83. **Jain, L., D. Vidyasagar, M. Xanthou, V. Ghai, S. Shimada, and M. Blend.** 1989. In vivo distribution of human milk leucocytes after ingestion by newborn baboons. Arch. Dis. Child. **64**:930-933.
84. **Jain, N., N. B. Mathur, V. K. Sharma, and A. M Dwarkadas.** 1991. Cellular composition including lymphocyte subsets in preterm and full term human colostrum and milk. Acta Paediatr Scand. **80**:395-399.
85. **Jensen, A.R., J. Elnif, D. G. Burrin, and P. T. Sangild.** 2001. Development of intestinal immunoglobulin absorption and enzyme activities in neonatal pigs is diet dependent. J Nutrition. **131**:3259-3265.
86. **Jeppesen, D. L., H. Hasselbalch, I. M. Lisse, A. K. Ersbøll, and M. D. Engelmann.** 2004. T-lymphocyte subsets, thymic size and breastfeeding in infancy. Pediatr Allergy Immunol. **15**:127-132.
87. **Johnson, H. M., J. K. Russell, and C. H. Pontzer.** 1991. Staphylococcal enterotoxin superantigens. Proc Soc Exp Biol Med. **198**:765-771.
88. **Karllsson, B. W.** 1966a. Immunoelectrophoretic studies on relationships between proteins of porcine colostrum, milk and blood serum. Acta Pathol Microbiol

Scand. **67**:83-101.

89. **Karllsson, B. W.** 1966b. Immunochemical studies on changes in blood serum proteins in piglets after colostrum ingestion during neonatal and juvenile development. Acta Pathol. Microbiol. Scand. **67**:237-256.
90. **Käser, T., W. Gerner, S. E. Hammer, M. Patzl, and A. Saalmüller.** 2008. Phenotypic and functional characterisation of porcine CD4+CD25high regulatory T cells. Vet Immunol Immunopathol. **122**:153-158.
91. **Kimman. T. G., F. Westenbrink, and P. J. Straver.** 1989. Priming for local and systematic antibody memory responses to bovine respiratory syncytial virus: effect of amount of virus, viral replication, route of administration and maternal antibodies. Vet Immunol Immunopathol. **22**:145–160.
92. **King, C. L., I. Malhotra, P. Mungai, A. Wamachi, J. Kioko, J. H. Ouma, and J. W. Kazura.** 1998. B Cell sensitization to helminthic infection develops *in utero* in humans. J Immunol. **160**:3578-3584.
93. **Kitikoon, P., D. Nilubol, B. J. Erickson, B. H. Janke, T. C. Hoover, S. A. Sornsen, and E. L. Thacker.** 2006. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. Vet Immunol Immunopathol. **112**:117–128.
94. **Klagsbrun, M.** 1978. Human milk stimulates DNA synthesis and cellular proliferation in cultured fibroblasts. Proc. Natl. Acad. Sci. **75**:5057–5061.
95. **Kleinman, R. E., and W. A. Walker.** 1979. The enteromammary immune system: an important new concept in breast milk host defense. Dig Dis Sci. **24**:876-882.
96. **Klinkenberg, D., R. J. M. Moormann, A. J. De Smit, A. Bouma, and M. C. M De Jong.** 2002. Influence of maternal antibodies on efficacy of a subunit vaccine: transmission of classical swine fever virus between pigs vaccinated at 2 weeks of age. Vaccine. **20**:3005–3013.
97. **Klobasa, F., E. Werhahn, and J. E. Butler.** 1981. Regulation of humoral immunity in the piglet by immunoglobulins of maternal origin. Res Vet Sci.

31:195–206.

98. **Klobasa, F., J. E. Butler, E. Werhahn, and F. Habe.** 1986. Maternal-neonatal immunoregulation in swine. II. Influence of multiparity on de novo immunoglobulin synthesis by piglets. Vet. Immunol. Immunopathol. **11**:149–159.
99. **Kohl, S., L. K. Pickering, and L. S. Loo.** 1982. Virus-induced colostral cell cytokine stimulation of human leukocyte natural killer cytotoxicity. Infect Immun. 36:691-695.
100. Kohl, S., L. K. Pickering, L.S. Loo. 1982. Virus-induced colostral cell cytokine stimulation of human leukocyte natural killer cytotoxicity. Infect Immun. **36**;691-695.
101. **Kono, Y., S. Suzuki, T. Muika, K. Okazaki, E. Honda, and T. Yamashiro.** 1993. Detection of specific systemic and local IgG and IgA antibodies of pigs after infection with Bordetella bronchiseptica by ELISA. J Vet Med Sci. **56**:249-253.
102. **Korotkov, M., E. Telemo, L. A. Hanson, and B. Strandvik.** 2004. Modulation of neonatal immunological tolerance to ovalbumin by maternal essential fatty acid intake. Pediatr. Allerg Immunol. **15**:112-122.
103. **Kritas, S. K., H. J. Nauwynck, M. B. Pensaert, and S. C. Kyriakis.** 1997. Effect of the concentration of maternal antibodies on the neural invasion of Aujeszky's disease virus in neonatal pigs. Vet Microbiol. **55**:29-36.
104. **Kumar, S. N., G. L. Stewart, W. M. Steven, and L. L. Seelig**. 1989. Maternal to neonatal transmission of T-cell mediated immunity to Trichinella spiralis during lactation. Immunol. **68**:87-92.
105. **Kumura, B. H., T. Sone, K. Shimazaki, and E. Kobayashi**. 2000. Sequence analysis of porcine polymeric immunoglobulin receptor from mammary epithelial cells present in colostrum. J Dairy Res. **67**:631–636.
106. **Labeta, M. O., K. Vidal, L. E. Nores, M. Arias, N. Vita, B. P. Morgan, J. C. Guillemot, D. Loyaux, P. Ferrara, D. Schmid, M. Affolter, L. K. Borysiewicz, A. Donnet-Hughes, and E. J. Schiffrin.** 2000. Innate recognition

of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. J Exp Med. **191**:1807–1812.

107. **Le Jan, C.** 1994. A study by flow cytometry of lymphocytes in sow colostrum. Res Vet Sci. **57**:300-304.

108. **Le Jan, C.** 1996. Cellular components of mammary secret ions and neonatal immunity: a review. Vet. Res. **27**:403–417.

109. **Le Jan, C., and C. Chevaleyre.** 1996. Reduced expression of SLA class 1 antigens by intestinal epithelium of newborn piglets. Vet Immunol Immunopathol. **50**:167-172.

110. **Le Potier, M. F., M. Monteil, C. Houdayer, and M. Eliot**.1997. Study of the delivery of the gD gene of pesudorabies virus to one-day-old piglets by adenovirus or plasmid DNA as ways to by-pass the inhibition of immune response by colostral antibodies. Vet. Microbiol. **55**:75–80.

111. **LeBouder, E., J. E. Rey-Nores, N. K. Rushmere, M. Grigorov, S. D. Lawn, and M. Affolter**. 2003. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. J Immunol. **171**:6680–6689.

112. **Lee, W. J., J. L. Farmer, M. Hilty, and Y. B. Kim.** 1998. The protective effects of lactoferring feeding against endotoxin lethal shock in germfree piglets. Infect Immun. **66**;1421-1426.

113. **Leece, J. G.** 1973. Effect of dietary regimen on cessation of uptake of macromolecules by piglet intestinal epithelium (closure) and transport to the blood. J Nutr. **103**:751-756.

114. **Lemke, C. D., J. S. Haynes, R. Spaete, D. Adolphson, A. Vorwald, K. Lager, and J. E. Butler.** 2004. Lymphoid hyperplasia resulting in immune dysregulation is caused by PRRSV infection in pigs, J. Immunol. **172**:1916–1925.

115. **Lin, S. J., P. J. Cheng, Y. J. Huang, and M. L. Ming-Ling Kuo**. 2004. Evaluation of cytotoxic function and apoptosis in interleukin (IL)-12/IL-15-treated umbilical cord or adult peripheral blood natural killer cells by a

propidium-iodide based flow cytometry. Ped Allergy & Immunol. **15**;79-85.

116. **Linghua, Z., G. Yong, T. Xingshan, and Z. Fengzhen.** 2006. Co-administration of porcine-specific CpG oligodeoxynucleotide enhances the immune responses to pseudorabies attenuated virus vaccine in newborn piglets in vivo. Dev Comp Immunol. **30**:589–96.

117. **Loeffen, W. L., P. P. Heinen, A. T. Bianchi, W. A Hunneman, and J. H. Verheijden.** 2003.Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infectionwith an influenza H1N1 virus. Vet Immunol Immunopathol. **92**:23–35.

118. **MacPherson, A. J., D. Gatto, E. Sainbury, G. R. Harriman, H. Hengartner, and R. M. Zinkernagel.** 2000. A primitive T cell independent mechanism of mucosal IgA responses to commensal bacteria. Science **288**:2222–2226.

119. **Magnussen, U.** 1999. Longitudinal study of lymphocyte subsets and major histocompatibility complex class II expressing cells in mammary glands of sows. Am. J. Vet. Res. **60**:546–548.

120. **Manickan, E., Z. Yu, and B. T. Rouse**. 1997. DNA immunization of neonates induces immunity despite the presence of maternal antibody. J Clin Invest. **100**:2371-2375.

121. **Marcatti Neto, A.** 1986 Effect of cross-fostering on piglet preweaning performance. Arquivos Brasileiros de Medicina Veterinaria e Zootecnia **38**;413-417.

122. **Marchalonis, J. J., S. F. Schuter, R. M. Bernstein, and V. S. Hohman.** 1998. Antibodies of sharks: revolution and evolution. Immunol Rev. **166**:103–122.

123. **Mawhinney, I. C., and M. R. Burrows.** 2005. Protection against bovine respiratory syncytial virus challenge following a single dose of vaccine in young calves with maternal antibody. Vet Rec. **156**:139–143.

124. **McDermott, M. R., and J. Bienenstock.** 1979. Evidence for a common

mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory, and genital tissues. J. Immunol. **122**:1892–1989.

125. **Menge, C. H., B. Neufeld, W. Hirt, R. Bauerfeind, G. Baljer, and L. H. Wieler.** 1998. Phenotypical characterization of peripheral blood leucocytes in the newborn calf. Z Veterinarmed [B] **46**:559–565.

126. **Messier, S., R. Ross and P. Paul.** 1990. Humoral and cellular immune responses of pigs inoculated with *Mycoplasma hyopneumoniae*. Am J Vet Res. **15:**52-58.

127. **Milligan, B. N., D. Fraser, and D. L. Kramer**. 2001. The effect of littermate weight on survival, weight gain, and suckling behaviour of low-birth-weight piglets in cross fostered litters. Journal of Swine Health and Production. **9**:161–166.

128. **Mokuno, Y., T. Matsuguchi, M. Takano, H. Nishimura, J. Wasizu, T. Ogawa, O. Takeuchi, S. Akira, Y. Nimura, and Y. Yoshikai.** 2000. Expression of toll-like receptor 2 on γδ T cells bearing invariant Vγ6/Vδ1 induced by Escherichia coli infection in mice, J. Immunol. **165**:931–940.

129. **Mold, J. E., J. Michaëlsson, T. D. Burt, M. O. Muench, K. P. Beckerman, M. P. Busch, Tzong-Hae Lee, D. F. Nixon, and J. M. McCunel.** 2008. Maternal Alloantigens Promote the Development of Tolerogenic Fetal Regulatory T Cells in Utero. Science. **322**:1562-1565.

130. **Moon, H. W.** 1972. Vacuolated vilous epithelium of the small intestine of young pigs. Vet. Pathol. **9**:3–21.

131. **Moughan, P. J., M. J. Birtles, P. D. Cranwell, W. C. Smith, and M. Pedraza.** 1992. The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. World Rev Nutr Diet. **67**:40–113.

132. **Neal, S. M. and K. M. Irvin.** 1991. The effects of crossfostering pigs on survival and growth. J An Sci. **69**;41-46.

133. **Newby, T. J., C. R. Stokes, and F. J. Bourne**. 1982. Immunological activities of milk. Vet. Immunol. Immunopathol. **3**:67–94.

134. **Nguyen, T. V., L. Yuan, M. S. Azevedo, K. I. Jeong, A. M. Gonzalez, C. Iosef, K. Lovgren-Bengtsson, B. Morein, P. Lewis, and L. J. Saif.** 2006a. Low titer maternal antibodies can both enhance and suppress B cell responses to a combined live attenuated human rotavirus and VLP-ISCOM vaccine. Vaccine. **24**:2302–2316.

135. **Nguyen, T. V., L. Yuan, M. S. Azevedo, K. I. Jeong, A. M. Gonzalez, C. Iosef, K. Lovgren-Bengtsson, B. Morein, P. Lewis, and L. J. Saif.** 2006b. High titers of circulating maternal antibodies suppress effector and memory B-cell responses induced by an attenuated rotavirus priming and rotavirus-like particle-immunostimulating complex boosting vaccine regimen. Clin Vaccine Immunol. **13**:475–85.

136. **Nguyen, T. V., L. Yuan, S. P. Azevedo, K. I. Jeong, A. M. Gonzalez, and L. J. Saif.** 2007. Transfer of maternal cytokines to suckling piglets: in vivo and in vitro models with implications for immunomodulation of neonatal immunity. Vet Immunol Immunopathol. **117**:236–248.

137. **Ogra, S. S., and P. L. Ogra.** 1978. Immunologic aspects of human colostrum and milk. II. Characteristics of lymphocyte reactivity and distribution of E-rosette forming cells at different times after the onset of lactation. J Pediatr. **92**:550-555.

138. **Okada, M., T. Asai, M. Ono, T. Sakano, and S. Sato.** 2000. Cytological and immunological changes in bronchoalveolar lavage fluid and histological observation of lung lesions in pigs immunized with Mycoplasma hyopneumoniae inactivated vaccine prepared from broth culture supernate. Vaccine. **18**;2825-2831.

139. **Okaragoz, F., H. B. Rudloff, S. Rajaraman, A. A. Mushtaha, F. C. Schmalsteig, and A. S. Goldman**. 1988. The mobility of human milk macrophage in collagen gels. Pedtr Res. **23**:449-452.

140. **Olin, M.R., K. Hwa Choi, J. Lee, and T. W. Molitor.** 2005a. Gamma delta T-lymphocyte cytotoxic activity against Mycobacterium bovis analyzed by

flow cytometry. J Immunol Methods. **297**;1-11.

141. **Olin, M.R., L. Batista, Z. Xiao, S. A. Dee, M. P. Murtaugh, C. C. Pijoan, and T. W. Molitor**. 2005b. Gamma delta lymphocyte response to porcine reproductive and respiratory syndrome virus. Viral Immunol. **18**:490-949.

142. **Olin, M.R., K. Choi, J. Lee. and T. W. Molitor**. 2007. Morphine modulates gamma delta lymphocytes cytolytic activity following BCG vaccination. Brain Behav Immun. **21**:195-201.

143. **Opriessnig, T., A. R. Patterson, J. Elsener. X. J. Meng, and P. G. Halbur.** 2008. Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental infection with PCV2. Clin Vaccine Immunol. **15**:397–401.

144. **Osterlundh, I., H. Holst, and U. Magnusson**. 1998. Hormonal and Immunologic changes in blood and mammary secretions in the sow at parturition. Theriogenology. **50**:465-477.

145. **Owen, R. L., and M. H. Heyworth**. 1985. Lymphocyte migration from Peyer's patches by diapedesis through M cells into the intestinal lumen. Adv Exp Med Biol. **186**:647-654.

146. **Palmer, D. J. and M. Makrides**. 2006. Diet of lactating women and allergic reations in their infants. Curr Opin Nutr Metab Car. **9**:284-288.

147. **Palzer, A., C. Straus, M. Ritzmann, and K. Heinritzi.** 2006. Effect of sow and piglet vaccination against *Mycoplasma hyopneumoniae* on serology and lung quality. Proceedings of the Allen D. Leman Swine Conference, Saint Paul, USA, Volume 33. p.16.

148. **Papadogiannakis, N.** 1997. Traffic of leukocytes through the maternofetal placental interface and its possible consequences. Cur Top Microbiol Immunol. **222**:141-157.

149. **Parchariyanon, S., U. Tantaswasdi, W. Pinyochon and P. Methiyapun.** 1994. Immunity against swine fever vaccine. II. Immunity against swine fever vaccine in piglets and protection level of maternal immunity in piglets

before vaccination. J Thai Vet Med Assoc. **45**:37–45.

150. **Park, Y. H., L. K. Fox, M. J. Hamilton, and W. C. Daws**. 1992. Bovine mononuclear leukocyte subpopulations in peripheral blood and mammary gland secretions during lactation. J Dairy Sci. **75**:998-1006.

151. **Parmely, M. J., A. E. Beer, and R. E. Billingham**. 1976. In vitro studies on the T-lymphocyte population of human milk. J Exp Med. **144**:358-370.

152. **Payne, L. C. and C. L. Marsh**. 1962. Gamma globulin absorption in the baby pip: The nonselective absorption of heterologous globulins and factors influencing absorption time. Journal of Nutrition **76**;151-158.

153. **Pertmer, T. M., A. E. Oran, J. M. Moser, C. A. Madorin, and H. L. Robinson.** 2000. DNA vaccines for influenza virus: differential effects of maternal antibody on immune responses to hemagglutinin and nucleoprotein. J Virol. **74**:7787-7793.

154. **Piffer, I.A. and R. F. Ross**. 1984. Effect of age on susceptibility of pigs to Mycoplasma hyopneumoniae pneumonia. Am J Vet Res. **45**:1122-1126.

155. **Piotrowski, P., and B. A. Croy**. 1996. Maternal cells are widely distributed in the murine fetus in utero. Biol Reprod. **54**:1103-1110.

156. **Pollack, M. S., D. Kirkpatrick, N. Kapoor, B. Dupont, and R. J. O'Rilley**. 1982. Identification of HLA typing of interauterine-derived maternal T cells in four patients with severe combined immunodeficiency. N Engl J Med. **307**:662-666.

157. **Pomorska-Mól, M., and I. Markowska-Daniel**. 2010. Interferon-gamma secretion and proliferative responses of peripheral blood mononuclear cells after vaccination of pigs against Aujeszky's disease in the presence of maternal immunity. FEMS Immunol Med Microbiol.**58**:405-11.

158. **Porter, P.** 1969. Transfer of immunoglobulins IgG, IgA and IgM to lacteal secretions in the parturient sow and their absorption by the neonatal piglet. Biochim. Biophys. Acta. **181**:381–392.

159. **Porter, P.** 1988. Immune system in: Leman AD, Straw B, Glock RD, et

al., eds. Diseases of swine (6th Edition). Iowa State University Press, Ames, pp 44-57.

160. **Putnova, L., A. Knoll, V. Dvorak, J. and Dvorak.** 2003. A novel porcine microsatellite panel for the identification of individuals and parentage control in the Czech Republic. Czech J. Anim. Sci. **8**:307-314.

161. **Radu, D. L., S. Antohi, A. Bot, A. Miller, A. Mirarchi, and C. Bona.** 2001. Effect of maternal antibodies on influenza virus-specific immune response elicited by inactivated virus and naked DNA. Scand J Immunol. **53**:475:482.

162. **Rahman, M. J., I. R. Degano, M. Singh, and C. Fernandez**. 2010. Influence of maternal gestational treatment with mycobacterial antigens on postnatal immunity in an experimental murine model. PLoS ONE. **5**:3.

163. **Rautiainen, E., and P. Wallgren.** 2001. Aspects of the transmission of protection against Mycoplasma hyopneumoniae from sow to offspring. J Vet Med **48**:55-65.

164. **Reber, A. J., A. R Hippen, and D. J. Hurley.** 2005. Effects of the ingestion of whole colostrum or cell-free colostrum on the capacity of leukocytes in newborn calves to stimulate or respond in oneway mixed leukocyte cultures. Am. J. Vet. Res. **66**:1854–1860.

165. **Reber, A. J., A. Lockwood, A. R. Hippen, and D. J. Hurley.** 2006. Colostrum induced phenotypic and trafficking changes in maternal mononuclear cells in a peripheral blood leukocyte model for study of leukocyte transfer to the neonatal calf. Vet Immunol Immunopath. **109**:139-150.

166. **Reber, A. J., D. C. Donovan, J. Gabbard, K. Galland, M. Aceves-Avila, K.A. Holbert, L. Marshall and D. J. Hurley**. 2008. Transfer of maternal colostral leukocytes promotes development of the neonatal immune system: I. Effects on monocyte lineage cells. Vet Immunol Immunopathol. **123**:186-196.

167. **Redman, D. R., E. H. Bohl, and R. F. Cross**. 1987. Intrafetal inoculation of swine with transmissible gastroenteritis virus. Am J Vet Res. **39**:907–911.

168. **Richie, E. R., K. D. Steinmetz, M. L. Meistrich, I. Ramirez, and J. K.**

Hilliard. 1980. T lymphocytes in colostrum and peripheral blood differ in their capacity to form thermostable rosettes. J Immunol. **125**:2344–2346.

169. **Ridpath, J. E., J. D. Neill, J. Endsley, and J. A. Roth**. 2003. Effect of passive immunity on the development of a protective immune response against bovine viral diarrhea virus in calves. Am J Vet Res. **64**:65–69.

170. **Riedel-Caspari, G., and F. W. Schmidt**, 1991. The influence of colostral leukocytes on the immune system of the neonatal calf. III Effect on phagocytosis. Dtsch Tierarztl Wschr. **98**:330-334.

171. **Rifkind, D., J. A. Frey, E.A Petersen, and M. Dinowitz**.1976. Delayed hypersensitivity to fungal antigens in mice. II. Molecular classes in immunogenic RNA extracts that transfer delayed hypersensitivity. J Infect Dis. **133**:523-532.

172. **Rivas, R. A., A. A. el Mohaandes, and I. M. Katona**. 1994. Mononuclear phagocytitc cells in human milk: HLA-DR and Fc gamma R ligand expression. Biol Neonate. **66**:195-204.

173. **Roberts, D. H.** 1973. Preliminary studies on the cell-mediated immune response in pigs to Mycoplasma hyopneumoniae. Br. Vet J. **129**:427-438.

174. **Roberts, S. A., and M. W. Turner**. 1983. Specific suppression of rat IgE responses with milk from immunized females and with feeds of serum antibody. Immunology. **48**:195-199.

175. **Rooke, J. A., and I. M. Bland**. 2002. The acquisition of passive immunity in the new-born piglet. Livestock Prod Sci. **78**:13-23.

176. **Ross, R. F.** 1999. Mycoplasmal Diseases, p. 495-510. In Straw, B., S. D'Allaire, W. Mengeline, D. Taylor (ed.), Diseases of Swine, Iowa State Univ Pr., Ames, Iowa.

177. **Ruiz, A.R., V. Utrera and C. Pijoan**. 2003. Effect of *Mycoplasma hyopneumoniae* sow vaccination on piglet colonization at weaning. J Swine Health Prod. **11**:131-135.

178. **Saalmuller, A., M. J. Reddehase, H. J. Buhring, S. Jonjic, and U. H. Koszinowski.** 1987. Simultaneous expression of CD4 and CD8 antigens by a

substantial proportion of resting porcine T lymphocytes. Eur J Immunol. **17**:1297–1301.

179. **Saalmuller, A., T. Werner, and V. Fachinger**. 2002. T-helper cells from naive to committed. Vet Immunol Immunopathol. **87**:137–145.

180. **Saji, F., Y. Samejima, S. Kamiura and M. Koyama**. 1999. Dynamics of immunoglobulins at the feto–maternal interface. Rev Repro. **4**:81–89.

181. **Salerno-Gonçalvesa R., and M. B. Sztein**. 2006. Cell-mediated immunity and the challenges for vaccine development. Trends in Microbiol. **14**:536-542.

182. **Salmon, H.** 1987. The intestinal and mammary immune system in pigs. Vet Immunol Immunopath. **17**:367-388.

183. **Salmon, H.** 2000. Mammary gland immunology and neonate protection in pigs. Homing of lymphocytes into the MG. Adv Exp Med Biol. **480**:279–286.

184. **Salmon, H., M. Berri, V. Gerdts, and F. Meurens**. 2008. Humoral and cellular factors of maternal immunity in swine. Dev Comp Immunol. **33**:384-393.

185. **Sanglid, P.T.** 2003. Uptake of colostral immunoglobulins by the compromised newborn farm animal. Acta Veterinaria Scandinavica. Supplementum. **98**:105-122.

186. **Schlesinger, J. J., and H. D. Covelli**. 1977. Evidence for transmission of lymphocyte responses to tuberculin by breast-feeding. Lancet. **10**:529-532.

187. **Schnorr, K. L., and L. D. Pearson**. 1984. Intestinal absorption of maternal leucocytes by newborn lambs. J. Reprod. Immunol. **6**:329–337.

188. **Schnulle, P. M. and W. L. Hurley**. 2003. Sequence and expression of the FcRn in the porcine mammary gland. Vet. Immunol. Immunopathol. **91**:227–231.

189. **Seiler, P., M. Bründler, C. Zimmermann, D. Weibel, M. Bruns, H. Hengartner, and R. M. Zinkernagel**. 1998. Induction of protective cytotoxic T cell responses in the presence of high titers of virus-neutralizing antibodies: Implications for passive and active immunization. J Exp Med. **187**:649-654.

190. **Sestak, K., I. Lanza, S. K. Park, P. A. Weilnau, and L. J. Saif**. 1996.

Contribution of passive immunity to porcine respiratory coronavirus to protection against transmissible gastroenteritis virus challenge exposure in suckling pigs. Am J Vet Res. **57**:664-671.

191. **Shahriar, F., J. R. Gordon, and E. Simko**. 2006. Identification of lipopolysaccharide-binding proteins in porcine milk. Can J Vet Res. **70**:243–250.

192. **Siegrist, C. A.** 2001. Neonatal and early life vaccinology. Vaccine. **19**:3331-3346.

193. **Siegrist, C. A.** 2003. Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. Vaccine. **21**:3406-3412.

194. **Siegrist, C. A., C. Barrios, X. Martinez, C. Brandt, M. Berney, M. Cordova, J. Kovarik, and P. H. Lambert**. 1998. Influence of maternal antibodies on vaccine responses: inhibition of antibody but not T cell responses allows successful early prime-boost strategies in mice. Euro J Immunol. **28**:4138-4148.

195. **Simister, N. E.** 2003. Placental transport of immunoglobulin G. Vaccine. **21**:3365-3369.

196. **Simpson-Morgan, M. W., and T. C. Smeaton**. 1972. The transfer of antibodies by neonates and adults. Adv. Vet . Sci. Comp. Med. **16**: 355–386.

197. **Sinkora, M., J. Sinkora, Z. Rehakova, I. Splichal, H. Yang, R. M. E. Parkhouse, and I. T** . 1998. Prenatal ontogeny of lymphocyte subpopulations in pigs. Immunology. **95**:595–603.

198. **Šinkora, M., J. Šinkorová, W. and Holtmeier**. 2005. Development of γδ thymocyte subsets during prenatal and postnatal ontogeny. Immunol. **115**:544–555.

199. **Sirota L., R. Straussberg, I. Notti, and H. Bessler**. 1995. Effect of human colostrum on interleukin-2 production and natural killer cell activity. Arc Dis Child Fet **Neonat Ed. 73:F99-102.**

200. **Skansen-Saphir, U., A. Lindfors, and U. Andersson**. 1993. Cytokine

production in mononuclear cells of human milk studied at the single-cell level. Pediatr Res. **34**:213-216.

201. **Stastny, P.** 1965. Accelerated graft rejection in the offspring of immunized mothers. J Immunol. **95**:929-936.

202. **Stavnezer, J.** 1995. Regulation of antibody production and class switching by TGF-beta. J Immunol. **155**:1647-1651.

203. **Stewart, T. S. and M. A. Diekman.** 1989. Effect of birth and fraternal litter size and cross-fostering on growth and reproduction in swine. J An Sci. **67**:635-640

204. **Stirling, C. M., B. Charleston, H. Takamatsu, S. Claypool, W. Lencer, R. S. Blumberg, and T. E. Wileman**. 2005. Characterization of the porcine neonatal Fc receptor—potential use for trans-epithelial protein delivery. Immunology. **114**:542–53.

205. **Stokes, C., and J. F. Bourne**. 1989. Mucosal immunity, pp 178–181, in: R. E. W. Halliwell, N.T. Gorman, eds. Veterinary clinical immunology. WB Saunders Co, Philadelphia.

206. **Straw, B. E.** 1997. Veterinary Practice: Art, science, and politics, in Proceedings of the American Association of Swine Practitioners, Quebec, CA, March 1 to 4, 1997; p 1-31.

207. **Straw, B. E., C. E. Dewey, and E. J. Burgi**. 1998.Patterns of cross-fostering and piglet mortality on commercial US and Canadian swine farms. Preventative Veterinary Medicine **33**:83-89.

208. **Sun, J., C. Hayward, R. Shinde, R. Christenson, S. P. Ford, and J. E. Butler.** 1998. Antibody repertoire development in fetal and neonatal piglets. I. Four VH genes account for 80% of VH usage during 84 days of fetal life. J Immunol. **161**:5070–5078.

209. **Suradhat, S., and S. Damrongwatanapokin**. 2003. The influence of maternal immunity on the efficacy of a classical swine fever vaccine against classical swine fever virus, genogroup 2.2, infection. Vet Microbiol. **92**:187-194.

210. **Tajima, M., T. Yagihashi, T. Nunoya, A. Takeuchi, and F. Ohashi.** 1983. *Mycoplasma hyopneumoniae* infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. Am J Vet Res. **45**:1928-1932.

211. **Takamatsu, H.H., M. S. Denyer, C. Stirling, S. Cox, N. Aggarwal, P. Dash, T. E. Wileman, and P. V. Barnett**. 2006. Porcine γδ T cells: Possible roles on the innate and adaptive immune responses following virus infection. Vet Immunol and Immunopathol. **112**:49-61.

212. **Tanaka, Y., S. Sano, E. Nieves, G. De Libero, D. Rosa, R. L. Modlin, M. B. Brenner, B. R. Bloom, and C. T. Morita**. 1994. Nonpeptide ligands for human γδ T cells. Proc Natl Acad Sci. **91:**8175–8179.

213. **Thacker E. L., B. J. Thacker, T. B. Boettcher, and H. Jayappa**. 1998. Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. Swine Health Prod. **6**:107-112.

214. **Thacker E. L., B. J. Thacker, M. Kuhn, P. A. Hawkins, and W. R. Waters.** 2000. Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. Am J Vet Res. **61**:1384-1389.

215. **Thacker, E.** 2006. Mycoplasmal Diseases. In Diseases of Swine. 9th edn. Eds B. E. Straw, J. J. Zimmerman, S. D'Allaire & D. J. Taylor. Ames, USA, Blackwell Publishing, pp 701-717.

216. **Thode Jensen, P.** 1978. Trypsin inhibitor in sow colostrum and it s function. Ann. Rech Vet. **2**:225–228.

217. **Thode Jensen, P., and K. B. Pedersen**. 1982. The influence of sow colostrum trypsin inhibitor on the iimmunoglobulin absorption in newborn piglets. Acta Vet. Scand. **23**:161–168.

218. **Tlaskalova-Hogenova, H., L. Mandel, I. Trebichavsky, F. Kovaru, R. Barot, and J. Sterzl**. 1994. Development of immune responses in early pig

ontogeny. Vet Immunol Immunopathol. **43**;135-142.

219. **Tuboly, S., G. Bernath, R. Glavits, and I. Medveczky**. 1988. Intestinal absorption of colostral lymphoid cells in newborn pigs. Vet Immunol Immunopath, **20**:75-85.

220. **Tuboly, S., S. Bernath, R. Glavits, A. Kovacs, and Z. Megyeri**. 1995. Intestinal absorption of colostral lymphocytes in newborn lambs and their role in the development of immune status. Acta Vet Hung. **43**:105-115.

221. **Tuboly, S., and S. Bernath,** 2002. Intestinal absorption of colostral lymphoid cells in newborn animals. Advan Exp Med Biol. 503:107-114.

222. **Tuboly, S., S. Bernath, R. Glavits, and I. Medveczky**. 1988. Intestinal absorption of colostral lymphoid cells in newborn piglets. Vet. Immunol. Immunopathol. **20**:75–85.

223. **USDA**. (2007) Swine 2006, Part I: Reference of Swine Health and Management Practices in the United States, 2006, USDA: APHIS: VS, CEAH. Fort Collins, CO, #N475.1007.

224. **Vaerman, J. P., J. B. Arbuckle, and J. Heremans**. 1970. Immunoglobulin A in the pig. II. Sow colostral and milk IgA: quantitative studies and molecular size estimation. Int. Arch. Allergy. **39**:22-29.

225. **Van Oirschot, J. T.** 1987. Intranasal vaccination of pigs against Aujeszky's disease: comparison with one or two doses of attenuated vaccine in pigs with high maternal antibody titres. Res Vet Sci. **42**:12-16.

226. **Van Rooij, E. M., H. W. Moonen-Leusen, Y. E. de Visser, W. G. Middel, W. J. Boersma, and A. T. Bianchi**. 2006. A DNA vaccine coding for gB and gD of pseudorabies virus (suid herpes type 1) primes the immune system in the presence of maternal immunity more efficiently than conventional vaccines. Vaccine. **24**:1264–1273.

227. **Vekemans J., A. Amedei, M. O. Ota, M. D'Elios, T. Goetghebuer, J. Ismaili, M. J. Newport, G. F. Del Prete, M. Goldman, J. McAdam and A. Marchant.** 2001. Neonatal bacillus Calmette-Guérin vaccination induces adult-

like IFN- production by CD4+ T lymphocytes. Eur. J. Immunol. **31**:1531-1535.

228. **Verhasselt, V., V. Milcent, J. Cazareth, A. Kanda, S. Fleury, D. Dombrowicz, N. Glaichenhaus, and J. Valérie**. 2008. Breastmilk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. Nature Med. **14**:170-175.

229. **Wagstrom, E. A., K. J. Yoon, and J.J. Zimmerman**. 2000. Immune component in porcine mammary secretions. Viral Immunology. **13**:383-397.

230. **Wallgren, P., G. Bolske, S. Gustafsson, S. Mattsson, and C. Fossum**. 1988. Humoral immune responses to Mycoplasma hyopneumoniae in sows and offspring following an outbreak of mycoplasmosis. Vet Micro. **60**:193-205.

231. **Wang, Y., Z. Xiang, S. Pasquini, and H. C. J. Ertl**. 1998. Effect of passive immunization or maternally transferred immunity on the antibody response to a genetic vaccine to rabies virus. J Virol.**72**:1790-1796.

232. **Watson, D. L.** 1980. Immunological functions of the mammary gland and its secretion—comparative review. Aust. J Biol Sci. **33**:4013–4022.

233. **Weaver, E. A., H. Tsuda, A.S. Goldblum, and C. P. Davis**. 1982. Relationship between phagocytosis and immunoglobulin release from human macrophages. Infect Immun. **38**:1073-1077.

234. **Weiler, I. J., W. Hickler, and R. Sprenger**. 1983. Demonstration that milk cells invade the suckling neonatal mouse. Am J Reprod Immunol. **4**:95-98.

235. **Werhahn, E., F. Klobasa, and J. E. Butler**. 1981. Investigation of some factors which influence the absorption of IgG by the neonatal piglet. Vet Immunol Immunopathol. **2**:35–51.

236. **Williams, P.** 1993. Immunomodulating effects of intestinal absorbed maternal colostral leukocytes by neonatal pigs. Can J Vet Res. **57**:1–8.

237. **Williams, P. P., S. P. Targowski, and M. V. S. Rao**. 1985. Organ culture of piglet intestinal explants: growth of virusus, incorporation of methyl-3H thymidine, and uptake of fluorescein isothiocyanate-labelled colostral cells. Microecol Ther. **15**:57-70.

238. **Wilson, E., and E. F. Butcher**. 2004. CCL28 controls immunoglobulin (Ig)A plasma cell accumulation in the lactating mammary gland and IgA antibody transfer to the neonate. J Exp Med. **200**:805–809.

239. **Wilson, M. R.** 1974. Immunologic development of the neonatal pig. J Anim Sci. **38**:1018–1021.

240. **Wittman, G., and V. Ohlinger**. 1987. Aujeszky's disease vaccination and infection of pigs with maternal immunity: effects on cell- and antibody-mediated immunity. Arch Virol. **92**:87-101.

241. **Wongyanina, P., S. Buranapraditkuna, K. Chokeshai-usahaa, R. Thanawonguwechb and S. Suradhat**. 2010. Induction of inducible CD4+CD25+Foxp3+ regulatory T lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV). Vet Immunol Immunopathol. **133**:170-182.

242. **Yabuhara, A., H. Kawai, and A. Komiyama.** 1990. Development of natural killer cytotoxicity during childhood: marked increases in number of natural killer cells with adequate cytotoxic abilities during infancy to early childhood. Pediatr Res. **28**:316-22.

243. **Yang, H. and R. M. Parkhouse.** 1997. Differential expression of CD8 epitopes amongst porcine CD8-positive functional lymphocyte subsets. Immunol. **92**:45–52.

244. **Yang, H., and R. M. E. Parkhouse**. 1996. Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues. Immunol. **89**:76-83.

245. **Yoshioka, Y., S. Kudo, H. Nishimura, T. Yajima, K. Kishihara, K. Saito, T. Suzuki, Y. Suzuki, S. Kuroiwa, and Y. Yoshikai**. 2005. Oral administration of bovine colostrum stimulates intestinal intraepithelial lymphocytes to polarize Th1-type in mice. International Immunopharmacol. **5**:581-590.

246. **Zizka, J., J. Hrdy, R. Lodinova-Zadnikova, I. Kocourkova, O. Novotna, I. Sterzl, and L. Prokesova**. 2007. Effect of breast milk of healthy and

allergic mothers on in vitro stimulation of cord blood lymphocytes. Ped Allerg Immunol. **18**:486-494.

247. **Zuckermann, F.A., and R. J. Husmann**. 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunol. **87**:500–512.

Appendix 1

AMERICAN SOCIETY FOR MICROBIOLOGY LICENSE TERMS AND CONDITIONS

Jul 20, 2010

This is a License Agreement between Meggan M Bandrick ("You") and American Society for Microbiology ("American Society for Microbiology") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and condition provided by American Society for Microbiology, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number 2473141165965
License date Jul 20, 2010
Licensed content publisher American Society for Microbiology
Licensed content publication Clinical and Vaccine Immunology
Licensed content title Passive Transfer of Maternal Mycoplasma hyopneumoniae-Specific Cellular Immunity to Piglets

Licensed content author Meggan Bandrick, Thomas W. Molitor
Licensed content date Mar 1, 2008
Volume 15
Issue 3
Start page 540
End page 543
Type of Use Dissertation/Thesis
Format Print and electronic
Portion Full article
Order reference number
Title of your thesis / dissertation
Maternal influences on neonatal immune development in pigs

Expected completion date Aug 2010
Estimated size(pages) 225
Total 0.00 USD

Terms and Conditions

Publisher Terms and Conditions

The publisher for this copyrighted material is the American Society for Microbiology (ASM). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

1. ASM hereby grants to you a non-exclusive license to use this material. Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process; any form of republication must be completed within 1 year from the date hereof (although copies prepared before then may be distributed thereafter). The copyright of all material specified remains with ASM, and permission for reproduction is limited to the formats and products indicated in your license. The text may not be altered in any way without the express permission of the copyright owners.

2. The licenses may be exercised anywhere in the world.
3. You must include the copyright and permission notice in connection with any reproduction of the licensed material, i.e. Journal name, year, volume, page numbers, DOI and reproduced/amended with permission from American Society for Microbiology.
4. The following conditions apply to photocopies:
a. The copies must be of high quality and match the standard of the original article.
b. The copies must be a true reproduction word for word.
c. No proprietary/trade names may be substituted.
d. No additional text, tables or figures may be added to the original text.
e. The integrity of the article should be preserved, i.e., no advertisements will be printed on the article.
f. The above permission does NOT include rights for any online or other electronic reproduction.
5. The following conditions apply to translations:
a. The translation must be of high quality and match the standard of the original article.
b. The translation must be a true reproduction word for word.
c. All drug names must be generic; no proprietary/trade names may be substituted.
d. No additional text, tables or figures may be added to the translated text.
e. The integrity of the article should be preserved, i.e., no advertisements will be printed on the article.
f. The translated version of ASM material must also carry a disclaimer in English and in the language of the translation. The two versions (English and other language) of the disclaimer MUST appear on the inside front cover or at the beginning of the translated material as follows:
The American Society for Microbiology takes no responsibility for the accuracy of the translation from the published English original and is not liable for any errors which may occur. No responsibility is assumed, and responsibility is hereby disclaimed, by the American Society for Microbiology for any injury and/or damage to persons or property as a matter of product liability, negligence or otherwise, or from any use or operation of methods, products, instructions or ideas presented in the Journal. Independent verification of diagnosis and drug dosages should be made.
Discussions, views, and recommendations as to medical procedures, choice of drugs and drug dosages are the responsibility of the authors.
g. This license does NOT apply to translations made of manuscripts published ahead of print as "[ASM Journal] Accepts" papers. Translation permission is granted only for the final published version of the ASM article. Furthermore, articles translated in their entirety must honor the ASM embargo period, and thus may not appear in print or online until 6 months after the official publication date in the original ASM journal.
6. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by ASM or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. In addition, permission granted is contingent upon author permission, which you MUST obtain, and appropriate credit (see item number 3 for details). If you fail to comply with any material provision of this license, ASM shall be entitled to revoke this license immediately and retain fees paid for the grant of the license. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and ASM reserves the right to take any and all action to protect its copyright in the materials.
7. ASM reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. ASM makes no representations or warranties with respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Billing and Payment terms and conditions for this licensing transaction.
9. You hereby indemnify and agree to hold harmless ASM and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
10. This license is personal to you, but may be assigned or transferred by you to a business associate (or to your employer) if you give prompt written notice of the assignment or transfer to the publisher. No such assignment or transfer shall relieve you of the obligation to pay the designated license fee on a timely basis (although payment by the identified assignee can fulfill your obligation).
11. This license may not be amended except in a writing signed by both parties (or, in the case of ASM, by CCC on ASM 's behalf).
12. Objection to Contrary terms: ASM hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions.
These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and ASM (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
13. The following terms and conditions apply to Commercial Photocopy and Commercial Reprint requests and should be considered by requestors to be additional terms. All other ASM terms and conditions indicating how the content may and may not be used also apply.
Limitations of Use:
The Materials you have requested permission to reuse in a commercial reprint or commercial photocopy are only for the use that you have indicated in your request, and they MAY NOT be used for either resale to others or republication to the public. Further, you may not decompile, reverse engineer, disassemble, rent, lease, loan, sell, sublicense, or create derivative works from the Materials without ASM's prior written permission.
14. Revocation: This license transaction shall be governed by and construed in accordance with the laws of Washington, DC. You hereby agree to submit to the jurisdiction of the federal and state courts located in Washington, DC for purposes of resolving any disputes that may arise in connection with this licensing transaction. ASM or Copyright Clearance Center may, within 30 days of issuance of this License, deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will ASM or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to ASM and/or Copyright Clearance
Center for denied permissions.
v1.5
Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.
If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10818137. Once you receive your invoice for this order, you may pay your invoice by credit card.
Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center