EFFECT OF BOVINE LACTOFERRIN ON T LYMPHOCYTE CELL PHENOTYPE AND FUNCTION AND THE RESPONSE TO VACCINATION IN THE NEONATAL PIGLET

BY

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THESIS

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Abstract

Deficiencies of the immune system, both innate and adaptive immunity, can weaken host defense and increase susceptibility to infection. The immune system exists to guard the host against pathogenic infections, particularly at stressful time periods, such as birth and weaning. Proper nutrition and vaccination have positive effects for infant immune maturation. Lactoferrin (Lf), an iron binding glycoprotein found in breastmilk has been shown to have numerous physiological and immune properties that are nutritionally advantageous for infants.

The goal of this thesis research was to discover how early nutrition influences immune maturation by investigating the addition of bovine Lf (bLf) to infant formula. The overall hypothesis of this research was that immune development is disparate between formula-fed and sow-reared piglets and that addition of bLf would enhance vaccination response and T-cell function in formula-fed piglets. To address this hypothesis, two specific aims were undertaken. N

Specific Aim 1 investigated the effect of bLf supplemented to infant formula on the response to immunization against influenza virus and serum Ig profiles compared to piglets that were sow-reared or fed unsupplemented formula. Newborn piglets were obtained at 4h of age and were fed either medicated sow milk replacer (FF; n=22) or sow milk replacer formula with 1g/L bovine lactoferrin (bLf) (LF; n=22). A reference control group remained with the sow (SR; n=25). Half of the piglets were further randomized into two groups to receive an IM injection of Fluzone™ on d7 and d14. On d21 postpartum, piglets in each group were euthanized and blood and secondary lymphoid tissue samples were collected. Intestinal weight and length was collected immediately following euthanasia and serum was collected for analysis of Fluzone™-IgG and total IgG and IgM concentrations. Formula intake between FF and LF piglets did not
differ. However, the body weight of the piglets was influenced by diet early in the study. Sow-reared piglets weighed more than both FF groups at day 3 and for the duration of the study (p<0.0001). LF piglet intake was approximately 0.38g/kg BW of Lf for the duration of the study. Also, at day 21 LF piglets weighed more than FF piglets (p<0.0001). When intestinal length was normalized by body weight (cm/kg), SR piglets had less normalized intestinal length in comparison to FF and LF. Similarly intestinal weights (g), which were normalized by body weight (g/kg) and intestinal length (g/cm), were significantly lower in SR piglets in comparison to FF and LF piglets (p<0.0001). A trend was observed in antibody response in dietary treatments, where SR piglets had greater Fluzone™-IgG versus the FF and LF groups (p=0.0953). However, LF had no impact on total IgG and IgM profiles.

**Specific Aim 2** examined the effect of bLf supplemented to infant formula on T-lymphocyte phenotypes and cytokine secretion following ex vivo stimulation. T-lymphocyte populations in peripheral blood mononuclear cells (PBMC), mesenteric lymph nodes (MLN) and spleen were stained with specific antibodies and identified by using flow cytometry. T-cell and cytokine responses to ex vivo stimulation were assessed by ELISA. Both dietary (SR vs. LF vs. FF) and vaccination effects on immunological development were observed. Few differences in T-cell subpopulations were observed in PBMC, MLN and spleen. Yet, when vaccination was removed from the model, memory T-cells from PBMC were greater in FF piglets than LF piglets, but not different between SR piglets (p=0.0388). After ex vivo stimulation, numerous diet and vaccination effects were identified. First, changes in PBMC T-cells were observed after culturing. FF piglets had higher amounts of CD8+ T-cells than LF or SR (p<0.0001). In contrast, SR piglets had higher T-helper cells (CD4+) than LF and FF piglets (p<0.0001). Also, LF piglets had decreased memory (CD4+CD8+) T-cells when compared to FF or SR piglets after
stimulation (p=0.0400). In the spleen, SR piglets had greater CD8+ T-cells than LF or FF piglets (p=0.0054). Yet, FF piglets had greater CD4+ T-cells than SR piglets, while LF was an intermediate (p=0.0006). Again, diet differences were observed in the spleen, where SR piglets had higher amounts of CD4+CD8+ T-cells than LF or FF piglets (p=0.0004). In MLN, vaccinated piglets had greater CD8+ T-cells when stimulated than non-vaccinated piglets (p=0.0293). Yet in MLN supernatants, dietary differences were observed in FF piglets who secreted more IL-12 than LF or SR piglets from cells that were unstimulated or stimulated with PHA and PMA (p=0.0041). When vaccination was removed, spleen IL-12 cytokine production following Fluzone™ stimulation was affected by diet. LF piglets had higher IL-12 secretion than SR piglets, while FF was an intermediate (p=0.0394). Overall, vaccination and serum Ig profiles in the neonatal piglets were minimally affected by dietary supplementation of 1.0 g/L bLf. Dietary bLf was found to have little impact on T-cell phenotype or cell-mediated immune responses. More specifically, bLf decreased the percentage of memory T-cells in PBMC in comparison to SR and FF piglets. Dietary bLf was also found to have no negative impacts on development, dietary tolerance or immune characteristics analyzed. However, this data demonstrates differences that exist between SR piglets in comparison to FF and LF piglets and provide a framework for future dietary supplementation studies.
To my Friends and Family
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bLf</td>
<td>Bovine lactoferrin</td>
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<tr>
<td>BW</td>
<td>Body weight</td>
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<tr>
<td>d</td>
<td>Day</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
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<td>GOS</td>
<td>Galactooligosaccharide</td>
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<tr>
<td>FF</td>
<td>Formula-fed</td>
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<tr>
<td>FV</td>
<td>Fluzone-vaccinated</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>IBD</td>
<td>Infectious bural disease</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>IFN</td>
<td>Interferron</td>
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<tr>
<td>IFN-γ</td>
<td>Interferron-gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>LAIV</td>
<td>Live attenuated influenza vaccination</td>
</tr>
<tr>
<td>Lf</td>
<td>Lactoferrin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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min       Minutes
mAbs      Monoclonal antibodies
NK        Natural killer
NV        Non-Vaccinated
OD        Optical density
PBMC      Peripheral blood mononuclear cells
pLf       Porcine lactoferrin
SD        Standard deviation
SIV       Swine influenza virus
SR        Sow-reared
TLR       Toll like receptor
TIV       Trivalent influenza vaccination
V         Vaccinated
Chapter 1
Literature Review

Introduction to the Immune System

The immune system is divided into two categories: innate and adaptive. The innate immune system is comprised of cells and mechanisms that defend the host from infection in a non-specific manner, acting as the first line of defense. It also functions to activate the adaptive immune system through a process known as antigen presentation through natural killer cells, T cells, and phagocyte cells such as macrophages, neutrophils and dendritic cells. The adaptive immune system is composed of highly specialized cells and process to eliminate or prevent pathogenic growth. Bridging innate and adaptive immunity, the major function of macrophages and T-cells is antigen recognition and clearance, thus coordinating the response of B-cells. The collaboration of the two systems is exceedingly effective at identifying pathogenic organisms from the host in order to function appropriately.

Neonatal Immune Development

The humoral immunity of the neonate is moderately underdeveloped, therefore, the infant must rely on maternal passive immunity prenatally and via colostrum and milk and proper nutrition (Calder et al., 2006). This immaturity of the immune system leaves infants vulnerable to foreign external stimuli and infection at an already stressful time of birth and weaning. In addition, the neonate’s inability to respond to external cues from ‘self’ does not the immune system to develop appropriate tolerogenic responses when necessary. Food antigens are also a
common source of benign antigens that modulate immune maturation and preservation of
tolerance as early as in the mother’s womb (Warner et al., 1996).

Throughout pregnancy, immunological changes occur due to the polarization of a T\(_{H2}\) bias between the mother and fetus (Reinhard et al., 1998). This response is necessary for the mother to not abort the infant during pregnancy; however, it yields a T\(_{H2}\) dominance in the fetus. After delivery, the T\(_{H2}\) bias must be down-regulated in order for the infant to not mount an allergen-specific IgE response to environmental or food-derived stimuli. Deficiency in the T\(_{H1}\) T-cell associated cytokines also leaves the newborn susceptible to bacterial and viral infections due to the failure to actively attack foreign antigens (Reinhard et al., 1998). Furthermore, the switch to a more favorable T\(_{H1}\) environment in the neonate decreases its risk of developing future hypersensitivity by effectively responding to the environment.

Immunological factors that the infant is exposed to during delivery or obtains from breastmilk encourage maturation of the neonatal gut-associated and systemic immune systems. Nutrition, introduced through breastmilk, formula or solid foods, influences the microbial environment of the maturing intestine. Constant stimuli from food or environmental antigens affect maturation of the neonatal immune system and immune responses (Laubereau et al., 2005).

**Neonatal Piglet Immune System**

The piglet is an excellent model for investigative studies of infant immune development. Unlike rodents, the piglet has a body size that facilitates surgical manipulations and long-term dietary treatment protocols (Guilloteau et al., 2010; Meurens et al., 2012). In addition, the piglet has a more immature digestive system immediately after birth, yet similar nutrition and
gastrointestinal tract (GIT) development characteristics to human neonates (Sangild, 2003; Guilloteau et al., 2010). During the perinatal period, maturation of the GIT is highly influenced by the introduction of new foods. Dietary changes can be reflected in rapid intestinal growth, nutrient absorption, GIT immune function and bacterial colonization (Sangild, 2003).

Piglets are also at risk for similar disease and pathogens to those that affect humans (Meurens et al., 2012). For example, swine influenza virus (SIV) is similar to human influenza virus. SIV is a highly contagious respiratory disease that disposes pigs to secondary infections that result in morbidity and mortality as high as 10% (Gao et al., 2012). More importantly, pigs with SIV are clinically analogous to influenza infected humans (Reeth et al., 2002).

The immune system of swine is equivalent to that of humans as early as day 26 of gestation when innate immunity and inflammatory protein mediators are detectable (Calder et al., 2006; Šinkora et al., 2009). Piglets are also an excellent model for compromised immunity at birth, due to the low level of stimuli by external antigens during fetal development when leads to low levels of memory T cells and mature B cells at birth (Šinkora et al., 1998). This immaturity is consistent with underdeveloped lymph nodes and Peyers patches, and low levels of peripheral lymphocytes observed in newborn piglets (Šinkora et al., 2009).

In contrast to human infants, piglets have no placental transfer of immunoglobulins from mother to fetus and are dependent upon uptake of colostral antibodies for passive immunity (Sangild 2003, Šinkora et al., 2009). Porcine colostrum contains very high levels of immunoglobulin-G (IgG). However, once the effective transfer of IgG has completed, gut closure occurs at 36-48h postpartum, which decreases the neonate’s ability to absorb macromolecules. After the initial immunoglobulin (Ig) transfer, the composition of Ig resembles that of human colostrum and milk, predominating in immunoglobulin-A (IgA) (Bulter et al.,
Antibody transfer from limited amounts of colostrum has shown to be sufficient to provide protection against a pertussis challenge in newborn piglets (Elahi et al., 2006). However, mutating viruses such as influenza require oral or intramuscular vaccination for protection and these vaccinations have shown to be effective in piglets (Inskeep et al., 2010).

**Infant Response to Influenza Vaccination**

Infants are at increased risk for life-threatening complications caused by influenza, a respiratory tract infection. This contagious infection, which can progress to pneumonia, hypercytokinemia, acute lung injury, respiratory failure and death, kills ~36,000 Americans every year (Thompson et al., 2003; Fiore et al., 2008). Epidemics of influenza have greatly increased hospitalization rates of high-risk children under two-years-of-age at an average of 1,181 per 100,000 persons annually. Patients considered high-risk for influenza infection, include patients with asthma, cardiovascular disease (CVD) or infants born prematurely. Even healthy infants have a 12-times greater likelihood of contracting influenza than older children ages 5-17 (Izurieta et al., 2000), increasing the urgency for influenza protection for vulnerable infants.

The influenza virus consists of three different types: A, B and C. Influenza A viruses can be separated into two subtypes based on two proteins on the virus’s cell surface: hemagglutinin (H) and the neuraminidase (N). Although different subtypes for influenza type B do not exist, this type can be divided by strain. Influenza A (H1N1), A (H3N2) and influenza B viruses are included in each year’s influenza vaccine because they cause seasonal epidemics of the disease every winter in the United States (CDC http://www.cdc.gov/flu/about/viruses/types.htm accessed 2009).
Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infections in infants and young children. Although both viral infections occur seasonally, RSV dominates during late winter months, after the influenza season typically has come to an end (Izurieta et al., 2000).

For people of all ages, influenza vaccines are the mainstay of efforts to prevent seasonal influenza. Intramuscular injection has been confirmed to be 70-90% successful among children and healthy adults, although slightly lower among the elderly (Fiore et al., 2009). One of the most commonly administered vaccines is Fluzone™,(Sanofi Pasteur, Swiftwater, PA) a trivalent inactivated vaccination that contains one influenza A (H3N2) virus, one seasonal influenza A (H1N1) virus, and one influenza B virus. This vaccine is administered by IM injection. Strains selected for the upcoming vaccine are thought to have the capacity to evade the immune system through mechanisms of mutation called antigenic drift, or variations of surface protein expression called antigenic shift (CDC http://www.cdc.gov/flu/professionals/vaccination/virusqa.htm accessed March 21, 2012). Vaccination is recommended for the general population and strongly encouraged for children 6 months and older, pregnant mothers and the elderly (United States Department of Health and Human Services). Vaccination effectiveness varies depending on the ability of the person receiving the vaccination to produce a vaccine-induced immunity, yet limited research has been done in populations less than 6 months-of-age. Recently, a randomized, double-blind, placebo-controlled trial conducted in the U.S. demonstrated that Fluzone™ administered to young infants (n=1,375) beginning at 6 to 12 weeks of age was safe and immunogenic (Englund et al., 2010).

Two novel routes of vaccination against the flu have emerged in recent years: Fluzone Intradermal™ (Sanofi Pasteur) and FluMist™ (MedImmune, Gaithersburg, MD). Fluzone
Intradermal™ is a trivalent inactivated vaccination that requires a less invasive procedure for administration by only puncturing the skin layer. It is currently only available for adults 18-64 years of age. FluMist™ is a live trivalent vaccine offering defense against the seasonal flu, without the use of a needle, recommended for healthy people ages 2-49 years of age (CDC [http://www.cdc.gov/flu/protect/vaccine/index.htm](http://www.cdc.gov/flu/protect/vaccine/index.htm) accessed March 22, 2012). Although Fluzone Intradermal™ and FluMist™ are capable of inducing protective immunity, recent studies have demonstrated that FluMist® was more protective than the other trivalent inactivated vaccines in children aged 6 months to 18 years of age (Ambrose *et al.*, 2010).

**Nutritional Regulation of Immune Function**

Breastfeeding is essential to complete postnatal development by promoting and priming neonatal immunologic, gastrointestinal and central nervous systems. Human milk contains its own immune system and a wide range of soluble and cellular factors, which likely facilitate the infant’s immune development (Field, 2005). These factors include anti-microbial, anti-inflammatory compounds such as immunoglobulins, cytokines, peptides, hormones, growth factors and a variety of important immune cells (Field, 2005). Recent studies have shown that breastfeeding compared to formula feeding induces differences in circulating T-cell phenotypes, including reduced CD4+:CD8+ T-cell ratio and an increase in natural killer (NK) cells (Hawkes *et al.*, 1999).

In the neonate, diet is a significant source of antigens to which the immune system must become tolerant. In addition, diet provides nutrients that shape intestinal flora, thereby also affecting antigen exposure, immune maturation and the subsequent responses. The mucosal and epithelial surfaces form the first line of defense to the neonate, which emphasizes the importance
for proper intestinal development (Newburg and Walker, 2007). The critical mechanisms through early nutrition highly influence immune competence, the ability to develop tolerogenic responses, and the development of immunological disorders (Calder et al., 2006). Epidemiological data provides strong evidence for a connection between undernutrition and life-threatening infections in infants due to the functional immaturity of their innate immune system, and lack of adaptive immunity (Jones et al., 2010).

Cell-mediated immunity to infectious pathogens has been shown to be inefficient during the neonatal period, further decreasing the neonate’s immune defense (Fadel and Sarzotti, 2000). In newborns, successful T<sub>H</sub>1 responses are compromised due to decrease production of T<sub>H</sub>1 type cytokines produced by CD4+ T-cells and mononuclear phagocytes. In addition, neonatal macrophages have decreased response for IFN-γ production upon stimulation (Marodi, 2006). Specifically, a T<sub>H</sub>1 cytokine, IL-12, has been shown to selectively promote the differentiation of naïve CD4+ T-cells into effector T<sub>H</sub>1 CD4+ T-cells, thereby increasing IFN-γ. Upon LPS stimulation, a powerful pathogen-derived activator, neonatal mononuclear cells were less responsive than cells of an adult (Marodi, 2006).

Two key factors that prevent disease and increase infant survival are breastfeeding and vaccination. In addition, a longer duration of breastfeeding elicits a rise of serological protection after vaccination. Exclusively breastfed infants for at least 90 days had an increased protection against *Haemophilus influenza* B vaccine (Hib) (Silfverdal et al., 1999). Since the rise of vaccines in the past 20 years, the correlation of the increased prevalence of allergic diseases and vaccinations has been of a focus of research and debate. Yet, the weight of evidence of the association suggests that vaccination alone cannot increase atopic disorders (Anderson et al.,
2001). Also, breastfeeding has an analgesic effect on vaccination compared to formula-fed infants (Dilli et al., 2009).

Many studies have shown that exclusive and even partial breastfeeding enhances immunity of the infant. Breastfeeding exclusively for 4 months, compared with feeding formula made with intact cow milk protein, has been shown to prevent or delay the incidence of atopic dermatitis, cow milk allergy and wheezing in infancy (Greer et al., 2008). In developing countries where infants are at risk for pneumonia, research has shown that exclusive or even partial breastfeeding decreases the incidences of pneumonia infections and subsequently hypoxemia (Chisti et al., 2011).

**Bioactive Components in Human Milk**

Human colostrum contains many immune-competent cells and soluble proteins that are beneficial to the infant and aid in immune maturation. For example, colostrum stimulates the production of the cytokine IL-1, IL-3 and IL-6 from peripheral mononuclear cells (PBMC) (Bessler et al., 1996). More specifically, exosomes found in colostrum have been suggested to participate in directing immune responses in the infant. Upon isolation, functional analysis revealed exosomes inhibited non-lymphocyte production of IL-2 and IFN-γ from allogeneic and autologous PBMC (Admyre, 2007). Thus, providing optimal nutrition for infants unable to breastfed is critical for appropriate immunological maturation.

Human milk is comprised of lipids, proteins and carbohydrates. Some of these nutrients function as bioactive components that have physiological benefits. Some of these bioactive components found in breastmilk have recently been under investigation. Formula-fed piglets supplemented with nucleotides had higher poliovirus type 1 neutralizing antibody (PV-VN1)
responses after vaccination than their formula-fed controls (Schaller et al., 2004). Galactooligosaccharide (GOS) when supplemented to infant formula for 3 months has shown to significantly increase bifidobacteria, lactobacilli, stool acetic acid content and stool frequency compared to infants with no GOS, although statistically similar to breastfed infants (Ben et al., 2008).

Lactoferrin (Lf), an iron binding glycoprotein in breastmilk has been shown to exert a variety of immune functions. Lf is produced by mammary tissue, grandular tissue on mucosal surfaces and can be found in secretory granules of polymorphonuclear leukocytes (Lönnerdal and Iyer, 1995). In nearly all organisms, iron serves as a cofactor for many important biological redox reactions, thus is an essential requirement for cell survival (Weingberg and Weingberg, 1995). At mucosal surface where invading bacteria can inhabit, the iron can be bound by Lf, thereby decreasing growth of Gram-positive and Gram-negative bacteria (Moshynskyy et al, 2003; Wakabayashi et al., 2009).

In the first 5 days of lactation, Lf is in highest concentration in colostrum and decreases by approximately 50%. Before 5 days of lactation, the concentration of Lf has been shown to be as high as 5.8g/L and 3.1g/L between 6 and 14 days. After the initial 14 days of lactation, lactoferrin further decreases to approximately 2g/L (Montagne et al., 1976). Yet, it is unclear if dietary, cultural or genetic factors influence the differences in Lf concentrations. Although the Lf concentration found in human milk is not depend on maternal iron status or iron supplementation (Lönnerdal and Iyer, 1995).

Research has shown that Lf additionally has numerous physiological properties and immune-modulating effects. Oral administration of Lf increased IgG and IgA concentrations in the small intestine of mice and increased serum IgG in weaned piglets (Debbabi et al., 1998, Lee
et al., 2010). Lf has additionally been shown to have antioxidant activity from oral supplementation in human males. In addition to an increased T-cell activation after 14 days supplementation, the subjects had a statistically significant increase in hydrophilic antioxidant capacity (Mulder et al., 2008).

The presence of specific cellular receptors enables many biological activities of Lf. Lactoferrin receptors have been discovered in the small intestine, liver and bone in addition to monocytes, lymphocytes, platelets and fibroblasts (Suzuki et al., 2005). Recently, Lf has been illustrated as a glycoprotein with a double face, composed of an external surface robustly cationic and prone to interact with many negatively charged macromolecules. The internal portion is highly conserved among species and is dedicated to binding iron, which is sequestered in the two identical lobes. Lf’s ability to bind and release iron alters its functional properties of iron scavenging, thus the structure and dynamics are altered by its present iron status (Baker and Baker, 2009). However, lactoferrin’s receptor binding ability does not appear to be influenced by cellular iron status, unlike transferrin (Yamada et al., 1987).

The innate immune response is the first line of defense against environmental insults, such as infectious pathogens, and it is mediated by pattern recognition receptors, especially Toll-like receptors (TLR), present on specialized cells such as macrophages and dendritic cells (DCs). Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria commonly induces septic like inflammation by binding of TLR-4. Lf has a LPS-binding domain, thereby down regulating the signal cascade that results in a release of pro-inflammatory mediators. In the presence of LPS, Lf injection increased survivability of mice by decreasing neutrophilia and subsequent TNF-α production (Yajima et al., 2005). Bovine lactoferrin (bLF) treated monocyte derived dendritic cells (MD-DC) failed to undergo activation in response to TLR stimulation.
This *in vitro* research demonstrates that bLF acts as a potent anti-inflammatory agent on monocytes by creating a tolerogenic-like program during differentiation into DCs (Puddu *et al.*, 2011). Lf has additionally been shown to have antimicrobial effects when orally administered. Weaned piglets fed Lf transgenic rice for 28 days exhibited lower aerobic and anaerobic bacteria, as well as coliform counts in cecal contents than the control group (Lee *et al.*, 2010).

Because Lf is resistant to digestion and excreted in high levels in the stool of breast-fed infants, its effect on viral replication has been widely researched. It has been established that Lf is a potent inhibitor of the growth of several naked viruses such as rotavirus, adenovirus and enterovirus (Seganti *et al.*, 2004). *In vitro* experimentation using a human enterocyte-like cell line showed that Lf is capable of reducing rotavirus attachment to cell receptors, therefore preventing the virus’s ability to replicate (Superti *et al.*, 1997). Recently, Lf has been studied as an additive to oral rehydration solutions (ORS) for enhancing treatment of virus induced diarrhea and subsequent hospitalizations was studied. Peruvian children receiving Lf-lysozyme supplemented ORS (1g/L lactoferrin and 0.2g/L lysozyme) had a significantly reduced duration of diarrhea (Zavaleta *et al.*, 2007).

Lf additionally has been shown to stimulate proliferation of immune cells. Weaned piglets fed Lf (50mg/kg BW) isolated from transgenic rice had a higher number of lymphocytes, an increased percentage of T-cells and a decreased number in B lymphocytes (Lee *et al.*, 2010). In addition, IP injection of Lf in C57BL/6NCr mice induced substantial infiltration of neutrophils 4 hours after injection and macrophages after 24 hours (de la Rosa *et al.*, 2008).

Recent studies using porcine lactoferrin (pLf) and bLf have suggested the potential for Lf to enhance vaccination response. In chickens, pLf (2.0% w/w) was used as a feedstuff additive to examine its impact on peripheral lymphocyte proliferation and serum antibody titers in chickens
vaccinated against the infectious bursal disease (IBD). The addition of pLf also led to significant increases in total serum IgA and IgG and in IBD-specific antibody titers. Moreover, the expression of IFN-γ and IL-12 from T-cells was increased, suggesting that pLf enhanced cell mediated immunity and augmented the ability of the vaccination to strengthen subsequent antiviral responses (Hung et al., 2010).

The immune modulatory activities of Lf include its ability to promote antigen specific cell-mediated immunity in C57BL/6 and BALB/c mice immunized with *Mycobacterium bovis bacillus Calmette Guerin* (BCG) with recombinant human Lf. BCG/Lf-immunized mice had a significant decrease in the development in pulmonary pathologies compared to immunized groups following *Mycobacterium tuberculosis* infection (MTB) (Hwang et al., 2009a). In addition, the splenocytes of these animals had significantly more CD4+ T-cells producing intracellular interferon-gamma (IFN-γ) in a group in which Lf was used as an adjuvant (<0.2 E.U./mg). This is important because *in vivo* studies have shown that type I interferons (IFN) are an influential vaccine adjuvant for increasing immune protection against influenza virus. IFN cytokines promote differentiation, survival and function of immune cells and enhance antibody response. The cellular immune response is crucial in order to clear the virus infection or respond to a vaccine, including innate NK cells and adaptive T-cells. In children (6 months to 4 years of age) the production of IFN-γ by T-cells and NK cells has been determined before and after vaccination. Peripheral blood mononuclear cells (PBMCs) from vaccinated animals stimulated *in vitro* with live influenza A virus had significantly higher IFN-γ producing cells from CD4+ T-cells and NK cells, suggesting a relationship between innate and adaptive immunity for viral infections and vaccination (Proiette et al., 2002).
In addition, under *ex vivo* stimulation of LPS, Lf increased the ratio of IL-12:IL-10 cytokines from murine leukocytes. *In vitro* experimentation with the addition of bLf to BCG infected macrophages also increased the IL-12:IL-10 ratio (Hwang *et al.*, 2006). Thus, Lf has been shown to promote T\(_H\)1 mediated response.

It is known that bioactive compounds found in breastmilk help direct and educate the infant’s immune system and maturation (Dorea, 2009). Research has proven numerous immunological benefits of Lf, however experiments conducted with antigen-specific immunity only investigate Lf either mixed with the vaccine, injected or added *in vitro*. Numerous sources and dosages of Lf has been researched (*Table 1.1*), but the ability of bLf to stimulate the influenza vaccination response *in vivo* has not been analyzed. In addition, T cell phenotype differentiation has not been assessed upon activation *ex vivo*. 
Table 1.1. Concentrations and sources of lactoferrin used as a food additive or adjuvant in research.

<table>
<thead>
<tr>
<th>Lf Source and Dose</th>
<th>How Administered</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLf: 2.0% w/w</td>
<td>Oral supplementation in chickens</td>
<td>Increased serum IgA and IgG; Increased IBD-antibody specific concentration</td>
<td>Hung et al. 2010</td>
</tr>
<tr>
<td>bLf: 0.2 E.U./mg</td>
<td>Used as an adjuvant.</td>
<td>Increased percentage of IFN-γ producing CD4+ T-cells</td>
<td>Hwang et al., 2009a</td>
</tr>
<tr>
<td>Recombinant Lf: 50mg/kg BW</td>
<td>Oral supplementation to weaned piglets</td>
<td>Increased concentrations of lymphocytes and T-cells Decreased concentration of B-cells</td>
<td>Lee et al., 2010</td>
</tr>
<tr>
<td>Recombinant Lf: 1g/L</td>
<td>Additive in ORS to children</td>
<td>Reduced duration of diarrhea</td>
<td>Zavaleta et al., 2007</td>
</tr>
</tbody>
</table>
Chapter 2
Research Objectives, Specific Aims, and Hypothesis

Influenza infections are a major cause of morbidity and mortality in infants each year. Proper nutrition and vaccination are two key factors that increase protection from infection and improve infant survival. Maternal milk provides the best nutrition by enhancing immunity of the infant. For examples, infants who are exclusively breastfed for at least 90 days had a better response to vaccination (Silfverdal et al., 1999). Although there are many evident immunological benefits of breast feeding, only 3 of 4 mothers initiate breastfeeding, and of that only one-third of infants are exclusively breast fed up to 3 months (CDC, 2011). Thus, identifying bioactive components for that could support proper immune development for formula-fed infants is under constant investigation.

The overall goal of this thesis research was to discover how early nutrition influences immune maturation by investigating the addition to bLf to infant formula. The overall hypothesis of this research was that immune development is disparate between formula-fed and sow-reared piglets and that addition of bLf would enhance vaccination response and T-cell function in formula-fed piglets. To address this hypothesis, two specific aims were undertaken.

Specific Aim 1 investigated the effect of bLf supplemented to infant formula on the response to immunization against influenza virus and serum Ig profiles compared to piglets that were sow-reared (SR) or fed unsupplemented formula (FF). The working hypothesis was that supplementation of bLf would promote a greater immunoglobulin response to immunization, and higher concentrations of total serum IgG and IgM than FF piglets and would be more comparable to SR piglets. Chapter 3 compared Fluzone™-specific IgG and total IgG and IgM
concentrations in the serum of vaccinated and non-vaccinated piglets in the three dietary treatment groups.

Specific Aim 2 examined the effect of bLf supplemented to infant formula on T-cell populations, T-cell and cytokine responses to ex vivo stimulation compared to FF and SR piglets. The working hypothesis of this specific aim was that supplementation of formula with bLf would elicit a more robust immunological development of piglets compared to those fed formula alone, which would be shown through greater percentages of memory T-cell populations. Furthermore, dietary bLf would lead to higher expression of T\(_{H1}\) cytokines upon stimulation. It was also hypothesized that T-cell phenotype, cell-mediated immune response and cytokine production would be affected by vaccination.
Chapter 3

Impact of Bovine Lactoferrin on Serum Immunoglobulins and Response to Influenza Vaccination in the Neonatal Piglet

Abstract

Neonatal immune development is vital in order to protect the infant from pathogens in the external environment, exposure to which could lead to an unfavorable and potentially life-threatening response. Lactoferrin, an iron-bind protein has been shown to have numerous immune modulating effects. We hypothesized that bovine lactoferrin-fed piglets would develop immunoglobulin profiles parallel to that of sow-reared piglets, but different from formula-fed piglets. Newborn piglets fed colostrum for 4h were randomized into three groups by litter and body weight. Dietary groups consisted of a non-medicated sow milk replacer (FF; n=22), the milk replacer supplemented with 1g/L bovine lactoferrin (LF; n=22) or the piglets remained with the sow (SR; n=25) throughout the duration of the study. On d7 and d14, half the piglets in each group were further randomized to receive Fluzone™ vaccination at the pediatric dose (0.25mL) by IM injection. Blood samples were collected on d7 and d14 by jugular puncture. On d21 postpartum, the piglets were euthanized and blood and tissue samples were collected. Total intestinal length and weight was taken immediately and measured from the pyloric sphincter to the ileocecal valve. In serum, total IgG and IgM concentrations and Fluzone™-specific IgG levels were analyzed by enzyme linked immunoabsorbent assays (ELISA). Formula intake between FF and LF piglets did not differ. However, the body weight of the piglets was influenced by diet early in the study. Sow-reared piglets weighed more than both FF groups at day 3 and for the entire duration of the study. Also, at day 21 LF piglets weighed more than FF
piglets. When absolute intestinal length (cm) was normalized by body weight (cm/kg), SR piglets had shorter intestinal length compared to FF and LF. However, FF and LF piglets showed no difference in intestinal length. Similarly intestinal weights (g), which were normalized by body weight (g/kg) and intestinal length (g/cm), were significantly lower in SR piglets in comparison to FF and LF piglets. In the LF piglets, daily bLf intake increased from 0.46g/d at d1 to 2.4g/d at d21. Normalized by BW, the average bLf intake was 0.38g/kg BW throughout the study. Identified by ELISA, vaccinated piglets had a higher Fluzone™-specific IgG levels at d21 than non-vaccinated and tended to have higher Fluzone™-IgG than FF and LF groups. Total IgG concentration was greater in all dietary treatments at d21 in comparison to d7 and d14. However, no vaccination or dietary effects were found. Total IgM concentration on d7 for all dietary treatments was greater than d14 and d21. On d14, SR piglets had higher amounts of IgM than LF piglets, both were not different from the FF piglets. Diet differences were also seen on d21 as SR piglets had higher serum IgM than LF and FF piglets. The findings of this study have established the impact of formula supplemented with bovine Lf and its implications on IgG, IgM and Fluzone™-specific IgG. Furthermore, these data demonstrated that differences exist between SR piglets in comparison to FF piglets and provide a framework for future dietary supplementation studies to increase the infant’s ability to develop an antigen-specific antibody response for protection.
Introduction

After birth, humoral immunity of the neonate is moderately underdeveloped making the infant rely primarily on maternal immunity and proper nutrition. An ongoing challenge for the medical field today is to provide proper nutrition for infants who are unable to breastfeed. Therefore, research is necessary to identify compounds found in breastmilk but absent in formula, to ensure optimal development of the neonate. Throughout this period of rapid development, the infant is dependent on their immature immune system for protection to defend themselves against foreign external stimuli. Nutrition is also a common source of these benign antigens that modulate immune maturation and preservation of tolerance throughout infancy (Calder et al., 2006). Thus, we investigated the effects of a lactoferrin (Lf), a bioactive breastmilk protein, on the innate immune system in the neonatal piglet as a model for the human infant.

Lf is an iron-binding glycoprotein that has been shown to have various physiological properties that affect immune system development and function. Lf has been shown to have antibacterial and anti-viral properties. On the mucosal surface where invading bacteria can inhabit, Lf has the ability to bind iron thus minimizing cell growth of Gram-positive and Gram-negative bacteria (Moshynskyy et al., 2003; Wakabayashi et al., 2009). In addition, Lf demonstrated its ability to reduce rotavirus attachment to cell receptors in vitro, therefore preventing the virus’s ability to replicate (Superti et al., 1997).

In addition to the immunomodulatory effects of Lf, oral administration of this glycoprotein has been shown to enhance total and antibody-specific immunoglobulin maturation. Oral supplementation of Lf increased IgG and IgA concentrations from small intestinal mucosa in mice and higher IgG in serum of weaned piglets (Debbabi et al., 1998; Lee et al., 2010).
Studies using porcine (pLf) and bovine (bLf) lactoferrin have suggested the potential for Lf to enhance vaccination response. The addition of pLf led to significant increases in total serum IgA and IgG and in infectious bursal disease (IBD) virus-specific antibody titers (Hung et al., 2010).

Despite the positive findings of Lf on antibody enhancement, minimal research has been done to investigate the impact of orally supplemented Lf on antibody-specific immunoglobulin development and T-cell responses in a neonatal model. Therefore, the presented research explores the effects of dietary Lf on the immunological development during vaccination in piglets less than three weeks of age. Outcomes included body weight, Lf intake, intestinal weight and length, and circulating concentrations of antibody specific and total immunoglobulins.
Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Bovine Lactoferrin

bLf was obtained from FrieslandCampina, Netherlands. The protein content of the bLf was 98% and iron content is 120mg/kg. Assuming all iron was bound, the iron saturation of Lf was 11.9%.

Dietary Treatment and Animal Protocol

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois. Pregnant sows were purchased from Midwest Research Swine (Gibbon, MN) and transported to the Edward R. Madigan Laboratory (ERML) at the University of Illinois one month prior to farrowing. Upon arrival, sows were vaccinated with LitterGuard LT-C (Pfizer Animal Health, Exton PA), Respisure (Pfizer Animal Health), and BPE (Intervet Inc., Millsboro, De), followed by a booster vaccination two weeks prior to farrowing and were placed on gestation diet. The sows were allowed to farrow naturally unless induction with Lutalyse (Pfizer, 2mL intramuscular (IM) injection [10mg]; Pfizer Animal Health, Fort Dodge, IA) was necessary. Piglets were allowed to suckle for 4h to receive colostrum before being randomized to receive a non-medicated sow milk replacer formula (FF; n=22) (Advance Baby Pig Liquiwean, Milk Specialities Global Animal Nutrition, Dundee, IL) or formula supplemented with 1g/L bLf (LF; n=22). Piglets were individually housed in environmentally controlled rooms (25°C) in cages that maintained three piglets separated by
Plexiglas partitions. Piglets were weighed each morning and formula was provided by a pump in 22 equal feedings at a total volume of 360 ml/kg BW/day. The remaining piglets (n=25) were sow-reared (SR) and were allowed to nurse *ad libitum* and were not given creep feed, although by d21 piglets were observed consuming minimal feed from the sow’s bin.

**Vaccination**

On d7, half of the piglets in each dietary treatment were randomized equally into two groups, vaccination (V) and non-vaccinated (NV) [FF-NV; n= 11, FF-V; n=11, LF-NV; n=10, LF-V; n=11, SR-NV; n=13, SR-V; n=11]. On d7 the FF-V, LF-V and SR-V groups were vaccinated with the pediatric dose (0.25mL) of Fluzone™ (Sanofi Pasteur, Swiftwater, PA) by IM injection and were given a booster on d14. Blood samples were obtained by jugular puncture at d7 and d14, or following euthanasia at d21.

**Sample Collection**

On d21 postpartum, piglets were sedated with an IM injection of Telazol (3.5 mg/kg SW each Tiletamine HCl and Zolazepam HCl, Pfizer Animal Health, Fort Dodge, IA). After sedation, blood was collected by cardiac puncture into non-coated vacuum tubes (BD Biosciences, Franklin Lakes, NJ) for serum isolation. Piglets were then euthanized by an intravenous injection of sodium pentobarbital (72 mg/kg BW Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). After euthanasia, the small intestine was removed from the pyloric sphincter and to the ileocecal valve. The small intestine was measured and cut at 10% and 85% from the proximal end to give 3 segments corresponding to the duodenum, jejunum and
ileum, respectively. Each section was weighed and snap frozen in liquid nitrogen. Spleen and mesenteric lymph nodes (MLN) were then collected for isolation of mononuclear cells.

**Assessment of Serum Immunoglobulin Levels**

Serum IgG and IgM concentrations were quantified using a porcine specific ELISA Quantification Set (Bethyl Laboratories, Montgomery, TX). Briefly, 96-well, flat-bottomed plates (Nune, Rochester, NY) were coated with 100μl of coating antibody (μg concentration used was advised by the manufacturer diluted in 0.05M carbonate-bicarbonate buffer, pH 9.6) then incubated at 4°C overnight. Once the coating antibody was decanted, the plate was washed three times with PBS/0.05% Tween20. The plates were blocked with 300μl of 3% BSA/PBS for 1 h at room temperature (RT). The plates were washed as before and serum samples were serially added to the wells in duplicate (100μl), diluted in 0.05% gelatin/PBS and incubated for 1 h at RT. The quantification set included samples for standard curves, which were used as directed. Plates were washed as before, and 100μl of HRP-conjugated detection antibody (concentration recommended by the manufacturer) in 0.05% gelatin/PBS was added to each well. Plates were removed from light and incubated for 1 h at RT. Plates were then washed three times with PBS-Tween 20. Next, 100μl TMB reagent solution (OptEIA, BD Biosciences, San Diego, CA) was added to each well and allowed to develop protected from light at RT for the recommended time by the manufacturer. The reaction was stopped with 100μl 2N H₂SO₄ per well and the absorbance was read at 450nm with 570nm correction using a microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA). Total immunoglobulin values were determined based on a standard curve that was run on each plate.
Assessment of Antibody Response

An enzyme-linked immunosorbent assay (ELISA) kit was developed in the Donovan laboratory to detect porcine Fluzone™-specific IgG. Flat bottomed plates (Nunc, Rochester, NY) were coated with dialyzed 50μl dialyzed Fluzone™ (1:80 dilution) in coating buffer [0.5M Carb/Bicarb Buffer, pH 9.6] and incubated overnight at 4°C. After the incubation, 200μl of PBS/10% FBS was added to each well to block non-specific binding for 1 h at RT. Following incubation, the plates were washed three times with PBS/0.05%Tween 20 and 50μl of serially diluted serum was added to each well and incubated for 1 h at 37°C. The plates were then washed three times with PBS/Tween and 50μl of goat anti-pig IgG conjugated HRP (Bethyl Laboratories) was added to each well at a 1:400 dilution in PBS/10% FBS then incubated for 1 h at 37°C. After washing as before, 50μl of TMB (BD Biosciences, San Diego, CA) was added and allowed to develop at RT. After 20 min, the reaction was stopped by 50μl 1M sulfuric acid. The plate was then analyzed on a microplate spectrophotometer (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) at 450nm and 570nm for correction. Positive stock serum from an SIV infected sow was run on each plate in dilutions ranging from 1:100-1:50,000 and used as the standard curve. Fluzone™-specific IgG was expressed as optical density (O.D.) from a 1:100 dilution.

Statistical Analysis

Analyses were completed using PROC GLM (general linear model) procedure within SAS (Version 9.2, SAS Institute, Cary, NC). The initial model tested was replicate, litter, diet and vaccination; however if the replicate was not significant, it was removed from the model. Diet included three groups: FF, LF and SR. The final model was vaccination, diet, and
vaccination*diet interaction. Diet included three groups: FF, LF and SR. If lactoferrin was not significant, LF and FF piglets were combined. If the vaccination was not significant, vaccinated and non-vaccinated were combined. Daily weight gain was analyzed by Chi-squared test using the PROC MIXED procedure within SAS. Statistical significance was defined as p<0.05, with trends reported as p<0.10. All data are expressed as means ± SD.

**Results**

*Formula Intake and Body Weight*

Formula intake ([Figure 3.1](#)) and body weight ([Figure 3.2](#)) were measured daily to determine whether bLf affected piglet growth. Formula intake was similar among the two diets and averaged 1076 ± 189 and 1088 ± 178 mL/day for the FF and LF, respectively, over the 21-day study period. Vaccination showed no effect on intake or body weight gain. Although average body weight in each diet group was not different at d0, from d3 to d21 body weight of SR was greater (p<0.05) than those of FF and LF. This can also be observed from the first two weeks, where average weight gain was significantly greater (p<0.001) in SR than FF or LF piglets. Yet, on day 21 body weight of LF was greater (p<0.05) than FF on d21. Therefore, during week 3, average weekly weight gain of SR and LF did not differ, but was significantly greater (p<0.01) than those of FF. At d21, weights averaged 5.46±1.14kg for FF-NV, 5.51±1.31kg for FF-V, 6.09±0.91kg for LF-NV, 5.85±0.78kg for LF-V, 7.68±1.3 for SR-NV, and 7.33±1.37kg for SR-V piglets.
Lactoferrin Intake

Lf intake was not different between vaccinated and non-vaccinated groups (p=0.5941) (Figure 3.3). Over the 21 day feeding period, bLf intake increased from ~0.5 to 2g/d. Daily average bLf intake per kg BW was approximately 0.38g/kg BW for the duration of the study.

Intestinal Weight and Length

Total intestinal length (Figure 3.4a) and weight (Figure 3.4b) were not significant between dietary and vaccination groups (p=0.8832 and p=0.4456), respectively. However, absolute intestinal length, normalized to body weight (cm/kg) was lower (p<0.05) decreased in SR piglets compared to FF and LF (Figure 3.5a). However, FF and LF piglets showed no difference in intestinal length. Intestinal lengths normalized by body weight at d21 by dietary treatments were: 148.83±21.73 cm/kg for FF, 141.219±17.8 cm/kg for LF, and 114.08±17 cm/kg for SR piglets. Similarly intestinal weights normalized by body weight (g/kg) and intestinal length (g/cm), were significantly lower in SR piglets in comparison to FF and LF piglets (Figure 3.5b).

Assessment of Antibody Response

A Fluzone™-specific IgG ELISA was developed to establish whether bLf increased antibody titers in response to the vaccination. Only samples from November and December 2011 replicates were used for this analysis. Serum concentrations were evaluated at d7, d14 and d21 to analyze antibody production over time. Vaccinated piglets had a significantly higher IgG titers at d21 than non-vaccinated (p<0.0001). There were differences in Fluzone™-IgG versus SR piglets and the FF and LF groups, but not statistically different (p=0.0953) (Figure 3.6).
Total Serum Immunoglobulin Levels

Total IgG and IgM was assessed by ELISA kit to investigate dietary and vaccination treatments on immunoglobulin concentrations. For total IgG concentration, d21 was statistically different from d7 and d14 (p<0.0001) (Figure 3.7). When days were analyzed separately, d7 had a trend (Model: p=0.0560) in vaccination (p=0.0562) for V piglets to have higher IgG. Also, a trend was seen for dietary treatments (p=0.0579) for FF piglets to have higher IgG, than SR and LF fed piglets. No statistical differences were observed on d14 or d21 (p=0.3395 and (p=0.3122 respectively). When all days were run using PROC MIXED, an interaction was seen between diet and day (p=0.0460) for total IgG. FF-d7 and SR-d14 are statiscally different than all diet groups at d21.

Total IgM concentration was also assessed by ELISA kit (Figure 3.8). Day 7 was found to be statistically different from d14 and d21 (p<0.0001). Diet significance was found when days were analyzed separately on d14 (Model: p=0.0396) and d21 (Model: p=0.0151) but not on day d7 (Model: p=0.7160). On d14, SR piglets significantly greater amounts of IgM than LF piglets, both not different from the FF piglets (p=0.0085). On d21, diet differences were again seen. SR piglets had statistically higher quantities of serum IgM than LF and FF (p=0.0016). When all days were analyzed together using PROC MIXED, a vaccination effect was not seen, but diet differences were. SR piglets had greater IgM than LF piglets, but were not different from
FF piglets (p=0.0294)
Figure 3.1. Average Daily Formula Intake (mL/d) of Formula-Fed Piglets. Neither bLF nor vaccination affected formula intake. Data are expressed as means±SD.
Figure 3.2. Average Daily Body Weights (kg) of Piglets Between Birth and d21 Postpartum. At d3, SR were significantly heavier than FF and LF fed groups. At d21, LF piglet weight is significantly greater than that of FF piglets. Data are expressed as means±SD.
Figure 3.3. Daily Lf Intake (g/d) Between d1 and d21. Lactoferrin intake was not affected by vaccination (p=0.5941). Data are expressed as means±SD.
Neither weight nor length was affected by diet or vaccination ($p=0.8832$ and $p=0.4456$ respectively). Data are expressed as means±SD.

**Figure 3.4.** Total Intestinal Length (top panel) and Weight (bottom panel) on d21. Neither weight nor length was affected by diet or vaccination ($p=0.8832$ and $p=0.4456$ respectively). Data are expressed as means±SD.
Figure 3.5. Average Intestinal Length (top panel) and Weight (bottom panel) Normalized by Body Weight on d21. Intestinal length (cm/kg) was shorter in SR piglets than FF or LF groups (p<0.0001). Intestinal weight of SR piglets was lower than FF and LF groups (p<0.0001). Vaccination had no effect on normalized intestinal weight or length. Data are expressed as means±SD. Different letter superscripts indicate significant differences.
Figure 3.6. Serum Fluzone™ IgG Concentration on d21. Fluzone™ IgG was undetectable in non-vaccinated piglets (data not shown). SR piglets had a trend towards higher Fluzone™ IgG than FF and LF piglets, but values were not statistically different. Data are expressed as means±SD.
Figure 3.7. Total IgG Concentration in Serum on d7, 14 and 21. Values on day 21 were statistically different from d7 and d14 (p<0.0001). An interaction was seen between diet and day. Using LS Means, FF-d7 and SR-d14 were denoted as A. LF-d7, LF-d14, FF-d14 and SR d7 are AB, and all diet groups at d21 are denoted as C. Data are expressed as means±SD.
Figure 3.8. Serum IgM Concentration on d7, 14 and 21. Concentrations on day 7 were statistically higher than d14 and d21 (p<0.0001). SR piglets had greater IgM than LF piglets, but not different than FF piglets (p=0.0294). There was no effect of vaccination. Data are expressed as means±SD.
Chapter 4

Impact of Bovine Lactoferrin and Vaccination on T Lymphocyte Phenotype and Response to Mitogen Stimulation Ex Vivo in the Neonatal Piglet

Abstract

Neonates are born in a $T_H2$ bias environment and the early postnatal switch to a more favorable $T_H1$ T-cell phenotype decreases the risk of developing future hypersensitivity by effectively responding to the environment. Immunological factors from delivery and breastmilk, such foreign antigens, are key aspects that encourage maturation of the neonatal gut-associated and systemic immune systems. Lactoferrin (Lf), a glycoprotein found in human milk has been shown to affect T-cell proliferation and activation. Peripheral blood mononuclear cells (PBMCs), mesentery lymph nodes (MLN) and spleen were collected for immune cell isolation from the study described in Chapter 3. T-lymphocyte subpopulations were identified using flow cytometry. Immune cells were also placed into culture and stimulated with mitogens and Fluzone™ ex vivo for 48hr and 72hr. T-lymphocyte subpopulations were again determined by flow cytometry and supernatants were collected for cytokine analyses by ELISA. Both dietary (SR vs. LF vs. FF) and vaccination effects on immunological development were observed. No effect of vaccination on T-cell subpopulations was observed in PBMC, MLN or spleen but memory T-cells from PBMC were greater in FF piglets than LF piglets, but not different between SR piglets. After ex vivo stimulation, both diet and vaccination effects were observed. Stimulation PMA was consistently different from other stimulants (unstimulated, Fluzone™, PHA and LPS). In PBMC, FF piglets had higher amounts of CD8+ T-cells than LF or SR after 48 hr in culture. In contrast, SR piglets had higher T-helper (CD4+) cells than LF and FF piglets.
after stimulation. Also, LF piglets had lower memory (CD4+CD8+) T-cells when compared to FF or SR piglets after stimulation. Although vaccination had little effect on spleen T-cell subpopulations after stimulation, diet differences were seen. In the spleen, SR piglets had greater CD8+ T-cells than LF or FF piglets. Yet, FF piglets had greater CD4+ T-cells than SR piglets, but LF did not differ between the two groups. Again, diet differences were observed for CD4+CD8+ T-cells in the spleen where SR piglets had higher amounts than LF or FF piglets. In MLN, no dietary differences were observed, but vaccination had an impact on CD8+ T-cells after stimulation. Vaccinated piglets had greater CD8+ T-cells when stimulated than non-vaccinated piglets. However, no differences were seen in MLN T-helper cells or memory T-cells from stimulation. Cytokine production was analyzed by ELISA from ex vivo stimulated MLN and spleen cell supernatants. Vaccination and dietary differences were not detected, but cytokines IL-10 and IL-12 differences were seen between stimulation in the spleen supernatants. When vaccination was removed, spleen IL-12 cytokine production from Fluzone™ stimulation had significant diet effects. LF piglets had higher secretion of IL-12 than SR piglets, but FF piglets did not differ between the two groups. In MLN, stimulation differences were detected in IL-10. MLN IL-12 cytokine production, vaccination differences were not observed, yet diet effects were observed. MLN T-cells from FF piglets secreted more IL-12 than those from LF or SR piglets under stimulation. The outcomes of this study have established the dietary impact of formula supplemented with bLf and its effect on T-cell subpopulations, cell-mediated immune responses and subsequent cytokine production. Also, these data demonstrated that differences exists between SR piglets in comparison to LF and FF piglets and provide a framework for future dietary supplementation studies to heighten the infant’s capacity to elicit a proper immune response.
Introduction

During infancy, rapid development is occurring and the infant is dependent on their immune system for protection against foreign environmental stimuli. Early nutrition, such as human colostrum, includes numerous immune-competent cells and soluble proteins that are valuable to the infant, and contribute to immune development (Calder et al., 2006). Research has revealed that breastfeeding stimulates variations in T-cell phenotypes, including greater percentages of CD8+ T-cells, lower percentages of CD4+ T-cells and more natural killer (NK) cells in comparison to formula-fed infants (Hawkes et al., 1999). Although no differences were observed in cytotoxic T-cell, helper T-cell, and memory T-cell subpopulations, the findings suggest that breastfed infants have greater maturity in the development of their immune system. Further compromising immune protection, cell-mediated immunity against harmful pathogens is not effective during infancy (Fadel and Sarzotti, 2000). The T_H2 bias during fetal development also compromises T_H1 responses in newborns. Research has shown that neonates are deficient in T_H1 associated cytokines, such as IL-12 and IFN-γ, produced by CD4+ T-cells and macrophages, respectively (Marodi, 2006). This immaturity of an infant is also consistent with underdeveloped lymph nodes and Peyers patches, and low quantities of peripheral T-cells seen in newborn piglets (Šínikora et al., 2009). Also, piglets are an excellent model for an immature immune system at birth due to lack of external antigens and passive immunity during fetal development, thereby decreasing levels of memory T-cells and mature B-cells at birth (Šínikora et al., 1998). For infants that are not breastfed, ongoing research is necessary to identify compounds found in breastmilk but absent in formula, to provide the most advantageous nutritional components to ensure optimal development of the neonate. Thus, we explored the
effects of a bioactive component found in breast milk, Lf, on the innate immune system of neonatal piglets as a model for human infants.

In addition to Lf positive effects against harmful pathogens, Lf has been shown to be a mediator between innate and adaptive immunity (Seganti et al., 2004; Superti et al., 1997). The innate immune system is the first line of defense to environmental stimuli and is mediated by pattern recognition receptors, such as especially Toll-like receptors (TLR). Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria commonly induces septic like inflammation by binding of TLR-4, although the LPS-binding domain found on Lf reduces the release of pro-inflammatory mediators (Yajima et al., 2005). The existence of receptors enables numerous activities of Lf, such as Lf’s ability to stimulate the innate immune system thereby improving the infant’s ability to fight infection (Suzuki et al., 2005).

Research has shown that orally supplemented piglets fed Lf had an increased percentage of lymphocytes, a decreased amount of B lymphocytes but a higher number of T-cells (Lee et al., 2010). When encountered with infectious pathogens, Lf has been shown to decrease rotavirus attachment in vitro (Superti et al., 1997). Under LPS stimulation ex vivo, Lf enhanced cytokines IL-12:IL-10 ratio from murine leukocytes (Hwang et al., 2006). The addition of bLf to BCG, the tuberculosis vaccine, infected macrophages also increased the IL-12:IL-10 ratio in vitro (Hwang et al., 2009b). Thus, Lf has been shown to promote Th1 mediated response.

Limited research has been done to explore oral supplemented Lf’s on T-cell subpopulations and cellular T-cell responses after stimulation. Thus, this research examines the effects of dietary Lf on the T-cell development during vaccination in the neonatal piglet. Characteristics analyzed include T-cell subpopulations, T-cell responses and cytokine responses after ex vivo stimulation.
Materials and Methods

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood was drawn into heparin-containing vacuum tubes and layered onto Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ). PBMCs were collected from the gradient interface and washed three times. The isolated cells were washed three times in wash buffer after lysing the red blood cells. Cells were counted using a Countess automated cell counter (Invitrogen, Carlsbad, CA). The number of viable cells was assessed by counting after staining with trypan blue (Gibco Invitrogen, Grand Island, NY).

Isolation of Mononuclear Cells from Tissues

Spleen and MLN samples were collected by cutting tissues into pieces followed by dissociation using a Gentle MACS Dissociator (Miltenyi Biotec, Auburn, CA). The chopped tissue solutions were strained through a 100μm (BD Falcon, San Jose, CA) followed by a 40μm cell strainer (BD Falcon, San Jose, CA) to form single cell suspensions. The isolated cells were washed three times in wash buffer after lysing the red blood cells. Cells were counted using a Countess automated cell counter (Invitrogen). The number of viable cells was assessed by counting after staining with trypan blue (Gibco Invitrogen).

Fluzone™ Preparation for Ex Vivo Analyses

Prior to use in ex vivo assays, Fluzone™ was dialyzed to remove additives that inhibit cell proliferation. Fluzone™ was placed into Spectra/Por® dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA) and submerged into PBS for 24h at 4°C. A Bradford
Assay (Bio-Rad Quick Start Bradford, Bio-Rad, Hercules, CA) was performed to establish the final protein concentration of Fluzone™, which was 18μg protein/mL.

Assessment of T-cell subpopulations

A total of $1 \times 10^7$ mononuclear cells per well from PBMC, spleen and MLN were plated in 96-well plates in a final volume of 200μL culture medium. Cells immediately underwent centrifugation and pellets were collected and resuspended in PBS/1% BSA/0.1% sodium azide until stained.

Assessment of Cell-Mediated Response

A total of 200,000 mononuclear cells per well from PBMC, spleen and MLN were plated in 96-well plates in a final volume of 200 μL culture medium for 48 or 72h at 37 °C under 5% CO₂. Cultures were stimulated at 0h with PHA (2.5μg/mL), Fluzone (10μg/mL), PMA + A23187 (125ng/mL and 1.25μg/mL respectively) or LPS (2μg/ml). After centrifugation, supernatants were collected from the 72h time point. Cultures from the 48 hr time point were suspended in PBS/1% BSA/0.1% sodium azide until stained. Ex vivo stimulations contain only piglets from the December replicate.

Phenotypic Identification of Mononuclear Cells from PBMC’s and Tissues for T-cells

The phenotypes of T lymphocytes subpopulations from PBMC, spleen and MLN were collected immediately after euthanasia and after ex vivo stimulation. Cell populations were determined by flow cytometry using a panel of fluorescently labeled mAbs. T lymphocytes were identified by a mouse anti-pig CD3:PE-Cy5 (Clone, Southern Biotec, Birmingham, AL). To
further differentiate T-cell populations, cells were stained with mouse anti-pig CD4:FITC (Clone, Southern Biotech) and mouse anti-pig CD8:PE (Clone, Southern Biotech). Staining procedures took place on ice and samples were removed from light when possible. In brief, each well were blocked with 5% mouse serum (Southern Biotec) and 200μg/mL purified mouse IgG (Invitrogen) for 5 min each. After centrifugation, CD3 was added to the wells and incubated for 20 min (50μL: CD3:PE-Cy5) and centrifuged again. CD4:FITC and CD8:PE were added (10μL each) and incubated for an additional 15 min until centrifuged. Cells were washed with PBS/1% BSA/0.1% sodium azide and then fixed with 2% paraformaldehyde. Cells were assessed using a LSRII flow cytometer (BD™, Biosciences, San Jose, CA). The percentage of T-cell subpopulations was determined using FlowJo 7.9 software (FlowJo, Ashland, OR) (Table 4.1). CD3+ events were considered T-cells. CD3+CD4+CD8- events were considered T-helper cells. CD3+CD4-CD8+ were considered cytotoxic T-cells. CD3+CD4+CD8+ were considered memory T-cells.

**Cytokine Production from Ex Vivo Stimulation**

An enzyme-linked immunosorbent assay (ELISA) was used to detect porcine IL-4, IL-6, IL-10, and IL-12/IL-23p40 from supernatants collected after 24h and 72h from the *ex vivo* stimulation. DuoSet’s of each ELISA were purchased from R&D Systems (Minneapolis, MN) and all concentrations of antibodies used were suggested by manufacturer. Flat bottomed plates (Nunc, Rochester, NY) were coated with 100μL coating antibody in coating buffer PBS [7.2 pH] and incubated overnight at 4°C. After incubation, the plates were washed three times with PBS/0.05% Tween 20. Then, 300μL of 1%BSA/PBS was added to each well to block non-specific binding for 2 h at RT. Following incubation, the plates were washed as before and
100μL of diluted supernatants and cytokine standard (provided by manufacturer) were added to each well and incubated for 2h at RT. Once the incubation passed, the plates were washed three times with PBS/Tween and 100μL detection antibody was added. After washing as before, 100μL of Streptavidin-HRP diluted in 1%BSA/PBS was added to each well for 20 min at RT and covered from light. After washing as described above, 100μl of TMB (BD Biosciences) was added and allowed to develop at RT. After 20 min, the reaction was stopped with 1M sulfuric acid. The plate was then analyzed on a microplate spectrophotometer (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) at 450nm and 570nm for correction. Concentrations of cytokines were calculated from the linear portion of the standard curve in pg/mL.

Statistical Analysis

Analyses were completed using PROC GLM (general linear model) procedure within SAS (Version 9.2, SAS Institute, Cary, NC). The initial model was replicate, diet and vaccination; however, the replicate was not significant and removed from the model. Diet included three groups: formula-fed (FF), lactoferrin-fed (LF) and sow-reared (SR) piglets. The model included vaccination, diet, and vaccination-diet interaction. If the vaccination was not significant, vaccinated and non-vaccinated were combined. If Lf was not significant, LF and FF piglets were combined. Daily weight gain was analyzed by Chi-squared test using the PROC MIXED procedure within SAS. Statistical significance was defined as p<0.05, with trends reported as p<0.10. All data are expressed as means ± SD.
Results

*T Lymphocyte Populations*

T-cell phenotype was analyzed by flow cytometry in PBMC, spleen and MLN T-cell populations. Representative images of lymphocytes gating is shown in **Figure 4.1.** In PBMC, cytotoxic T-cells (CD3+CD4-CD8+) and T-helper cells (CD3+CD4+CD8-) did not differ between dietary treatments, or vaccination (**Figure 4.2**). Given that vaccination did not have an effect on memory T-cells (CD3+CD4+CD8+), when NV and V were pooled, FF piglets did have a significantly greater percentage of memory T-cells than LF piglets (p<0.0388) (**Figure 4.3**). However, the percentage of memory T-cell in SR piglets was not significantly different from the two diet groups. Neither MLN (**Figure 4.4**) nor spleen (**Figure 4.5**) T-cell profiles were affected by diet or vaccination.

*Cell-Mediated Immune Response*

To determine cell-mediated immune responses, PBMC T-cell subpopulation differences were determined 48h after *ex vivo* stimulation at 37°C at 5% CO₂. CD8+ T-cell differences were seen between diet groups. FF piglets had higher amounts of CD8+ T-cells than LF or SR (p<0.0001). Average %CD8+ T-cells grouped by diet were: FF (8.96±2.81), LF (7.08±2.42) and SR (6.14±2.77). In addition, vaccinated piglets (7.67±2.78%) exhibited a trend towards increased percentage of CD8+ T-cells than NV piglets (6.82±2.72%) when all stimulations were combined (p=0.0529). Between stimulations, differences were only seen between PMA and unstimulated, PHA, Fluzone™ and LPS stimulations (p<0.0001). A trend was also observed for an interaction between vaccination and diet (p=0.0681). Specifically, FF-V was significantly different than LF-V, LF-NV, SR-NV and SR-V, and FF-NV was intermediate (**Figure 4.6**).
T-helper (CD4+) PBMC differences were also seen after *ex vivo* stimulation; however, vaccination did not have an impact on these changes. Significant differences were seen between each diet group after stimulation (p<0.0001): FF (49.30±15.84), LF (56.03±13.33) and SR (62.38±10.85). PMA was again significantly dissimilar from the following stimulations: unstimulated, PHA, Fluzone™ and LPS (p<0.0001). Additionally, an interaction was seen between vaccination and diet (p=0.0320). SR-V was significantly different LF-V, FF-NV and FF-NV; and SR-NV and LF-NV were intermediate (*Figure 4.7*).

Alterations in PBMC memory T-cells (CD3+CD4+CD8+) were also seen after *ex vivo* stimulation. Although vaccination had no effect on memory T-cell changes, memory T-cell populations were affected by diet (p=0.0400). LF piglets had decreased memory T-cells (7.83±4.40%) in comparison to FF (9.38±6.50%) and SR (9.21±5.39%) (*Figure 4.8*). Memory T-cell changes were also observed between stimulations, with PMA being significantly different from unstimulated, PHA, Fluzone™ and LPS (p<0.0001).

A cell-mediated response was seen from lymphocytes in the spleen. CD8+ T-cell populations were unaffected by vaccination, but differences were seen in diet groups. SR piglets (12.40±6.44%) had significantly greater amount of CD8+ T-cells in spleen (p=0.0054) than LF or FF piglets (9.93±4.65 and 9.77±4.48%, respectively) (*Figure 4.9*). Stimulation differences were seen between PMA and unstimulated, PHA, Fluzone™ and LPS (p<0.0001). A shift in T-helper cell population was also observed in the spleen by dietary treatments, although vaccination had no effect. FF piglets had a greater percentage of CD4+ T-cells (71.24±7.81) than SR piglets (59.40±14.27) but LF piglets did not differ between the two (65.61±13.88). PMA was again significantly dissimilar from the following stimulations: unstimulated, PHA, Fluzone™ and LPS (p<0.0001) (*Figure 4.10*). Additionally, memory T-cell differences were seen between
diet groups. SR piglets had higher amounts of memory T-cells than LF or FF (p=0.0004). Average percent memory T-cells grouped by diet are: FF (7.50±1.52), LF (6.52±1.77) and SR (10.48±7.21). Between stimulations, differences were seen only between PMA from unstimulated, PHA, Fluzone™ and LPS stimulations (p<0.0001). An interaction involving vaccination and diet (p=0.0681) was also observed. FF-V was statistically different from SR-NV and SR-V, while LF-V and LF-NV were intermediates (Figure 4.11).

MLN CD8+ T-cell differences were seen after ex vivo stimulation in vaccination and stimulation. While diet had no effect, vaccinated piglets (6.94±3.10%) had higher cytotoxic T-cells when stimulated than non-vaccinated piglets (5.98±3.13) (p=0.0293). Stimulation with PMA was also significantly different from unstimulated, PHA and LPS (p<0.0001). An interaction between vaccination and diet was also seen (p=0.0480). FF-V was statistically different from FF-NV, while groups SR-NV, SR-V, LF-NV and LF-V are intermediates (Figure 4.12). However, no significant differences were seen in MLN T-helper cells (Figure 4.13) or memory T-cells (Figure 4.14) due to stimulation (Model: p=0.2275, p=0.8275 respectively).

Cytokine Production from Cell-Mediated Immune Response

To further investigate cell-mediated immune responses, cytokines IL-4, IL-10, IL-12 and IL-6 were analyzed after ex vivo stimulation in spleen and MLN tissues. Cytokines not included were below detectable limits, and therefore not added to the model. After stimulation, IL-4 was below detectable limits from spleen and MLN tissues. In addition, cytokine levels after 24hr stimulation were undetectable, therefore only 72h is reported. For spleen IL-10 cytokine production, vaccination and diet differences were not observed; however, differences were
detected between PHA and PMA stimulations (p<0.0001) (Figure 4.15). Although vaccination and diet differences were not observed, IL-12 cytokine production from spleen supernatants was significantly different among stimulations PHA, PMA and LPS (p<0.0001). Additionally, an interaction between diet and vaccination was observed (p=0.0370). SR-NV were statistically different from FF-NV, while groups SR-V, LF-NV, LF-V, FF-V are intermediates (Figure 4.16). Separately, IL-12 cytokine production after Fluzone™ stimulation had significant diet effects, if vaccination was removed (p=0.0394) (Figure 4.17). LF animals had higher secretion of IL-12 than SR piglets, but FF piglets did not differ from either. IL-6 production in spleen was detected after Fluzone™ and LPS stimulations, however no differences were seen (p=0.0632) (Figure 4.18).

MLN supernatants also saw cytokine differences between groups. Although vaccination and diet differences were not observed, IL-10 cytokine production from MLN supernatants were significantly different between PHA and PMA stimulation (p<0.006) (Figure 4.19). MLN IL-10 was below detectable limits in unstimulated, Fluzone™ and LPS stimulations. For MLN IL-12 cytokine production, vaccination differences were not observed, yet diet effects were detected (p=0.0041) (Figure 4.20). Importantly, IL-12 was detected from unstimulated cells, but not from Fluzone™ or LPS, yet significance was seen by stimulation (p<0.0001). FF piglets were statistically different that SR piglets, while LF was an intermediate (p=0.0041). No differences were observed from MLN IL-6 cytokine production (p=0.9802) when stimulated with PMA. MLN IL-6 was also below detection for unstimulated, PHA, Fluzone™ and LPS samples (Figure 4.21).
Figure 4.1. Example of flow cytometry T-lymphocyte separation and gating. T lymphocytes were identified by a mouse anti-pig CD3:PE-Cy5 and mouse anti-pig CD4:FITC and mouse anti-pig CD8:PE to further differentiate T-helper and cytotoxic T-cells, respectively. In the forward side scatter (FSC) and side scatter (SSC), the lymphocytes were gated and selected (panel a). Then, the CD3+ T-cells were selected (panel b). After selecting within this population, a graph was generated for the CD4+ and CD8+ T-cells (panel c) using anti-CD4 and anti-CD8. Based on single stains, the gating was set and percentages of these populations are obtained. CD8+ T-cells are shown in the upper left quadrant, CD4+ T-cells are shown in the lower right quadrant, and double positive memory T-cells are shown in the upper right quadrant.
Figure 4.2. **Average PBMC T-cell populations.** No statistical differences were seen between vaccination and diet groups in CD4+, CD8+ and CD4+CD8+ T-cells. Data are expressed as means±SD.
Figure 4.3. PBMC Memory T-cell Populations. T-cell populations are expressed as a percent of CD3+ events. The memory T-cell population in PBMC was affected by diet, when vaccination was removed from the model (p=0.0388). FF piglets had significantly higher memory T-cells than LF piglets, but SR piglets did not differ between the two. Data are expressed as means±SD. Different letter superscripts indicate statistical differences.
Figure 4.4. Average T-cell Populations in MLN. T-cell populations are expressed as a percent of CD3+ events. No significant differences were seen between vaccination or diet groups in CD4+, CD8+ and CD4+CD8+ T-cells. Data are expressed as means±SD.
Figure 4.5. T-cell Populations in Spleen. No significant differences were seen between vaccination or diet groups in CD4+, CD8+ and CD4+CD8+ T-cells. Data are expressed as means±SD.
Figure 4.6. PBMC CD8+ T-Cell Populations following 48hr Stimulation. T-cell populations are expressed as a percent of CD3+ events. FF piglets had significantly greater CD8+ T-cells after stimulation than LF or SR piglets (p<0.0001). In addition, PMA was statistically different from all other treatments (p<0.0001). Data are expressed as means±SD. Different letter superscripts indicate statistical differences.
Figure 4.7. PBMC CD4+ T-Cell Populations following 48hr Stimulation. T-helper cell differences were seen due to diet and stimulation after ex vivo stimulation. T-cell populations are expressed as a percent of CD3+ events. Denoted by letters, FF piglets had the least percentage of CD4+ T-cell compared to LF and then SR piglets (p<0.0001). PMA was statistically different than all stimulations (p=0.0002). An interaction between diet and vaccination (p=0.0320) are denoted by LSM: SR-V is A, SR-NV and LF-NV are denoted AB, and LF-V, FF-NV and FF-NV are denoted B. Data are expressed as means±SD. Different letter superscripts indicate statistical differences.
Figure 4.8. PBMC Memory T-Cell Populations following 48hr Stimulation. PBMC memory T-cell differences were seen due to diet and stimulation after ex vivo stimulation. T-cell populations are expressed as a percent of CD3+ events. Although diet differences were seen (p=0.0400), LSM denoted all groups A. PMA was statistically different from all other stimulations (p<0.0001). Data are expressed as means±SD.
Figure 4.9. CD8+ T-Cell Populations in Spleen following 48hr Stimulation. Spleen CD8+ T-cell differences were seen due to diet and stimulation after *ex vivo* stimulation. T-cell populations are expressed as a percent of CD3+ events. While vaccination had no effect, SR piglets had greater CD8+ T-cells than LF or FF piglets (p=0.0054). In addition, PMA stimulation was statistically different from unstimulated, PHA, Fluzone and LPS (p<0.0001). Data are expressed as means±SD. Different letter superscripts indicate statistical differences.
Figure 4.10. CD4+ T-Cell Populations in Spleen following 48hr Stimulation. Spleen CD4+ T-cell differences were seen due to diet and stimulation after ex vivo stimulation. T-cell populations are expressed as a percent of CD3+ events. While vaccination had no effect, SR (A) piglets had greater CD4+ T-cells than LF(AB), even more so than FF (B) piglets (p=0.0006). Data are expressed as means±SD. Different letter superscripts indicate statistical differences.
Figure 4.11. Memory T-Cell Populations in Spleen following 48hr Stimulation. Spleen Memory T cell differences were seen due to diet and stimulation after ex vivo stimulation. T-cell populations are expressed as a percent of CD3+ events. While vaccination had no effect, SR (A) piglets had greater memory T cells than LF and FF (B) piglets (p=0.0004). PMA was also significantly different (p<0.0001) from stimulations: unstimulated, PHA, Fluzone and LPS. A diet and vaccination interaction was additionally seen. The interaction was observed in SRNV piglets (A), which were statistically different from CN, CV, LFNV, LFV, and SRV groups (B) (p=0.0147). Data are expressed as means±SD. Different letter superscripts indicate statistical differences.
Figure 4.12. CD8+ T-Cell Populations in MLN following 48hr Stimulation. MLN CD8+ T-cell differences were seen vaccination and stimulation after ex vivo stimulation. T-cell populations are expressed as a percent of CD3+ events. While diet had no effect, vaccinated piglets had higher cytotoxic T-cells when stimulated than non-vaccinated piglets (p=0.0293). Stimulation PMA was also significantly different from unstimulated, PHA and LPS (p<0.0001). An interaction between vaccination and diet was additionally seen (p=0.0480). Data are expressed as means±SD.
Figure 4.13. CD4+ T-Cell Populations in MLN following 48hr Stimulation. MLN CD4+ T-cell differences were not observed after ex vivo stimulation (p=0.2275). T-cell populations are expressed as a percent of CD3+ events. Data are expressed as means±SD.
Figure 4.14. Memory T-Cell Populations in MLN following 48hr Stimulation. MLN memory T-cell differences were not observed after ex vivo stimulation (p=0.8275). T-cell populations are expressed as a percent of CD3+ events. Data are expressed as means±SD.
Although vaccination and diet differences were not observed, IL-10 cytokine production from spleen supernatants were significantly different from one another by stimulation (p<0.0001). Unstimulated and Fluzone™ samples were below detection. Data are expressed as means±SD.

Figure 4.15. IL-10 Secretion by Spleen T-cells.
Figure 4.16. IL-12 Secretion by Spleen T-cells. Although vaccination and diet differences were not observed, IL-12 cytokine production from spleen supernatants were significantly different by stimulation (p<0.001). In addition, an interaction between diet and vaccination was seen (p=0.037). Data are expressed as means±SD.
Figure 4.17. IL-12 Secretion by Spleen T-cells differed by Diet. When vaccination was removed from the model, diet differences were observed in IL-12 production from spleen tissues when stimulated with Fluzone™ (p=0.0394). Data are expressed as means±SD. Different letter superscripts indicate statistical differences.
Figure 4.18. IL-6 Secretion by Spleen T-cells. No statistical differences were observed of IL-6 production from spleen tissues after ex vivo stimulation (Model: p=0.0632). Data are expressed as means±SD.
Figure 4.19. IL-10 Secretion by MLN T-cells. Although vaccination and diet differences were not observed, IL-10 cytokine production from MLN supernatants were significantly different by stimulation (p=0.006). Data are expressed as means±SD.
Figure 4.20. **IL-12 Secretion by MLN T-cells.** Vaccination differences were not observed in MLN IL-12 cytokine production, yet diet differences were detected (p=0.0041). Importantly, IL-12 was detected from unstimulated cells, but not from Fluzone™ or LPS. Data are expressed as means±SD. Different letter superscripts indicate statistical differences.
Figure 4.21. IL-6 Secretion by MLN T-cells. No differences were observed from MLN IL-6 cytokine production (Model: p=0.9802). Unstimulated, PHA, Fluzone™ and LPS were below detection. Data are expressed as means±SD.
Discussion

Lactoferrin (Lf) is a glycoprotein expressed in biological fluids to transport iron in milk and other secretions functioning as a major component to the innate immune system (Gonzalez-Chavez et al., 2009). It has many biological implications, including immune response function as an antioxidant (Mulder et al., 2008) and protection against microbial (Moshynskyy et al., 2003) and viral pathogens (Hung et al., 2010). In addition, Lf has been shown to exhibit anti-inflammatory properties (Puddu et al., 2011). This study examined infant immune development using the neonatal piglet model to further investigate bLf on vaccination and T-cell phenotype responses. Overall, vaccination and serum Ig profiles in the neonatal piglets were minimally affected by dietary treatment of bLf. Additionally, bLf was found to have little impact on T-cell phenotype or cell-mediated immune responses. bLf was also found to have no negative impacts on development, dietary tolerance or immune characteristics analyzed.

One major difference between the human infant and neonatal piglet is the absence of immunoglobulin transfer during prenatal development in piglets, leaving their immune system vulnerable to infection after birth (Sangild, 2003). Therefore, piglets are dependent on colostrum and milk as a source of external antigens for maturation of the immune system. Limited amounts of stimuli have been shown to decrease memory T-cells and mature B-cells at birth in piglets (Šinkora et al., 2009). A difference in the piglet immune system from humans and various other species is the high percentage of double positive CD4+CD8+ T-cell subpopulations. This subpopulation, which is considered to represent memory T-cells, have been shown to rise with age and maturation of the immune system (Zuckermann and Gaskins, 1996). Although dietary treatments and vaccination had minimal impact on the percentage of memory T-cells in PBMCs, MLN and spleen, a dietary impact was found in PBMC when vaccination was removed. Dietary bLf decreased the percentage of memory T-cells in PBMC in comparison to SR and FF piglets.
Although no research has been examined memory T-cell populations from orally supplementation of Lf, vaccination has induced generation of memory T lymphocytes in swine (Ober et al., 1998).

Infants are born with a T<sub>H</sub>2 bias, which must be down-regulated successfully to a T<sub>H</sub>1 response since the T<sub>H</sub>1 associated cytokines are necessary to defend the infant effectively against bacterial and viral infections by aggressively attacking foreign antigens (Reinhard et al., 1998). The decline in neonatal T<sub>H</sub>1 responses are observed from decreased T<sub>H</sub>1 associated cytokines, such as IL-12 and IFN-γ, produced by CD4+ T-cells and macrophages (Marodi, 2006). Recently, in vivo models have shown that orally supplementation of pLf increased T<sub>H</sub>1 cytokines such as IFN-γ and IL-12 suggesting that Lf could increase cell-mediated immunity (Hung et al., 2010). Hung and researchers used concentrations 2.0% w/w pLf as a feedstuff additive, which is greater than used in this study.

The ability of Lf to modulate antigen presentation to affect T-cell activity is associated with the cell types that have Lf receptors (Suzuki et al. 2005). For example, monocytes and lymphocytes, which contain Lf receptors, aid in the maintenance and generation of antigen-specific B and T-cells. Lf has been shown to affect the contribution of lymphocytes to function as antigen-presenters to activate CD4+ T-cells to produce IFN-γ (Hwang et al. 2007). In addition, Lf has been shown to increase IBD vaccination response in chickens when orally supplemented (Hung et al., 2010). In this study, oral supplementation of Lf had no effect on vaccination response in neonatal piglets, although a trend was observed that suggests SR enhances antibody response (p=0.0953). The ability of breastmilk to enhance antibody responses has been previously demonstrated in infants (Silfverdal et al., 1999). Although dietary Lf had no impact of IgM and IgG concentration in serum, it has been observed that Lf can enhance Ig
concentrations on mucosal surfaces (Debbabi et al., 1998). Differences in total IgM that were identified, in comparison to total IgG, could be due to low concentrations of IgM in serum.

Although no dietary differences in PBMC T-cell subpopulations were observed, changes in T-cell populations upon ex vivo stimulation were observed. FF piglets had decreased CD4+ T-cells after mitogen stimulation compared to LF or SR piglets (p<0.0001). This is interesting because an increase in CD4+ T-cell suggests that Lf may be contributing to increase antigen presentation. In addition, FF piglets exhibited greater PBMC CD8+ T-cell populations after ex vivo stimulation (p<0.0001) compared to LF and SR piglets, suggesting that Lf may be modulating the cell survival of CD8+ T-cells. This increased PBMC CD4+ T-cell survivability upon stimulation could be caused by T<sub>H</sub>1 associated cytokines promoting differentiation of naïve CD4+ T-cells into effector CD4+ T-cells, thereby increasing protection against infectious pathogens (Marodi, 2006).

Hwang and colleagues (2009a) have shown that Lf used as an adjuvant with BCG vaccine up-regulated CD4+ T-cells producing IFN-γ in splenocytes of animals. Again in this study, T-cell subpopulations of splenocytes were unaffected by dietary Lf; however, under mitogen stimulation ex vivo dietary differences were observed. CD4+ T-cells in splenocytes were greater in SR piglets versus FF piglets, but LF piglets were not statistically different from either group (p=0.0006). This is interesting because supplementation of bLf may be modulating CD4+ T-cell survival in splenocytes upon activation. In addition, CD8+ splenocytes were greater in SR in comparison to FF and LF piglets (p=0.0054) which is consistent with higher percentages of CD8+ T-cells in mononuclear cells from breastfed infants (Hawkes et al., 2010). Our research showed that Fluzone™ stimulation of splenocytes increased IL-12 secretion in LF piglets compared to SR piglets. In addition, SR piglets had greater IL-12 secretion than FF in
MLN under stimulation, but LF piglets were not significantly different from either dietary treatment. These data suggest that Lf is promoting $T_H1$ cytokines, thereby enhancing the neonate’s ability to fight infection (Marodi, 2006).

Little vaccination effects were observed, although piglets that were vaccinated had higher CD8+ T-cells than piglets than were non-vaccinated in MLN after stimulation ($p=0.0293$). Intranasal immunization of mice suggests that antigen-specific proliferating T cells can be found is distal lymph nodes, suggesting migration of locally primed T-cells (Ciabattini et al., 2011).
Chapter 5

Conclusions and Future Directions

This study focused on the impact of Lf, a bioactive component found in human milk, on vaccination response, serum IgG profiles and the T-cell phenotype response after mitogen stimulation. Our first objective was to assess the ability of Lf to influence antibody production in a neonatal model against vaccination versus FF piglets. It is critical to investigate the differences in formula additives to enhance our understanding of how breastfeeding is able to attribute to immune development and maturation. The implication of this research can be extensive, especially when applied to improving the quality of the neonate’s immune system and its capability to fight infectious pathogens. Generally, our findings showed that Lf had little impact on vaccination response, serum Ig profiles and T-cell subpopulations, but did impact T-cell populations after stimulated conditions.

Future investigation involving Lf on vaccination response could be focused in mucosal immune response. Because Lf is produced by mammary and found in mucosal secretions, it may have a greater impact on the response to vaccination with FluMist®, the live attenuated influenza vaccination (LAIV) (Lonnerdal and Iyer, 1995). It has also been shown that LAIV vaccination in children 6-months to 4-years of age are able to develop virus specific IFN-γ CD4+ and CD8+ T-cells, similar to the currently used trivalent influenza vaccination (TIV). In addition, more research could examine various infection models in order to further the understanding of whether Lf can impact immunological differences in antibody response and T-cell subpopulations. This would aid in identification the characteristics necessary to heighten the infant’s ability to elevate the appropriate immune response to a vaccination.
Chapter 6

References


Appendix A

Supplemental Information: T-cell Population Changes after 48 hour in Culture and T-cell Population Summaries

Alterations in PBMC Subpopulations after 48h Culture

T-cell subpopulation changes were seen after 48hr culture. PBMC CD8+ T-cells were significantly different after 48 incubation (p<0.0001). Before culture, the combined T-cell population was 18.357±3.32 %CD8+ T-cells and after culture the CD8+ T-cells decreased to 7.785±2.711% (Figure A.1). Opposing, CD4+ T-cells significantly decreased after 48h in culture (p<0.0184): T-cell population 37.25±12.68 %CD4+ T-cells and 48h culture is 50.025±16.64 % CD4+ T-cells (Figure A.2). T-cell subpopulation changes were also seen in memory T-cells (CD3+CD4+CD8+). After 48h, memory T-cells significantly decreased (p<0.0001) from 8.638±3.319 % memory T-cells to 5.886±2.811% memory T cells (Figure A.3).

T-Cell Populations

Summarization of spleen (Figure A.4) and MLN (Figure A.5) T-cell populations, no significance was observed.

Fluzone IgG Concentration

Fluzone IgG concentration from August 2011 replicate only. Data is expressed as optical density from the 1:100 dilution from the sample. Data could not be combined with the November and December 2011 replicates due to sow-reared piglets response to Fluzone™ vaccination (Figure A.6). On d21, vaccinated piglets were greater than non-vaccinated (p<0.0001). SR
piglets had higher Fluzone IgG compared to FF and LF groups (p<0.0001). Also, an interaction was observed between vaccination and diet (p<0.0001). LSM denoted SR-V as A, and all groups were denoted B.
Figure A.1. CD8+ PBMC T-cell Population Changes After 48hr in Culture. CD8+ T cells decreased more than half after 48h in culture (Model: p<0.0001). Data are expressed as means±SD.
Figure A.2. CD4+ PBMC T-cell Population Changes After 48hr in Culture. CD4+ T cells increased by ~13% after 48h in culture (Model: p=0.0529). Data are expressed as means±SD.
Figure A.3. Memory PBMC T-cell Population Changes After 48hr in Culture. Memory T cells decrease by ~3% after 48h in culture (Model: p=0.0017). Data are expressed as means±SD.
Figure A.4. Spleen T-cell Subpopulations after *ex vivo* stimulation. Data are expressed as means±SD.
Figure A.5. MLN T-cell Subpopulations after *ex vivo* stimulation. Data are expressed as means±SD.
Figure A.6. Circulating Fluzone™ IgG Concentration from August 2011 replicate on d21. On d21, vaccinated piglets were greater than non-vaccinated (p<0.0001, not shown). SR piglets had higher Fluzone IgG compared to FF and LF groups (p<0.0001). Also, an interaction was observed between vaccination and diet (p<0.0001). Data are expressed as means±SD.
Appendix B

Supplemental Information: Incubation of T-cells with Fluzone™ Peptides has no Effect on T-cell Ratio

Fluzone Peptide T-cell Stimulation

In March 2011, a pilot was conducted to stimulate PBMC from piglets vaccinated with Fluzone™ on d7 and d14 then euthanized on d21 as previously performed. After isolation of the PBMC, 1 x 10⁶ cells/ml were plated. Only vaccinated piglets were analyzed for this pilot. Samples were unstimulated, or stimulated with: Fluzone™ (Sanofi Pasteur, Swiftwater, PA) at concentrations 1μg/ml, 5μg/ml or 10μg/ml. PBMC from vaccinated piglets were additionally stimulated with peptides: ProMix Influenza A NP Protein, ProMix Influenza A HA Brisbane, ProMix Influenza A MP2 Protein (thinkpeptides, Oxford, UK) at concentrations 1μg/ml, 5μg/ml, 10μg/ml, 50μg/ml and 100μg/ml. In same concentrations, the individual peptides were mixed equally and used to stimulate the cells. In comparison to unstimulated PBMC, Fluzone™ stimulation showed a marked increase in CD4+:CD8+ T-cell ratio in concentrations 5μg/ml and 10μg/ml. Thus, our methods in Chapter 4 stimulated cells from PBMC and spleen with Fluzone™ at 10μg/ml. Although, no differences were observed when cells were stimulated with individual peptides or mixed together.
Figure B.1. Ratio of CD4+:CD8+ T-cells After Fluzone™ Peptide Stimulation. 48hr Fluzone peptide stimulation had no impact on CD4+:CD8+ ratio (Model: p<0.7804).