ABSTRACT

KICK, ANDREW ROBERT. Effects of Stress on the Adaptive Immune System in Pigs. (Under the direction of Glen William Almond.)

The purpose of this study was to investigate the effects of stress on the adaptive immune system in pigs. A preliminary study, using a Mycoplasma vaccination after weaning, was conducted in order to validate the use of flow cytometry. This study provided critical information required to refine the timing of blood collection and flow cytometry procedures for subsequent studies.

The first study used nineteen crossbred pigs that were weaned at 18 days of age. Blood samples were collected when pigs were 11, 17, 19, 20, 21 and 25 days of age and were analyzed for peripheral blood cell percentages and concentrations of neutrophils, lymphocytes, T cell subsets, mature B cells and cortisol concentrations. Cortisol concentrations increased at weaning ($p<.0001$); lymphocyte concentrations tended to decrease at weaning ($p=.091$); and the N:L ratio ($p<.0001$) and CD4:CD8 ratio ($p=.0459$) both increased between day 20 and day 21. Age-related changes in the percentages of T cell subsets and B cells were consistent with previous reports. It was concluded that weaning is a stress-inducing event; however, weaning did not affect the composition of T cell subsets and B cells in the peripheral blood.

The effects of a chronic stress induced by 5 days of mixing and crowding on the adaptive immune system were evaluated when the pigs were 47 days of age. Pigs were randomly assigned into one of two treatments: STRESS (n=9 pigs) and CONTROL

(n=10 pigs). Peripheral blood was examined on days 47, 52, 53, 54, 55 and 62 for the same measures described previously and additionally for in-vitro IFN-γ and IL-4 production following ConA stimulation. Though there were some significant differences between the treatments upon conclusion of the stress, the STRESS pigs did not appear to be immunologically compromised.

The second study used twenty-three crossbred pigs and examined the effects of weaning age on the adaptive immune system. Pigs were randomly assigned to one of three treatments: weaning at 14 (n=8 pigs), 21 (n=7 pigs) or 28 (n=8 pigs) days of age. Peripheral blood was obtained when pigs were 13, 15, 20, 22, 27, 29 and 35 days of age and analyzed for the same stress-related variables evaluated in the first study. In all treatments, weaning affected cortisol concentrations ($p<.0001$) and body weight percent change ($p<.017$). Lymphocyte concentrations displayed a treatment effect for pigs weaned at 14 days of age ($p=.0744$) and at 28 days of age ($p=.0139$). Other significant differences occurred between treatments; however, the differences were not directly associated with weaning. Based upon the immunological measures utilized in this study, there was not an explicit benefit to the adaptive immune system for any weaning age.

In summary, chronic stress and early weaning do not negatively affect the adaptive immunological competence of pigs as determined by changes in populations of immune cells and cortisol concentrations. Additional studies comparing chronic stress or weaning age in a commercial herd and its effect on disease frequency are required in order to adjudicate for issues of animal welfare.

Effects of Stress on the Adaptive Immune System in Pigs

by
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BIOGRAPHY

Andrew Robert Kick was born on June 10, 1977, to Stephen and Kay Kick in Ohio. He graduated from Loudonville High School in Loudonville, Ohio, in 1996. He graduated from the University of Dayton in 2000 with a bachelor of science in Biology. Upon graduation, he was commissioned as an officer in the United States Army. His military duty locations include Fort Leonard Wood, MO; Camp Casey, Republic of Korea (ROK); Fort Huachuca, AZ; Fort Drum, NY; Baghdad, Iraq; Pittsburgh, PA; Yongsan Garrison, ROK; and NC State University. His positions have included battalion chemical officer, smoke platoon leader, company executive officer, brigade assistant intelligence officer, battalion intelligence officer, assistant professor of military science, brigade assistant operations officer, company commander and currently graduate student. Upon completion of his thesis, he will become an instructor at the United States Military Academy at West Point, NY.

Andrew married Veronica Lee Kick in 2003. They have three children: Mark, Maryann and Matthew. Andrew hopes that through the pursuit of this thesis, God, the Creator and Sustainer of all life, and Jesus Christ, his Lord and Savior, would be glorified.

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LITERATURE REVIEW

Introduction

Stress and animal welfare continues to be a major food industry and political issue. Groups, such as the Humane Society of the United States, support legislation that would significantly change many current practices in the US commercial food industry and particularly in the swine industry. In the European Union, weaning pigs before 28 days of age is normally prohibited (ECCD, 2001). Much of the debate centers on perceived stress that occurs in animals due to established husbandry practices and presumed degradation of animal welfare. One potential indication of decreased animal welfare is a greater occurrence of disease within an observed herd, as a result of a compromised immune system.

The two primary immune responses to micro-organisms and their antigens include the innate and adaptive (acquired) immune systems. In general, the adaptive immune response, coordinated by the bone marrow derived lymphocytes (B cells) and thymus-derived lymphocytes (T cells), is the most important component of the host response to pathogenic viruses, bacteria, and vaccination.

Various studies endeavored to assess the effects of stress on pigs from gestation through finishing. Several stressors included social interaction (crowding/mixing) (Morrow-Tesch et al., 1994), temperature variation (Hicks et al., 1998), restraint (Tuchscherer et al., 2002), transportation (Sutherland et al., 2009), food deprivation

(Webel et al., 1997), weaning (Blecha et al., 1983), and light/dark cycles (Niekamp et al., 2006). In addition to utilizing common stress hormones (i.e. cortisol) to quantify whether the animals were stressed, several common methods were used to examine the immune function: neutrophil to lymphocyte ratios (Hyun et al., 2005), B-cell development and immunoglobulin levels (Blecha and Kelley, 1981a), flow cytometry (Tuchscherer et al., 2009), and lymphocyte proliferation (Tuchscherer et al., 1998). The results of these studies varied in documenting whether the animals were stressed, what the general effects were on the porcine immune system and in particular, which cells of the adaptive immune system were affected.

The primary goal of this literature review is to describe the stress response in pigs, the common types of perceived stress associated with U.S. swine husbandry practices, the immunological and corticosteroid effects by the stress, and then in greater detail provide an overview on T cells in swine and how stress affects those critical cells of the adaptive immune system.

Stress

Characteristics of Stress

Stress is now a common term in reference to humans, animals, structures and geology. Hans Selye was a pioneer in the 20th century as he investigated the hypothalamic-pituitary-adrenal (HPA) axis and attempted to describe the relationship between stress, the HPA axis and disease (Selye, 1955). He described the stress response

in three stages: alarm, adaptation and exhaustion (Selye, 1998). Stress is the result of any number of changes in a controlled variable or stimuli which affects an animal's homeostasis and results in an error signal being generated by a sensor. The error signal is processed by an integrator and the correcting signal is executed by an effector. The reflex response from the effector provides negative feedback to minimize the change in the controlled variable (Grossfeld, 2004). Stress also may be defined as the relationship between a stressor, the brain's perception of the stressor and the integrated physiological response to the stressor (Dhabhar, 2002). Allostasis and allostatic load are an additional description of the body's response to stress. Allostasis is the physiological response in order to maintain homeostasis and allostatic load is the effect of maintaining homeostasis when physiological systems no longer operate as effectively (Sterling and Eyer, 1988; McEwen, 1998).

Within physiologically tolerable ranges, changes in controlled variables result in quick adjustments to maintain homeostasis in the animal. Numerous studies have attempted to prove that controlled stress (mental and physical) is beneficial to animals (Tuchscherer et al., 1998; Ernst et al., 2006; Krebs and McGlone, 2009). More significantly, numerous researchers over the past 50 years have endeavored to quantify the negative effects of stress on domestic animals. Acute stress is of a limited duration normally lasting seconds to hours, and the animal is usually able to quickly re-establish homeostasis with minimal long-term effects (McEwen, 1998; Dhabhar, 2002). Chronic stress extends over a greater duration of time (several hours to days or months) and causes extended adaptation to maintain homeostasis in the stressed animal. The effects of

chronic stress are of great medical and veterinary consequence and are linked to immunosuppressive or immunomodulatory function (McEwen, 1998; Dhabhar, 2000; Dhabhar, 2002).

Biological Effects of Stress

The biological effects of stress in domestic animals are diverse. Minton (1994) extensively reviewed the effects of acute stress on the HPA axis and the sympathetic nervous system. The HPA axis consists of the following glands and primary peptides produced by the glands. The paraventricular nucleus of the hypothalamus produces corticotropin-releasing hormone (CRH) and activates the anterior lobe of the pituitary gland producing adrenocorticotropic hormone (ACTH). ACTH activates the adrenal cortices of the adrenal glands which in turn release cortisol. Cortisol has many effects and targets once it enters the periphery blood to include negative feedback on the hypothalamus and pituitary gland, cardiac function, digestion, water/salt balance, brain function and immunity.

Additionally, beyond the HPA axis, stress affects other biological systems. Though difficult to test due to the rapid fluctuation in epinephrine, several studies have observed increases in epinephrine upon application of a stressor (Minton, 1994). Additionally, stress affects the central nervous system, which integrates all sensory function and regulates biological function through the autonomic nervous system (McEwen, 1998).

In livestock, it is common to study animal behavior in order to assess whether animals are more stressed based upon the frequency with which they engage in different behaviors (Morrow-Tesch et al., 1994; Hicks et al., 1998).

Endocrine Effects of Stress

The endocrine effects of stress in domestic animals are diverse. In order to determine if the stress is significant, investigators examined several key determinants. Primarily, cortisol concentrations were used as a measure of stress in an animal. Cortisol concentrations are diurnal with a peak in the morning and a nadir in the afternoon, so it is critical that blood is sampled at the same time each day. Normal serum cortisol levels in weanling pigs range from 30-93 ng/ml (Niekamp et al., 2007; Sutherland et al., 2009). Normal serum cortisol in nursery pigs range from 30 – 97 ng/ml and the mean concentrations tend to be higher than in weanling pigs (Deguchi and Akuzawa, 1998; Hicks et al., 1998; Sutherland et al., 2005; Sutherland et al., 2006). When under stress, cortisol levels can be as high as 225 – 303 ng/ml (Heo et al., 2005). Additional measures of stress include corticosteroid-binding globulin (CBG), which regulates plasma cortisol concentrations (Heo et al., 2005), and adrenocorticotropic hormone (ACTH), which is released by the anterior lobe of the pituitary gland and stimulates cortisol synthesis by the adrenal glands (Minton, 1994).

Types of Stress Common to Pigs

Within commercial pig production, there are common practices that result in perceived stress in animals. Debatably, the most significant stress in a pig's life (other than birth) is weaning, which involves several key changes for the animal. Highlighting the changes, the pigs are removed from the sow, their food changes from milk to solid feed, the pigs are transported to a nursery pen or offsite facility, and the pigs are mixed with new penmates and forced to establish a new hierarchy. Several investigators have studied the various effects of weaning stress. Other common stressors and studies examining their effect are listed in Table 1.

Table 1: Types of stress common in pigs

Type of Stress	**Papers**
Weaning	(Blecha and Kelley, 1981b; Blecha et al., 1983; Borghetti et al., 2006; Davis et al., 2006; Kojima et al., 2008)
Social Interaction (crowding/ mixing / isolation)	(McGlone et al., 1993; Morrow-Tesch et al., 1994; Deguchi and Akuzawa, 1998; Tuchscherer et al., 1998; de Groot et al., 2001; Kanitz et al., 2004; Tuchscherer et al., 2004; Heo et al., 2005; Sutherland et al., 2006; Tuchscherer et al., 2006; Sutherland et al., 2007)
Restraint	(Tuchscherer et al., 2002)
Transportation	(McGlone et al., 1993; Ekkel et al., 1996; Hicks et al., 1998; Kojima et al., 2008; Sutherland et al., 2009)
Food deprivation	(Webel et al., 1997; Toscano et al., 2007)
Light/dark cycles	(Niekamp et al., 2006; Niekamp et al., 2007)
Novel environments	(Lewis et al., 2008; Krebs and McGlone, 2009)
Disease	(Webel et al., 1997; de Groot et al., 2001; Sutherland et al., 2007)
Temperature	(Blecha and Kelley, 1981a; Blecha and Kelley, 1981b; Minton et al., 1988; Morrow-Tesch et al., 1994; Hicks et al., 1998; Carroll et al., 2001; Heo et al., 2005; Hyun et al., 2005; Sutherland et al., 2007)

Adaptive Immune System

Types of Lymphocytes and T-Lymphocytes

There are three primary classes of lymphocytes in the pig: Natural Killer (NK) cells, B cells, and T cells. NK cells are unique in their role within the innate immune system and their function continues to be better understood. T cells and B cells comprise the fundamental components of the adaptive immune response. Clusters of Differentiation (CD) are the primary method utilized for distinguishing different subsets of lymphocytes and all immune cells. NK cells are $CD2^+$ $CD3^-$. The ubiquitous marker for mature B cells is CD21. All T cells can be distinguished by the expression of CD3, which is part of the T cell antigen receptor (TCR) (Murphy et al., 2008).

Within pigs, there are multiple unique populations of T cells located in the peripheral blood. T cells are further divided by the TCR into αβ-T Cells and γδ-T Cells based upon the specific domains of the TCR chains. The distinguishing co-receptor molecules further differentiate T cells with mature cells for the most part either expressing CD4 or CD8 (Murphy et al., 2008). Unique to pigs when compared to humans or mice, porcine peripheral T cells may also be "double positive" expressing both CD4 and CD8 (Gerner et al., 2009). In mice and humans, double positive T cells are found only when maturing in the thymus. T cells with αβ-TCRs express both co-receptor molecules CD4 or CD8. $CD4^+$ T cells are called T helper cells. $CD8^+$ T cells are called cytotoxic T cells. For γδ-T Cells, the majority do not express either CD4 or CD8; however, there are some γδ-T Cells that express CD8 (Gerner et al., 2009). There are many additional markers to differentiate the different T cell phenotypes or activation

states; however, Table 2 illustrates the different types of T cells found in the peripheral blood of pigs (Gerner et al., 2009), which are examined within this research.

Table 2: T cell Subsets and Phenotypes

T cell subset	Phenotype
Cytotoxic T cells	$CD3^{+}CD8\alpha^{+}$ TCR- $\alpha\beta^{+}$
T helper cells	$CD3^{+}CD4^{+}$ TCR- $\alpha\beta^{+}$
Double Positive T cells	$CD3^{+}CD4^{+}$ $CD8\alpha^{+}$ TCR- $\alpha\beta^{+}$
γδ-T Cells	$CD3^{+}CD8\alpha^{-}$ TCR- $\gamma\delta^{+}$
γδ-T Cells, $CD8\alpha^{+}$	$CD3^{+}CD8\alpha^{+}$ TCR- $\gamma\delta^{+}$

Functions of T-Lymphocytes

T cells function to protect organisms from invading pathogens and to regulate inflammation and autoimmunity. As observed in Table 2, there are several phenotypes of T cells. There are many additional phenotypes discussed in immunological research; however, they are not within the scope of the present research.

Cytotoxic T cells (CTLs) are $CD8^{+}$ and engage Major Histocompatibility Complex (MHC) Class I molecules on antigen presenting cells (APCs), such as dendritic cells and macrophages. When presented antigenic peptides with high affinity and under inflammatory conditions, CTLs will proliferate, differentiate, target and kill, with perforin and granzymes, infected cells presenting the same antigen peptides in their MHC Class I molecules. CTLs produce cytokines, which are immunological signaling molecules. The primary cytokine produced by CTLs is interferon-γ (IFN-γ) (Murphy et al., 2008).

T helper cells (T_H) are $CD4^+$ and engage MHC Class II molecules on APCs and B cells. T helper cells when presented antigen peptides with which they have high affinity and under inflammatory conditions will proliferate, differentiate, produce stimulatory or regulatory cytokines, and activate different cell types. Traditionally, within $CD4^+$ T cells, they have been further classified into different types of T helper cells based upon their cytokine production. T_H1 cells are macrophage activating, and the primary cytokine produced to stimulate macrophages is IFN-γ. T_H2 cells are B cell activating, and the primary cytokine produced to stimulate B cells is IL-4 (Murphy et al., 2008). Over the past several years, additional phenotypes of T_H have been discovered; however, significant evidence for the plasticity within T_H differentiation has also been reported. Additionally, due to this plasticity, it may be more appropriate to distinguish T_H responses based upon their cytokine production versus the supposed T_H phenotype (Gor et al., 2003; Locksley, 2009).

Peripheral blood double positive T cells ($CD4^+CD8^+$) are unique in pigs compared with other commonly studied mammals. In mice and humans, double positive T cells are primarily restricted to the thymus. Double positive T cells were demonstrated to be able to recall antigen (Summerfield et al., 1996). Additionally, their percentage of the peripheral blood lymphocytes increases as the pig ages (Yang and Parkhouse, 1996). Their function has not been examined extensively, but they are believed to be activated and/ or memory T helper cells (Saalmuller et al., 2002).

Lastly, γδ-T cells comprise a unique portion of T cells in the peripheral blood making up a much greater portion of peripheral blood than in mice or humans (Binns et

al., 1992). The overall knowledge of their function is limited for pigs. As reviewed by Gerner et al. (2009), the percentage of γδ-T cells in peripheral blood decreases with age in pigs. Their functions possibly include responding to non-peptide antigens, memory, cytolytic activity, antigen presenting and overall critical importance in juvenile swine, which decreases with age, based upon the overall decrease in the percentage of peripheral blood lymphocytes (PBL) (Gerner et al., 2009).

Characteristics of T-Lymphocytes in Pigs

Due to the wide variety of pig breeds tested, tremendous variations among individual pigs, variability in gating of flow cytometry, sample size and health status of the pigs tested, the percentage of T cell subsets in peripheral blood varies considerably among studies. Described below are generalities of several recent studies attempting to quantify the age related lymphocyte composition of swine peripheral blood.

$CD4^-CD8^+$ T cells increase in percentage of PBL and in total number from birth (28% [day 4] or 10%; 3.0×10^5 cells/ml, respectively) to 40-41 days of age (65% or 25%; 3.0×10^6 cells/ml, respectively) (Solano-Aguilar et al., 2001; Borghetti et al., 2006) and increase with age with a consistent percentage of PBL of adult populations being reached by six months of age (15-30%) (Yang and Parkhouse, 1996; Zuckermann and Gaskins, 1996; Sinkora et al., 1998). One conflicting study showed $CD4^-CD8^+$ T cells remaining between 6 – 10% of PBL from birth to adulthood (Stepanova et al., 2007).

$CD4^+CD8^-$ T cells decreased or remained consistent in their percentage of PBL from birth (14% [day 4] or 40%, respectively) to 40-41 days of age (15%, respectively);

however, their total number remained consistent after day 19 (1.9 x 10^6 cells/ml) (Solano-Aguilar et al., 2001; Borghetti et al., 2006). Several studies confirm $CD4^+CD8^-$ T cells decrease as a percentage of peripheral blood as pigs age with a consistent percentage of adult populations being reached by eight months of age (5-15%) (Yang and Parkhouse, 1996; Zuckermann and Gaskins, 1996; Sinkora et al., 1998; Stepanova et al., 2007)

$CD4^+CD8^+$ T cells increased in their number (absent) and percentage (3.9%) of PBL from birth to day 40-41 (8.5% or approximately 7.5 x 10^5 cells/ml, respectively) (Solano-Aguilar et al., 2001; Borghetti et al., 2006) and the increase in percentage of PBL continued into adulthood (between 4-14%) (Yang and Parkhouse, 1996; Zuckermann and Gaskins, 1996; Sinkora et al., 1998; Stepanova et al., 2007).

There are a wide range of reports on the percentage of γδ-T Cells in pigs as they age. One study reported the overall number in peripheral blood increased from birth (approximately 2.5 x 10^5 cells/ml) to 41 days of age (approximately 1.8 x 10^6 cells/ml (Borghetti et al., 2006). In a separate study, the percentage remained between 12 – 21% of PBL from 4 – 47 days of age (Solano-Aguilar et al., 2001). When comparing neonatal levels to adult levels, the percentage of PBL remained between 20 to 40% and did not show a consistent trend (Yang and Parkhouse, 1996; Sinkora et al., 1998). However, two different studies observed PBL γδ-T Cells at different ages reaching levels as high as 70% of PBL (Brown et al., 2006; Stepanova et al., 2007).

T cells have increased ability to respond to mitogenic stimulation as pigs age. The blastogenic response of PBL in pigs at 28 days of age was significantly higher for Phytohemagglutinin (PHA) and Pokeweed mitogen (PWM) and tended to be higher for

Concanavalin A (ConA) compared with pigs one or 16 days of age (Becker and Misfeldt, 1993). This also was observed with PHA for 14 and 18 day old pigs compared with seven day old pigs (Brown et al., 2006). Lymphocyte proliferation with ConA and PHA from 0.5 to six weeks of age increased linearly for pigs at 0.5, one, three and six weeks of age (Hoskinson et al., 1990).

T-cell in-vitro cytokine production (IL-2 and IL-4) when stimulated with ConA was not significantly different at ages of 14 or 18 days (Brown et al., 2006). When comparing age related differences of cytokine production (IFN-γ, IL-10 and IL-4) in T cells stimulated with PHA using ELISPOT and ELISA, an age related increase in ELISPOT was observed for IFN-γ, but ELISA results did not correlate with the increase. IL-4 was largely undetectable in their results (Diaz and Mateu, 2005).

Effects of Stress on Immunological Measures

The general effects of stress on the immune system in pigs were thoroughly investigated over the past 30 years. Utilizing the different types of stress as described in the previous section, researchers used common methods to quantify the effects of stress on the immune system. The results of these different methods were extremely varied with numerous factors affecting the results, namely, animal age, type of stress, animal breed, investigator, method, variability/error, and laboratory replication of biological processes. Table 3 summarizes the immunological measures and overall observed results.

Table 3: Immunological measures utilized in pigs

Immunological Measure	**Studies**	**Stress Treatment (Environmental, Disease, Hormone, Weaning) Effect Observed**
Immunoglobulin level assays or after injection of Sheep Red blood cells	(Blecha and Kelley, 1981a; Minton, 1994; Morrow-Tesch et al., 1994; Hicks et al., 1998; Tuchscherer et al., 1998; de Groot et al., 2001; Tuchscherer et al., 2002; Kanitz et al., 2004; Bilandzic et al., 2005; Ernst et al., 2006; Sorrells et al., 2006; Sutherland et al., 2006; Niekamp et al., 2007; Rudine et al., 2007; Kojima et al., 2008; Otten et al., 2008; Torrey et al., 2009)	9 of 17
In-vivo cell mediated immunity utilizing PHA	(Blecha et al., 1983; Ekkel et al., 1996)	2 of 2
In-vitro cell mediated immunity using PHA and/or ConA for T-lymphocytes and PWM and Lipopolysaccharide (LPS) for B-lymphocytes or viral strain for T-lymphocytes	(Blecha et al., 1983; Johnson et al., 1994; Morrow-Tesch et al., 1994; Salak-Johnson et al., 1997; Deguchi and Akuzawa, 1998; Hicks et al., 1998; Tuchscherer et al., 1998; de Groot et al., 2001; Tuchscherer et al., 2002; Kanitz et al., 2004; Davis et al., 2006; Ernst et al., 2006; Sutherland et al., 2006; Niekamp et al., 2007; Otten et al., 2007; Rudine et al., 2007; Sutherland et al., 2007; Tuchscherer et al., 2009)	5 of 18 (PHA/ConA) and 9 of 13 (PWM/LPS)
Endotoxin / LPS challenge	(Webel et al., 1997; Carroll et al., 2001; Tuchscherer et al., 2004; Tuchscherer et al., 2006)	4 of 4
Serum cytokine levels	(Sorrells et al., 2006; Sutherland et al., 2007; Tuchscherer et al., 2009)	1 of 3
Stimulated in-vitro cytokine production	(de Groot et al., 2001; Davis et al., 2006; Tuchscherer et al., 2009)	1 of 3
Monocyte/Macrophage Phagocytosis	(Davis et al., 2006; Sutherland et al., 2006; Niekamp et al., 2007; Rudine et al., 2007; Sutherland et al., 2007; Lewis et al., 2008; Sutherland et al., 2009)	4 of 7
Differential leukocyte concentrations and/or Neutrophil to Lymphocyte ratio	(Minton et al., 1988; McGlone et al., 1993; Morrow-Tesch et al., 1994; Salak-Johnson et al., 1997; Hicks et al., 1998; Scroggs et al., 2002; Hyun et al., 2005; Davis et al., 2006; Ernst et al., 2006; Sutherland et al., 2006; Niekamp et al., 2007; Rudine et al., 2007; Sutherland et al., 2007; Kojima et al., 2008; Lay et al., 2008; Lewis et al., 2008; Damgaard et al., 2009; Krebs and McGlone, 2009; Sutherland et al., 2009)	11 of 19
NK Cell Assay	(McGlone et al., 1993; Salak-Johnson et al., 1997; Hicks et al., 1998; Tuchscherer et al., 2002; Sutherland et al., 2006; Niekamp et al., 2007; Sutherland et al., 2007)	4 of 7
Neutrophil Chemotaxis and Chemokinesis	(Salak-Johnson et al., 1997; Hicks et al., 1998; Sutherland et al., 2006; Niekamp et al., 2007; Lewis et al., 2008; Sutherland et al., 2009)	1 of 6
Flow Cytometry	(McGlone et al., 1993; Tuchscherer et al., 2009)	2 of 2

Peripheral Immunoglobulins

There is considerable variety in the methods utilized to measure peripheral Ig; however, the purpose of the measure is to determine the competency of B cells in producing antibodies and in class-switching.

Environmental related stress was largely not significant in its effect on Ig levels in peripheral blood with only six of 12 studies obtaining significant results among treatments. Disease (zero of one) and hormone injections (two of three) were mixed in their effect on Ig levels. Lastly, differences in weaning age resulted in significant Ig differences. The studies are summarized in Table 4.

Table 4: Stress effects on peripheral Immunoglobulin

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Cold (2.5 hrs at 10° C)	14.5 hrs old	IgG lower in stress trt	(Blecha and Kelley, 1981a)
Heat / cold / transportation	6-week old	No trt effect. Dominant pigs IgG lower than intermediate or submissive	(Hicks et al., 1998)
Heat	6 week old	IgG lower in stress trt	(Morrow-Tesch et al., 1994)
Fluctuating temperature	nursery	No treatment effect	(Minton et al., 1988)
Heat / crowding / mixing	7 week old / different breeds	No treatment effect or breed x trt effect	(Sutherland et al., 2006)
Mixing / restricted food access	12 week old	IgG higher in dominant pigs	(Tuchscherer et al., 1998)
Indoor / Outdoor rearing	6 – 9 weeks old	Higher SRBC titers in outdoor reared	(Rudine et al., 2007)
Neonatal Processing	1 or 3 days old	No interaction between trt and age of trt	(Torrey et al., 2009)
Mental stimulation - Call-feeding stations	Not specified	IgG higher in stimulation trt	(Ernst et al., 2006)
Isolation – 9 days, 2 hrs per day	12 day old	No treatment effect	(Kanitz et al., 2004)
Sows gestation housing	Birth to 35 days old	No treatment effect	(Sorrells et al., 2006)
Sow daily restraint – late gestation	1, 3, and 35 days old	IgG lower in stress trt at 1 and 3 d/o and higher at 35 d/o	(Tuchscherer et al., 2002)
Disease			
Mixing Pseudo-rabies Virus (PRV) vaccinated / challenged 42 days after vaccination	6 – 12 weeks old	No treatment effect	(de Groot et al., 2001)
Hormone Administration			
Sow treated w/ ACTH during late gestation	1 day old	No treatment effect	(Otten et al., 2007)
ACTH Admin.	6-7 month old Boars	IgG higher in stress trt	(Bilandzic et al., 2005)
Somatotropin Admin.	24 day old	IgM higher in treatment	(Kojima et al., 2008)
Weaning			
Weaning age	14, 21 or 28 days old	IgG higher in 14 d/o than 21 or 28 d/o	(Niekamp et al., 2007)

In-vivo cell mediated immunity utilizing PHA

This method has limited use in swine. The purpose of it is to measure the initial response of T cells to PHA stimulation in-vivo. It quantifies the T cell response as cytokines are produced and inflammation results.

Stress treatments in both cases had significantly lower responses to PHA. The studies are highlighted below. Pigs weaned at 2 -3 weeks of age had lower PHA skin test responses than those not weaned; however, there was no difference in response between pigs weaned and not weaned at 5 weeks of age (Blecha et al., 1983). Eight week-old pigs mixed and transported had significantly decreased responses at 12 and 18 hours after PHA injection compared to pigs not transported or mixed (Ekkel et al., 1996).

In-vitro cell mediated immunity using PHA and/or ConA

Measuring T cell proliferation by stimulating with PHA and ConA quantifies the overall T cell response since it is not TCR specific. It measure T cell functionality by whether proliferation has been down regulated by glucocorticoid receptors or other cell signaling mechanisms.

Environmental stress resulted in a lower lymphocyte proliferation in three of 10 studies. Mixed results for lower proliferation in the treatment group were also seen for models of disease (zero of two), hormone injection (one of three), and weaning (one of three). The studies are summarized in Table 5.

Table 5: Stress effects on T cell blastogenesis

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Heat / cold / transportation	6-week old	No treatment effect. Only dominance effect	(Hicks et al., 1998)
Heat	6 week old	No treatment effect	(Morrow-Tesch et al., 1994)
Heat / crowding / mixing	7 week old / different breeds	No treatment effect	(Sutherland et al., 2006)
Mixing / restricted food access	12 week old	Proliferation higher in dominant pigs	(Tuchscherer et al., 1998)
Mixing for 19 days	9 week old	Proliferation lower in stress trt	(Deguchi and Akuzawa, 1998)
Indoor / Outdoor rearing	6 – 9 weeks old	No treatment effect. Proliferation higher in dominant pigs	(Rudine et al., 2007)
Mental stimulation - Call-feeding stations	Not specified	Proliferation higher in stimulation trt	(Ernst et al., 2006)
Isolation – 9 days, 2 hrs per day	12 day old	Proliferation lower in stress trt	(Kanitz et al., 2004)
Isolation – 4 hrs	7, 21 or 35 days old	No treatment effect	(Tuchscherer et al., 2009)
Sow daily restraint – late gestation	1, 3, and 35 days old	Proliferation lower in stress trt	(Tuchscherer et al., 2002)
Disease			
Porcine Reproductive and Respiratory Syndrome (PRRS) virus / Heat	7 week old	No treatment effect	(Sutherland et al., 2007)
Mixing PRV vaccinated / challenged 42 days after vaccination	6 week old at vaccination	No treatment effect for mixed/control or dominance. Increase in proliferation observed post-challenge due to antigen recall	(de Groot et al., 2001)
Hormone Administration			
Sow treated w/ ACTH during late gestation	1 day old	Proliferation lower in stress trt	(Otten et al., 2008)
CRH Admin.	8 week old	No treatment effect	(Salak-Johnson et al., 1997)
CRH Admin.	finishing	Proliferation lower in stress trt	(Johnson et al., 1994)
Weaning			
Weaning age	14, 21 or 35 days old	Proliferation lower in weaned than not weaned at 14 and 21 d/o. No trt effect at 35 d/o	(Blecha et al., 1983)
Weaning age	14 or 21 days old	No treatment effect	(Davis et al., 2006)
Weaning age	14, 21 or 28 days old	No treatment effect	(Niekamp et al., 2007)

In-vitro cell mediated immunity using Pokeweed Mitogen or LPS

B cell proliferation is induced by LPS. PWM is a mitogen measuring B cell proliferation after activation by T cells. Both mitogens are utilized to evaluate the overall level of B cell proliferation, not Ig specific.

Environmental stress resulted in higher lymphocyte proliferation in one study and lower proliferation in five of eight studies. A disease model (zero of one) did not affect proliferation while ACTH injection (one of one) did result in lower proliferation. The effects of weaning were conflicting with weaning at 14 days of age resulting in lower proliferation, higher proliferation or no effect in three separate studies. The studies are summarized in Table 6.

Table 6. Stress effects on B cell or B cell / T cell blastogenesis

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Heat	6 week old	No treatment effect. Dominance effect.	(Morrow-Tesch et al., 1994)
Heat / crowding / mixing	7 week old / different breeds	Proliferation higher in stress trt. No breed x trt interaction	(Sutherland et al., 2006)
Mixing / restricted food access	12 week old	Proliferation higher in dominant pigs	(Tuchscherer et al., 1998)
Mixing for 19 days	9 week old	Proliferation lower in stress trt	(Deguchi and Akuzawa, 1998)
Mental stimulation - Call-feeding stations	Not specified	Proliferation higher in stimulation trt	(Ernst et al., 2006)
Isolation – 9 days, 2 hrs per day	12 day old	Proliferation lower in stress trt	(Kanitz et al., 2004)
Isolation – 4 hrs	7, 21 or 35 days old	No treatment effect	(Tuchscherer et al., 2009)
Sow daily restraint – late gestation	1, 3, and 35 days old	Proliferation lower in stress trt	(Tuchscherer et al., 2002)
Disease			
Porcine Reproductive and Respiratory Syndrome (PRRS) virus / Heat	7 week old	No treatment effect	(Sutherland et al., 2007)
Hormone Administration			
Sow treated w/ ACTH during late gestation	1 day old	Proliferation lower in stress trt	(Otten et al., 2007)
Weaning			
Weaning age	14, 21 or 35 days old	Proliferation lower at 14 d/o in weaned pigs	(Blecha et al., 1983)
Weaning age	14 or 21 days old	No treatment effect	(Davis et al., 2006)
Weaning age	14, 21 or 28 days old	Proliferation higher in pigs weaned at 14 d/o vs. 21 or 28 d/o. No difference at 6-10 weeks of age	(Niekamp et al., 2007)

Endotoxin / LPS Challenge

LPS injection is a method to trigger a rapid innate immune response. Other immunological measures are often measured in conjunction with LPS injection in order to evaluate the overall innate response.

In all studies (four), the stress treatment affected the immune response to LPS injection. Studies are summarized in Table 7.

Table 7. Stress effects on response to LPS injection

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Cold (18° C for 3 hrs) / LPS injection	neonatal	Higher BW loss in stress trt	(Carroll et al., 2001)
Food deprivation	nursery	LPS increased TNF-α and IL-6	(Webel et al., 1997)
Isolation – 2 hrs daily from 3 – 11 d/o	12 and 56 days old	TNF-α lower in stress trt 56 d/o	(Tuchscherer et al., 2004)
Isolation – 3 hrs daily from 3 – 11 d/o	12 and 56 days old	TNF-α lower in stress trt 56 d/o. IgG lower in stress trt at 12 and 56 d/o	(Tuchscherer et al., 2006)

Serum cytokine levels

Measuring serum cytokine levels provides a measure of the in-vivo immune response or ability of the animal to maintain immune homeostasis. Differences in serum cytokine levels in a stress treatment reveal an immunomodulatory or immunosuppressive response in the stress treatment.

In two of the four studies, the stress treatment had a significant effect on cytokine production. Studies are summarized in Table 8.

Table 8. Stress effects on serum cytokine levels

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Sows gestation housing	Birth to 35 days old	No treatment effect for TNF-α	(Sorrells et al., 2006)
Isolation – 4 hrs	7, 21 or 35 days old	TNF-α lower in stress trt. No treatment effect for IL-6	(Tuchscherer et al., 2009)
Disease			
Porcine Reproductive and Respiratory Syndrome (PRRS) virus / Heat	7 week old	No treatment effect for temperature for IL-10 or IFN-γ. IL-10 higher in PRRSV challenged	(Sutherland et al., 2007)

Stimulated in-vitro cytokine production,

Mitogenic stimulation of immune cells to measure in-vitro cytokine production provides an illustration of changes in relative cytokine production in vivo. The method is imperfect as there are so many additional factors in-vivo that contribute to cytokine production; however, it is the best method for in-vitro examination of specific lymphocyte functionality.

Environmental stress resulted in a treatment effect while neither mixing / disease challenge or weaning resulted in a treatment effect. The studies are summarized in Table 9.

Table 9: Stress effects on in-vitro cytokine production

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Isolation – 4 hrs	7, 21 or 35 days old	IL-1β and IL-10 lower in stress trt at 7 and 21 d/o (LPS stimulation)	(Tuchscherer et al., 2009)
Disease			
Mixing PRV vaccinated / challenged 42 days after vaccination	6 – 12 weeks old	No treatment effect (PRV stimulation)	(de Groot et al., 2001)
Weaning			
Weaning age	14 or 21 days old	No treatment effect for IFN-γ (ConA stimulation)	(Davis et al., 2006)

Neutrophil/monocyte/macrophage phagocytosis

This method measures the ability of the innate immune cell to phagocytos bacteria in a controlled assay.

Environmental stress (two of four), disease (one of one) and weaning stress (one of two) resulted in observed treatment effects; however, the effects were conflicting with

phagocytosis being higher or lower among stress treatments. The studies are summarized in Table 10.

Table 10: Stress effects on Neutrophil/monocyte/macrophage phagocytosis

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Heat / crowding / mixing	7 week old / different breeds	No treatment effect or breed x treatment interaction	(Sutherland et al., 2006)
Transportation – space allowance	weaned	No treatment effect	(Sutherland et al., 2009)
Indoor / Outdoor rearing	6 – 9 weeks old	Phagocytosis higher in outdoor reared pigs	(Rudine et al., 2007)
Novel Environment – alley / ramp course	finishing	Phagocytosis lower in trained pigs	(Lewis et al., 2008)
Disease			
Porcine Reproductive and Respiratory Syndrome (PRRS) virus / Heat	7 week old	Phagocytosis lower in PRRSV challenged pigs. No effect for temperature.	(Sutherland et al., 2007)
Weaning			
Weaning age	14 or 21 days old	No treatment effect	(Davis et al., 2006)
Weaning age / Photoperiod	14, 21 or 28 days old	Phagocytosis higher in short day weaned at 14 d/o and lower in long day weaned at 28 d/o	(Niekamp et al., 2007)

Differential Leukocyte concentrations and Neutrophil to Lymphocyte ratios

Analyzing neutrophil, lymphocytes, or other immune cells concentration in the peripheral blood is the simplest method to measure immune status in an animal. Additionally, neutrophil:lymphocyte (N:L) ratios are commonly examined as during stress the N:L ratio is expected to increase (Dhabhar et al., 1995).

Environmental stress resulted in a treatment effect of increased N:L ratio in six of 14 studies. Treatment effects resulting in higher N:L ratios were observed for the disease model, hormone injection and weaning. The studies are summarized in Table 11.

Table 11: Stress effects on Leukocyte concentrations and N:L ratios

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Heat / cold / transportation	6 week old	No treatment effect	(Hicks et al., 1998)
Heat / crowding / mixing – additive combination	finishing	Higher N:L ratios with high temperature and multiple concurrent stressors	(Hyun et al., 2005)
Heat	6 week old	No treatment effect. N:L ratio higher in dominant vs. subordinate pigs upon mixing	(Morrow-Tesch et al., 1994)
Heat / crowding / mixing	7 week old / different breeds	No treatment effect or breed x treatment interaction	(Sutherland et al., 2006)
Fluctuating temperature	nursery	No treatment effect	(Minton et al., 1988)
Mixing – associated with split marketing	8 week old	N:L ratio higher 72 hrs after mixing. No treatment effect for split marketing	(Scroggs et al., 2002)
Transportation (4 hours)	nursery	N:L ratio higher in stress trt	(McGlone et al., 1993)
Transportation – space allowance	weaned	N:L ratios higher for smallest space allowance. N:L ratio increased after transport for all trts	(Sutherland et al., 2009)
Indoor / Outdoor rearing	6 – 9 weeks old	No treatment effect	(Rudine et al., 2007)
Dynamic / Static Group – Stress response	4 – 18 weeks old	N:L ratio higher at 7, 10, 13 weeks old in dynamic group	(Damgaard et al., 2009)
Mental stimulation - Call-feeding stations	Not specified	No treatment effect	(Ernst et al., 2006)
Novel environment – simulated slaughter chute training	finishing	N:L ratio lower in trained pigs	(Krebs and McGlone, 2009)
Novel Environment – alley / ramp course	finishing	No treatment effect	(Lewis et al., 2008)
Sows rough treatment or ACTH Admin. – mid gestation	Weaning	No treatment effect	(Lay et al., 2008)
Disease			
Porcine Reproductive and Respiratory Syndrome (PRRS) virus / Heat	7 week old	N:L ratio higher for PRRSV challenged. No effect for temperature	(Sutherland et al., 2007)
Hormone Administration			
Somatotropin Admin.	24 day old	Neutrophil conc. higher in treated pigs 4 hrs after weaning. Lymphocyte conc. lower in treated pigs 4 hrs after weaning.	(Kojima et al., 2008)
CRH Admin.	8 week old	Neutrophils higher and lymphocytes lower in CRH injected animals	(Salak-Johnson et al., 1997)
Weaning			
Weaning age	14 or 21 days old	Higher lymphocyte conc in 21 d/o than 14 d/o pigs after weaning	(Davis et al., 2006)
Weaning age	14, 21 or 28 days old	N:L ratio higher in 14 d/o weaning vs. pigs weaned at 21 or 28 d/o	(Niekamp et al., 2007)

NK Cell Cytotoxicity

NK cells are unique in their function being lymphocytes but a component of the innate immune system. NK cell cytotoxicity measures the ability of NK cells to kill target cells.

Environmental stress results were conflicting with the stress treatment increasing cytotoxicity in two of four studies. There was no effect observed for the disease model. For hormone injection and weaning, the stress treatment resulted in lower NK cell cytotoxicity. The studies are summarized in Table 12.

Table 12: Stress effects on NK Cell Cytotoxicity

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Heat / cold / transportation	6 week old	NK cytotoxicity higher in cold stress	(Hicks et al., 1998)
Heat / crowding / mixing	7 week old / different breeds	NK cytotoxicity higher in stress trt. No breed x treatment interaction	(Sutherland et al., 2006)
Transportation (4 hours)	nursery	No treatment effect. NK cytotoxicity higher in dominant pigs than intermediate or submissive	(McGlone et al., 1993)
Sow daily restraint – late gestation	1, 3, and 35 days old	No treatment effect	(Tuchscherer et al., 2002)
Disease			
Porcine Reproductive and Respiratory Syndrome (PRRS) virus / Heat	7 week old	No treatment effect	(Sutherland et al., 2007)
Hormone Administration			
CRH Admin.	8 week old	NK cytotoxicity lower in stress trt one hour after injection; no difference two hours after injection	(Salak-Johnson et al., 1997)
Weaning			
Weaning age	14, 21 or 28 days old	NK cytotoxicity lower at 14 d/o wean than 21 or 28 d/o wean	(Niekamp et al., 2007)

Neutrophil Chemotaxis and Chemokinesis

This method determines the ability of neutrophils to migrate, which is critical in their function to move into inflamed or infected areas.

Environmental stress (four of four) and weaning age (one of one) had no treatment effect on neutrophil chemotaxis or chemokinesis. Only hormone injection resulted in a treatment effect for neutrophil chemotaxis. The studies are summarized in Table 13.

Table 13: Stress effects on Neutrophil Chemotaxis and Chemokinesis

Stress	Age of Pig	Effect	Study
Environmental Stressors			
Heat / cold / transportation	6 week old	No treatment effect	(Hicks et al., 1998)
Heat / crowding / mixing	7 week old / different breeds	No treatment effect or breed x treatment interaction	(Sutherland et al., 2006)
Transportation – space allowance	weaned	No treatment effect	(Sutherland et al., 2009)
Novel Environment – alley / ramp course	finishing	No treatment effect	(Lewis et al., 2008)
Hormone Administration			
CRH Admin.	8 week old	Chemotaxis lower in stress trt	(Salak-Johnson et al., 1997)
Weaning			
Weaning age	14, 21 or 28 days old	No treatment effect	(Niekamp et al., 2007)

Flow cytometry

Flow cytometry quantifies specific immune cell subsets in the peripheral blood or organ of interest. Utilizing established antibodies for different clusters of differentiation, flow cytometry quantifies the percentages of specific immune cells and phenotypes.

Flow cytometry in swine has limited use for examining the effects of stress. The majority of flow cytometry in examining pig immune cell types was utilized to

characterize changes in cell types during aging and changes as the result of vaccination. Two studies examined stress with flow cytometry. For piglets isolated at seven, 21 or 35 days of age for four hours, isolation resulted in a lower percentage of $CD4^+$ cells and higher percentage of $CD8^+$ cells at all three time points. Isolation did not affect the percentage $CD4^+CD8^+$ (Tuchscherer et al., 2009). When comparing four hours of transportation on growing pigs, the percentage of B cells of the PBL was lower following transportation; however, no effect was observed for $CD4^+$ or $CD8^+$ T cells (McGlone et al., 1993).

Effects of Vaccination on Immunological Measures

There is extensive literature both in the public and private domains on the effects of vaccination on the immune system. The most common vaccinations in pigs are designed to protect against *Mycoplasma hyopneumoniae*, PRRSV, Porcine Circovirus Type 2 and PRV (prior to 2000). Sows are frequently vaccinated and vaccination regimens normally begin in young pigs around the time of weaning. Most pig vaccination studies compared vaccinated and control groups; overall health of the groups to include histological examination at the time of slaughter and antibody titers to the pathogen (Okada et al., 1999; Pallares et al., 2000; Haruna et al., 2006). Fewer studies evaluated the effects of vaccination on immunological measures. The next section will highlight several studies that quantify vaccination effects with immunological measures.

The immunological measures examined in vaccination studies in swine are limited. Vaccination and challenge with PRRSV resulted in changes in $CD4^+$ and $CD8^+$ percentages of PBL. In a separate study, PRRSV vaccination resulted in higher IFN-γ staining and expression. Additionally, an immunostimulant of Porcine granulosa cell walls and *E. coli* lipopolysaccharide (PG/LPS) in conjunction with other common swine vaccines resulted in higher lymphocyte proliferation. Conversely, *Hemophilus parasuis* vaccination had no effect on the percentage of T cell subsets. Studies are summarized in Table 14.

Table 14: Immunological measures for vaccination studies

Pathogen	Immune Measure	Pig Age	Effect	Study
PRRSV vaccinated / challenged 35 days after vaccination	Flow Cytometry	5 week old at vaccination	Post-vaccination: $CD4^+$ decrease and $CD8^+$ increase compare to unvaccinated in first two weeks. Post-challenge: $CD4^+$ higher in vaccinated trt	(Martelli et al., 2007)
PRRSV vaccinated / challenged 45 days after vaccination	Flow Cytometry	5 week old at vaccination	Post-vaccination: $CD8^+$ higher in vaccinated trt Post-challenge: $CD8^+$ higher in vaccinated trt. γδ T cells higher in vaccinated trt	(Martelli et al., 2009)
	ELISPOT		IFN-γ secreting cells higher in vaccinated animals after challenge	
PRRSV vaccinated / challenged 55 days after vaccination	Intracellular IFN-γ staining. CD25+ staining after stimulation	3 week old at vaccination	All vaccination treatments higher for IFN-γ expression. No treatment effect for CD25	(Charerntantanakul et al., 2006)
	Flow cytometry of T cell subsets expressing IFN-γ or CD25		All vaccination treatments higher for IFN-γ expression. No treatment effect for CD25 except for one of 6 vaccination types	
Classical swine fever virus (CSFV) and M. hyopneumoniae with PG/LPS injection	In-vitro cell-mediated immunity (ConA)	6 week old at vaccination	Lymphocyte proliferation higher at 7 and 14 days post-injection compare to controls not injected PG/LPS	(Lo et al., 2009)
H. parasuis vaccinated / challenged 37 days after vaccination	Flow cytometry	8 week old at vaccination	Vaccination did not affect percentage of peripheral $CD4^+$, $CD8^+$ or $CD4^+CD8^+$	(de la Fuente et al., 2009)

Effects of Acute / Chronic Stress on T-Lymphocytes

The most commonly practiced method for determining the effects of stress on T cells is measuring proliferation following mitogenic stimulation. The results of several studies were discussed earlier and obtaining significant results was elusive and is summarized briefly below.

Early weaning (14 or 21 days vs. 28+ days) decreased lymphocyte proliferation (Blecha et al., 1983) or had no effect (14 vs. 21 days) (Davis et al., 2006) but no long-term effect was observed at six, eight, or ten weeks (Niekamp et al., 2007). Combinations or separate treatments of commonly perceived animal welfare stress (heat, cold, transportation, mixing, isolation, and indoor/outdoor) largely did not affect lymphocyte proliferation (Morrow-Tesch et al., 1994; Hicks et al., 1998; Tuchscherer et al., 1998; Sutherland et al., 2006; Rudine et al., 2007; Sutherland et al., 2007; Tuchscherer et al., 2009); however, dominance order did affect the response in two studies (Hicks et al., 1998; Tuchscherer et al., 1998). In a few separate studies, common welfare concerns were found to have significant effects; mixing (Deguchi and Akuzawa, 1998) and isolation (Kanitz et al., 2004) both decreased proliferation, while cognitive stimulating activity increased lymphocyte proliferation (Ernst et al., 2006). Injection of CRH decreased proliferation (Johnson et al., 1994) or was observed to have no effect (Salak-Johnson et al., 1997). In both cases, late gestational stress on sows resulted in lower lymphocyte proliferation in offspring (Tuchscherer et al., 2002; Otten et al., 2007).

For both papers where PHA was injected into pigs to measure stress effects, the stress treatment pigs (early weaning or mixing/transportation) had significantly decreased responses to PHA (Blecha et al., 1983; Ekkel et al., 1996).

Isolation in weaning age pigs resulted in lower in-vitro T cell cytokine production and a lower CD4:CD8 ratio with flow cytometry (Tuchscherer et al., 2009). Four hours of transportation decreased the percentage of B cells of the PBL; however, no effect was observed for $CD4^+$ or $CD8^+$ T cells (McGlone et al., 1993).

Lymphocyte Trapping

Though not studied in pigs, an observable immune effect of stress in mammals is lymphocytic trapping. Lymphocytic trapping refers to a short-term decrease in lymphocyte levels in peripheral blood following increased corticosteroid levels in the animal's blood. When prednisolone was injected into steroid sensitive normal or thymectomized rats, PBL levels decreased equivalently in both sets of animals after injection but returned to pre-injection levels within 24 hours (Lundin and Hedman, 1978). In an extensive study on rats exposed to two hours of restraint in their active or inactive diurnal period, PBL levels for T cells, B cells and NK cells all were significantly reduced within the restraint period but returned to pre-stress levels by five hours after completion of the stress. Additionally, in the stress period, $CD8^+$ cells were preferentially trapped over $CD4^+$ cells based upon the increased CD4/CD8 ratio during stress. Following the stress period, the ratio returned to pre-stress levels within five hours. The effect was determined to be dependent on the HPA axis with adrenalectomy eliminating or reducing the effect of the stress for all measures (Dhabhar et al., 1995).

Dhabhar (Dhabhar, 2000) proposed that lymphocytic trapping in response to acute stress is beneficial for animals resulting in a greater percentage of lymphocytes in sites of potential pathogen invasion, while chronic stress has an immunosuppressive effect. McEwen proposed allostasis, maintaining homeostasis through external and internal changes, and allostatic load, the effort the body must exert to maintain homeostasis, as a method to describe the effects of stress on bodily systems and particularly the immune system. Though the animal is designed to respond to acute stress with lymphocytic

trapping in order to be prepared to counter pathogen invasion, if the allostatic load is overwhelmed through repeated stress, then the stress will degrade the animal's immunological response (McEwen, 1998).

Though not within the scope of this study, researchers have evaluated other molecules that result in lymphocytic trapping from the peripheral blood. In mice with experimentally induced colitis and fed shiitake mushroom extract, $CD8^+$ T cells were trapped in the liver from the peripheral. Additionally, in-vitro IFN-γ ELISPOT was lower in shiitake fed mice. The overall effect was reduced colitis in those animals (Shuvy et al., 2008). Braude et al. (1999), reviewed redistribution of lymphocytes in response to increased testosterone levels in peripheral blood.

Summary and Conclusions

As summarized earlier, there are several common methods for analyzing both stress and the immune system in swine. Measuring HPA axis related hormones and critically analyzing observed animal behavior are the most common methods for measuring stress. The wide variety of immunological tests provides measureable data by which overall immunological conclusions can be made. Unfortunately, many of the immunological assessments are performed in-vitro, and so they do not replicate the actual performance of the target animal. Only, large-scale studies examining stress treatments and overall animal health would truly quantify the effect of different treatments on animal immunological function. Regardless, based upon the constraints of research, there are several models by which to adequately test the effect of stress on immunological function in pigs.

First, there is considerable research on dominance and its interaction with stress and immune response. Despite documented differences, because a new practice in swine husbandry is not possible to improve animal welfare and minimize effects of dominance, these biological differences will not be further examined in this research.

Second, behavior assessment between treatments is beneficial to quantify perceived stress; however, it often does not correlate with physiological differences in the blood. Behavior does not necessarily correlate with stress and due to limits in research funding and time, it will not be further examined in this research.

Third, cortisol levels continue to be the standard by which stress is measured in animals. This research will utilize cortisol as the primary means to determine whether the treatment stimulated the HPA axis resulting in a stress response from the animal. Mixing, space allowance, and weaning age are three husbandry practices that are perceived to affect animal welfare and farm management has the direct ability to control. These common practices are also often targeted by animal rights interests and will be the stress model for this research.

Fourth, as evidenced by the literature review, there is extensive research into the effect of stress on common immunological tests as described earlier. In measuring T cell response, the most common methods have been measuring N:L ratios and lymphocyte proliferation index. The scope of this research is focused on the stress response, and so N:L ratio will be examined. Additionally, changes in T cell subsets in peripheral blood are documented based on changes in age, but not as a response to stress. Accordingly, flow cytometry was utilized to examine the effects of stress on T cell phenotypes. Lastly, a new question to be addressed is does stress cause a different T_H1 (IFN-γ) or T_H2 (IL-4) response (Salak-Johnson and McGlone, 2007). Stimulating lymphocytes with ConA and examining the supernatant to determine changes in IFN-γ or IL-4 production is a potential method to examine these differences.

In conclusion, there is tremendous effort in animal research and in political action to determine what effects stress have on animal health and welfare. As evidenced by the tremendous depth and breadth of research that has already been devoted to stress responses in swine, swine are very adept at maintaining homeostasis through a wide

variety of stresses. The best available method to assess the effect of stress on T cells in pigs is to determine what changes occur in peripheral blood T cell phenotypes and determine what changes in function occur within those cell types. Ultimately, large-scale pathogen and stress challenge studies at a commercial pig facility will provide the most meaningful research on whether stressed pigs are more susceptible to disease. It is fully expected within this research that the stress treatments may have a short-term immunological effect on treatment groups; however, the duration of the stress will not impede the long-term growth and success of the animal. Lastly, observable environmental conditions that a human observer believes are stress inducing in pigs may not actually cause stress to the animal or harm its overall health and welfare.

MATERIALS AND METHODS

Preliminary Study

Animals, Housing and Experimental Design

The North Carolina State University Institutional Animal Care and Use Committee approved all experimental procedures involving animals.

Twenty-four piglets were selected from three crossbred sows all second parity or greater at the NC State University Teaching Animal Unit. Experiments were conducted from January through March. Sows were maintained in farrowing stalls with ad libitum feed and access to nipple waterers. Farrowing room temperatures were environmentally controlled to remain between 21 and 26° C. Supplemental heat for the baby pigs was provided by two heat lamps in each crate. Farrowing pens had concrete slats underneath the sows and tenderfoot flooring in the piglet areas. Piglet processing occurred within 72 hours after birth and included iron injection, tail docking, teeth clipping, castration and ear notching.

Nursery rooms had tribar flooring with nipple waterers and ad libitum food through feeders (Hog Slat, Newton Grove, NC). Ventilation and temperature were controlled with a side-wall baffle system that relied on two, variable speed exhaust fans for cooling and normal air circulation and a gas-heater located in the end of each room. Feed during nursery phase was designed to meet or exceed established NRC guidelines (National Research Council . Subcommittee on Swine Nutrition, 1998).

Pigs were weaned at 16 (±1) days of age (day 0 of the study). Pigs (n=16) in the treatment group (**VAC**) were vaccinated with Mycoplasma hyopneumoniae bacterin, (PneumoSTARTM Myco Novartis, East Hanover, NJ). Control (**CNTL**) pigs (n=8) were not vaccinated. Pigs were randomly assigned to treatment group. VAC pigs were placed in one of three nursery pens, and CNTL pigs were placed in one nursery pen. All nursery pens were in the same nursery room, and there were approximately 12 pigs per pen (some pigs per pen were not included in the study). Pigs remained in the same pens for the remainder of the study.

Piglets were restrained and bled in a supine position with blood samples collected by vena cava puncture into Vacutainer tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ). Twelve ml of blood was collected at each bleeding. Pigs were bled prior to weaning and vaccination on day 0 and then on days 3, 7, 14, 30 and 60. Complete blood count and Flow Cytometry were completed at each bleeding.

Differential Blood Leukocyte Concentrations

All blood samples were submitted to NCSU Veterinary Hospital Clinical Pathology service for Complete Blood Count. Differential blood leukocyte concentrations were obtained for porcine blood and reported as absolute numbers of neutrophils and lymphocytes.

Flow cytometry

Flow cytometry was performed in order to assess differences in immune cell populations in peripheral blood based upon *Mycoplasma* vaccination. The following antibodies were utilized in one or two color flow cytometry and at the noted dilutions to

identify the following cell types: $CD4^+$ T cells (CD4α, mouse IgG_{2b}, MCA1749F, AbD Serotec, Oxford, UK) at 1:100 dilution with R-phycoerythrin (R-PE) and $CD8\alpha^+$ T cells (CD8α, mouse IgG_{2a}, MCA1223, AbD Serotec, Oxford, UK) at 1:100 dilution with Fluorescein (FITC); $CD3^+$ T cells (CD3e, mouse $IgG_{2b}\kappa$, BB238E6, Southern Biotech, Birmingham, AL) at 1:50 dilution with Allophycocyanin (APC); mature B cells (CD21, mouse $IgG_1\kappa$, BB6-11C9.6, Southern Biotech, Birmingham, AL) at 1:200 dilution per tube with R-PE; activated T and B cells (CD25, mouse IgG_{2b}, MCA1736, AbD Serotec, Oxford, UK) at 1:200 dilution with FITC; macrophages/granulocytes (CD172a, mouse IgG_{2b}, 74-22-15A, VMRD, Pullman, WA); granulocytes (PG68A, mouse IgG_1, VMRD, Pullman, WA); and IgG Fc Receptor (AAI41F, goat IgG, AbD Serotec, Oxford, UK) at 1:100 dilution with FITC.

Briefly, antibodies were diluted at the listed dilutions in Phosphate Buffered Saline (PBS) to a volume of 100 µl. Whole blood was centrifuged for 10 min at 400 x g and serum was removed. Blood cells were reconstituted to original volume with PBS. Then, 100 µl was added to each staining series as well as a cells only tube with only PBS. Blood was incubated for 20 minutes in the dark at room temperature. Cells were washed with 3 ml PBS and centrifuged for 10 min at 400 x g. Red blood cells were lysed with 1 ml FACS Lysing solution (Beckton Dickinson, San Jose, CA) for 10 min at room temperature twice and washed with 2 ml PBS after each lyse and centrifuged for 10 min at 400 x g. Cells were resuspended in 200 µl PBS/2% Fetal Bovine Serum (FBS). Samples were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose,

CA) and analyzed using CELLQuest Pro software with 20,000 events and gating based upon forward and side scattering.

Statistical Analysis

All data were analyzed utilizing the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The initial statistical model included time, treatment, and the treatment by time interaction. The individual variation within animals, ID(trt), was not statistically different from the residual error ($p > 0.10$) for the study. This indicated that the variations among samples within pig were not a significant source of variation in the study and samples taken from the same animal over time were essentially independent. Consequently, a statistical model examining the effect of time and treatment as independent sources of variation was used. Next, all data were tested for the interaction between time and treatment. Main effects of only treatment and time were analyzed when the interaction was not significant ($p>.10$). Data were analyzed for the main effect of treatment at each time point. All data are presented as least squares means ± SEM.

Part I. Effects of Weaning and Chronic Stress on the Adaptive Immune System

Animals, Housing and Experimental Design

The North Carolina State University Institutional Animal Care and Use Committee approved all experimental procedures involving animals (project #09-069).

Twenty-four piglets were selected from nine crossbred sows, all fourth parity or greater at the NC State University Swine Educational Unit. Experiments were conducted

from June through August. Sows were maintained in farrowing stalls with ad libitum feed and access to nipple waterers. Farrowing room temperatures were environmentally controlled to remain between 21 and 26° C. Supplemental heat for the baby pigs was provided by two heat lamps in each crate. Farrowing pens had slatted metal flooring (Tri-bar, Hollam, Inc., Fort Wayne, IN). Piglet processing occurred within 72 hours after birth and included iron injection, tail docking, teeth clipping, castration and ear notching. Nursery rooms had raised metal flooring (Tri-bar) with two nipple waterers and ad libitum food through feeders. Pen dimensions were 1.52 m x 1.83 m (0.46 m^2 / pig). Ventilation and temperature were controlled with a side-wall baffle system that relied on two, variable speed exhaust fans for cooling and normal air circulation and a gas-heater located in the end of each room. Feed during the nursery phase was Renaissance Stage 3 Pellet 5820 for the first 14 days followed by ground nursery starter. Both were formulated to meet NRC guidelines for growing pigs (National Research Council . Subcommittee on Swine Nutrition, 1998).

For the weaning portion of the study, all pigs were weaned at 18±2 days of age (day 18 of study). Blood samples were collected at days 11, 17, 19, 20, 21 and 25. For the stress portion of study, which began on day 47, pigs were bled before mixing occurred on day 47, at the conclusion of stress treatment on day 52, and then on day 53, 54, 55 and 62. Pigs were restrained and bled in a supine position with blood samples collected by vena cava puncture into Vacutainer tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ). On days that complete blood count and flow cytometry were conducted, a minimum of 4 ml of blood was collected (day 11, 19, 21, 25, 53, 54

and 62). On days that lymphocyte stimulation occurred in addition to CBC and Flow cytometry, 20 ml of blood was collected (day 17, 20, 47, 52 and 55). All bleeding occurred between 6:30 – 8:00 am, and bleeding normally lasted less than one minute per pig. Pigs were weighed days 11, 19, 47, 52, and 55 prior to being bled.

In the nursery, pigs were randomly assigned to two treatment groups: stress treatment (**STRESS**, n=9 pigs) and control treatment (**CONTROL**, n=10 pigs). Barrows (n=9 pigs) and gilts (n=10 pigs) were evenly allocated between treatments. Five pigs not included in the above numbers were dropped from the study because their farrow date was 14 days different from the above group, which changed their physiological maturity. Piglets were assigned to pen based upon weight and treatment. There were two pens (6 pigs / pen) for STRESS and two pens (6 pigs / pen) for CONTROL. All pens were in the same nursery room. The model evaluated the pig's stress response to the effects of mixing and five days of crowding. At approximately seven weeks of age, pigs in the stress treatment were mixed by being combined into one pen and subjected to crowding by placing a gate in the pen to reduce the overall space allowance per pig from 0.46 m^2 to 0.174 m^2. The space reduction was 65% of the Pork Industry Handbook's recommendation of 0.27 m^2 / pig for 22.7 kg pigs (Brumm, 2008). Additionally, pigs in the stress treatment had access to one nipple waterer and one feeder.

Differential Blood Leukocyte Concentrations

All blood samples were submitted to Antech Diagnostics (Antech Diagnostics, Lake Success, NY) for Complete Blood Count. Differential blood leukocyte

concentrations were obtained for porcine blood and reported as absolute numbers of neutrophils and lymphocytes.

Flow cytometry

Flow cytometry was performed in order to assess the T cell and B cell composition of the peripheral blood. The following antibodies were utilized in one, two or three color flow cytometry and at the noted dilutions in the following series. Series 1 (three-color) contained antibodies for the following cells: γδ T cells (δ-chain, mouse IgG_1, PGBL22A, VMRD, Pullman, WA) at 1:800 dilution per tube with Allophycocyanin (APC, 115-135-205, Jackson Laboratories, West Grove, PA); $CD8\alpha^+$ T cells (CD8α, mouse IgG_{2a}, MCA1223PE – AbD Serotec, Oxford, UK) at 1:50 dilution per tube with R-PE; and $CD4^+$ T cells (CD4α, mouse IgG_{2b}, MCA1749F, AbD Serotec, Oxford, UK) at 1:100 dilution per tube with FITC. Series 2 (two-color) contained antibodies for the following cells: $CD8\alpha^+$ T cells (CD8α, mouse IgG_{2a}, MCA1223PE – AbD Serotec, Oxford, UK) at 1:100 dilution per tube with R-PE and $CD3^+$ T cells (CD3e, mouse $IgG_{2b}\kappa$, BB238E6, Southern Biotech, Birmingham, AL) at 1:50 dilution per tube with FITC. Series 3 (one-color) contained antibody for mature B cells (CD21, mouse $IgG_1\kappa$, BB6-11C9.6, Southern Biotech, Birmingham, AL) at 1:200 dilution per tube with R-PE.

Briefly, antibodies were diluted at the listed dilutions in PBS to a volume of 100 µl. Whole blood was centrifuged for 10 min at 400 x g and serum was removed. Blood cells were reconstituted to original volume with PBS. Then, 100 µl was added to each staining series as well as a cells only tube with only PBS. Blood was incubated for 20

minutes in the dark at room temperature. Cells were washed with 3 ml PBS and centrifuged for 10 min at 400 x g. (Order for Series 1 conjugation was as follows: incubate for δ-chain, wash, incubate for APC, wash, incubate for CD8α and CD4α, and wash). Red blood cells were lysed with 1 ml FACS Lysing solution (Beckton Dickinson, San Jose, CA) for 10 min at room temperature twice and washed with 2 ml PBS after each lyse for 10 min at 400 x g. Cells were resuspended in 200 µl PBS/2% FBS. Samples were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CELLQuest Pro software with 20,000 events and lymphocyte gating based upon forward and side scattering.

Cortisol

Whole blood was centrifuged for 10 min at 400 x g. Plasma was removed, frozen and stored at -20° C for use in the cortisol assay. Plasma samples were tested using a Coat-A-Count cortisol kit (Siemens, Los Angeles, CA) in accordance with the manufacturer's protocol and as previously conducted (Richards and Almond, 1994). The tubes were counted on a Cobra II Auto Gamma counter (Packard Instrument Company, Meriden, CT). For the weaning portion of the study, the intra-assay coefficient of variation was 1.4%. For the stress portion of the study, the intra-assay coefficient of variation was 3.8%. For each portion of the study, all samples were analyzed in the same assay.

T cell Functionality Test

T cell stimulation with ConA was performed in order to analyze IFN-γ and IL-4 cytokine production and whether stress treatment resulted in a shift of cytokine production. Lymphocytes were isolated in accordance with the protocol in the Histopaque 1077 product manual. To briefly describe the method, 15 ml of whole blood was layered onto 15 ml of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) in a 50-ml centrifuge tube (Beckton Dickinson, San Jose, CA) with both liquids at room temperature. Tubes were centrifuged for 30 minutes at 400 x g. After centrifugation, a glass Pasteur pipette was used to aspirate the plasma layer. The buffy coat layer of mononuclear cells was removed with a Pasteur pipette and transferred to a 15-ml centrifuge tube (Beckton Dickinson, San Jose, CA). Cells were washed with 10 ml Hanks' Balanced Salt Solution (HBSS) (Mediatech, Manassas, VA) with .05% EDTA, centrifuged for 10 minutes at 250 x g and the supernatant was aspirated. Cells were resuspended in 5 ml HBSS with .05% EDTA, centrifuged for 5 minutes at 250 x g and the supernatant was aspirated. The cells were resuspended to a total volume of 2 ml with Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich, St. Louis, MO) (with 24 mM sodium bicarbonate and 25 μg/mL of gentamicin sulfate) and supplemented with 10% FBS. Lymphocyte concentration was determined utilizing a Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA) (Hansen, 2001). Cells were diluted in order to obtain a final cell concentration of 5 x 10^6 cells / ml. One ml of cells per sample was added in triplicate to a 24-well plate. An additional cells only well was added per sample. One ml of 10 μg ConA / ml RPMI 1640 (Brown et al., 2006; Davis et

al., 2006) was added to the stimulation wells and one ml RPMI 1640 was added to the cells only well. The final cell concentration was 2.5 x 10^6 cells per well. Plates were incubated for 24 hours at 37º C with 5% CO_2. After incubation, plates were centrifuged for 5 minutes at 400 x g to adhere cells to the plates. Supernatant was removed from each stimulated well and stored at -80º C. Non-stimulated wells were counted to ensure greater than 90% of cells were viable after incubation.

An ELISA for IFN-γ and IL-4 (Invitrogen, Camarillo, CA) was performed in accordance with the manufacturer's protocol on all supernatants. Each supernatant sample was tested in duplicate. For the IL-4 ELISA, the intra-assay coefficient of variation was 10.6%, and the inter-assay coefficient of variation was 10.8%. For the IFN-γ ELISA, the intra-assay coefficient of variation was 4.8%, and the inter-assay coefficient of variation was 29.2%.

Statistical Analysis

All data were analyzed utilizing the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The initial statistical model included time, treatment, and the treatment by time interaction. The individual variation within animals, ID(trt), was not statistically different from the residual error ($p > 0.10$) for both phases of the study. This indicated that the variations among samples within pig were not a significant source of variation in the study and samples taken from the same animal over time were essentially independent. Consequently, a statistical model examining the effect of time and treatment as independent sources of variation was used. Next, all data were tested for the interaction between time and treatment. Main effects of only treatment and time were

analyzed when the interaction was not significant (p>.10). For the weaning portion of the study, there was only one treatment, so data were analyzed for the effect of time. For the stress portion, data were analyzed for the main effect of treatment at each time point. All data are presented as least squares means ± SEM. For cortisol concentrations during the stress portion, two comparisons were made. First, serum cortisol concentrations were compared by day for differences between treatment. Second, a comparison was made of relative cortisol concentrations compared to the pre-stress values by setting cortisol concentrations for day 47 equal to one and then dividing subsequent day concentration by the concentration on day 47. This method was done because of the apparent variation in pre-stress values between treatments.

Part II. Effects of Weaning Age on the Adaptive Immune System

Animals, Housing and Experimental Design

The North Carolina State University Institutional Animal Care and Use Committee approved all experimental procedures involving animals (project #09-069).

Twenty-four piglets were selected from four crossbred sows, all second parity or greater at the NC State University Swine Educational Unit. Experiments were conducted from October through November. Sows were maintained in farrowing stalls respectively with ad libitum feed and access to nipple waterers. Farrowing rooms temperatures were environmentally controlled and crates had two heat lamps. Farrowing pens had slatted metal flooring. Nursery rooms had raised metal flooring (tri-bar) with two nipple

waterers and ad libitum food through J feeders. Pen dimensions were 1.52 m x 1.83 m (0.46 m^2 / pig). Room temperature was maintained by a gas heating system, and cooling and ventilation were by exhaust fans and doors. Feed during the nursery phase was Renaissance Stage 3 Pellet 5820 for the first 14 days followed by ground nursery starter. Both were formulated to meet NRC guidelines for growing pigs (National Research Council . Subcommittee on Swine Nutrition, 1998). Piglet processing occurred within 72 hours after birth and included iron injection, tail docking, teeth clipping, castration and ear notching.

Farrowing dates for all piglets were within one day (day 0). On day 6, pigs were selected by sex in order to ensure equal number of barrow and gilts per treatment and were randomly assigned to treatment group with six pigs selected per sow with two pigs per treatment. Pigs were assigned to three treatment groups consisting of weaning on day 14 (**14D**, n=8 pigs), 21 (**21D**, n=7 pigs) or 28 (**28D**, n=8 pigs). One pig from 21D was dropped from the study because of sickness. Pigs from the same litter not in the study were also weaned on the same day but were not placed in the same nursery room. Upon weaning, pigs were placed in adjacent pens in the same nursery room.

Piglets were restrained and bled in a supine position with blood samples collected by vena cava puncture into Vacutainer tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ). Blood samples were collected from all pigs on days 13, 15, 20, 22, 27, 29 and 35. On days that complete blood count and flow cytometry were conducted, a minimum of 4 ml blood was collected (days 13, 20, 27). On days that lymphocyte stimulation occurred in addition to CBC and Flow cytometry, 16 ml blood was collected

on days 15 and 22, and 20 ml blood was collected on days 29 and 35 (only 12 ml of blood was used for lymphocyte stimulation in younger pigs in order to minimize the amount of time pigs were restrained). All bleeding occurred between 6:30 – 8:00 am, and bleeding normally lasted less than one minute per pig. Pigs were weighed on the following days of the study: days 6, 14, 21, 28, and 35 (prior to being bled).

Differential Blood Leukocyte Concentrations

All blood samples were submitted to Antech Diagnostics (Antech Diagnostics, Lake Success, NY) for Complete Blood Count. Differential blood leukocyte concentrations were obtained for porcine blood and reported as absolute numbers of neutrophils and lymphocytes.

Flow cytometry

Flow cytometry was performed in order to assess the T cell and B cell composition of the peripheral blood. The following antibodies were utilized in one, two or three color flow cytometry and at the noted dilutions in the following series. Series 1 (three-color) contained antibodies for the following cells: γδ T cells (δ-chain, mouse IgG_1, PGBL22A, VMRD, Pullman, WA) at 1:800 dilution per tube with Allophycocyanin (APC, 115-135-205, Jackson Laboratories, West Grove, PA); $CD8\alpha^+$ T cells (CD8α, mouse IgG_{2a}, MCA1223PE – AbD Serotec, Oxford, UK) at 1:50 dilution per tube with R-PE; and $CD4^+$ T cells (CD4α, mouse IgG_{2b}, MCA1749F, AbD Serotec, Oxford, UK) at 1:100 dilution per tube with FITC. Series 2 (two-color) contained antibodies for the following cells: $CD8\alpha^+$ T cells (CD8α, mouse IgG_{2a}, MCA1223PE – AbD Serotec, Oxford, UK) at 1:100 dilution per tube with R-PE and $CD3^+$ T cells (CD3e, mouse

$IgG_{2b}\kappa$, BB238E6, Southern Biotech, Birmingham, AL) at 1:50 dilution per tube with FITC. Series 3 (one-color) contained antibody for mature B cells (CD21, mouse $IgG_1\kappa$, BB6-11C9.6, Southern Biotech, Birmingham, AL) at 1:200 dilution per tube with R-PE.

Briefly, antibodies were diluted at the listed dilutions in PBS to a volume of 100 µl. Whole blood was centrifuged for 10 min at 400 x g and serum was removed. Blood cells were reconstituted to original volume with PBS. Then, 100 µl was added to each staining series as well as a cells only tube with only PBS. Blood was incubated for 20 minutes in the dark at room temperature. Cells were washed with 3 ml PBS and centrifuged for 10 min at 400 x g. (Order for Series 1 conjugation was as follows: incubate for δ-chain, wash, incubate for APC, wash, incubate for CD8α and CD4α, and wash). Red blood cells were lysed with 1 ml FACS Lysing solution (Beckton Dickinson, San Jose, CA) for 10 min at room temperature twice and washed with 2 ml PBS after each lyse for 10 min at 400 x g. Cells were resuspended in 200 µl PBS/2% FBS. Samples were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CELLQuest Pro software with 20,000 events and lymphocyte gating based upon forward and side scattering.

Cortisol

Whole blood was centrifuged for 10 min at 400 x g. Plasma was removed, frozen and stored at -20° C for use in the cortisol assay. Plasma samples were tested using a Coat-A-Count cortisol kit (Siemens, Los Angeles, CA) in accordance with the manufacturer's protocol and as previously conducted (Richards and Almond, 1994). The tubes were counted on a Cobra II Auto Gamma counter (Packard Instrument Company,

Meriden, CT). The intra-assay coefficient of variation was 2.2%. All samples were analyzed in the same assay.

T cell Functionality Test

T cell stimulation with ConA was performed in order to analyze IFN-γ and IL-4 cytokine production and whether weaning resulted in a shift of cytokine production. Lymphocytes were isolated in accordance with the protocol in the Histopaque 1077 product manual. To briefly describe the method, 15 ml of whole blood was layered onto 15 ml of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) in a 50-ml centrifuge tube (Beckton Dickinson, San Jose, CA) with both liquids at room temperature. Tubes were centrifuged for 30 minutes at 400 x g. After centrifugation, a glass Pasteur pipette was used to aspirate the plasma layer. The buffy coat layer of mononuclear cells was removed with a Pasteur pipette and transferred to a 15-ml centrifuge tube (Beckton Dickinson, San Jose, CA). Cells were washed with 10 ml HBSS (Mediatech, Manassas, VA) with .05% EDTA, centrifuged for 10 minutes at 250 x g and the supernatant was aspirated. Cells were resuspended in 5 ml HBSS with .05% EDTA, centrifuged for 5 minutes at 250 x g and the supernatant was aspirated. The cells were resuspended to a total volume of 2 ml with RPMI 1640 (Sigma-Aldrich, St. Louis, MO) (with 24 mM sodium bicarbonate and 25 μg/mL of gentamicin sulfate) and supplemented with 10% FBS. Lymphocyte concentration was determined utilizing a Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA) (Hansen, 2001). Cells were diluted in order to obtain a final cell concentration of 5×10^6 cells / ml. One ml of cells per sample was added in triplicate to a 24-well plate. An additional cells only well was added per sample. One ml

of 10 μg ConA / ml RPMI 1640 (Brown et al., 2006; Davis et al., 2006) was added to the stimulation wells and one ml RPMI 1640 was added to the cells only well. The final cell concentration was 2.5 x 10^6 cells per well. Plates were incubated for 24 hours at 37º C with 5% CO_2. After incubation, plates were centrifuged for 5 minutes at 400 x g to adhere cells to the plates. Supernatant was removed from each stimulated well and stored at -80º C. Non-stimulated wells were counted to ensure greater than 90% of cells were viable after stimulation.

For some animals particularly before weaning, lymphocyte concentrations were not sufficient to stimulate in three wells. In those cases, lymphocytes were stimulated in two wells and no cells only well was included.

An ELISA for IFN-γ and IL-4 (Invitrogen, Camarillo, CA) was performed in accordance with the manufacturer's protocol. Due to a shortage of supply of IL-4 ELISA kits, only 6 pigs per treatment and only supernatant from the first two stimulated wells was tested for cytokine production for both IL-4 and IFN-γ. Each supernatant sample was tested in duplicate. After completing those tests, the manufacturer made available more kits; however, due to the inconclusive results, a decision was made not to test the remainder of the supernatant samples. For the IL-4 ELISA, the intra-assay coefficient of variation was 8.8%, and the inter-assay coefficient of variation was 3.8%. For the IFN-γ ELISA, the intra-assay coefficient of variation was 6.9%, and the inter-assay coefficient of variation was 38.4%.

Statistical Analysis

All data were analyzed utilizing the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The initial statistical model included time, treatment, and the treatment by time interaction. The individual variation within animals, ID(trt), was not statistically different from the residual error ($p > 0.10$) for the study. This indicated that the variations among samples within pig were not a significant source of variation in the study and samples taken from the same animal over time were essentially independent. Consequently, a statistical model examining the effect of time and treatment as independent sources of variation was used. Next, all data were tested for the interaction between time and treatment. Main effects of only treatment and time were analyzed when the interaction was not significant ($p>.10$). Data were analyzed for the main effect of treatment at each time point. All data are presented as least squares means ± SEM.

RESULTS

Preliminary Study

The purpose of the preliminary study was to validate flow cytometry procedures and to determine if *Mycoplasma* vaccination affected the percentage and number of peripheral blood lymphocytes in pigs following weaning and vaccination or weaning only. Differences were observed between treatments before weaning and within seven days after weaning and vaccination for T cell subsets. No differences were observed for overall lymphocyte concentrations or for other lymphocytes throughout the study and for T cell subsets beyond the first seven days.

Differential Blood Leukocyte Concentrations

Neutrophil (p=.7415) and lymphocyte (p=.7327) concentrations did not differ between treatments (Figure 1). Additionally, there were no differences between treatments for neutrophils or lymphocytes at any time point.

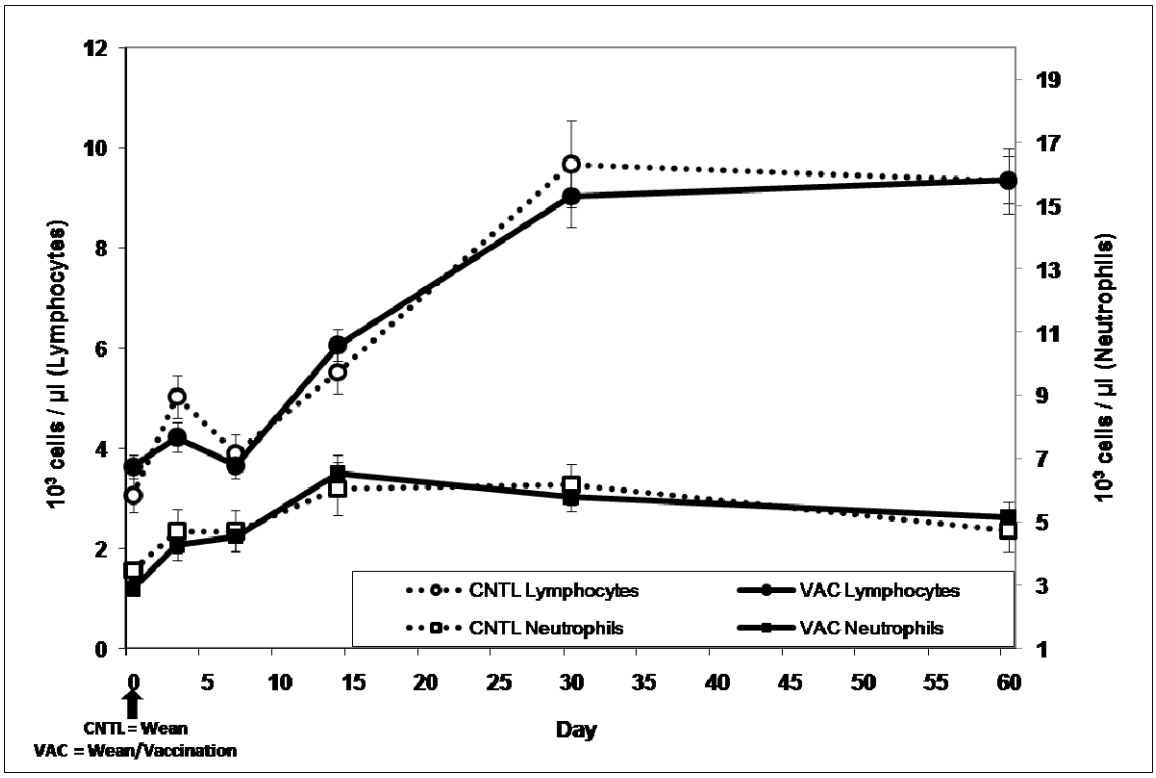

Figure 1. Preliminary study lymphocyte and neutrophil concentrations. Lymphocytes and neutrophil peripheral blood concentrations (lsmean ± SEM) from weaning and vaccination for CNTL (n=8 pigs) and VAC (n=16 pigs). Pigs were weaned at 16 ± 1 days of age (day 0). Within cell type and by day, there were no differences between treatments.

Flow cytometry

The percentage of $CD4^+$ T cells of the PBL tended to be different (p=.0663) between treatments, while treatments did not differ (p=.3562) for the number of $CD4^+$ T cells (Figure 2). Three days after weaning, the percentage of $CD4^+$ T cells of the PBL was lower (p=.0095) in the CNTL pigs than in the VAC pigs.

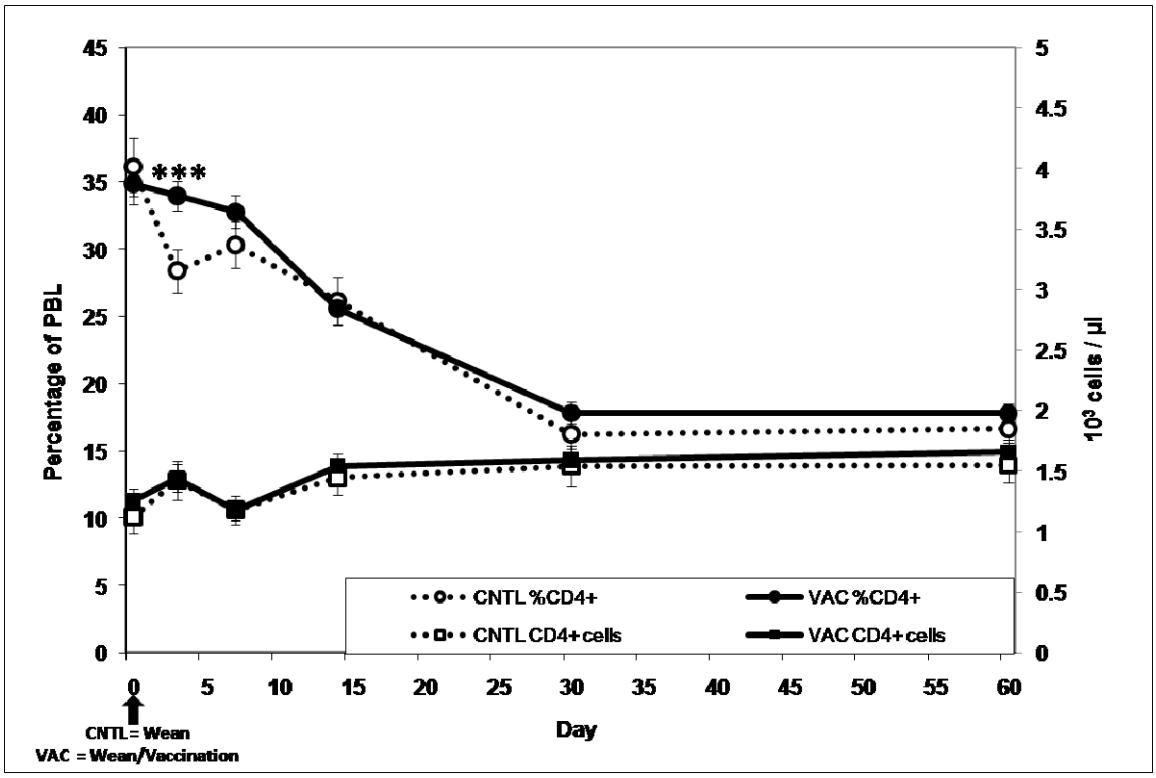

Figure 2. Preliminary study percentage and number of $CD4^+$ T cells. The percentage (lsmean ± SEM) of $CD4^+$ T cells of the PBL and number (lsmean ± SEM) of $CD4^+$ T cells in the peripheral blood from weaning and vaccination for CNTL (n=8 pigs) and VAC (n=16 pigs). Pigs were weaned at 16 ± 1 days of age (day 0). Within cell type and by day, differences between lsmeans of treatments designated: ***, $p<.01$.

For the percentage of $CD8^+$ T cells of the PBL ($p<.0001$) and the number of $CD8^+$ T cells ($p=.0431$), there was an interaction between time and treatment (Figure 3). There were differences between treatments for the percentage of $CD8^+$ T cells of the PBL on day 0 ($p=.029$), day 3 ($p=.0092$), and on day 7 ($p=.0031$). There were differences between treatments for the number of $CD8^+$ T cells in the peripheral blood on day 0 ($p=.05$), day 3 ($p=.0062$), and on day 7 ($p=.0458$).

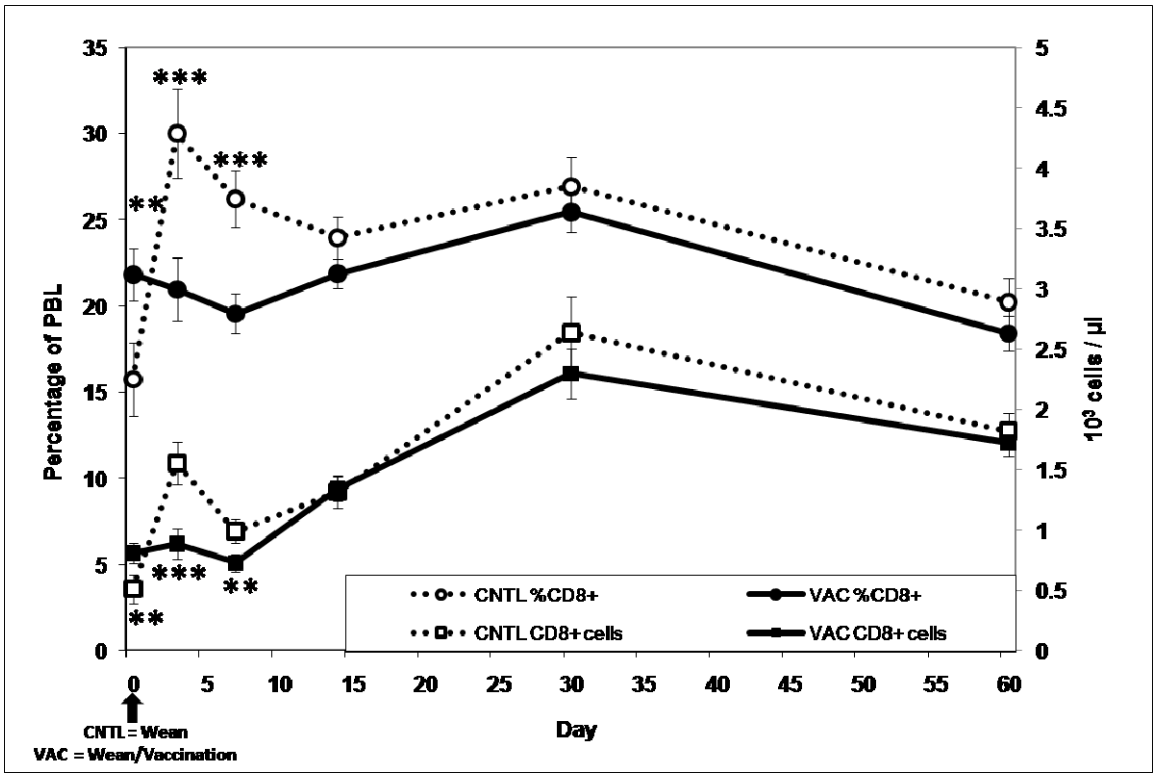

Figure 3. Preliminary study percentage and number of $CD8^+$ T cells. The percentage (lsmean ± SEM) of $CD8^+$ T cells of the PBL and number (lsmean ± SEM) of $CD8^+$ T cells in the peripheral blood from weaning and vaccination for CNTL (n=8 pigs) and VAC (n=16 pigs). Pigs were weaned at 16 ± 1 days of age (day 0). Within cell type and by day, differences between lsmeans of treatments designated: **, $p \leq .05$; ***, $p < .01$.

The percentage of $CD4^+CD8^+$ T cells of the PBL was not different (p=.1877) between treatments, while there tended to be a treatment effect (p=.0661) for the number of $CD4^+CD8^+$ T cells (Figure 4). The number of $CD4^+CD8^+$ T cells was higher in the CNTL pigs on day 3 (p=.0045) and on day 7 (p=.0404) than in the VAC pigs.

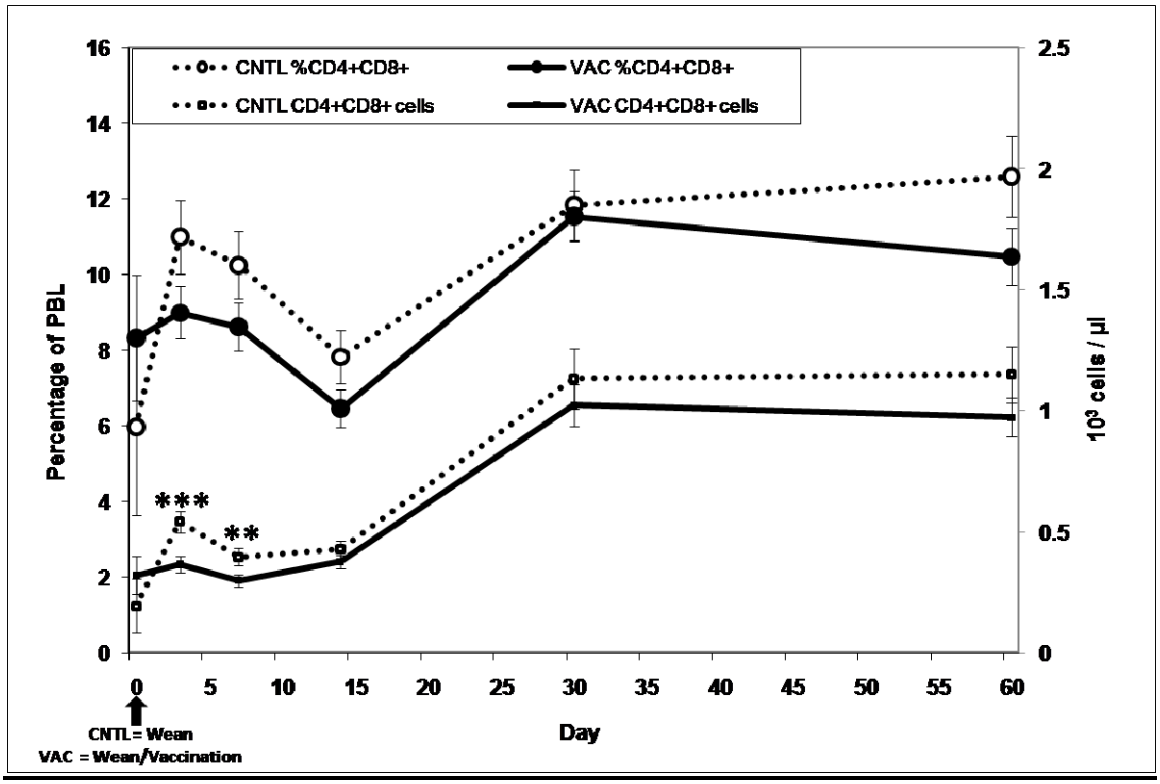

Figure 4. Preliminary study percentage and number of $CD4^{+}CD8^{+}$ T cells. The percentage (lsmean ± SEM) of $CD4^{+}CD8^{+}$ T cells of the PBL and number (lsmean ± SEM) of $CD4^{+}CD8^{+}$ T cells in the peripheral blood from weaning and vaccination for CNTL (n=8 pigs) and VAC (n=16 pigs). Pigs were weaned at 16 ± 1 days of age (day 0). Within cell type and by day, differences between lsmeans of treatments designated: **, $p<.05$; ***, $p<.01$.

The percentage of $CD21^{+}$ cells of the PBL differed (p=.0358) between treatments, while the number of $CD21^{+}$ cells in the peripheral blood did not differ (p=.2477) between treatments (Figure 5). There were no by day differences for $CD21^{+}$ cells.

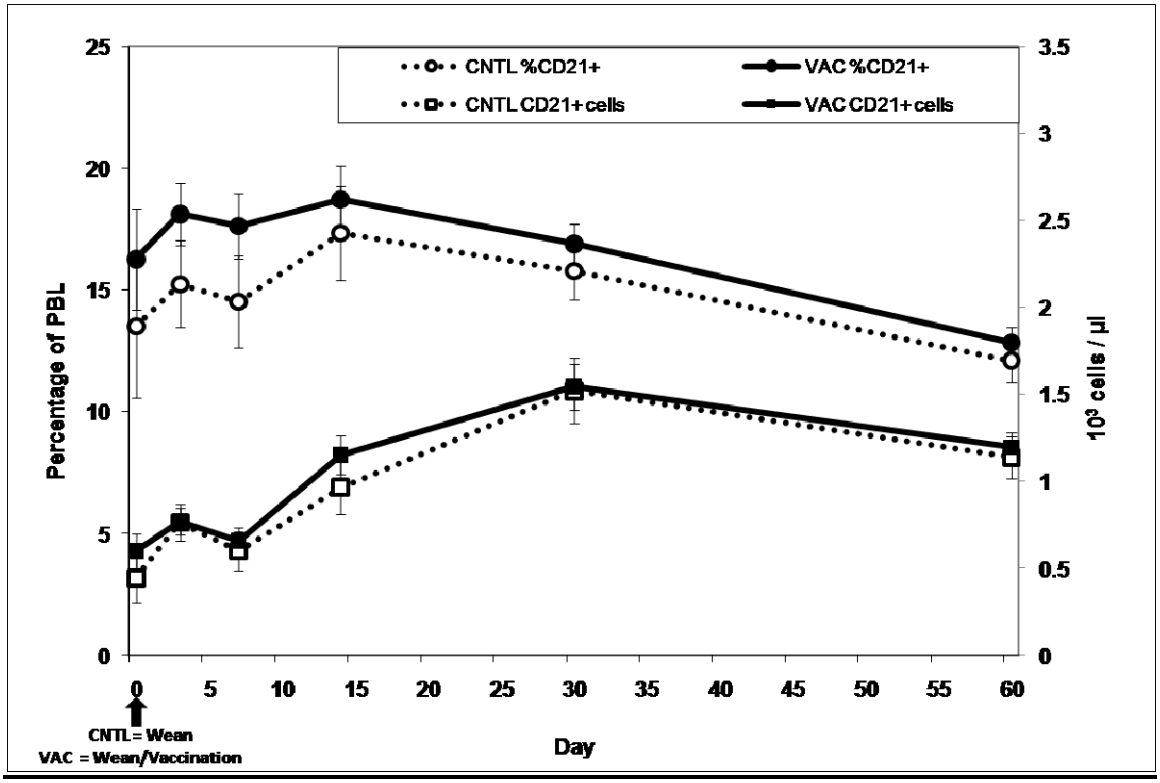

Figure 5. Preliminary study percentage and number of $CD21^+$ cells. The percentage (lsmean ± SEM) of $CD21^+$ cells of the PBL and number (lsmean ± SEM) of $CD21^+$ cells in the peripheral blood from weaning and vaccination for CNTL (n=8 pigs) and VAC (n=16 pigs). Pigs were weaned at 16 ± 1 days of age (day 0). Within cell type and by day, there were no differences between treatments.

Neither the percentage of $CD25^+$ cells of the PBL (p=.1665) or the number of $CD25^+$ cells in the peripheral blood (p=.2634) differed between treatments (Figure 6). On day 7, the percentage of $CD25^+$ cells of the PBL tended to be higher (p=.06) in VAC pigs than in the CNTL pigs.

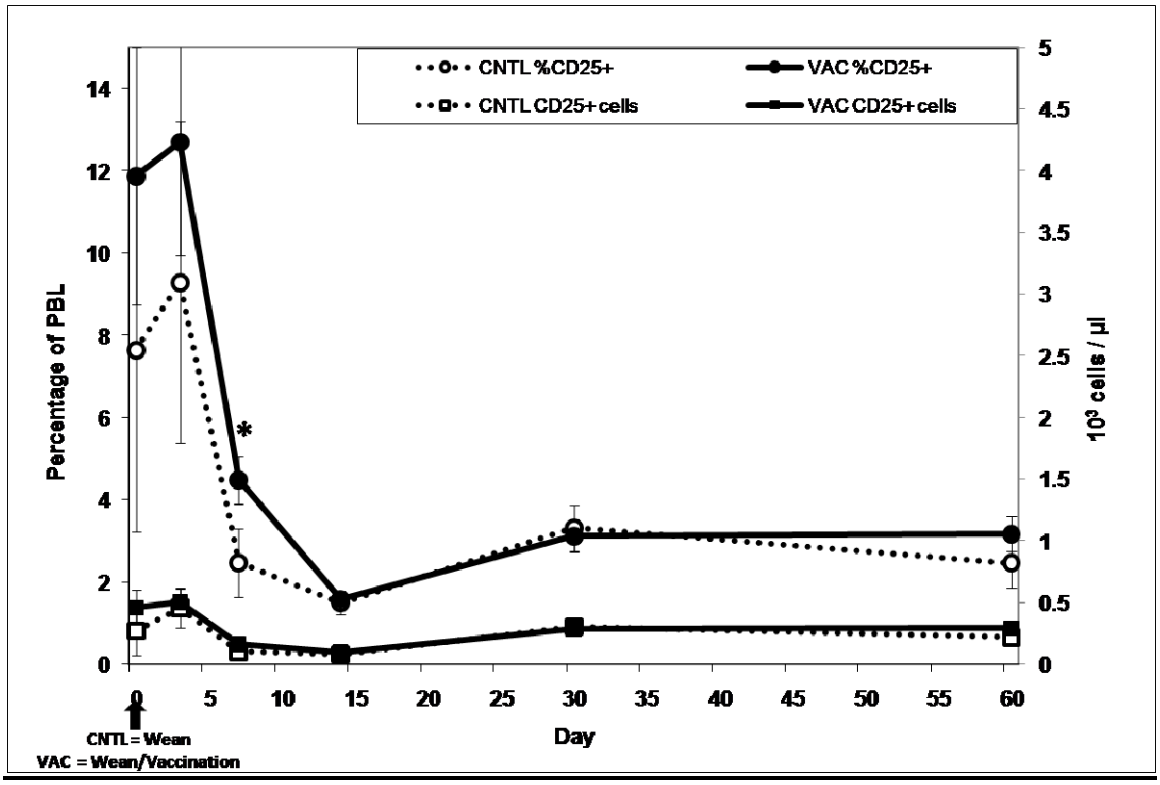

Figure 6. Preliminary study percentage and number of $CD25^+$ cells. The percentage (lsmean ± SEM) of $CD25^+$ cells of the PBL and number (lsmean ± SEM) of $CD25^+$ cells in the peripheral blood from weaning and vaccination for CNTL (n=8 pigs) and VAC (n=16 pigs). Pigs were weaned at 16 ± 1 days of age (day 0). Within cell type and by day, differences between lsmeans of treatments designated: *, p<.1.

The percentage of IgG $receptor^+$ lymphocytes ($IgGRL^+$) of the PBL tended to be different (p=.0711) between treatments, while a treatment effect was not observed (p=.1188) for the number of $IgGRL^+$ cells (Figure 7). On day 60, the percentage of $IgGRL^+$ cells of the PBL (p=.0919) and the number of $IgGRL^+$ cells (p=.0575) tended to be higher in VAC pigs compared to CNTL pigs.

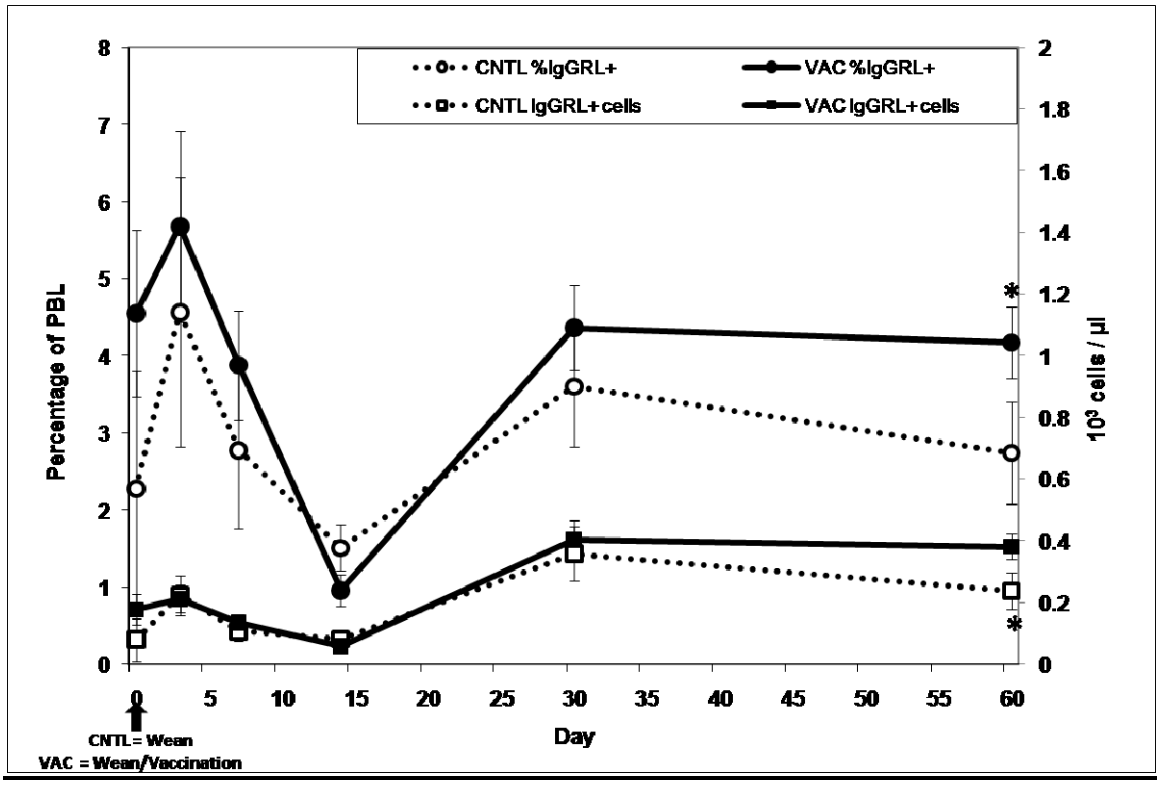

Figure 7. Preliminary study percentage and number of IgGRL$^+$ cells. The percentage (lsmean ± SEM) of IgGRL$^+$ cells of the PBL and number (lsmean ± SEM) of IgGRL$^+$ cells in the peripheral blood from weaning and vaccination for CNTL (n=8 pigs) and VAC (n=16 pigs). Pigs were weaned at 16 ± 1 days of age (day 0). Within cell type and by day, differences between lsmeans of treatments designated: *, p<.1.

Stress-related immune cell ratios

The N:L ratios did not differ (p=.5233) between treatments, while there was an interaction (p=.0001) between time and treatment for CD4:CD8 ratios (Figure 8). On day 0, the N:L ratio was lower (p=.0176) in the VAC pigs than in the CNTL pigs. On day 0 (p=.0336), day 3 (p=.0128) and day 7 (p=.0141), the CD4:CD8 ratio differed between the treatments.

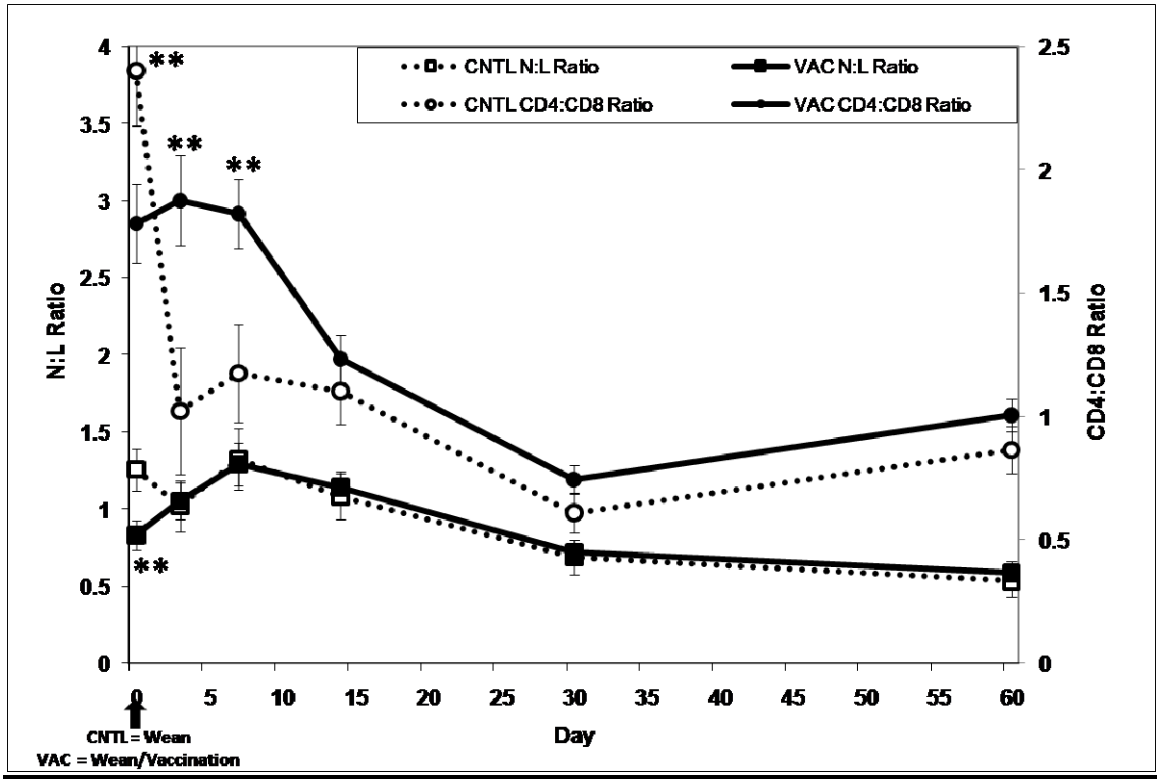

Figure 8. Preliminary study N:L and CD4:CD8 ratio. The N:L ratio (lsmean ± SEM) and the CD4:CD8 ratio (lsmean ± SEM) from weaning and vaccination for CNTL (n=8 pigs) and VAC (n=16 pigs). Pigs were weaned at 16 ± 1 days of age (day 0). Within ratio and by day, differences between lsmeans of treatments designated: **, $p<.05$.

Part Ia. Effects of Weaning on the Adaptive Immune System

The purpose of part Ia was to determine the effects of weaning on peripheral blood lymphocytes and on physiological measures. Weaning resulted in lymphocytic trapping 24 hours after weaning and increased peripheral neutrophil concentrations 72 hours after weaning. Weaning largely did not have an effect on percentages of peripheral blood lymphocytes. Changes in concentrations and percentages of peripheral blood

lymphocytes were age related. Weaning resulted in an increase in serum cortisol concentrations 24 hours after weaning.

Differential Blood Leukocyte Concentrations

Neutrophil ($p<.0001$) and lymphocyte ($p=.0233$) concentrations changed over time (Figure 9). Neutrophils were higher ($p<.001$) on day 21 than any other day. Lymphocytes were lower ($p=.025$) the day after weaning (day 19) than on day 11 and tended to be lower ($p=.091$) than on the day before weaning (day 17).

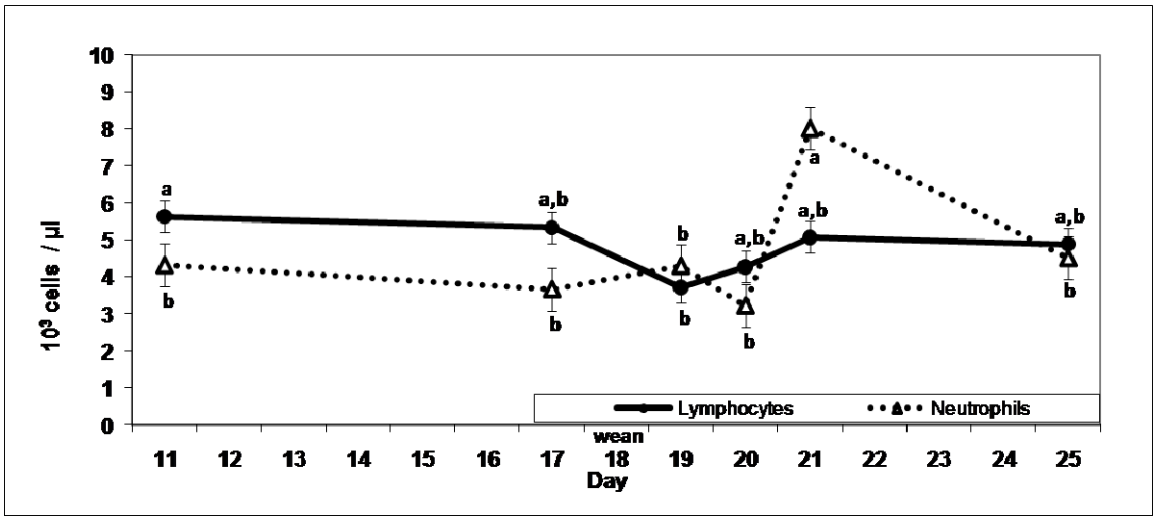

Figure 9. Part Ia lymphocyte and neutrophil concentrations. Lymphocyte and neutrophil concentrations in peripheral blood (lsmean ± SEM) before and after weaning (n=19 pigs). Pigs were weaned on day 18. Least squares means with differences ($p<.05$) designated by different letter (a,b).

Flow cytometry

For the percentage of T cell subsets and B cells of PBL on the days before and after weaning, there was only a difference after weaning ($p=.022$) for the percentage of $CD4^+CD8^+$ T cells, which increased. The numbers of T cell subsets and B cells did not

differ on the day before or after weaning. A greater effect was observed over time for changes in the percentage of PBL and cell numbers.

The percentage of $CD4^+$ T cells (p=.0159) and $CD4^+CD8^+$ T cells (p<.0001) of the PBL changed over time, while the percentage of CTLs did not change (Figure 10). The percentage of $CD4^+$ T cells on day 25 was lower than on day 11 (p=.0283) and day 20 tended to be lower than on day 11 (p=.088). The percentage of $CD4^+CD8^+$ T cells increased during the time period with day 25 higher (p<.01) than all other days except day 19. The percentage of $CD4^+CD8^+$ cells on day 19 was higher than day 17 (p=.022) and day 11 (p<.0001). The percentage of $CD4^+CD8^+$ cells on day 11 was lower (p<.001) than on all other days.

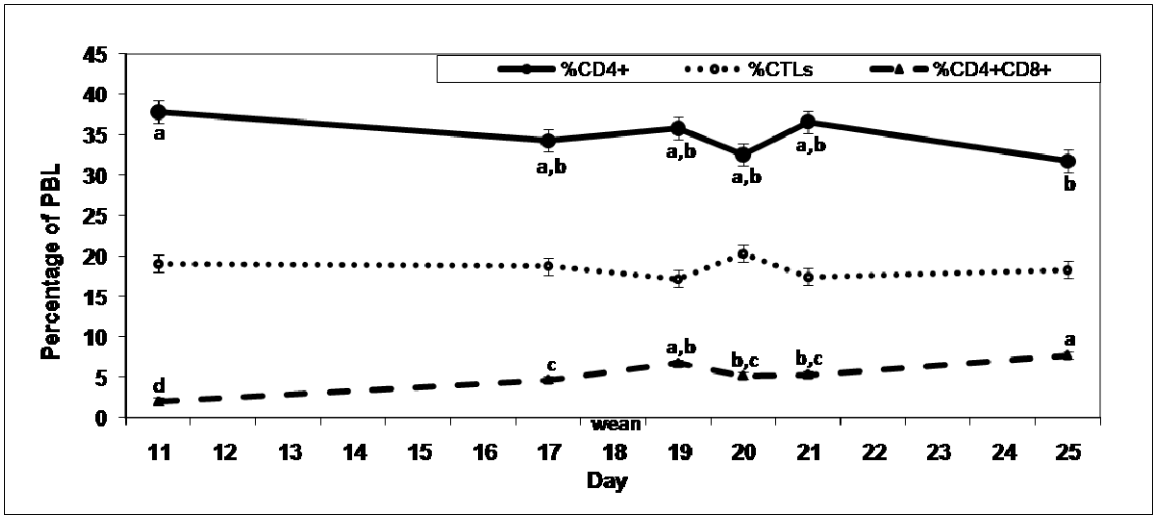

Figure 10. Part Ia percentage of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells. The percentage (lsmean ± SEM) of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells of the PBL before and after weaning (n=19 pigs). Pigs were weaned on day 18. Within cell type, least squares means with differences (p<.05) designated by different letter (a,b,c,d).

The number of $CD4^+$ T cells (p<.0001), CTLs (.0457) and $CD4^+CD8^+$ T cells (p<.0001) changed over time (Figure 11). The number of $CD4^+$ T cells decreased over time with day 11 higher than day 19 (p<.0001), day 20 (p=.0001) and day 25 (p=.0021). The number of CTLs only differed (p=.023) when day 11 was compared to day 19. The number of $CD4^+CD8^+$ T cells increased over time with day 25 higher than day 11 (p<.001), day 17 (p=.066) and day 20 (p=.015). The number of $CD4^+CD8^+$ cells was higher (p=.0153) on day 21 than on day 11.

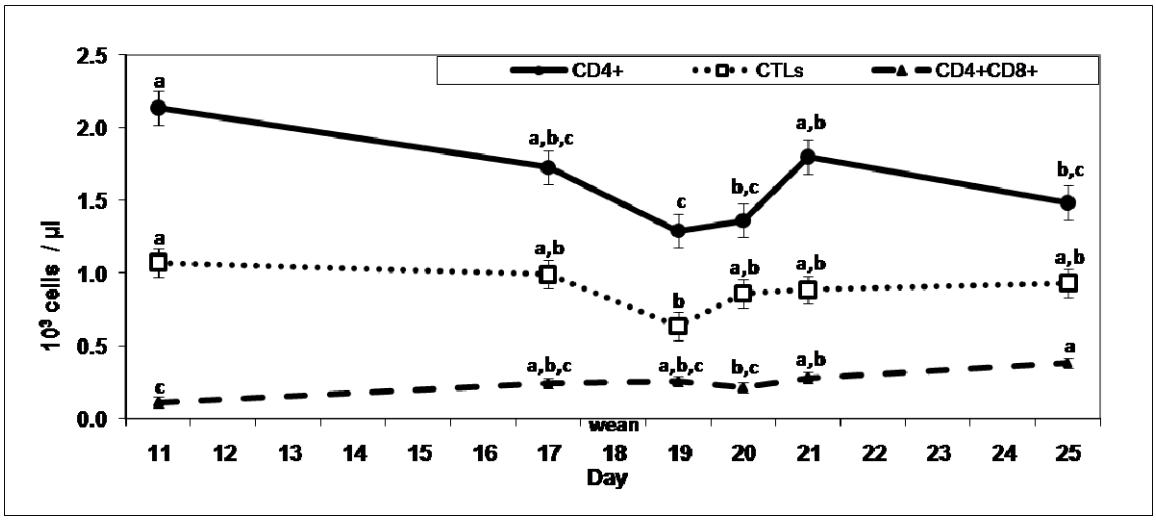

Figure 11. Part Ia number of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells. The number (lsmean ± SEM) of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells in the peripheral blood before and after weaning (n=19 pigs). Pigs were weaned on day 18. Within cell type, least squares means with differences (p<.05) designated by different letter (a,b,c).

The percentage of $CD21^+$ cells (p<.0001) and $\gamma\delta^+$-T cells (p=.007) increased in the PBL over time (Figures 12 and 13). The percentage of $CD21^+$ cells on days 20 (p=.0059), 21 (p=.0212) and 25 (p<.0001) were higher than on day 11. The percentage of $CD21^+$ cells on day 25 was higher (p=.0148) than on day 19. The number of $CD21^+$ cells did not differ among days. The percentage of $\gamma\delta^+$-T cells was higher on day 25

(p=.0097) than on day 19 and tended to be higher than on day 11 (p=.087). The number of $\gamma\delta^+$-T cells tended to be lower (p=.098) on day 19 compared to day 21.

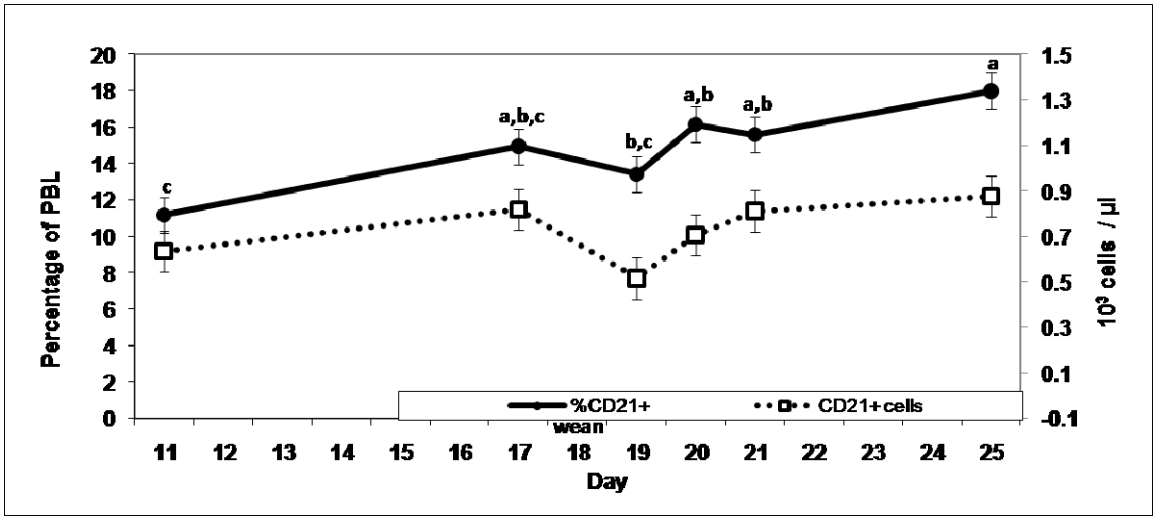

Figure 12. Part Ia percentage and number of $CD21^+$ cells. The percentage (lsmean ± SEM) of $CD21^+$ cells of the PBL and number (lsmean ± SEM) of $CD21^+$ cells in the peripheral blood before and after weaning (n=19 pigs). Pigs were weaned on day 18. Within cell type, least squares means with differences ($p<.05$) designated by different letter (a,b,c).

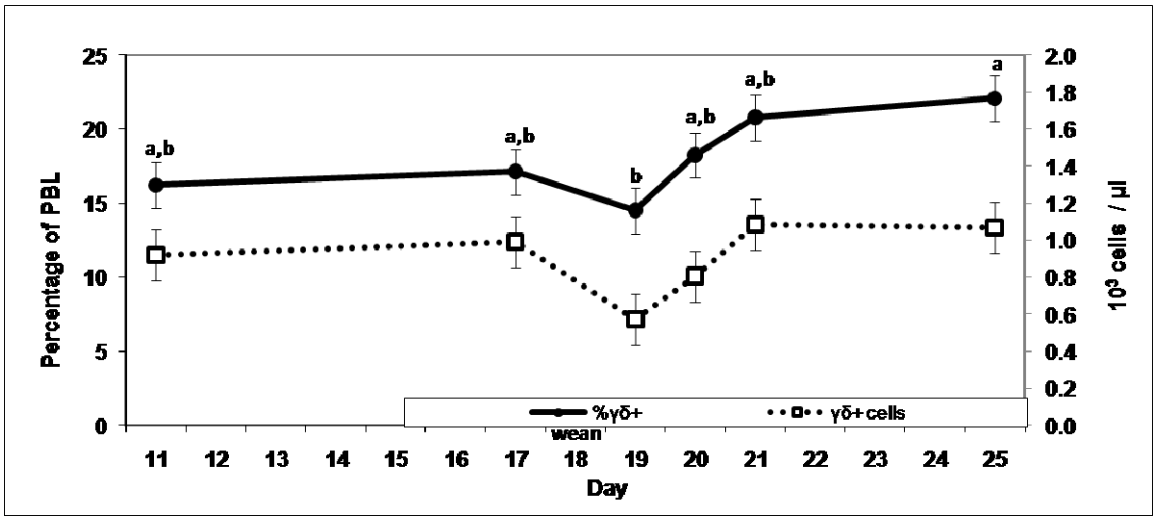

Figure 13. Part Ia percentage and number of $\gamma\delta^+$-T cells. The percentage (lsmean ± SEM) of $\gamma\delta^+$-T cells of the PBL and number (lsmean ± SEM) of $\gamma\delta^+$-T cells in the peripheral blood before and after weaning (n=19 pigs). Pigs were weaned on day 18. Within cell type, least squares means with differences ($p<.05$) designated by different letter (a,b).

Stress-related immune cell ratios

The N:L ratio ($p<.0001$) and CD4:CD8 ratio ($p=.0245$) differed over time (Figure 14). On day 21, the N:L ratio was higher ($p\leq.0126$) than on all other days, and the CD4:CD8 ratio was higher ($p=.0459$) than on day 20. The CD4:CD8 ratio is a commonly utilized comparison for immunology and stress (McGlone et al., 1993; Dhabhar et al., 1995). Due to presence of CD8α+, γδ-T cells in pig blood and the intent to compare ratios of $CD4^+$ T cells to CTLs, the traditional CD4:CD8 ratio is utilized for familiarity; however, the actual comparison is CD4:CTL. This same comparison of CD4:CTL is utilized throughout this study in place of CD4:CD8.

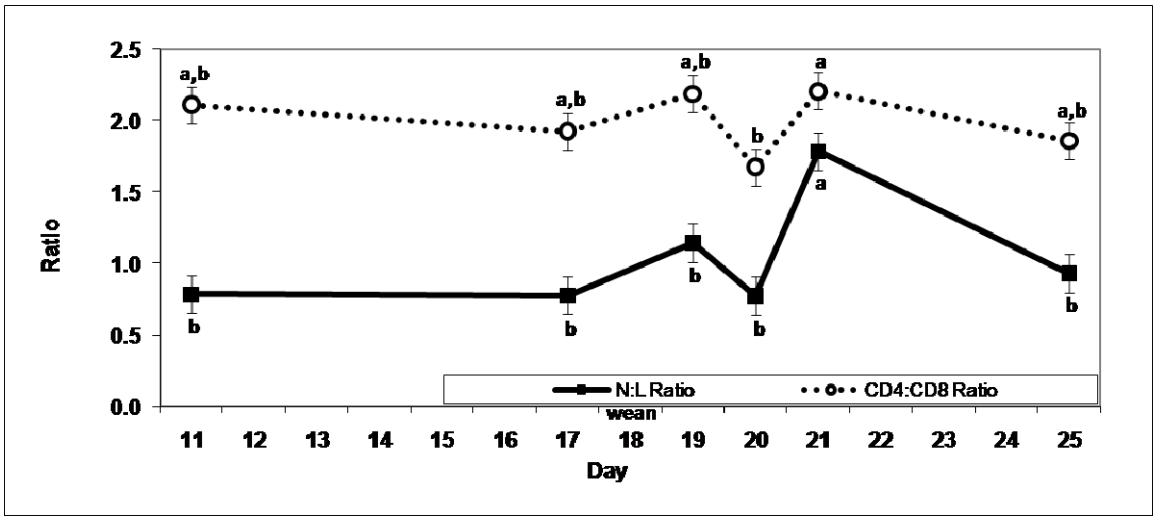

Figure 14. Part Ia N:L and CD4:CD8 ratio. The N:L ratio (lsmean ± SEM) and CD4:CD8 ratio (lsmean ± SEM) in the peripheral blood before and after weaning (n=19 pigs). Pigs were weaned on day 18. Within ratio, least squares means with differences ($p<.05$) designated by different letter (a,b).

Cortisol

Weaning caused an increase in plasma cortisol concentration (Figure 15). Plasma cortisol concentrations on day 19 were higher than on all other days ($p<.0001$) to include day 20 ($p=.0484$). Plasma cortisol concentrations on day 20 were higher than on days 11 ($p=.0225$) and 25 ($p=.0095$).

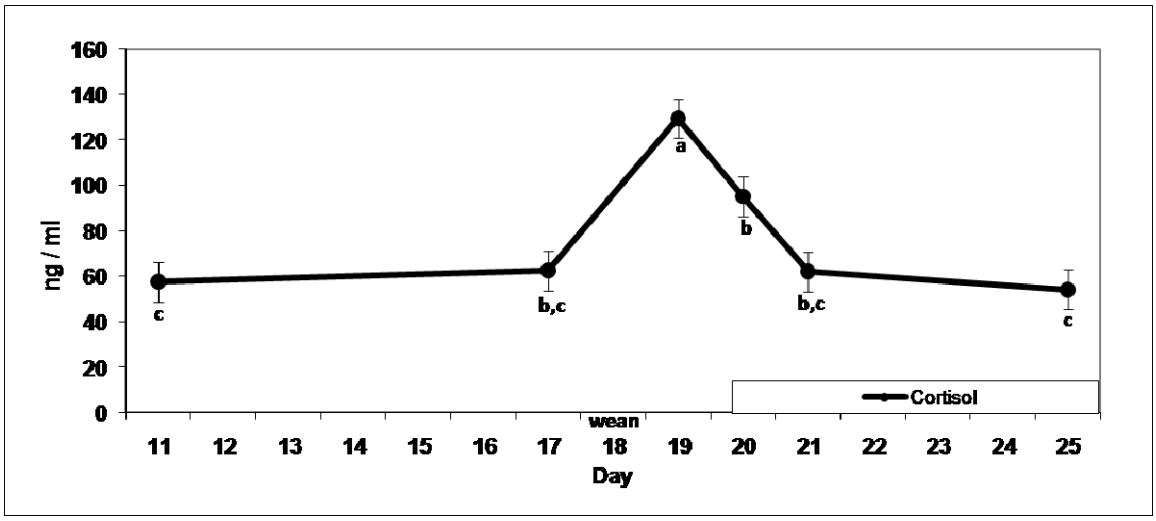

Figure 15. Part Ia cortisol concentrations. Serum cortisol concentrations (lsmean ± SEM) in the peripheral blood before and after weaning (n=19 pigs). Pigs were weaned on day 18. Least squares means with differences ($p<.05$) designated by different letter (a,b,c).

T cell Functionality Test

The T cell functionality was not completed for the weaning portion of the study. Not enough lymphocytes were isolated with Histopaque in order to stimulate in a minimum of duplicate.

Part Ib. Effects of Chronic Stress on the Adaptive Immune System

The purpose of part Ib was to determine the effects of chronic stress on peripheral blood lymphocytes and on physiological measures. The chronic stress of mixing and crowding for five days resulted in a difference between treatments for the percentage change in serum cortisol concentrations immediately following the stress period. The only differences between treatments observed for immunological measures occurred for

neutrophil concentrations at 72 hours after the stress and $CD21^+$ cell concentrations at 48 and 72 hours after the stress, with both higher in the STRESS pigs.

Differential Blood Leukocyte Concentrations

Neutrophil (p=.0031) and lymphocyte (p=.0109) concentrations were different between treatments (Figure 16). On day 54, neutrophils tended to be higher (p=.0999) in the STRESS pigs, and neutrophils on day 55 were higher in the STRESS pigs (p<.001) than in the CONTROL pigs. Though lymphocytes were different as a main effect of treatment, there were not differences by day.

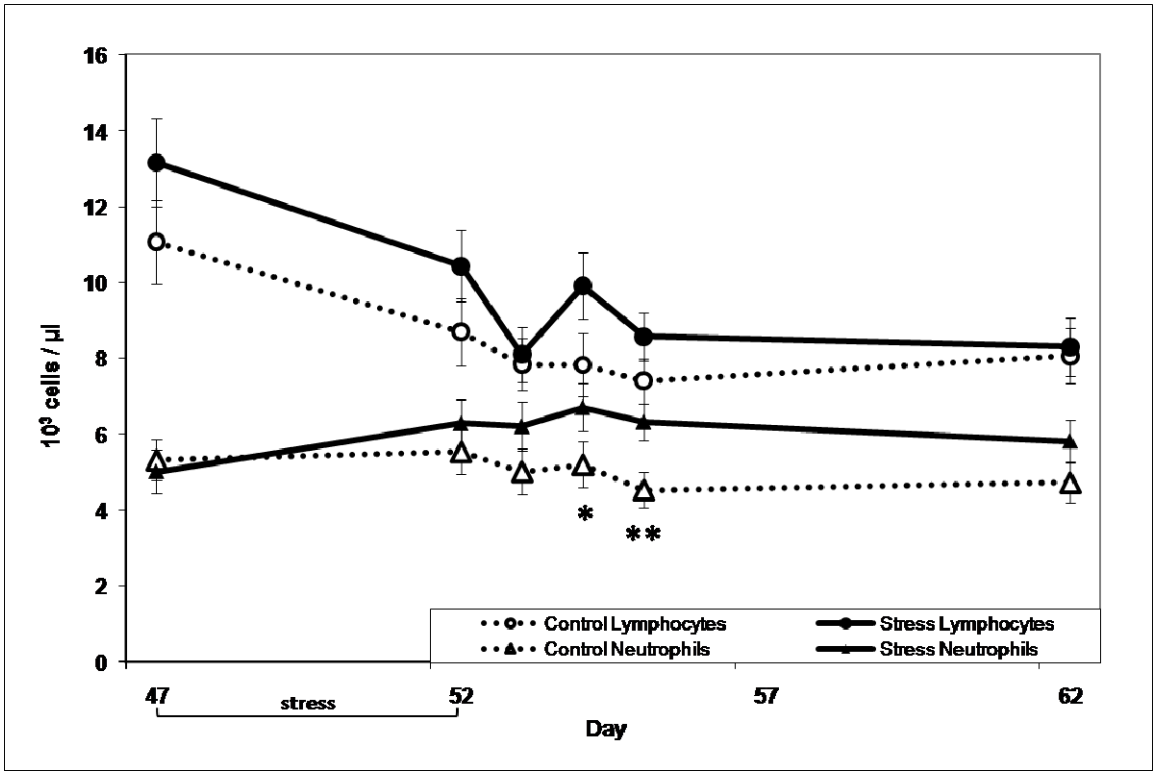

Figure 16. Part Ib lymphocyte and neutrophil concentrations. Lymphocyte and neutrophil peripheral blood concentrations (lsmean ± SEM) in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). Within cell type and by day, differences between lsmeans of treatments designated: *, p<.1; **, p<.05.

Flow cytometry

There were few differences by day between treatments in the percentage of T cell subsets and B cells of the PBL. There was a treatment effect for the percentage of PBL for T cell subsets and the number of cells in the peripheral blood for several cell types.

The number of $CD4^+$ T cells (p=.0219) in the peripheral blood and percentage of $CD4^+CD8^+$ T cells (p=.0458) of PBL and number of $CD4^+CD8^+$ T cells (p=.0282) differed between treatments (Figures 17 and 18). The number of CTLs also tended to differ between treatments (p=.0704). The number of $CD4^+$ T cells on day 52 tended to be higher (p=.0737) in the STRESS pigs than in the CONTROL pigs.

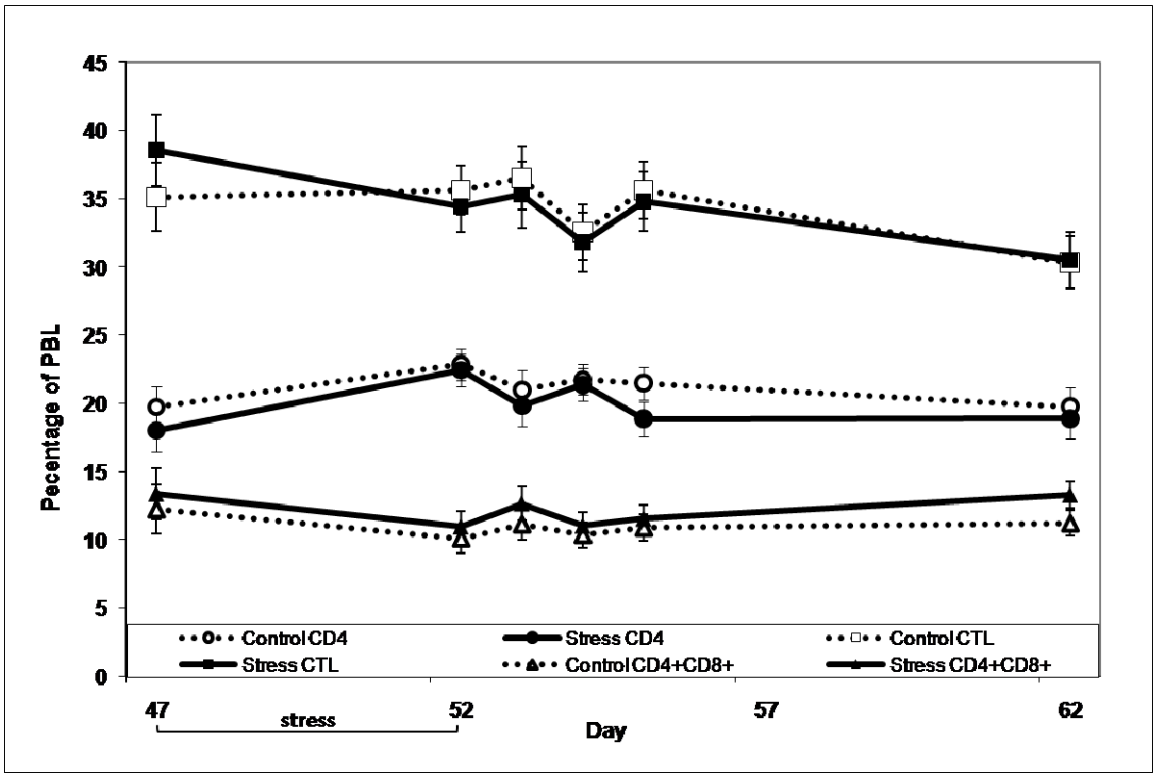

Figure 17. Part Ib percentage of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells. The percentage (lsmean ± SEM) of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells of the PBL in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). Within cell type and by day, there were no differences between treatments.

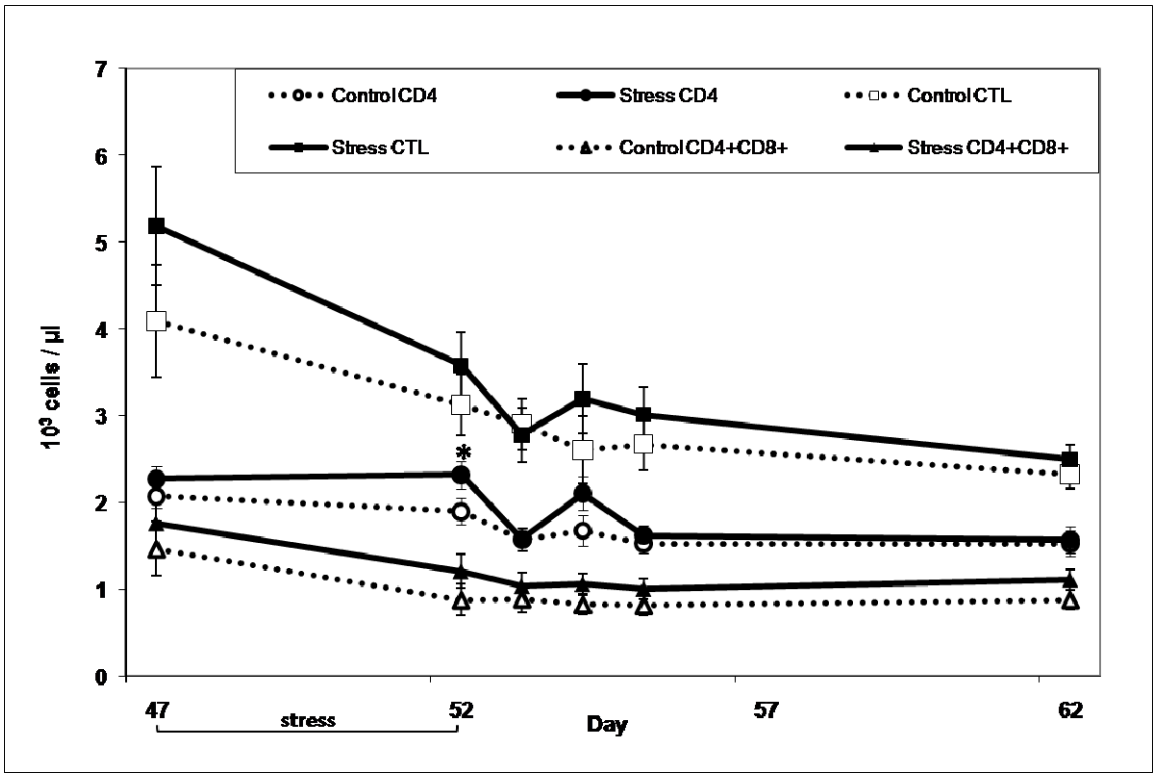

Figure 18. Part Ib number of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells. The number (lsmean ± SEM) of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells in the peripheral blood in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). Within cell type and by day, differences between lsmeans of treatments designated: *, p<.1.

The percentage of $CD21^+$ cells of the PBL (p=.0262) and the number of $CD21^+$ cells (p=.0009) differed between treatments, while there was not a difference (p=.4937) in the percentage or number of $\gamma\delta^+$-T cells (Figures 19 and 20). The percentage of $CD21^+$ cells on days 54 tended to be higher (p=.0875) in the STRESS pigs than in the CONTROL pigs. The number of CD21+ cells was higher in the STRESS pigs than in the CONTROL pigs on day 54 (p=.0079) and on day 55 (p=.0398).

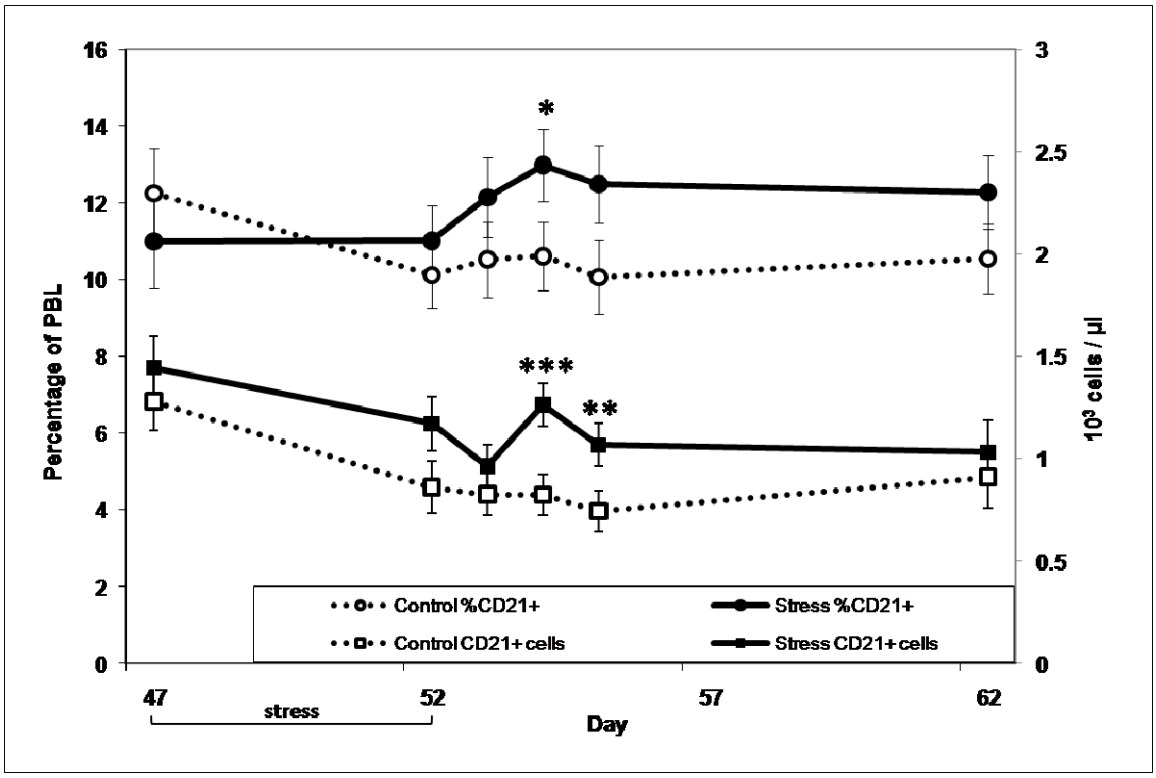

Figure 19. Part Ib percentage and number of $CD21^+$ cells. The percentage (lsmean ± SEM) of $CD21^+$ cells of the PBL and number (lsmean ± SEM) of $CD21^+$ cells in the peripheral blood in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). Within cell type and by day, differences between lsmeans of treatments designated: *, $p<.1$; **, $p<.05$; ***, $p<.01$.

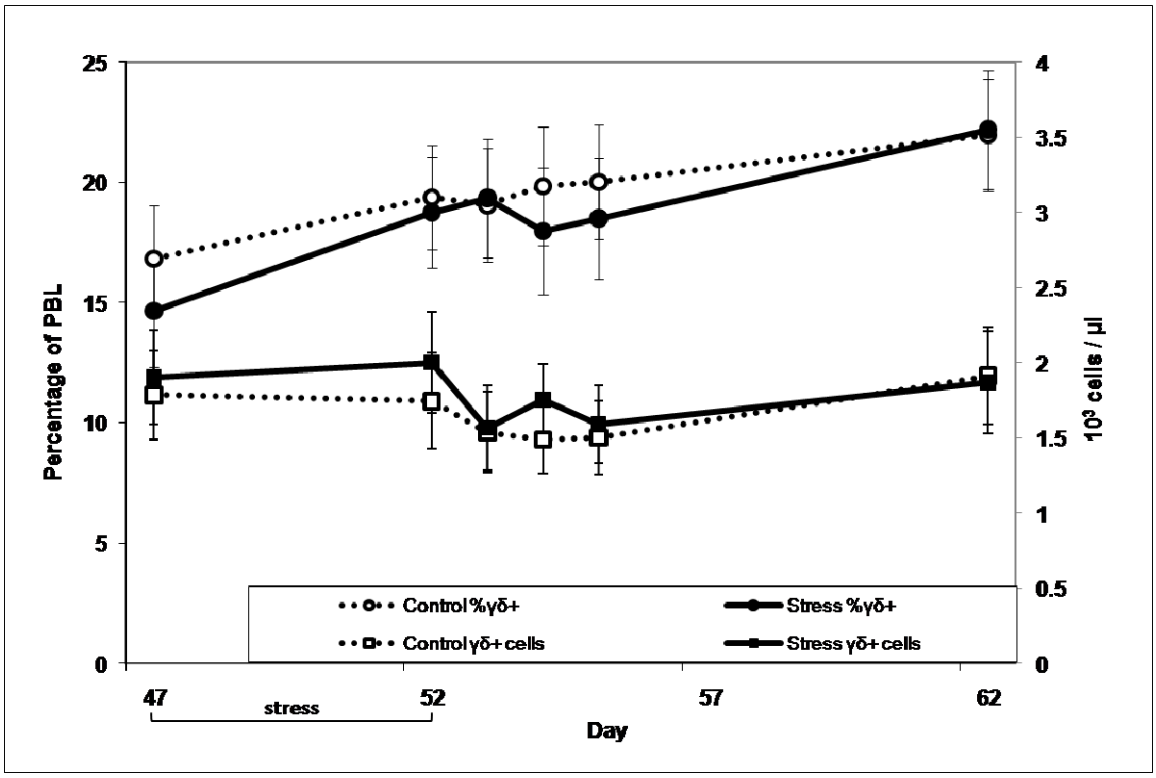

Figure 20. Part Ib percentage and number of $\gamma\delta^+$-T cells. The percentage (lsmean ± SEM) of $\gamma\delta^+$-T cells of the PBL and number (lsmean ± SEM) of $\gamma\delta^+$- T cells in the peripheral blood in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). Within cell type and by day, there were no differences between treatments.

Stress-related immune cell ratios

The N:L ratio (p=.4142) and the CD4:CD8 ratio (p=.1999) did not differ between treatments (Figure 21).

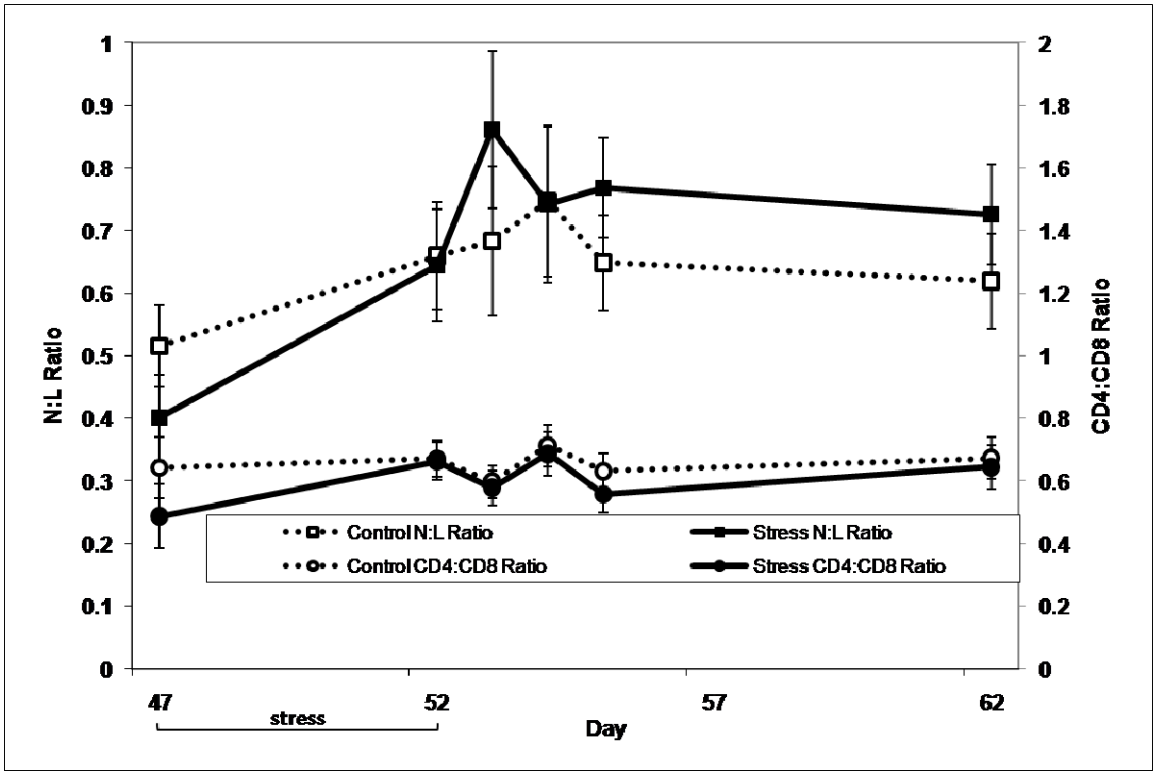

Figure 21. Part Ib N:L and CD4:CD8 ratio. The N:L ratio (lsmean ± SEM) and CD4:CD8 ratio (lsmean ± SEM) in the peripheral blood in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). Within ratio and by day, there were no differences between treatments.

Cortisol

There was not a difference (p=.1110) in serum cortisol concentrations between treatments (Figure 22a). There was a difference (p=.0316) between treatments on day 52 when comparing the by day relative concentration compared to the pre-stress concentration value on day 47 (Figure 22b).

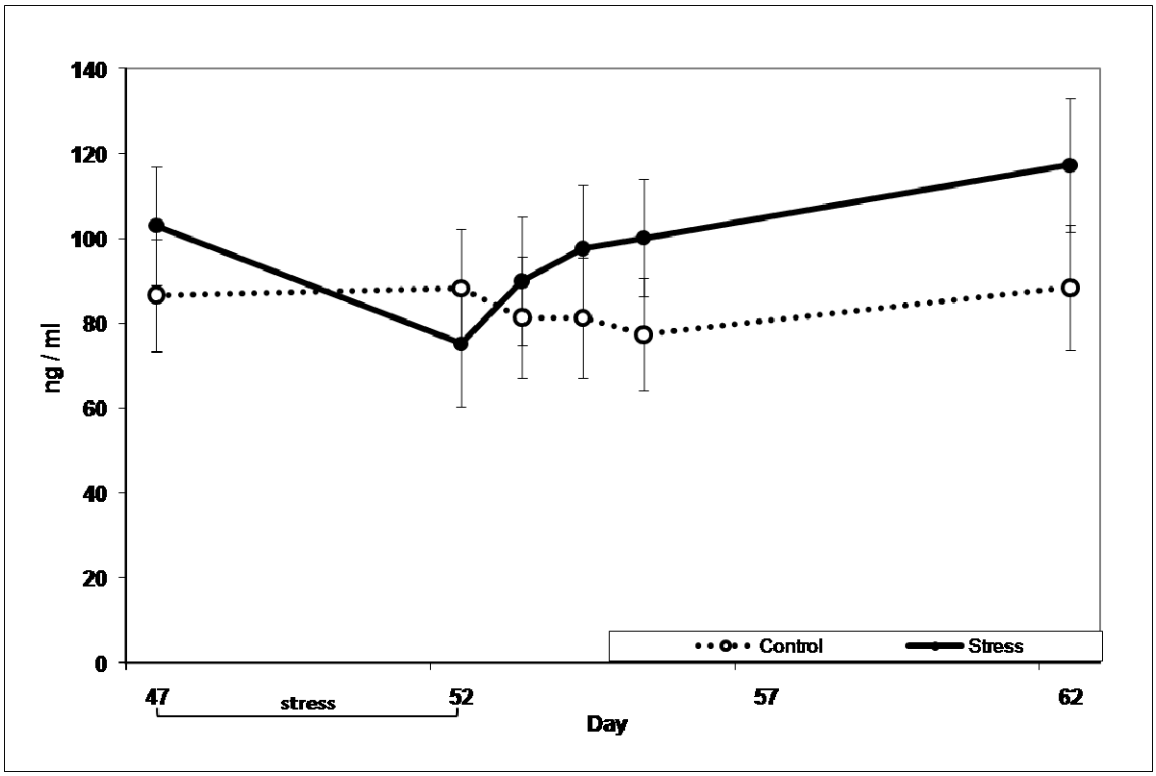

Figure 22a. Part Ib cortisol concentrations. Serum cortisol concentrations (lsmean ± SEM) in the peripheral blood in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). By day, there were no differences between treatments.

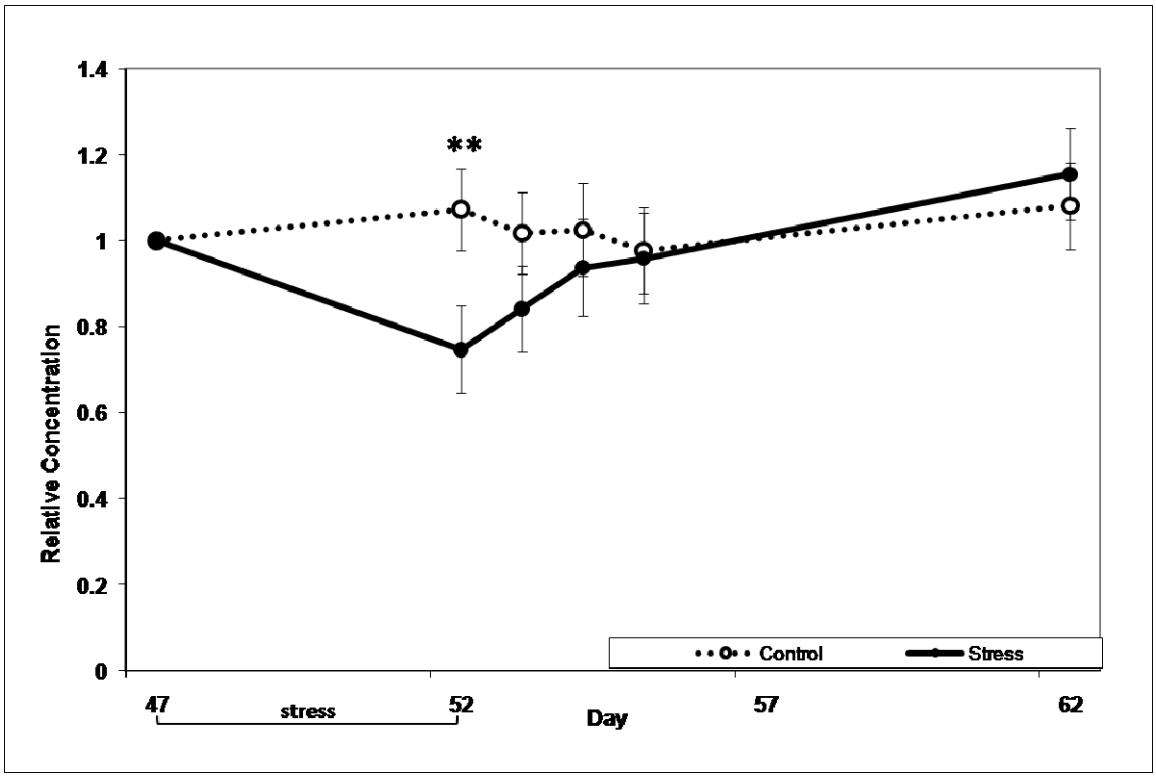

Figure 22b. Part Ib relative cortisol concentrations. cortisol concentrations (lsmean ± SEM) in the peripheral blood in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). By day, differences between lsmeans of treatments designated: **, $p<.05$.

Body weight

There was not a treatment effect (p=.2097) for body weight (BW). There were no differences observed in BW by day or for the percentage change in weight gain between days (Figure 23).

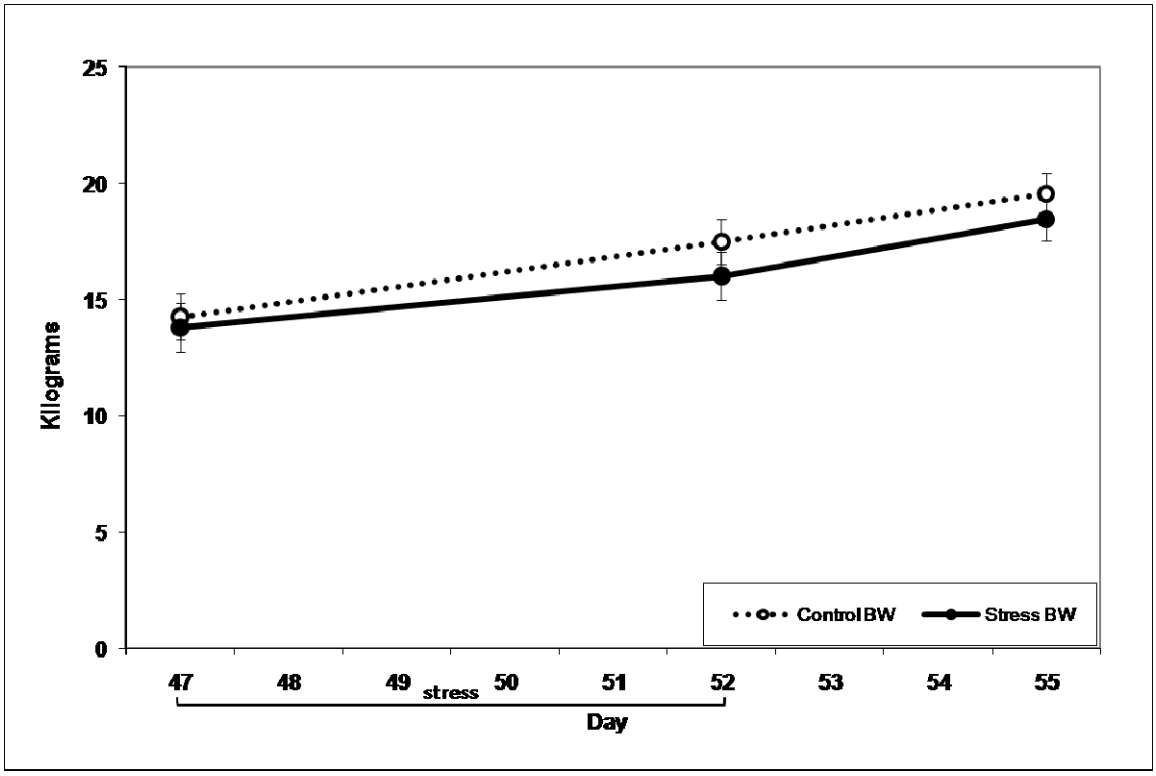

Figure 23. Part Ib body weights. The BW of pigs (lsmean ± SEM) in the CONTROL (n=10 pigs) and STRESS (n=9 pigs) before and after five days of chronic stress (crowding/mixing). By day, there were no differences between treatments.

T cell Functionality Test

There was not a difference between treatments for IL-4 (p=.7690) or IFN-γ (p=.7951) production nor were there any differences between days (Table 15).

Table 15. In-vitro cytokine production (lsmean ± SEM) following ConA stimulation in the control (n=10) and stress (n=9) treatment before and after five days of chronic stress (crowding/mixing).

Trt - Cytokine	Day 47	Day 52	Day 55
Control – IFN-γ (pg / ml)	260.0 ± 54.1	172.4 ± 27.1	278.2 ± 72.7
Stress - IFN-γ (pg / ml)	279.0 ± 57.0	185.7 ± 28.6	211.1 ± 72.7
p-value	.81	.74	.52
Control – IL-4 (pg / ml)	49.7 ± 12.0	71.9 ± 9.3	57.5 ± 8.5
Stress – IL-4 (pg / ml)	61.0 ± 12.6	80.5 ± 9.8	45.1 ± 8.5
p-value	.53	.53	.32

Part II. Effects of Weaning Age on the Adaptive Immune System

The purpose of part II was to determine the effects of weaning age on peripheral blood lymphocytes and on physiological measures. Weaning resulted in an increase in serum cortisol concentrations 24 hours after weaning and lower weight gain for seven days following weaning. Weaning resulted in lymphocytic trapping; however, percentages of peripheral blood lymphocytes were not affected by weaning. The pigs' age at weaning did not affect the overall similar effects of weaning observed among treatments.

Differential Blood Leukocyte Concentrations

Neutrophil (p=.1107) and lymphocyte (p=.1256) concentrations were not different among treatments (Figure 24). On day 27, neutrophils in the 14D pigs tended to be higher (p=.0971) than for the 28D pigs. On day 35, neutrophil concentrations for the 21D pigs tended to be higher (p=.0624) than the 14D pigs and were higher (p=.0385) than the 28D pigs. For lymphocytes, there tended to be a treatment effect of weaning on day 15 (p=.0744) and was a treatment effect on day 29 (p=.0139). On day 29, lymphocyte concentrations for the 28D pigs were lower than the 14D pigs (p=.0193) and the 21D pigs (p=.0423).

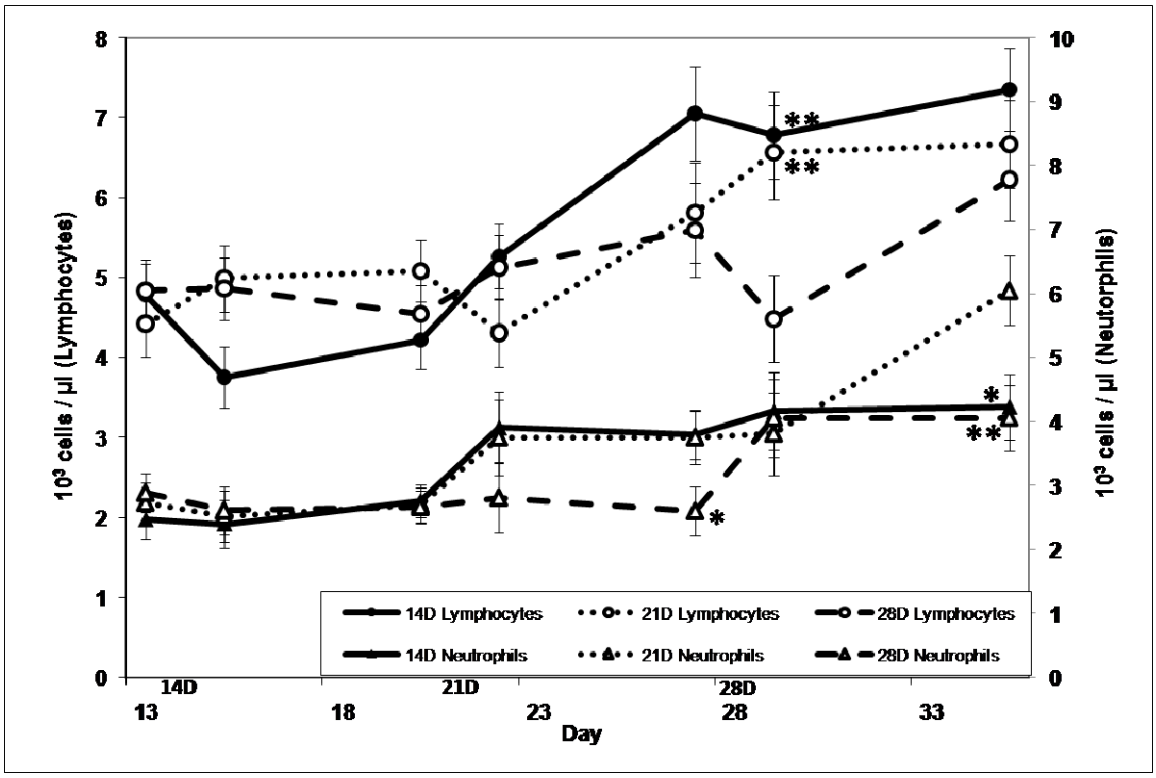

Figure 24. Part II lymphocyte and neutrophil concentrations. Lymphocyte and neutrophil peripheral blood concentrations (lsmean ± SEM) for different weaning ages: 14D (n=8 pigs), 21D (n=7 pigs) and 28D (n=8 pigs). xxD is the day xxD treatment was weaned. Within cell type and by day, differences among lsmeans of treatments designated: *, p<.1; **, p<.05.

Flow cytometry

The percentage of $CD4^+$ T cells (p=.0673) and CTLs (p=.0501) of the PBL tended to differ among treatments, while there was not a treatment effect (p=.2356) for the percentage of $CD4^+CD8^+$ T cells (Figure 25). The number of $CD4^+$ T cells (p=.1603) and CTLs (p=.5179) did not differ among treatments, while there was a treatment effect (p=.0483) for the number of $CD4^+CD8^+$ T cells (Figure 26). On day 27, the number of $CD4^+$ T cells was higher (p=.0086) for the 14D pigs compared to the 21D pigs, and the number tended to be higher (p=.0930) than the 28D pigs. On day 29, the number of

$CD4^+$ T cells was higher (p=.0194) for the 14D pigs than the 28D pigs. On day 20, the number of CTLs was higher (p=.0415) in 21D pigs than the 14D pigs. On day 29, the number of $CD4^+CD8^+$ T cells tended to be higher (p=.069) in the 14D pigs and the 21D pigs (p=.0821) compared to the 28D pigs. On day 35, the percentage (p=.0522) and number (p=.0351) of $CD4^+CD8^+$ T cells was higher for the 14D pigs compared to the 28D pigs.

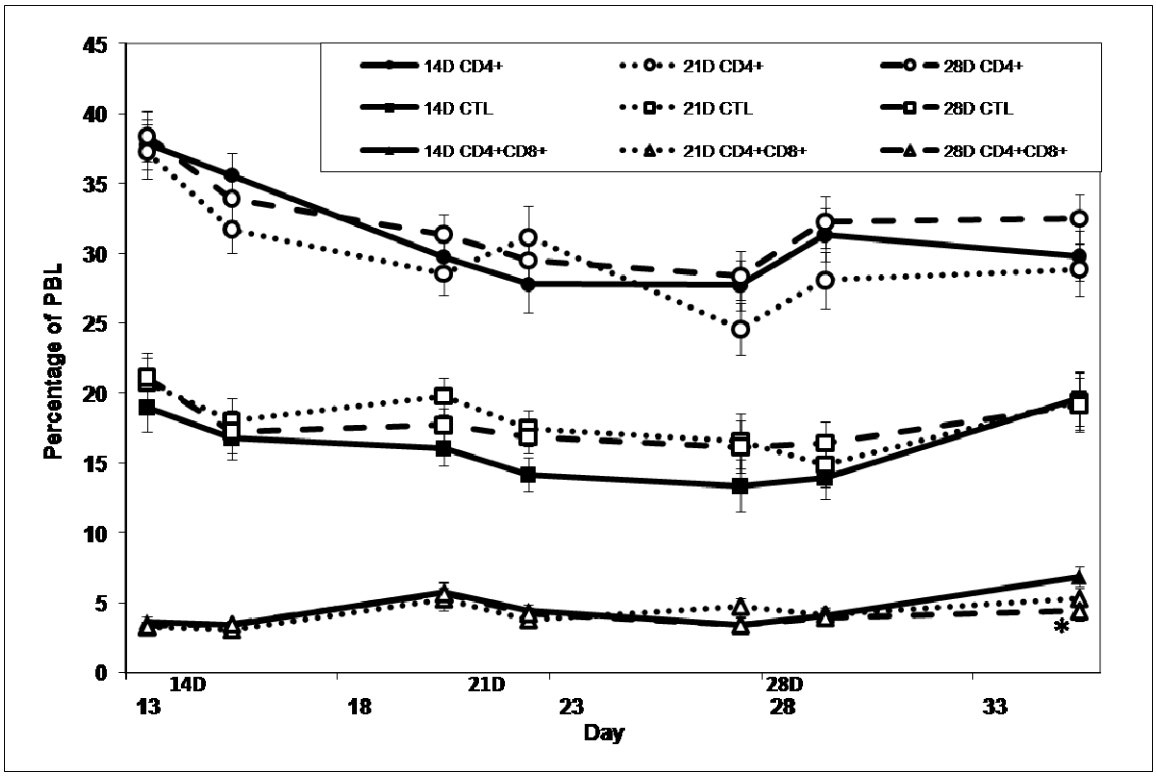

Figure 25. Part II percentage of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells. The percentage (lsmean ± SEM) of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells of the PBL for different weaning ages: 14D (n=8 pigs), 21D (n=7 pigs) and 28D (n=8 pigs). xxD is the day xxD treatment was weaned. Within cell type and by day, differences among lsmeans of treatments designated: *, p<.1.

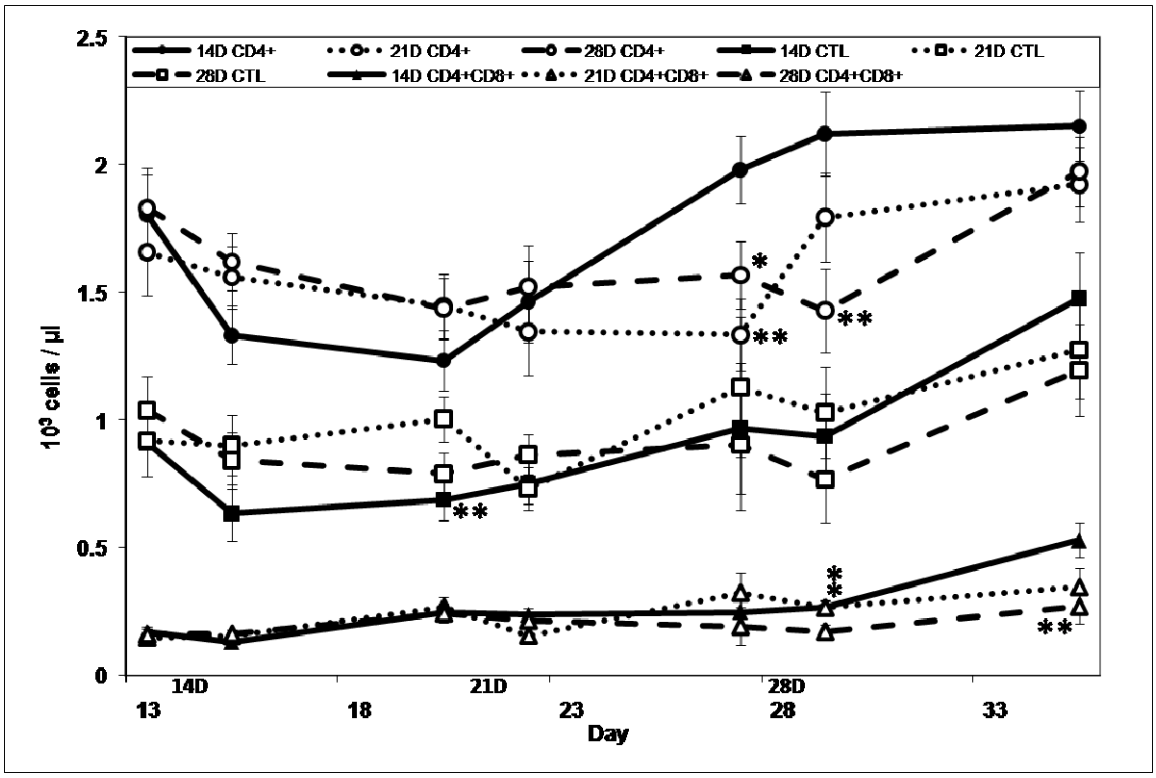

Figure 26. Part II number of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells. The number (lsmean ± SEM) of $CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells in the peripheral blood for different weaning ages: 14D (n=8 pigs), 21D (n=7 pigs) and 28D (n=8 pigs). xxD is the day xxD treatment was weaned. Within cell type and by day, differences among lsmeans of treatments designated: *, p<.1; **, p<.05.

The percentage of $CD21^+$ cells of the PBL differed (p=.0211) among treatments, while there was not a difference (p=.6834) in the percentage of $\gamma\delta^+$-T cells (Figures 27 and 28). On day 27, the number of $CD21^+$ cells was higher for the 14D pigs compared to the 21D pigs (p=.0012) and the 28D pigs (p=.0025). On day 29, the number of $CD21^+$ cells was lower for the 28D pigs compared to the 14D pigs (p=.0062) and the number tended to be lower than the 21D pigs (p=.0779). On day 21, the number of $\gamma\delta^+$-T cells tended to be lower (p=.0851) for the 28D pigs compared to the 21D pigs.

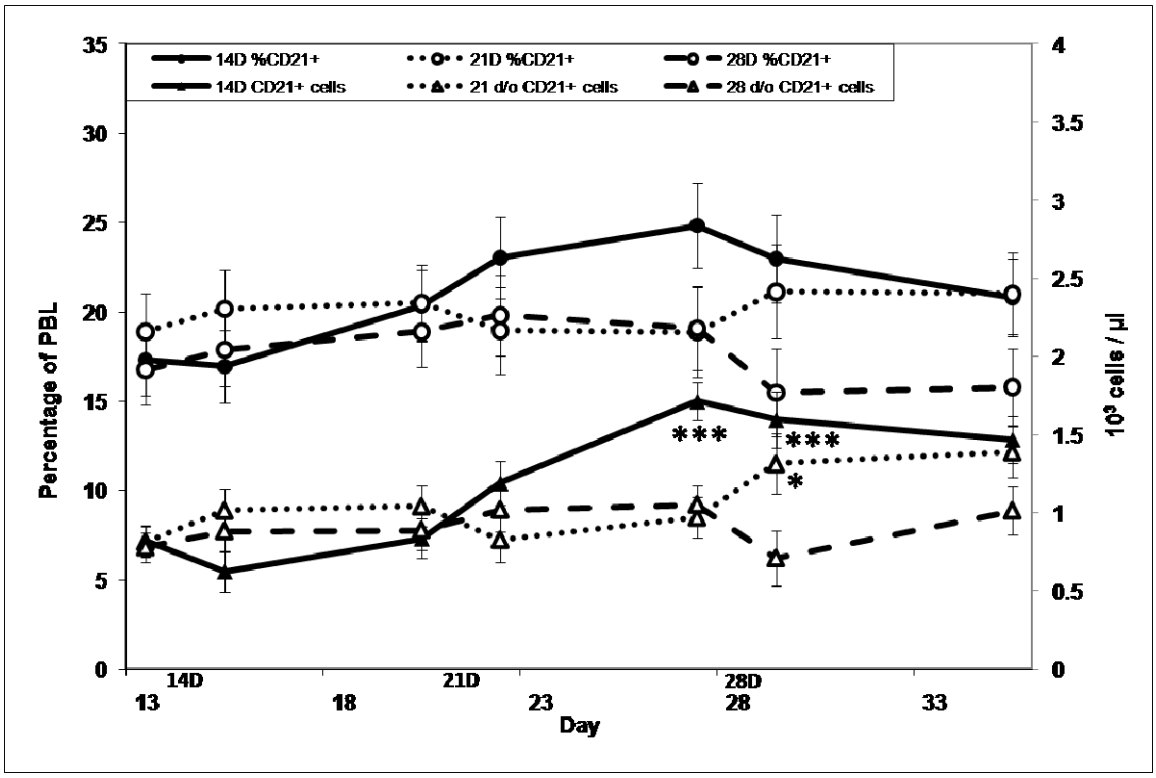

Figure 27. Part II percentage and number of $CD21^+$ cells. The percentage (lsmean ± SEM) of $CD21^+$ cells of the PBL and number (lsmean ± SEM) of $CD21^+$ cells in the peripheral blood for different weaning ages: 14D (n=8 pigs), 21D (n=7 pigs) and 28D (n=8 pigs). xxD is the day xxD treatment was weaned. Within cell type and by day, differences among lsmeans of treatments designated: *, $p<.1$; ***, $p<.01$.

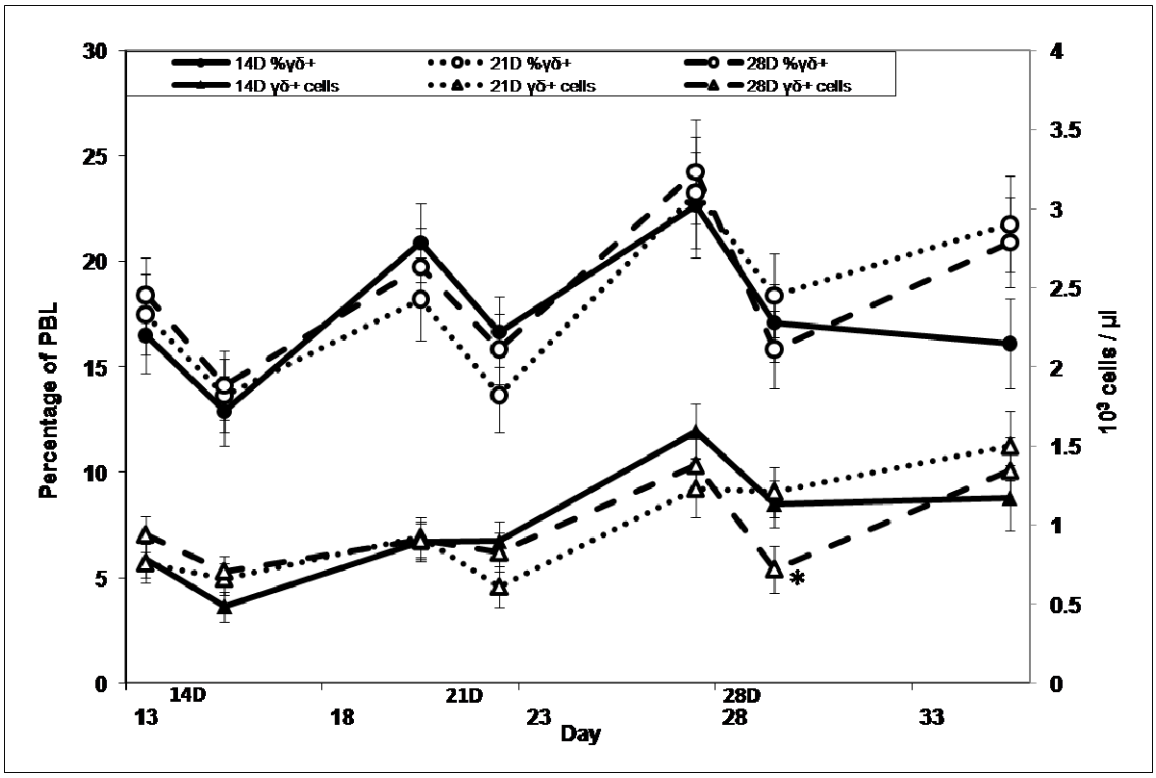

Figure 28. Part II percentage and number of $\gamma\delta^+$-T cells. The percentage (lsmean ± SEM) of $\gamma\delta^+$-T cells of the PBL and number (lsmean ± SEM) of $\gamma\delta^+$- T cells in the peripheral blood for different weaning ages: 14D (n=8 pigs), 21D (n=7 pigs) and 28D (n=8 pigs). xxD is the day xxD treatment was weaned. Within cell type and by day, differences among lsmeans of treatments designated: *, $p<.1$.

Stress-related immune cell ratios

The N:L ratio (p=.6060) did not differ among treatments, while the CD4:CD8 ratio (p=.0921) tended to be different among treatments (Figure 29). On day 20, the N:L ratio for the 14D pigs tended to be higher (p.0981) than for the 21D pigs. On day 35, the N:L ratio for the 21D pigs tended to be higher (p=.0544) than for the 14D pigs.

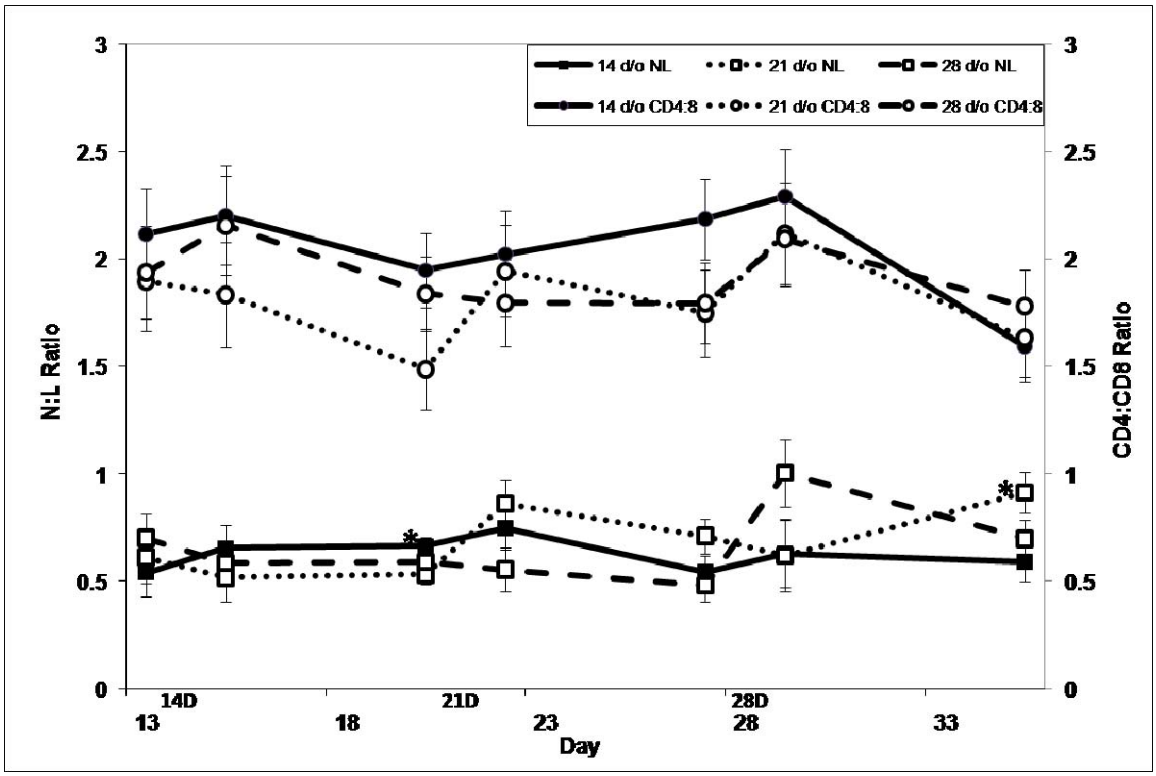

Figure 29. Part II N:L and CD4:CD8 ratio. The N:L ratio (lsmean ± SEM) and CD4:CD8 ratio (lsmean ± SEM) in the peripheral blood for different weaning ages: 14D (n=8 pigs), 21D (n=7 pigs) and 28D (n=8 pigs). xxD is the day xxD treatment was weaned. Within ratio and by day, differences among lsmeans of treatments designated: *, p<.1.

Cortisol

There was a treatment x time interaction for cortisol concentration ($p<.0001$), so the main effect of cortisol was not considered. On day 15 ($p<.0001$), day 22 ($p=.0027$) and day 29 ($p=.0023$), the weaned group had higher cortisol concentrations than the other groups (Figure 30).

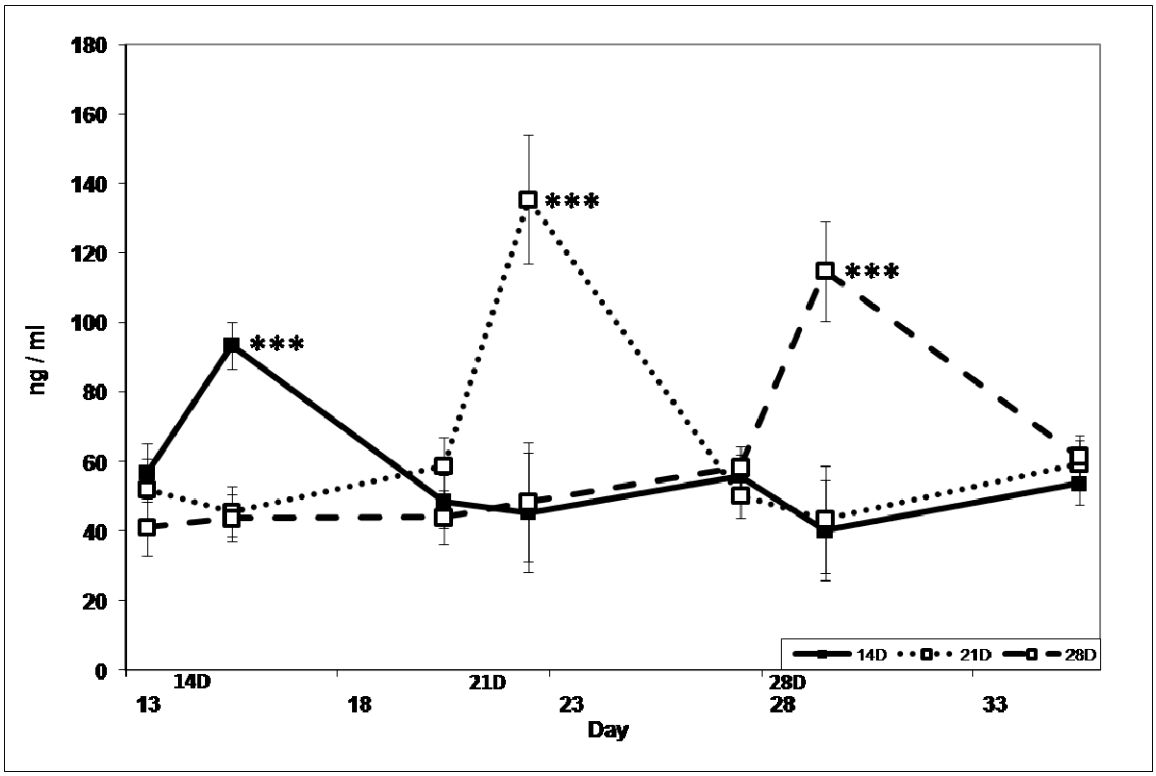

Figure 30. Part II cortisol concentrations. Serum cortisol concentrations (lsmean ± SEM) in the peripheral blood for different weaning ages: 14D (n=8 pigs), 21D (n=7 pigs) and 28D (n=8 pigs). xxD is the day xxD treatment was weaned. By day, differences among lsmeans of treatments designated: ***, $p<.01$.

Body weight

There was a treatment x time interaction for BW (p=.0152). For all three treatments following weaning, the percentage change in BW over the next seven days was lower than for those pigs not weaned (14D, p<.0001; 21D, p≤.017; 28D, p<.0001). Before weaning on days 6 and 14, and one week after all pigs were weaned on day 35, there was no difference in BW among treatments. On day 21 (p=.0159) and day 28 (p=.0103), BW differed among treatments (Figure 31).

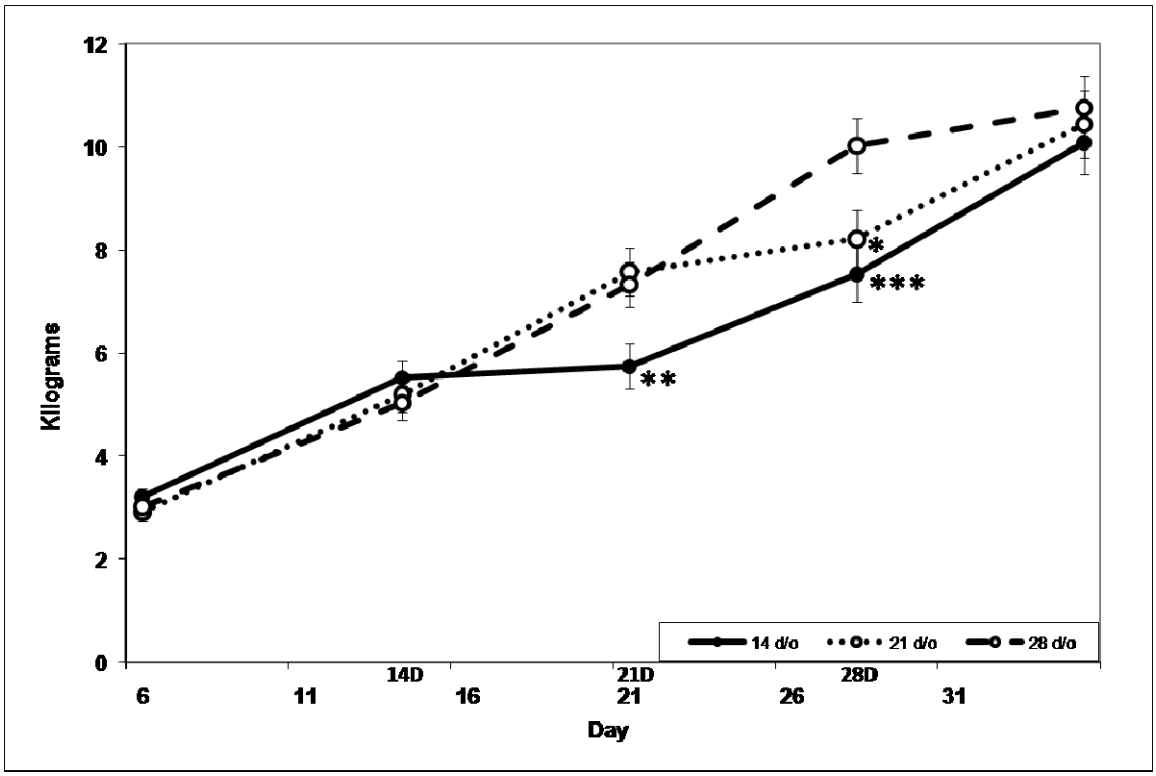

Figure 31. Part II body weights. The BW of pigs (lsmean ± SEM) for different weaning ages: 14D (n=8 pigs), 21D (n=7 pigs) and 28D (n=8 pigs). xxD is the day xxD treatment was weaned. By day, differences among lsmeans of treatments designated: *, p<.1; **, p<.05; ***, p<.01.

T cell Functionality Test

The results of the ELISAs are presented to provide reference values for future research. Due to the tremendous intra-assay and inter-assay variation, lymphocytes with no response to stimulation and variation within treatments, no conclusions are made from this data (Table 16). For day 29, though cells were viable upon completion of the incubation period, when the supernatant was tested, most supernatant was negative for IL-4 and IFN-γ. It is undetermined why the cells did not produce cytokines on that day.

Table 16. In-vitro cytokine production (lsmean ± SEM) following ConA stimulation for different weaning ages: 14D (n=8), 21D (n=7) and 28D (n=8). NSTR=Nothing Significant to Report.

Trt - Cytokine	Day 15	Day 22	Day 29	Day 35
14D – IFN-γ (pg / ml)	44.1 ± 46.1	36.1 ± 55.6	NSTR	136.1 ± 53.5
21D - IFN-γ (pg / ml)	103.2 ± 46.1	14.9 ± 55.6	NSTR	131.3 ± 53.5
28D - IFN-γ (pg / ml)	144.6 ± 46.1	248.0 ± 60.9	NSTR	110.3 ± 65.5
p-value	.33	0.03	NSTR	0.95
14D – IL-4 (pg / ml)	70.8 ± 16.9	35.4 ± 37.0	NSTR	36.4 ± 15.8
21D – IL-4 (pg / ml)	58.8 ± 18.6	79.4 ± 37.0	NSTR	46.1 ± 15.8
28D – IL-4 (pg / ml)	81.2 ± 16.9	100.5 ± 40.5	NSTR	35.2 ± 19.4
p-value	.68	.49	NSTR	0.88

DISCUSSION

Preliminary Study

The preliminary study was critical for validating the method of flow cytometry and the antibodies utilized for staining different T cell subsets and B cells. Additionally, blood collection was restricted to day 3 and day 7 after weaning, which left concerns regarding changes in PBL populations before weaning and in the first 48 hours after weaning. Also, it was determined that it was necessary to differentiate CD8α+, γδ-T cells from CTLs, so additional antibodies were validated in order to complete that task. Lastly, it was purposed to investigate functionality of T cells to analyze whether $CD4^+$ T cells were being modulated or suppressed by stress and whether the stress resulted in a shift in an IFN-γ or IL-4 response.

Percentages and numbers of T cells subsets and B cells in the peripheral blood at different ages were consistent with previous research (Yang and Parkhouse, 1996; Zuckermann and Gaskins, 1996; Sinkora et al., 1998; Solano-Aguilar et al., 2001; Borghetti et al., 2006; Stepanova et al., 2007). An increase in lymphocyte concentrations was observed with age. Additionally, the percentage of $CD8^+$ and $CD4^+CD8^+$ T cells of the PBL increased with age and the percentage of $CD4^+$ T cells of the PBL decreased with age.

Within the first seven days after weaning, differences occurred between treatments for $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ T cells. The percentage of both cell types in

CNTL pigs varied considerably; however, at the time it was unknown whether that was a normal consequence of weaning or whether the vaccination somehow stabilized immune cell populations through lymphocytic trapping. The results of study parts I and II subsequently did not reveal large changes in the percentage of PBL populations after weaning, which suggested that the CNTL pigs, for an unknown reason, had abnormal variation in their $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ T cells rather than stabilization of populations by the vaccination. This variation, probably unrelated to the vaccine, also was revealed with the difference in N:L ratio on day 0 and the different CD4:CD8 ratios within the first seven days. Though there are no published studies utilizing flow cytometry and a *Mycoplasma* vaccination, vaccination for PRRSV resulted in a decrease in $CD4^+$ T cells and an increase in $CD8^+$ T cells in the first two weeks (Martelli et al., 2007). There are considerable differences between the immunological response to the pathogens, namely, CTL directed against PRRSV and $CD4^+$ T cell directed against *Mycoplasma*; however, the purpose in discussing these differences solely is to be prudent about drawing inappropriate conclusion on the changes in T cell subsets.

A future *Mycoplasma* vaccination study should include the following additional elements in order to assess efficacy. First, blood should be drawn prior to weaning and daily after weaning for the first 96 hours and at 7 and 14 days post-vaccination in order to observe the initial immune response (Murphy et al., 2008). Additionally, either a controlled challenge or ideally a natural challenge in an infected commercial herd would provide observable evidence for whether the vaccination prevented disease. Furthermore, repeated blood sampling during the challenge would provide insight into changes in the

PBL populations to better understand differences in response based upon the vaccination. Cytokine production with the pathogen at challenge and then 30 days after challenge would hopefully illustrate greater resistance in the vaccinated pigs even after exposure. Since this pathogen affects the lung and airways, it also would be worthwhile to examine the local immune response associated with the vaccination and active infection.

Part Ia. Effects of Weaning on the Adaptive Immune System

Percentages and numbers of T cells subsets and B cells in the peripheral blood at different ages were consistent with previous research (Yang and Parkhouse, 1996; Zuckermann and Gaskins, 1996; Sinkora et al., 1998; Solano-Aguilar et al., 2001; Borghetti et al., 2006; Stepanova et al., 2007). Cortisol concentrations also were comparable to previously reported values for pigs of this age and following stress (Niekamp et al., 2007; Kojima et al., 2008; Sutherland et al., 2009; Tuchscherer et al., 2009).

First, was weaning a stressful event for the pigs? As a measure of stress, serum cortisol concentrations revealed that weaning was a stressful event for the pigs. The cortisol concentrations on the day after weaning were significantly higher than all other days and it took 48 hours for cortisol concentration to return to normal levels. Serum cortisol concentrations were expected to be significantly higher 24 hours after weaning (Kojima et al., 2008). A conclusion can be made based upon the cortisol concentrations that weaning was stressful for the pigs.

Second, expected changes were observed in the peripheral blood for lymphocytes and neutrophils as a result of stress. Lymphocyte concentrations were lower on day 19 following weaning and the N:L ratio was higher on day 21, 72 hours after weaning. Lymphocytic trapping is a documented response to a chronic stress with beneficial effects for the animal (Dhabhar, 2000). A higher N:L ratio as the result of stress is also commonly observed (McGlone et al., 1993; Sutherland et al., 2009).

Comparing the percentage of T cell subsets and B cells of the PBL, weaning largely did not have an effect. The only difference comparing percentages 24 hours before and after weaning was for the percentage of $CD4^+CD8^+$ T cells. Overall, changes in percentages of subsets were the result of age-related changes. The numbers of T cell subsets revealed greater differences as they couple changes in lymphocyte concentrations with percentages of PBL. Notably, $CD4^+$ T cells and CTLs on day 19 were lower than on day 11, while no change occurred for $CD4^+CD8^+$ T cells.

Interestingly, the N:L ratio and CD4:CD8 ratio both were higher at 72 hours after weaning than at 48 hours after weaning. A physiological change occurred during this period as the result of weaning or the new environment. Scroggs et al. (2002) observed a higher N:L ratio in pigs 72 hours after mixing. In contrast, Dhabhar et al. (1995) observed a higher CD4:CD8 ratio in rats following 2 hours of restraint; however, the ratio returned to normal within three hours after the stress. Species differences, at least in part, may account for differences in this response to stress.

In summary, weaning was a stressful event for the pigs and changes in immunological measures were observed out to at least 72 hours after weaning; however,

the composition of T cell subsets largely did not change as a result of weaning. The largest influence in changes in peripheral blood lymphocyte populations was increased age.

Part Ib. Effects of Chronic Stress on the Adaptive Immune System

Percentages and numbers of T cells subsets in the peripheral blood at 40 – 60 days of age were consistent with previous reports (Solano-Aguilar et al., 2001; Borghetti et al., 2006; Gerner et al., 2009). Cortisol concentrations also were comparable to previously reported values for pigs of this age and following stress (Deguchi and Akuzawa, 1998; Hicks et al., 1998; Sutherland et al., 2005).

In answering the first question of whether the treatment of mixing and crowding was indeed stressful, there was not a significant difference in cortisol concentrations between treatments or by day within treatments; however, there was a difference in the relative cortisol concentration between treatments immediately following the chronic stress on day 52 with STRESS pigs' relative concentrations being lower than the CONTROL pigs. Due to the nature of a chronic stress, blood samples were not collected until after five days of stress, so whether initial changes in cortisol occurred in the STRESS pigs upon mixing and crowding is unknown. Mixing after one hour resulted in significantly higher cortisol concentrations; however, after 24 hours cortisol concentrations returned to basal levels (Deguchi and Akuzawa, 1998). From the data, it is possible that chronic stress resulted in abrogation of the cortisol diurnal pattern as a

result of chronic stimulation of the hypothalamo-hypophyseal-adrenal axis. In contrast, Mormède et al. (2007) reviewed considerable conflicting results in swine as to the effects of chronic stress on serum cortisol concentration and concluded chronic stress had a non-predictable effect on basal porcine cortisol levels. Accordingly, it was not determined whether crowding and mixing in the present study were sufficient to affect the animal's welfare based upon cortisol concentrations.

Secondly, BW and the percentage change in BW were not significantly different between treatments. A stress treatment of 28 days of heat reduced BW gain (Morrow-Tesch et al., 1994); however, separately, acute or chronic temperature stress had no effect on BW (Minton et al., 1988; Hicks et al., 1998). Following 14 days of a chronic stress (heat, crowding and mixing), BW was significantly lower in the stress treatment compared with the control (Sutherland et al., 2006). Likely, the duration of the stress was not sufficient to reduce BW gain in the STRESS pigs in the present study.

The chronic stress resulted in limited differences between treatments for immunological measures. Neutrophils were higher at 72 hours after the conclusion of the treatment in STRESS pigs compared to CONTROL pigs. The number of $CD21^+$ cells was also higher in STRESS pigs at both 48 hours and 72 hours after conclusion of the treatment. For an acute stress of 4 hours of isolation, isolation resulted in a lower percentage of $CD4^+$ T cells and higher percentage of $CD8^+$ T cells at 7, 21 and 35 days of age (Tuchscherer et al., 2009). Shipping stress in growing pigs did not affect the percentage of $CD4^+$ or $CD8^+$ T cells of the PBL, but did decrease the percentage of B cells of the PBL (McGlone et al., 1993). There are no studies that utilized flow

cytometry to analyze a chronic stress in pigs. N:L ratios were commonly increased in stress treatments that included mixing (Scroggs et al., 2002; Hyun et al., 2005; Damgaard et al., 2009); however, chronic stress can also have no effect on N:L ratios (Sutherland et al., 2006). The effects of mixing on T cell blastogenesis was also varied, having no effect (de Groot et al., 2001; Sutherland et al., 2006) or decreasing blastogenesis (Deguchi and Akuzawa, 1998).

The results of the in-vitro cytokine production were more consistent in this study than in part II; however, particularly for the IFN-γ ELISA, the large inter-assay CV made it difficult to draw conclusions. When comparing other stress treatments, investigators used three different mitogens/pathogens to stimulate the lymphocytes and targeted differing cytokines, and only one investigator (Tuchscherer et al., 2009) saw decreased cytokine production from the stress treatment (de Groot et al., 2001; Davis et al., 2006; Tuchscherer et al., 2009).

In summary, five days of crowding and mixing, though it appeared to be stressful to the observer, did not affect immunological measures in the STRESS pigs. Previous research demonstrated differences in particularly N:L ratios in stress treatments; however, for this chronic stress of five days, the STRESS pigs quickly adapted to the crowding and mixing and were not different than the CONTROL pigs.

Part II. Effects of Weaning Age on the Adaptive Immune System

Percentages and numbers of T cells subsets and B cells in the peripheral blood at different ages were consistent with previous research (Solano-Aguilar et al., 2001; Borghetti et al., 2006). Cortisol concentrations also were comparable to previously reported values for pigs of this age and following stress (Niekamp et al., 2007; Kojima et al., 2008; Sutherland et al., 2009; Tuchscherer et al., 2009).

Similar to part Ia, cortisol concentrations of the pigs one day after weaning compared to the other treatments, demonstrated that weaning was a stressful event for the 14D, 21D and 28D pigs. Serum cortisol concentrations were expected to be significantly higher 24 hours after weaning (Kojima et al., 2008). Comparing spikes associated with weaning would not be useful since serum cortisol concentrations change with age. Blood samples were not collected daily following weaning like in part Ia; however, by 6 days after weaning serum cortisol concentrations returned to normal levels for all treatments affirming the limited duration of weaning stress at each weaning age.

Body weight changes also demonstrated that weaning was a stressful event with significant decreases in the percent change in BW for the seven days following weaning for all treatments. Notably, by day 35, there were no differences in BW among treatments; however, it is unknown what longer term effects weaning age would have had on BW since the study was completed on day 35. In a study comparing BW through the nursery phase, pigs weaned at 21 days of age gained weight faster than those weaned at 14 days of age (Davis et al., 2006). At 10 weeks of age, BW of pigs weaned at 28 days of age was higher than pigs weaned at 14 days of age. In contrast, pigs weaned at 21

days of age did not differ between either 14 or 28 day old weaning (Niekamp et al., 2007).

Lymphocytic trapping was most pronounced in pigs weaned at 28 days of age with 14D and 21D pigs' lymphocyte concentrations significantly higher than 28D pigs. Neutrophils were higher at day 35 in 21D pigs; however, there was not a clear reason for this difference. An increase in lymphocyte concentration was observed when comparing pigs weaned at 21 days of age versus 14 days of age; however, this difference could also have been simply age related increases (Davis et al., 2006). N:L ratios differed among weaning age; however, again age related differences in leukocyte concentrations were not accounted for (Niekamp et al., 2007).

Statistically significant difference were observed among treatments for the number of T cell subsets ($CD4^+$ T cells, CTLs and $CD4^+CD8^+$ T cells) and the percentage of $CD21^+$ cells in the peripheral blood; however, these differences did not correlate with weaning. The percentages of $\gamma\delta^+$-T cells and accordingly the numbers of $\gamma\delta^+$-T cells on days 15, 22, 29 and 35 were lower than the respective total on days 13, 20, 27 because the blood on those days (15, 22, 29 and 35) was refrigerated for 24 hours before flow cytometry was conducted, so that lymphocyte stimulation could occur immediately following bleeding. The observable trend that the $\gamma\delta^+$-T cells begin to die in refrigeration was not known until after all flow cytometry was completed.

In-vitro cytokine production was unreliable for this portion of the study for a number of possible reasons. A discussion on that method and the collected results is

reserved for Appendix A. Weaning at 14 or 21 days of age had no treatment effect on ConA stimulated IFN-γ production during the nursery phase (Davis et al., 2006).

In this study, physiological and immunological changes were observed with weaning for each treatment; however, those differences were eliminated by day 35, one week after all pigs were weaned. There was not a definitive immunological or physiological benefit among weaning ages, which conflicts with previous research. For physiological measures, as previously discussed, older weaning age correlated with increased BW in studies that lasted longer time periods (Davis et al., 2006; Niekamp et al., 2007). Utilizing different immunological measures, T cell blastogenesis at 24 hours after weaning was lower in pigs weaned at 14 or 21 days of age than those not weaned; however, no difference was observed at a weaning age of 35 days (Blecha et al., 1983). T cell blastogenesis was not affected by weaning age in two other studies (Davis et al., 2006; Niekamp et al., 2007). The available laboratory tests remain ill-equipped to definitively decide on whether weaning age affects the immunological competence of pigs.

In summary, weaning at 14, 21 or 28 days of age was a stressful event and immunological changes were observed particularly lymphocytic trapping at 14 and 28 days of age. Demonstrated differences in the percentage of T cell subsets and B cells were not present. By day 35, pigs from 14D, 21D and 28D appeared to be equally physiologically and immunologically competent. Based upon this data, it cannot be concluded that weaning at 14, 21 or 28 days of age compromises the welfare of pigs.

CONCLUSION

The purpose of this thesis was to gain a better understanding of the effects of stress on the adaptive immune system in pigs. Twelve months ago when the experimental design was envisioned, it was hoped the peripheral blood and particularly the IFN-γ and IL-4 ELISAs would demonstrate a clear effect of stress. A year later, like so many researchers before, drawing conclusions based upon the results was difficult.

Clearly, weaning was a stressful event in the pig's life causing a significant increase in serum cortisol concentrations that lasted 48 hours. Additionally, the percent change in BW was negatively affected by weaning over the next seven days. Immunologically, the pigs were not compromised by the weaning stress. Lymphocytic trapping occurred, which was shown by Dhabhar et al. (2000) to be beneficial by increasing lymphocyte presence at likely pathogen entry points. Weaning did not have a clear effect on T cell subsets in the peripheral blood at 24 or 48 hours after weaning, though changes in the CD4:CD8 ratio could have been missed based upon the rapid return to normal levels demonstrated by Dhabhar et al. (1995). Additionally, weaning age did not have a different immunological effect among treatments. Based upon this information, there is not an immunological reason to restrict weaning to pigs 28 days or older for animal welfare concerns (ECCD, 2001).

The chronic stress probably did not compromise the pigs immunologically. After the initial mixing and hierarchy was established in the first 24 hours (McGlone, 1986),

the crowding though probably enough to restrict weight gain (Brumm, 2008) was not immunologically unfavorable, at least by the measures utilized in this study. Producers should not crowd pigs for decreased performance measures; however, this study did not demonstrate a decrease in immunological performance. Like in weaning, this study did not produce any immunological reason from the peripheral blood why crowding and mixing decrease animal welfare.

In conclusion, this thesis did not produce evidence in the peripheral blood of immunological decreases in animal welfare as the result of a chronic stress or different weaning ages. The Swine Educational Unit is a high health status herd. The next step to further this research is to conduct the same treatments for a chronic stress and weaning age comparison at a commercial herd with a lower health status. Even if more predictable immunological trends were observed as a result of the stressors in this study, ultimately what really matters to animal welfare is do stressed pigs have a higher occurrence of disease? If the stress treatment, either mixing/crowding or early weaning does not cause an increase in disease in the group, then there is not an immunological case for animal welfare concerns. Scientific immunological tests and measures are critical to increase understanding of the immense complexity of the interaction between stress and the immune system; however, when determining animal husbandry practices either privately or publicly, stress resulting in increases in disease should be the proof required in order to conclude a practice is harmful to animal welfare.

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APPENDIX

APPENDIX A

Discussion on ELISA Results

It was anticipated that the results from the IFN-γ and IL-4 ELISAs would further our understanding of the effects of stress on an IFN-γ or IL-4 response (Salak-Johnson and McGlone, 2007). Though the time effort was extensive and cost significant, the results were not significant and were unreliable, particularly for part II. Results for part Ib for IL-4 production were very consistent and except for the interassay variation, IFN-γ results were sufficient to at least feel confident in declaring there were no significant differences between treatments. Recently, other reports utilized ELISAs for in-vitro cytokine production with mixed success.

Davis et al. (2006) tested IFN-γ production by stimulating lymphocytes with ConA in nursery pigs and finishing pigs. No treatment effects were observed; however, replication was consistent and SE were less than 25% of values. Brown et al. (2006) tested IL-2 and IL-4 production by lymphocytes with ConA stimulation in pigs at 7, 14 and 18 days of age and had tremendous variation and SE from 70% to greater than 100% of the values. In addition, Diaz et al. (2005), using PHA stimulation, observed considerable changes in IFN-γ production with age (1 – 22 weeks of age) with SE ranging from 25% - 100% of totals. IL-4 was largely undetectable in their results. de Groot et al. (2001) obtained expected results for IFN-γ and IL-10 production when stimulating with PRV after vaccination and challenge, and SE were less than 20% of totals.

Though it is not possible to determine the precise causes of the poor results for the ELISA part II, there are several possible sources of error. First, the blood from pigs that were not weaned had high levels of cholesterol, which co-located with the buffy coat after centrifugation with Histopaque. This made obtaining enough cells difficult for some pig samples. For the ELISA in part Ib, pigs were a minimum of 47 days of age, while the pigs were 15 – 35 days of age for part II. Potentially, there was a difference in ConA utilized between part Ib and part II; however, though ordered separately, the ConA was all of the same lot. Third, cell viability after 24 hours of stimulation was consistent throughout all four iterations of the experiment, so it is unlikely that there was a problem in incubation particularly on day 29. Also, samples from both day 29 and 35 were on the same plates of the ELISA, so the poor results were not caused by a manufacturer error. The remaining source of error was laboratory error, which could have contributed to the poor results.

Due to the cost associated with porcine ELISA kits, production of ELISA kits in our lab should be considered before conducting additional research. Additionally, a more thorough validation of the ELISA kits for pigs at different ages must be conducted before beginning new research. Lastly, deviations from the Histopaque product manual should be investigated in order to improve isolation and recovery of lymphocytes.