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Effects of Lipopolysaccharide Induced Inflammatory Response on Early Embryo Survival in Ewes

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**Effects of Lipopolysaccharide Induced Inflammatory Response
on Early Embryo Survival in Ewes**

Megan Renee Graham

**Thesis submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Master of Science
in
Reproductive Physiology**

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Division of Animal and Nutritional Sciences

**Morgantown, West Virginia
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**Keywords: Early Embryonic Loss, Lipopolysaccharide, Acute Phase Response,
Sheep, Inflammation, Tumor Necrosis Factor-Alpha, Dexamethasone**

Abstract

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Megan Graham

Early pregnant ewes, 5 or 6 day post coitus (dpc) were used as a model to study early embryonic loss via gram-negative bacterial infections, such as mastitis. Ewes 5/6 dpc were injected with a gram-negative bacteria cell wall component, lipopolysaccharide (LPS) or tumor necrosis factor-alpha (TNF- α), to induce an innate immune system acute phase response (APR). The induction of the APR and its reactant molecules, such as TNF- α and acute phase proteins (APP), haptoglobin (Hp) and serum amyloid A (SAA), were initiated to study their effects on embryonic loss. In addition, ewes were also injected with LPS plus dexamethasone (Dex) to study its effects on indirectly altering embryonic loss through toll-like receptor 4 (TLR4). Thirty-eight Dorset x Texel ewes were synchronized for estrus and bred by fertile rams. On 5 or 6 dpc, ewes were assigned to one of four treatment groups per pen and received via the jugular vein either 2.5 mL of 0.1% BSA/PBS (controls, n=9), 2.5 mL of 2.5 μ g/kg of LPS (n=9), 5 mL of 1 μ g/kg of TNF- α (n=10) in two bolus injections given thirty minutes apart, or 2.5 mL of solution containing 2.5 μ g/kg of LPS after having received 3.5 mL of solution containing 0.14 mg/kg BW im dexamethasone (Dex, n=10) at -12 and 0 hours. Plasma was collected from the jugular vein before challenge, followed by post challenge samples every 30 minutes until 3 hours and every hour until 12 hours, and once at 24, 36, and 48 hours. In addition, behavioral changes and rectal temperature were also documented before challenge injections followed by every hour for 12 hours post challenge, and processed soon after collection for total white blood cell counts, and plasma was stored at -80°C. A white blood cell differentiation was determined by staining and counting one hundred cells classified among monocyte, lymphocyte, eosinophil, neutrophil, or basophil cell types. Assays were conducted for APR reactants, TNF- α , SAA, Hp. Jugular samples were collected in EDTA treated tubes on days 9 or 10 and 25 or 26 pc for determination of concentrations of progesterone (P₄) for evaluation of luteal function. At day 25 or 26 pc, detection for pregnancy was examined. Intoxication of day 5 or 6 pregnant ewes treated with LPS, TNF- α , or LPS+Dex did not differ in pregnancy status among treatment groups (p=0.298) and more control ewes plus ewes treated with TNF- α tended to remain pregnant than ewes treated with LPS or LPS+Dex (p=0.05). Total white blood cell count differed by treatment (p<0.0001), hour (p<0.001), and treatment by hour (p<0.0001). There was an effect of treatment on lymphocytes (p<0.0001) and monocytes (p=0.0103). There was an effect of hour on lymphocytes (p<0.0001), neutrophils (p<0.0001), and monocytes (p<0.0001). There was an effect of treatment by hour on neutrophils (p=0.0047). There was no difference in Hp concentration by treatment (p=0.0859), hour (p=0.4317), and treatment by hour (p=0.0996). There was a difference in SAA concentration by treatment (p<0.001), hour (p<0.001), and treatment by hour (p<0.001). The inflammatory response and APR was elicited via treatment with LPS. Although LPS treatment affected pregnancy, treatment with TNF- α did not. Dexamethasone did attenuate the inflammatory response but did not increase pregnancy outcome.

Table of Contents

Abstract	ii
Table of Contents	iii
List of Figures	iv
Acknowledgements	1
Literature Review	2
Introduction.....	2
Early Pregnancy.....	4
Embryogenesis.....	6
Maternal Recognition of Pregnancy.....	8
Bacteria.....	10
Innate Immunity.....	14
Adaptive Acquired Immunity.....	31
Glucocorticoid Action.....	33
Summary.....	37
Objectives	41
Materials and Methods	41
Synchronization Protocol for Ewes.....	41
Treatment Assignments and Preparations, Blood Collection (5/6 dpc).....	42
Assays.....	43
Statistical Analyses.....	44
Results	45
Rectal Temperatures.....	52
Mucosal Responses and Lethargy.....	53
Total White Blood Cell Count in Jugular Blood.....	54
Concentrations of Lymphocytes, Monocytes, and Neutrophils in Jugular Blood.....	55
Percent of Lymphocytes, Monocytes, and Neutrophils in Jugular Blood.....	56
Concentrations of TNF- α , Hp, and SAA in Jugular Plasma.....	57
Progesterone Concentrations on days 9 and 10 and 25 and 26 pc.....	58
Pregnancy Status on days 25 and 26 pc and Lambing Percentage.....	59
Discussion	60
References	71

List of Figures

Figure 1. Innate and Acquired/Adaptive Immune Response Summary.....	39
Figure 2. Effects of Acute Gram-Negative Bacterial Immune Challenge on Early Embryonic Survival Summary.....	40
Figure 3. Rectal Temperatures in Dorset Texel ewes after treatment.....	52
Figure 4. Contingency Analyses of Mucosal Response and Lethargy in Dorset Texel Ewes after Treatment.....	53
Figure 5. Total White Blood Cell Count in Jugular Blood in Dorset Texel Ewes after Treatment.....	54
Figure 6. Concentrations of Lymphocytes, Monocytes, and Neutrophils in Jugular Blood in Dorset Texel Ewes after Treatment.....	55
Figure 7. Percent of Lymphocytes, Monocytes, and Neutrophils in Jugular Blood in Dorset Texel Ewes after Treatment.....	56
Figure 8. Concentrations of TNF- α , Hp, and SAA in Jugular Plasma in Dorset Texel Ewes after Treatment.....	57
Figure 9. Progesterone Concentrations on Days 9 and 10 and 25 and 26 Post Coitus in Dorset Texel Ewes after Treatment.....	58
Figure 10. Contingency Analyses of Pregnancy Status on days 25 and 26 Post Coitus and Lambing Percentage in Dorset Texel Ewes after Treatment.....	59

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Literature Review

Introduction

For most species of domesticated mammals, receptivity to a male, ovulation, and fertilization are efficient processes; however, embryonic and fetal losses are relatively high: cattle and sheep lose up to 30-35% (Moore et al., 2005). In cattle, most embryonic losses occur during the first 2 weeks of gestation with some evidence of greater losses before day 8 (Diskin and Morris, 2008); in human beings, about 50% of embryos are lost before implantation (Kline, 1989); while in sheep, conceptuses are lost throughout gestation (Dixon et al., 2007). Embryonic and fetal losses not only have negative effects on the dam but also have significant economic impacts on animal agriculture. Senger (2012) stated that a reduction in embryonic loss by just 2.8% would result in the additional production of 135 thousand sheep, 1 million beef cattle, 3.2 million pigs, and 3.5 million gallons of milk per year.

Many factors can contribute to early embryonic loss, ranging from genetic abnormalities of the conceptus to external environmental factors like nutrition, weather, and disease. In cattle, for example, Ealy et al. (1993) showed that heat stress during the period of oocyte maturation or early embryonic development leads to reduced embryonic survival. Loureiro and colleagues (2007) observed that the zona pellucida alone is unable to protect the embryo from apoptosis during heat stress or innate immune challenges that result in secretion of pro-inflammatory cytokines. Although the conceptus in sheep was extremely vulnerable to increased maternal temperature between days 0 and 4 of gestation (Dutt, 1963), the sheep embryo was less sensitive to adverse effects of high temperature after the eighth day of development, allowing the embryo to be less susceptible to inflammatory factors such as cytokines, as well as other hypothalamic-pituitary-adrenal-axis disturbances that could result in a decrease in embryonic survival.

Bacterial infections have been implicated in the ability of the developing conceptus to survive. Studies in cattle have led to emergence of the idea that infectious disease outside the reproductive tract can lead to reduced pregnancy rate. For example, mastitis, an inflammatory disease of the mammary gland, disrupts reproduction in dairy cattle possibly by initiating host immune responses that may have negative ramifications on early embryonic survival and death of the dam (Morris et al., 2002). Two toxic

components of bacteria, lipopolysaccharide (LPS) and peptidoglycan (PGN), have been used in attempts to define the mechanism between infection and early embryonic failure. Cullor (1993) indicated that LPS, a component of cellular walls of gram-negative bacteria, induced luteolysis and reduced conception rate and early embryonic survival through the release of inflammatory mediators. Then, Barker and colleagues (1998) observed a decrease in conception rate in cattle if mastitis occurred before first insemination, and that more inseminations were required to establish pregnancy if cows were infected with mastitis early after first insemination, but not in later pregnancy. Either of the first two situations resulted in a longer interval between calving and pregnancy. Furthermore, mated ewes inoculated with either peptidoglycan-polysaccharide (PG-PS), a cellular wall component of gram-positive bacteria, or killed *Streptococcus pyogenes*, a gram-positive bacteria, to mimic the effects of mastitis increased early embryonic losses (Stewart et al., 2003; Holásková et al., 2004). In addition, Dow and colleagues (2010) showed that injection of PG-PS on day 5 after mating resulted in fever; increased concentrations of the inflammatory cytokine, tumor necrosis factor-alpha (TNF- α), and acute phase proteins, haptoglobin (Hp) and serum amyloid A (SAA), on the day of and the day after the PG-PS challenge. These changes were followed by decreases in progesterone on days 14 and 21. Specifically, inference with establishment of early pregnancy in ewes inoculated with PG-PS appeared to be from local uterine inflammatory response, as shown by elevated TNF- α in the posterior vena cava. Therefore, the early immune response, i.e. inflammation associated with diseases such as bacterial derived mastitis, likely compromise establishment or maintenance of pregnancy (Soto et al, 2003; Hansen et al., 2004).

Oocyte maturation, fertilization, and early embryonic development could be disrupted by products of the microorganisms that colonize the mammary gland or by cytokines or other inflammatory mediators produced in response to infection. Among the molecules that could have negative ramifications on the developing embryo are endotoxins (LPS) whose effects on the oocyte and embryo have not been clarified. In one experiment, LPS reduced embryonic development of cultured mouse embryos (Dubin et al., 1995); while, in another experiment, LPS only reduced development if mouse embryos also were exposed to TNF- α (Randall et al., 1991). Other molecules produced

during inflammation that might compromise oocyte and embryo function are prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and nitric oxide (NO). In several tissues, including the endometrium, synthesis of $PGF_{2\alpha}$ is increased by LPS, by inflammatory cytokines such as $TNF-\alpha$ and interleukin- 1β (IL- 1β), and by NO (Davidson et al., 1995; Skarzynski et al., 2000). Because increased concentrations of LPS can have deleterious consequences on oocyte function and embryonic development, identification of the mechanism through which infectious diseases affect reproductive efficiency may lead to improving fertility in animals experiencing infectious disease or inflammation.

Early Pregnancy

Following copulation and deposition of semen into the female reproductive tract, spermatozoa are exposed to a series of different environments that alter their number and function. The fertile life-span after deposition in the female reproductive tract varies among species. In the cow, sperm are fertile for 1.5 to 2 days; while, in the human being, sperm are fertile for 5 to 6 days (Senger, 2012). After their deposition, some spermatozoa are damaged and/or destroyed by retrograde transport and phagocytized by leukocytes within the female tract (Senger, 2012). The remaining spermatozoa exit the cervix in a “trickle-like” effect from the reservoirs in the cervix and uterotubal junction where the spermatozoa can attach to the epithelium, known as sperm docking, along the entire oviduct (Senger, 2012). Spermatozoa then must traverse the cervix towards the uterus where they pass through the uterine horn via uterine contractions and fluids secreted in the lumen to the site of fertilization, the ampula/isthmus junction, in the oviduct. While the spermatozoa acquire maturity during epididymal transit in the testes, they are not completely fertile. Spermatozoa must remain in the female reproductive tract for a period of time to undergo alterations that will allow fertility. These changes are referred to as capacitation. Capacitation consists of enzymes in the female reproductive tract that remove adherent seminal plasma proteins that allow for binding of the sperm to the zona pellucida of the oocyte (Senger, 2012). Once capacitation is complete, the spermatozoa become progressively motile or hyperactive, marked by asymmetrical movement of the sperm tail within the oviduct (Yanagimachi, 1994). The hyperactivity seen in spermatozoa in the oviduct can be attributed to influxes of extracellular calcium and an increase in cyclic AMP (Nassar et al., 1999; Ho and Suarez, 2003). Hyperactive motility

not only allows for propulsion of sperm to the egg but also allows for penetration of the outermost egg coat, the zona pellucida.

Spermatozoa have specific proteins on their plasma membrane surfaces that must be exposed during capacitation to be able to bind specifically to zona pellucida proteins through multiple receptor-ligand interactions. Glycoproteins on the zona pellucida of the oocyte function as sperm receptors (Wassarman, 1999). There are several proteins on the surface of the sperm that bind to the zona pellucida glycoprotein receptors. Some investigators believe that there are at least two receptors, one for binding and the other for induction of acrosome reaction (Shur et al., 1997; Pang et al., 2011). The mechanical force from the sperm's moving tail, in combination with acrosomal enzymes released upon attachment to the zona pellucida, allow the sperm to digest a small hole through the zona where the sperm can pass. Upon complete penetration of the zona, the sperm reaches the space between the zona and the oocyte plasma membrane, termed the perivitelline space (Senger, 2012). Once fusion has occurred between the equatorial segment of the spermatozoon and the oocyte plasma membrane, the fertilizing spermatozoon is engulfed.

Prior to fertilization, mature oocytes are held in a developmentally quiescent state, arrested in metaphase of the second meiotic division. Upon binding of a sperm, the egg rapidly undergoes a number of metabolic and physical changes that collectively are called egg activation (Gadella and Evans, 2011). Prominent effects on the now fertilized oocyte include a rise in the intracellular concentration of calcium, completion of the second meiotic division and the cortical reaction. The cortical reaction prevents penetration by additional spermatozoa by exocytosis of its cortical granules into the perivitelline space. The contents of cortical granules include mucopolysaccharides, proteases, plasminogen activator, acid phosphatase and peroxidase (Senger, 2012). All of these components modify the zona pellucida so that it is no longer receptive to sperm (Wassarman, 1994). The result of exocytosis of the cortical granules is the zona block so that further sperm cannot penetrate the zona pellucida. The importance of the zona block is to prevent fertilization of an oocyte by more than one spermatozoon, which results in embryonic death. Some species, like the rabbit, have the ability to block polyspermy through the egg

plasma membrane block instead of through the zona reaction (Overstreet and Bedford, 1974).

After the fertilizing spermatozoon enters the oocyte cytoplasm the nucleus must separate so that the male chromosomes may pair up with the female chromosomes. This is accomplished by the reduction of many disulfide cross-links in the sperm nucleus by the cytoplasm of the oocyte (Senger, 2012). The fusion of the male and female pronuclei, referred to as syngamy, is the final step of fertilization. Following syngamy, the zygote enters the first stages of embryogenesis.

Embryogenesis

Upon completion of fertilization and the fusion of the male and female pronuclei, the resulting single-celled zygote is now termed an embryo. Next, approximately 24-30 hours after fertilization, the zygote undergoes a series of mitotic divisions called cleavage divisions to produce a two-celled embryo, the cells of which are called blastomeres. Each blastomere undergoes subsequent divisions, yielding 4, 8, and then 16 daughter cells in approximately 2-3 days after fertilization (Senger, 2012). Pre-implantation embryonic development to the blastocyst stage requires activation of the embryonic genome. This maternal-zygotic transition occurs at the 2-cell stage in mice, between the 8-and 16-cell stage in cows and sheep (2-3 days after fertilization), and between 4- and 8-cell stage in humans (Artley et al., 1992). The cleavage divisions continue within the oviduct and a four-celled embryo gives rise to an eight-celled embryo, which then forms a ball of cells, termed a morula, which is now located within the uterus approximately 3-4 days after fertilization. During this time, cells in the inner cell mass develop gap junctions while the outer cell mass develop tight junctions. The compaction process marks the beginning of the embryonic cell differentiation where a separation of cells forming an inner and outer cell mass occurs. The morula is now ready to leave the site of fertilization and enter into the uterus on day three in the human and mouse (Hardy and Spanos, 2002) and day 4-6 post fertilization in sheep and cattle (Rowson and Moor, 1966).

At the 32-cell stage of embryonic development, approximately 4-10 days after fertilization, a fluid-filled cavity begins to appear and signals the initiation of another developmental milestone from morula to blastocyst. The embryo is partitioned into two distinct cellular masses called the inner cell mass and the trophoblast, giving rise to the

body of the embryo and extra-embryonic membranes, respectively. At the blastocyst stage, the conceptus is still protected by the zona pellucida, which must be destroyed for the continuation of development and attachment. Importantly, while the zona pellucida becomes hardened after penetration of the sperm into the ovum, it is not impenetrable as discussed by Hastings et al. (1972) and Sinowatz et al. (2001). Specifically, Glass (1963) found that serum proteins can pass through the zona pellucida of mouse eggs and cleavage stages *in vivo*. The porous nature of the zona pellucida allows for penetration of relatively large molecules such as immunoglobins. On the other hand small molecules are prevented from penetrating the zona pellucida. The ability of molecules to pass this extracellular matrix does not primarily depend on the size of the penetrating molecule but also on other biochemical or physicochemical properties, for example surface charge (Shivers and Dunbar, 1997). In 2007, Loureiro and colleagues observed that during times of heat stress or innate immune challenges resulting in the secretion of the proinflammatory cytokine tumor necrosis factor- α (TNF- α), the zona pellucida alone is unable to protect the embryo from apoptosis. Specifically, based on results using heat shock, capacity for induced apoptosis becomes acquired between the 8- and 16-cell stages (Paula-Lopes and Hansen, 2002). Heat shock was used because effects of elevated temperature on embryonic survival have been implicated in the infertility experienced by lactating cows during heat stress (Hansen and Aréchiga, 1999). Also, actions of TNF- α were of interest because this cytokine may be a contributor to the reduced fertility associated with mastitis (Hansen et al., 2004). Hansen (2007) found that there is a decrease in vulnerability to environmental stresses for the embryo as it ages and develops. The exact mechanism for the decreased susceptibility on the embryo is yet to be fully understood, but it could be that as the embryonic genome is completely turned on, the embryo gains the protection of a very early innate immune system.

After hatching of the blastocyst from its zona pellucida on day 7-8 of gestation in the sheep, the embryo becomes free floating within the lumen of the uterus and is dependent on the uterine environment for survival. The embryo is without a blood supply and direct cell-to-cell contact within the reproductive tract (Böving, 1959). During this time the embryo continues to develop and differentiate while experiencing massive growth by receiving nourishment from uterine secretions, often termed histotrophs

(Roberts and Bazer, 1988). The continuation of blastocyst development and differentiation, attachment, recognition and maintenance of pregnancy require an effective maternal to embryonic connection. The timing of implantation varies among species but by day 16 of pregnancy in the sheep the initiation of attachment has begun (Böving, 1959; Boshier, 1969; Guillomot et al., 1993).

Early implantation of embryos involves two epithelial tissues: the embryonic trophoblast and the maternal uterine epithelium. However, many criteria must be met in order for successful attachment to occur. Based on a comparative implantation scheme proposed by Guillomot and colleagues (1981, 1993, 1995), the phases of implantation include shedding of the zona pellucida between days 8 and 9, precontact and blastocyst orientation in the uterus between days 9 and 14, apposition of the conceptus trophoblast becoming closely associated with the endometrial luminal epithelium on day 14, and adhesion on day 16 where the trophoblast begins to adhere firmly to the endometrial luminal epithelium. In ruminants, apposition is restricted to uterine epithelial glandular sites called aglandular caruncular and glandular intercaruncular tissues, which will be the superficial sites of attachment and placentation (Wimsatt, 1950).

Maternal Recognition of Pregnancy

In order for the events of early embryogenesis to continue into an established pregnancy, a critical series of events must occur by which the conceptus initially signals its presence to the dam and enables pregnancy to continue, referred to as maternal recognition of pregnancy. In addition, the conditions must be such that the fetus can survive and grow regularly in the maternal uterus despite the fetus bearing paternal alloantigens recognized as foreign by the mother (Girardi et al., 2006). Proteins physiologically secreted by the trophoblast of ovine and bovine conceptuses during a short period around implantation are known to mediate maternal recognition of pregnancy by maintaining the function of the corpus luteum during early pregnancy as demonstrated by Martal and colleagues (1979). As Lefèvre and Boulay (1993) demonstrated in the pig, uterine differentiation at the time of apposition, adhesion, and implantation is coordinated maternally by an increase in vascular permeability and the

secretion of progesterone and estrogen in a spatiotemporal manner, and embryonically by production of interferon- τ .

At day 13-21 of gestation of domestic livestock species, the trophoblastic cells begin to produce IFN- τ , an anti-luteolytic hormone that blocks uterine production of prostaglandin $F_{2\alpha}$ thereby prolonging the life of the corpus luteum and progesterone (P_4) production. Synchrony between the developing embryos and the uterus is essential, as well as optimal physiological conditions that will allow for acceptance and acknowledgement between the developing embryo and the dam (Roberts et al., 1996). The process of maternal recognition is a species specific event; in species such as humans, swine, sheep, and cattle the developing embryo provides the signal for maternal recognition of pregnancy via production of hormonal signals such as human chorionic gonadotropin (hCG), estradiol (E_2), and IFN- τ respectively (Senger, 2012). In pigs, the trophoblast reportedly produces estradiol that alters the direction of $PGF_{2\alpha}$ secretion away from the submucosal capillaries and toward the uterine lumen where luminal $PGF_{2\alpha}$ cannot cause luteolysis (Spencer et al., 2004). In comparison, maternal recognition of pregnancy in ruminants requires that the conceptus develops to produce IFN- τ , which is the pregnancy recognition signal that prevents endometrial secretion of $PGF_{2\alpha}$. However, in the human being, hCG is secreted from the human conceptus that acts on the corpus luteum to inhibit intraovarian luteolysis (Senger, 2012). Regardless of the origin or the type of signal itself, the purpose of the conceptus and uterine dialogue is to ensure the lifespan of the corpus luteum that continues to provide the hormone of pregnancy, P_4 .

In cattle and sheep, IFN- τ is an antiluteolysin that acts on the endometrium to prevent uterine release of luteolytic $PGF_{2\alpha}$ induced by oxytocin. Interestingly, IFN- τ can leave the uterus via the uterine vein to affect the ovary and circulating immune cells. Ongoing research suggests that IFN- τ stimulates circulating immune cells of the dam to defend against invading viral pathogens. In the ewe, trophoblast cells in the embryo synthesize and secrete IFN- τ between day 13 and 14 of gestation (Senger, 2012). Starting on day 11, the spherical blastocyst begins to elongate and by day 17, resembles a long filament where IFN- τ begins to be produced (Spencer et al., 2004).

Bacteria

Bacteria are single-celled prokaryotic organisms consisting of a cytoplasm surrounded by a lipid cytoplasmic membrane. Outside the cytoplasmic membrane is a thick, carbohydrate cell wall that holds the cell together and is found in all bacteria except mycoplasmas. The important chemical constituent of bacterial cell wall is peptidoglycan, whose percentage within the cell wall defines the organism as either gram-positive or gram-negative based on results from the negative stain technique. Peptidoglycan is a very large molecule composed of alternating *N*-acetyl glucosamine and *N*-acetyl muramic acid cross-linked by short peptide chains. The cell walls of gram-positive and gram-negative bacteria differ considerably. In gram-positive bacteria, the peptidoglycan layer is about 25 nm wide and contains an additional polysaccharide called teichoic acid. About 60 to 90 percent of the cell wall is peptidoglycan, and, therefore gram-positive bacteria's peptidoglycan will trap the crystal violet iodine complex in its many cross linkages giving the bacteria a blue-purple stain. In comparison, gram-negative bacteria have considerably less peptidoglycan in their cell walls, a layer that is only about 3 nm wide with no evidence of teichoic acid. The thin rigid layer of peptidoglycan between the outer membrane and the cytoplasmic membrane gives the wall its strength. The crystal violet-iodine complex in gram staining is lost partly because of the thinness of the cell wall, and, hence, bacteria would trap less of the iodine in the complex ultimately giving them a clear, orange-red stain. Common gram-negative bacteria include *Escherichia coli* and *salmonella* species. The cell wall in these bacteria is multi-layered and contains various polysaccharides, proteins, and lipids and is much more complex than the cell wall of gram-positive bacteria. The outer membrane contains a unique lipopolysaccharide (LPS) that is a complex molecule consisting of a lipid, called lipid A, and a polysaccharide. The LPS forms the external surface of the cell wall, and a section of the polysaccharide chain, called the O antigen, extends off the cell wall to provide a unique molecular fingerprint that can be used during the process of bacterial identification. Lipopolysaccharides of the outer membrane of gram-negative bacteria play a major role in diseases caused by gram-negative bacteria because they are extremely toxic. The outer cell wall LPS of gram-negative bacteria bind to toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) and induce the production of inflammatory cytokines when an animal is

infected. These cytokines cause fever and sickness, so bacterial LPS and other components of the cell walls of bacteria are called endotoxins, non-secreted constituents, found in *Escherichia coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, and *Bordetella pertussis*. These non-secreted constituents trigger phagocytes to release cytokines with local and systemic effects. Endotoxins manifest themselves by similar signs and symptoms. Usually an individual experiences an increase in body temperature, substantial body weakness and aches, and general malaise. A condition called endotoxic shock may occur when the tissues swell and the blood pressure drops due to a high concentration of endotoxin release as bacilli disintegrate. The biological activity of LPS can vary in comparison among different bacteria but, essentially, consists of the primary immunostimulatory lipid component, Lipid A, which consists of an oligosaccharide attached to a lipid and to a series of repeating trisaccharides (Tizard, 2013). From a reproductive standpoint, bacterial endotoxins induce fetal resorption in mice and rats (Zahl and Bjerknes, 1943; McKay and Wong, 1963). However, endotoxins do not appear to interrupt the normal endocrine functions of pregnancy and do not cross the placental barrier (Chedid et al., 1962; Parant and Chedid, 1964; Gasic et al., 1975). On the other hand, exotoxins are protein toxins secreted by either living bacteria or upon bacterial cell lysis, typically by gram-positive bacteria. Exotoxins are highly immunogenic proteins and can cause severe damage to host cell membranes (Wilson et al., 2002).

Infections from gram-negative bacteria like *Escherichia coli* (*E.coli*), *Pseudomonas* spp., *Pasteurella* spp., which have been linked to mastitis, cause detrimental health effects and even death of the host or developing embryo. Mastitis is considered the most costly disease of dairy cows worldwide (Yazdankhah et al., 2001; De Oliveira et al., 2000). Economic losses for the dairy industry itself are estimated at \$2 billion dollars per year in the United States alone (De Oliveira et al., 2000). In addition to the loss from discarded milk, mammary infections initiate immune responses that possibly have negative ramifications in early embryo loss and death of the dam (Morris et al., 2002). Early embryonic mortality accounts for most pregnancy losses in many mammalian species including 20-30% in cows and sheep (Tanabe and Casida, 1949; Hulet et al., 1956). For maximum profitability within a typical production year, dairy cows must be pregnant within 90 days after parturition. Maternal disease, like mastitis,

during early embryonic development can have negative ramifications on survival of the embryo and ability of the dam to conceive by 90 days of postpartum.

Two toxic components of bacteria, LPS and PG, have been used in attempts to define the mechanism between infection and early embryonic failure. Both have similar biologic actions and evoke common components of the nuclear factor-kappa B (NF- κ B) intracellular signaling pathway leading to secretion of inflammatory cytokines. Barker *et al.* (1998) found that clinical mastitis caused by either gram-negative or gram-positive pathogens reduces reproductive performance during early lactation. During treatment with PG or LPS, an inflammatory response was observed by Law (1923), including fever, in cows with bacterial infection of the udder. Cullor (1993) indicated that LPS induces luteolysis and reduces conception rate and early embryonic survival through the release of inflammatory mediators. Each type of pathogen may act through similar mechanisms to increase inflammatory mediators, such as cortisol, interleukin 1 (IL-1), and TNF- α , leading to reproductive failure. Injections of PG have long been implicated in a pyretic response in many different species. Both LPS and PG stimulate the immune system (Rotta, 1975; Lichtman *et al.*, 1994) by crossing the intestinal mucosa and entering the portal veins, subsequently stimulating to release TNF- α from Kupffer cells.

Increased secretion of PGF_{2 α} in response to PG would be expected to decrease pregnancy rate by inducing luteal regression. Buford *et al.* (1996) found that even with replacement therapy with progestagens, luteal regression during days 4-9 after estrus increased early embryonic death in cattle. Costine *et al.* (2001) showed that progestagen-supplemented ewes are similarly sensitive to the embryotoxic effect of PGF_{2 α} on days 4-7 after mating. Therefore, the early pregnant ewe should be a useful model for evaluation of the effects of inflammatory mediators on reproductive performance. Stewart *et al.* (2003) reported that pregnancy loss after treatment with PG on day 5 post-treatment was not due to fever, increased cortisol, decreased progesterone, luteinizing hormone (LH), and estradiol as pregnancies were maintained despite in these changes. However, pregnancy rate was reduced after administration of higher doses, 30 or 60 μ g/kg, of PG on day 5 after breeding (Stewart *et al.*, 2003). As a result, Stewart and colleagues (2003) indicated that inflammatory mediators such as PGF_{2 α} and TNF- α could act directly on the embryo, which might have detrimental effects on embryonic survival after PG treatment

in ewes. Holásková et al. (2004) reported that immunization of ewe lambs with PG-PS or killed *S. pyogenes* did not improve pregnancy maintenance thus concluding that the insult to early embryonic development occurs during the innate immune response rather than the adaptive. However, early embryonic loss in mice induced by a bacterial infection could be prevented by prior immunization with LPS, as long as the anti-LPS antibody titers remained above 1/500 (Baines et al., 1996).

Genito-urinary tract or systemic infections by gram-negative bacteria in pregnant women have been shown to cause abortions, preterm labor, and several other perinatal complications. The most potent antigenic component of the gram-negative bacterial cell wall, LPS, is known to modulate the expression of various pro-inflammatory cytokines. Kaushik et al. (2005) reported that LPS significantly altered the proliferation of the glandular epithelium, luminal epithelium, and stroma during the preimplantation period in mice affecting the uterine preparation for blastocyst implantation. Kabaroff and colleagues (2006) reported that sheep are impacted differently during pregnancy and lactation with moderate doses of the inflammatory stressor LPS. Within the same study, they reported that the temperature response in ewes to *E. coli* LPS is attenuated during early pregnancy and early lactation in comparison with nonpregnant animals. Breed differences within a species have also been reported. Four distinct breeds of chickens, for example, displayed different behavioral responses, body weight (BW) gain, organ development, and core temperatures in response to an intravenous injection of 5 µg of LPS/kg of BW (Cheng et al., 2004).

Microbes are highly diverse structures and can mutate and change many of their surface molecules very quickly. For this reason, the initial immune response does not attempt to recognize all possible microbial molecules but instead uses receptors that can bind and respond to many essential molecules that are common to a large variety of microorganisms but are absent from normal animal tissues. These PRRs found on sentinel cells like macrophages, dendritic cells, and mast cells within the immune system, can sense particular structures of the invading microorganisms by recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Both PAMPs and DAMPs initiate a well-orchestrated immune response (Kawai and Akira, 2010; Takeuchi and Akira, 2010). Upon binding of bacterial PAMPs

to the PRRs of the host defense cells, proinflammatory cytokines and chemokines are synthesized and secreted, which trigger local increases in blood flow, attract more defensive cells, and increase blood vessel permeability. Although secretion of cytokines and chemokines is helpful in triggering the body's innate defenses, the process can result in negative consequences like tissue damage and at high concentrations host death and potentially early embryonic loss.

Innate Immunity

Innate immunity refers to the host defense that is present in all animals upon birth and needs not be induced by prior exposure to an infectious agent. Innate immunity is activated immediately when a pathogen penetrates the epithelial barriers, ideally lasts for just a few hours, and is directed toward the rapid elimination of the pathogen. This host immunity lies behind most inflammatory responses, which are triggered in the first encounter of any pathogenic molecule by macrophages, leukocytes, and mast cells through their innate immune receptors.

Carried within the blood and lymph and populating the lymph organs are various white blood cells, or leukocytes, that participate in the immune response. All blood cells arise from the hematopoietic stem cell (HSC) within the bone marrow, which then give rise to either a lymphoid progenitor cell or myeloid progenitor cell. Myeloid progenitor cells give rise to erythrocytes, neutrophils, eosinophils, basophils, monocytes, mast cells, dendritic cells while lymphoid progenitor cells give rise to B, T, and natural killer (NK) cells or broadly called lymphocytes. The lymphocytes have antigen receptors that are crucial for adaptive immunity. As part of the innate immune system NK cells do not express the set of surface markers that characterize T or B cells. Instead, NK cells display cytotoxic activity against a wide range of tumor cells and some cells infected with viruses. The B lymphocyte binds antigen that matches its membrane-bound antibody and then divides rapidly into plasma cells, which produce the antibody in a form that can be secreted, and memory B cells. The T lymphocyte has a unique antigen binding molecule called the T-cell receptor (TCR) that only recognizes antigen bound to cell membrane proteins called major histocompatibility complex (MHC) molecules. There are three populations of T cells: T helper, T cytotoxic, and T regulatory.

The mononuclear phagocytic system includes circulating monocytes and macrophages in the tissue. There are an average of 200 monocytes/ μl in the sheep. Macrophage-like cells serve different functions in different tissues. For example, intestinal macrophages are in the gut, Kupffer cells are in the liver, and microglial cells are in the brain. Macrophages are activated by a variety of stimuli in the course of an immune response and ingest and digest exogenous antigens, like whole microorganisms. The granulocytes are classified as neutrophils, eosinophils, and basophils based on their cellular morphology and cytoplasmic-staining characteristics. Approximately 3000-6000 human neutrophils circulate per cubic millimeter of blood, accounting for about 65% of the total white blood cells. In the sheep, there are an average of 2,400 neutrophils circulate per microliter of blood. Neutrophils are usually the first to arrive at a site of inflammation. During some bacterial infections, the neutrophil population of the blood often doubles. Basophils and eosinophils together make up less than 5% of the white blood cells. There are on average 50 basophils/ μl and 400 eosinophils/ μl circulating in the sheep. The role of basophils is questionable; however, they do play a role in certain allergies. Eosinophils appear to intercede certain diseases caused by parasitic helminthic worms. Mast cell precursors are released in the blood as undifferentiated cells; they do not differentiate until they enter the tissues where they contain histamine. Lastly, dendritic cells are versatile and often present antigen to T cells in the lymph nodes. The cells of the adaptive immune system, T and B lymphocytes, are characterized by specificity developed by clonal gene rearrangements from a broad repertoire of antigen-specific receptors on the lymphocytes and eliminate pathogens in the late phase of infection and generate immunological memory. In comparison, innate immunity is the first line of host defense during infection and is antigen-nonspecific that relies on defense cells like macrophages, granulocytes, and antigen-presenting cells (APCs) to recognize conserved molecular structures produced by the bacteria but not by the host. These conserved structures or PAMPs such as LPS, PG, lipoteichoic acid (LTA), mannose, bacterial DNA, and glucans are all that is required to initiate the correct cascade and begin host immune attack (Janeway and Medzhitow, 2002).

When a pathogen breaches the host's epithelial barriers and initial antimicrobial defenses, the innate response occurs in three phases. First, antimicrobial enzymes digest

bacterial cell walls, and antimicrobial peptides lyse cell membranes directly. Second, the pathogen encounters a major component of innate immunity known as the complement system, or humoral innate immunity, which targets pathogens for lysis and for phagocytosis by bathing the pathogen in complement fragments that are recognized by macrophages and neutrophils. These soluble proteins present in blood and other body fluids are synthesized mainly by liver hepatocytes, but also blood monocytes, tissue macrophages, and epithelial cells of the gastrointestinal and genitourinary tracts. Particular complement proteins interact with each other to form several different pathways of complement activation, which all lead to the formation of the membrane-attack complex (MAC), which ultimately kills the pathogen, either directly or indirectly by facilitating its phagocytosis and activating additional inflammatory responses. The three main pathways differ in their initial complexes and proteins involved. The classical pathway begins with antigen-antibody binding, specifically C1 binds to antigen-antibody complexes. The alternative pathway is antibody-independent and can be activated in the presence of the pathogen alone and is initiated by binding of spontaneously generated C3b to activating surfaces such as microbial cell walls. Lastly, the lectin pathway, is activated by lectin-type proteins that recognize and bind to carbohydrates on pathogen surfaces and is initiated by binding of the serum protein mannose binding lectin (MBL) to the surface of a pathogen. All three pathways generate C3 and C5 convertases and bound C5b, which is converted into the macromolecular structure called MAC. This complex forms a large channel through the membrane of the target cell, enabling ions and small molecules to diffuse freely across the membrane. The MAC mediates cell lysis, while other complement components participate in the inflammatory response, opsonization of antigen, viral neutralization, and clearance of immune complexes (Kuby, 2003). The complement system is generally effective in lysing gram-negative bacteria. However, some gram-negative and most gram-positive bacteria have mechanisms for evading complement-mediated damage. For example, in *Escherichia coli*, resistance to complement is associated with the presence of long polysaccharide side chains in the cell wall LPS component (Kuby, 2003). The increased LPS in the wall of resistant strains may prevent insertion of the MAC into the bacterial membrane.

The release of high amounts of biologically active products during the activation cascade of the innate immune system could cause tissue damage directly via MAC or recalling circulating inflammatory cells through the activation molecules (i.e., C3a and C5a). The placental fetomaternal interface could represent a potential target of complement system where its physiological function might cause damage. In the placenta, the fetal and maternal layers are interdigitated in a strict vascular connection which could leave the fetal trophoblast exposed to maternal blood and be potentially attacked by maternal complement. Various authors demonstrated that both early (Cq and C4) and late components (C5, C6, and C9) of complement cascade are found in the placenta; in particular, early complement proteins are detected in the stroma of the villi around fetal vessels, while C3d and C9 are detected on trophoblasts' membranes (Girardi et al., 2006). In pregnant mice, a membrane-bound regulatory receptor, called Crry, selectively blocks C3 and C4 activation at the fetomaternal interface (Kim et al., 1995). Deficiency in Crry leads to embryonic loss, massive deposition of C3, and tissue damage at the placental level (Xu et al., 2000). However, recent findings showed that C3 has a physiological role in early phase pregnancy. Chow and colleagues (2009) showed that knock-out mice for C3 showed smaller blastocysts, higher resorption rates, and smaller sizes of placentae. In humans, complement could play a role in recurrent spontaneous abortions due to the observation of C3 deposition on decidual vessels in patients with recurrent fetal loss (Girardi et al., 2006).

In order to protect against infection, the host organism must be able to distinguish self from non-self by identifying molecules that are species and strain specific for a particular type of pathogen, as well as those molecules that are found within the host PAMP (Janeway and Medzhitow, 2002). The PRRs found on sentinel cells located throughout the body bind PAMPs located on the bacterial cell. Many different PRRs are used to cover as many PAMPs as possible. Most are cell-associated receptors found on cell membranes, within the cytosol, within cytoplasmic vesicles, and even circulating in the bloodstream. Once bound, PRRs functions include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis (Janeway and Medzhitow, 2002). Additionally, defense cells within a host, like macrophages, have PRRs located on their

cell surfaces called toll-like receptors (TLRs) and internally in the cytosol called nucleotide-binding oligomerization domain proteins (NOD) (Akira, 2004).

Intracellular signaling PRRs bind a diverse number of microbial molecules along with their associated PAMPs, both from bacterial and viral agents (Nakayama et al., 1993). The most notably and researched of the intracellular signaling PRRs are the TLRs, which detect cytokine and chemokine production by macrophages induced by an invading pathogen and launch a pathogen-killing reaction (Drexler and Foxwell, 2010). Signals generated when PAMPs bind TLRs activate sentinel cells and promote both synthesis and release of intercellular regulatory molecules such as cytokines and chemokines that activate the inflammatory process and propagate the development of effective innate immunity. The TLRs are much conserved among species from drosophila to humans and are membrane-spanning proteins that share a common structural element in their extracellular region, repeating segments of 24 to 29 amino acids containing leucine-rich repeats (LRRs). All TLRs contain several LRRs, and a subset of the LRRs make up the extracellular ligand-binding region and an interior domain call the TIR domain in the TLR. The TIR domains have three regions, highly conserved among all members of the TIR family, called boxes 1, 2, and 3, that serve as binding sites for intracellular proteins participating in the signaling pathways mediated by TLRs. Takeda and colleagues (2003) summarized the identification of 10 known members of the TLR family that can recognize a distinct set of PAMPs. Among these, the LTA of gram-positive bacterial cell walls and the LPS of the outer membrane of gram-negative bacteria are important in the recognition of bacteria by the innate immune system. The TLRs are found not only on immune cell surfaces like neutrophils, macrophages and dendritic cells, but also in the membranes in the cytosol. These TLRs can work independently or with one another depending on the PAMP presented. Work by Ozinsky and colleagues (2000) showed that TLR 2 and TLR 6 work together during recognition and binding of PG. The binding of a microbial pathogen to its corresponding extracellular TLR transmits a multi-protein signaling complex to the cytoplasmic side, where the highly conserved TIR structural motifs and the binding sites for other components of the pathway are found. The TLR signaling induces a diverse range of production of inflammatory cytokines by activating several different signaling pathways that each activate different transcription

factors. Initially, adaptor proteins interact with the TIR domain. The most common adaptor protein for TLRs is MyD88, which has two protein domains that are responsible for its function: a TIR domain and a death domain. The TIR domain of MyD88 interacts with the domain of the TLR while the MyD88 TIR domain interacts with two protein kinases, IRAK1 and IRAK4. Another protein kinase, TAK1, joins the complex which allows for it to perform the phosphorylation-mediated activation of two other signal transduction modules. One of these is the mitogen-activated protein kinase (MAP kinase) and the other is the NF κ B pathway. The latter is a transcription factor that initiates the transcription of many genes necessary for the effector functions of innate immunity. Additionally, members of the activator protein 1 (AP-1) family are activated by MAPK. The AP-1 and NF- κ B activate the genes for three major proteins, IL-1, IL-6, and TNF- α (Tizard, 2013). These proteins produced are classified as cytokines, which regulate the activities of cells involved in the defense of the body. Upon their production, cytokines bind to their receptors on other cells, including those of the defensive system, furthering the innate immunity cascade.

Cytokine and chemokine production by macrophages, neutrophils, and dendritic cells are the result of stimulation of signaling receptors on these cells by a wide variety of pathogen components. Of these receptors, TLR-4 is expressed by several types of immune-system cells including dendritic cells and macrophages that recognize bacterial LPS as a PAMP. Ectopic overexpression of TLR 4, the first mammalian TLR identified, was shown to cause induction of the genes for several inflammatory cytokines and co-stimulatory molecules (Medzhitov et al., 1997). Then, Hoshino and colleagues (1999) showed that TLR4-deficient mice generated by gene targeting are hypo-responsive to LPS, confirming that TLR 4 is an essential receptor for the recognition of LPS, although other molecules are involved in the recognition of LPS in addition to TLR 4. LPS can become detached from the outer membrane of gram-negative bacteria and be picked up by LPS-binding protein in blood and extracellular fluid in tissues. The LPS-LPS-binding protein complex is recognized by CD14, a glycosylphosphatidylinositol-anchored molecule expressed in macrophages and neutrophils (Takeda et al., 2003). The LPS stimulation is followed by increased physical proximity between CD14 and TLR 4, suggesting that CD14 and TLR 4 may interact in LPS signaling (Jiang et al., 2000; Da

Shilva et al., 2001). Similarly, the accessory protein MD-2 was identified as a molecule that associates with the extracellular portion of TLR 4 and enhances LPS responsiveness (Shimazu et al., 1999; Akashi et al., 2000). Chinese hamster ovary cell lines that are hypo-responsive to LPS have mutations in the MD-2 gene (Schromm et al., 2001). Generation of MD-2-deficient mice demonstrated its essential role in the response to LPS. Furthermore, MD-2 deficient mice are resistant to LPS-induced endotoxic shock, similar to TLR4-deficient mice (Nagai et al., 2002). The MD-2 associates with TLR 4 in the endoplasmic reticulum/cis Golgi and then the TLR4/MD-2 complex moves to the cell surface, where excess MD-2 is secreted (Visintin et al., 2001). Furthermore, whereas TLR 4 normally resides on the cell surface in wild-type cells, it is found in the Golgi apparatus in cells deficient for MD-2, indicating that MD-2 is essential for the intracellular distribution of TLR 4 (Nagai et al., 2002). Thus, several components are implicated in the recognition of LPS, indicating that the functional receptor forms a large complex.

However, cells of the immune system are not the only ones that possess TLRs. Herath and colleagues (2006) found that endometrial stromal and epithelial cells in cows express CD14 and TLR 4 mRNA and were able to respond to LPS by producing PGE₂ and PGF_{2α}, without the presence of immune cells *in vitro*. Within the same study, the functional response was TLR 4 mediated. Interestingly, E₂ and P₄ inhibited the production of prostaglandins by endometrial cells in response to LPS indicating a possible role for steroidal hormones in the response to LPS (Herath et al., 2006). Progesterone suppresses uterine immune responses and can result in the increased susceptibility of the uterus to infection.

In addition to TLR 4, TLR 2 has been associated with cellular responses to numerous microbial products, including LPS and bacterial lipoproteins (Yang et al., 1998). However, many preparations of LPS contain low concentrations of highly bioactive contaminants described as “endotoxin protein,” indicating that these contaminants could be responsible for the TLR 2 mediated signaling observed upon LPS stimulation (Hirschfeld et al., 2000). Hirschfeld and colleagues (2000) suggested that neither human nor murine TLR 2 plays a role in LPS signaling in the absence of contaminating endotoxin protein.

The TLRs are all located on cell surfaces or in the membranes of intracellular vesicles and sense the presence of extracellular pathogens. Another large family of receptors that use leucine-rich repeat (LRR) scaffold domains to detect pathogens are located in the cytoplasm. Nucleotide-binding oligomerization domains are cytosolic PRR proteins that allow intracellular recognition of bacterial peptidoglycan and muramyl dipeptide components and activate NF κ B to initiate the same inflammatory responses as TLRs. The NOD proteins are expressed in cells that are routinely exposed to bacteria including epithelial cells, macrophages, and dendritic cells. Macrophages and dendritic cells express TLRs as well as NODs and are activated by both pathways. The corresponding NOD receptors are referred to as NOD-like receptors (NLRs). The first NLRs reported to survey the cytosol for the presence of PG components were NOD 1 and NOD 2 (Fukata, et al., 2009). Binding to either NLR activates MAPK and NF- κ B pathway and triggers the production and release of proinflammatory cytokines (Park et al., 2007). The NOD 1 detects γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP), a breakdown product of PG of gram-negative bacteria and is an important activator of the innate immune system. On the other hand, NOD 2 detects muramyl dipeptide, which is present in the PG of most bacteria and seems to have a more specialized role, being strongly expressed in the Paneth cells of the gut, where it regulates the expression of potent antimicrobial peptides.

The NOD subfamily has an amino-terminal caspase recruitment domain (CARD), which is related to the death domain in MyD88. When NOD 1 or NOD 2 recognizes its ligand, it recruits the CARD-containing serine-threonine kinase RIPK2. The RIPK2 activates the kinase TAK1, which activates NF- κ B via the activation of IKK. Previous studies showed that NOD 1 can mediate MyD88-independent cellular responsiveness to LPS preparations from various gram-negative bacteria including *Escherichia coli* but not to PGN from the gram-positive bacterium *Staphylococcus aureus* (Inohara et al., 2001). Conversely, NOD 1 mediates the host response to PG from a subpopulation of bacteria, with iE-DAP as the minimal structure in PG capable of stimulating NOD 1 (Chamaillard et al., 2003). This is significant because although muramyl dipeptide (MDP), the moiety recognized by NOD 2, is present in PGNs from both gram-positive and gram-negative bacteria, iE-DAP exists only in specific bacteria including common gram-negative

bacteria, such as *E. coli*, and several gram-positive bacteria. Chamaillard and colleagues (2003) further demonstrated that the expression of NOD 1, NOD 2 or TLR 4 together with its cofactor MD-2 conferred responsiveness to LPS preparation from *E.coli*, whereas only NOD 2 induced the response to *S. aureus* PGN. Raetz and Whitfield (2002) suggested that the lipid A moiety of LPS is implicated in TLR4-MD-2 activation. Consistent with this finding, NOD 1 and NOD 2, but not TLR 4, mediated a response to detoxified LPS containing deacylated lipid A (Chamaillard et al., 2003). Conversely, TLR 4, but not NOD 1 and NOD 2, responded to purified intact lipid A (Chamaillard et al., 2003). These results indicated that NOD 1, NOD 2, and TLR 4 recognize different bacterial components. Therefore, NOD 1 mediates the host response to a subset of microbes, whereas NOD 2 can elicit a broader recognition of bacteria.

While TLRs utilize their TIR domain and NLRs their CARD for downstream signaling upon activation, NLRs recruit signaling adaptors through their pyrin (PYD) domain. This other large subfamily of NLR proteins have a pyrin domain at their amino termini and are known as the NLRP family. Humans have 14 NLR proteins containing pyrin domains. Active NLRs trigger multiple innate immune effector pathways, but the best established function of these PYD containing proteins is the assembly of inflammasomes, which form in response to infection and tissue damage. The inflammasome is a large, multiprotein complex that is responsible for the activation of inflammatory caspases, in particular caspase 1, which then cleaves IL-1 β and IL-18 into their mature, biologically active forms (Martinon et al., 2002). Active caspases then induce inflammatory cell death, maturation, and/or secretion of proinflammatory cytokines, IL-1 β and IL-18, and contribute to the release of IL-1 α (GroB et al., 2012; Fettelschoss et al., 2011). The best characterized is NLRP3, which is an important sensor of cellular damage or stress. In stressed cells, such as those exposed to a broad range of molecules, including bacterial and viral RNA, and bacterial products (e.g. LPS and muramyl dipeptide). NLRP3 assembles with an adaptor protein and the protease caspase 1 to form a complex called an inflammasome (Martinon et al., 2002). Caspase 1 is required for the proteolytic processing of some proinflammatory cytokines, which is needed before they can be secreted. Once enzymatically active, caspase 1 can go on to process the cytokines pro-IL-1 β into their mature secreted forms (Thornberry et al.,

1992). It is unknown how the NLRP3 inflammasome is able to respond to such a wide variety of agonists; however, it is widely accepted that low intracellular K^+ or the generation of reactive oxygen species (ROS) are triggers for activation (Pétrilli et al., 2007). However, the inflammasome has been implicated in the actions of several inflammation-inducing chemicals and in some inflammatory diseases. In contrast to other known inflammasomes, activation of the NLRP3 can be achieved by a wide range of structurally dissimilar agonists, including pathogens, pore-forming toxins, environmental irritants, endogenous damage associated molecular patterns, and PAMPs. Microbial activators include various gram-positive and -negative bacteria, fungi, RNA and DNA viruses, as well as protozoa (as reviewed by Ratsimandresy et al., 2013). Based on the diverse structural nature of NLRP3, one model assumes that intermediate factors may be involved in sensing these activators, rather than a direct receptor-ligand interaction. However, a two-step model in which both priming and activating signals are required to produce a functional inflammasome is generally accepted. Considering the potency of the products of inflammasome activation, the requirement for two signals to achieve activation represents an important regulatory checkpoint to avoid immune responses capable of harming the host. In general, step 1 consists of a priming stimuli that can include any whose receptor signaling results in the activation of the transcription factor NF- κ B, such as ligands for IL-1R1, TLRs, NLRs, and the cytokine receptors TNFR1 and TNFR2 (Bauernfeind et al., 2009). The activation of NF- κ B is critical for upregulating the transcription of both IL-1 β and NLRP3, as IL-1 β is not consistently expressed and basal levels of NLRP3 are inadequate for efficient inflammasome formation. In contrast, transcriptional modulation is not required to license the inflammasome components caspase 1 substrate pro-IL-18, as these are found at adequate concentrations in the steady state (Schroder et al., 2012). The second step in activation of the NLRP3 inflammasome is provided by one of a diverse group of agonists that triggers the specific activation of NLRP3, assembly of the inflammasome complex, and finally culminates in the activation of caspase 1.

Macrophages and dendritic cells are activated when PAMPs bind to their PRRs. As a result, they synthesize and secrete a mixture of molecules that trigger inflammation, inhibit microbial growth, and initiate the first steps in adaptive immunity that set up a

state of inflammation. These small proteins that are activated during an innate immune response are called cytokines and chemokines. Cytokines act as intracellular and intercellular signaling molecules that are synthesized and secreted by a variety of different cell types, as well as act on a range of target cells. A great example that shows the versatility of cytokine production and action is TNF- α , which is synthesized and secreted mainly by monocytes, macrophages, and mast cells. Red blood cells (RBC) are the only known cells that do not contain TNF- α receptors (Tizzard, 2013). Once soluble TNF- α is cleaved from its membrane-bound form, it can have many effects on many different cells including activating other cells (vascular endothelium, macrophages, lymphocytes, neutrophils), enhancing collagen synthesis and fibroblast growth, promotion of inflammation, and toxic effects (Tizzard, 2013). Some cytokines, like TNF- α , initiate and amplify the response, others sustain it, and some cause it to resolve (anti-inflammatory). These changes are induced by a variety of inflammatory mediators released as a consequence of the recognition of pathogens by macrophages, and later by neutrophils and other white blood cells. Both macrophages and neutrophils secrete lipid mediators of inflammation: prostaglandins, leukotrienes, and platelet-activating factor (PAF).

The cytokines secreted by activated dendritic cells and macrophages in response to activation of PRRs include a structurally diverse group of proteins. The various families of cytokines include the IL-1 family, the hematopoietins, the TNF family, and the type I interferons. Cytokines can be grouped by structure into different families, and their receptors can likewise be grouped. One method of distinguishing cytokines is by T-helper 1 (Th-1) or T-helper 2 (Th-2) cytokines named for their influence of the nature of the helper T cell response in the adaptive immune response. A more general method of categorizing the cytokines is by their role in innate or adaptive immunity. Mediators of natural immunity secreted by dendritic cells and macrophages in response to activation of PRRs include TNF- α , IL-1, IL-6, IL-12, IFN- α , IFN- β , IFN- γ , and chemokines. Mediators of adaptive immunity include IL-2, IL-4, IL-5, and TGF- β . As stated by Agarwal et al. (2000) a successful pregnancy is the result of a delicate balance between the various types of cytokines involved in growth, development, and maternal tolerance of the conceptus.

Cytokines such as IL-1, TNF- α , and IL-6 are stimulated immediately after initiation of the innate immune system (van Deventer et al., 1990). The biological effects of LPS are largely mediated by the pro-inflammatory cytokines, IL-1, IL-6, TNF- α . Upon activation of these cytokines, the liver produces acute-phase reactants (APR), and stimulates the hypothalamic-pituitary-adrenal axis to help control inflammation. The participating cytokines are considered to be pro-inflammatory because they induce other cytokines and chemokines, which function to amplify the initial immune response. Chemokines, like IL-8, are produced by macrophages or mast cells and attract neutrophils to the site of infection. Moreover, these same cytokines and chemokines not only play an integral role in the immune response, but they are also vital for homeostasis.

An important cytokine that triggers local containment of infection, TNF- α can also have detrimental effects on the host when released systemically. The TNF- α can be cleaved by a specific protease TACE and is released from the membrane as a soluble cytokine. The presence of infection in the bloodstream, or sepsis, is accompanied by a massive release of TNF- α from macrophages located throughout the body. However, the diversity of TNF- α actions have yet to have been fully understood. For example, Terranova and colleagues (1995) identified TNF- α binding sites on the ovaries of several mammalian species. Brannstrom and colleagues (1995) suggested that ovulation rates were enhanced by addition of TNF- α in rat ovaries. Murdoch et al. (1997) showed that TNF- α is expressed on thecal endothelial cells of ovine follicles destined to ovulate and that localized release of this cytokine is a prelude to programmed ovarian cell death and follicular rupture. These data suggest that TNF- α may also be a critical component of ovulation.

However, TNF- α may not always induce positive effects on the host or the developing embryo as shown by Gendron *et al.* (1990) who reported that increased production of TNF- α , induced by injection of LPS was associated with failure to establish pregnancy in mice. Within the same study, treatment of pregnant mice during early gestation with LPS resulted in fetoplacental resorption but resorption was significantly reduced when mice were pretreated with the TNF- α suppressing drug pentoxifylline. Placental necrosis has been demonstrated in rats treated with recombinant human TNF- α (Silen et al., 1989) and therefore the mechanism of LPS-induced fetoplacental resorption

may be due to the direct effect of TNF- α on the placental vasculature resulting in hemorrhage and necrosis (Gendron et al., 1990). TNF- α has also been demonstrated to inhibit DNA synthesis and proliferation in trophoblast cells (Hunt, 1989). Therefore, TNF- α may act through the inhibition of trophoblast proliferation of the developing embryo after LPS treatment. Similarly, addition of TNF- α in vitro to the cultures of mouse and cow blastocysts inhibited their development (Baines et al., 1996; Soto et al., 2003). Although peptidoglycan-polysaccharide (PG-PS) binds to different TLRs (TLR 2 and TLR 6) on macrophages than LPS (TLR 4), both PG-PS and LPS binding lead to activation of NF- κ B and, subsequently, secretion of inflammatory cytokines. Dow et al. (2010) showed that interference with establishment of early pregnancy in ewes inoculated with PG-PS appears to be from local uterine inflammatory response, as shown by elevated TNF- α in the posterior vena cava. Additionally, IL-6 is produced not only by immune and immune accessory cells, but also by many non-immune cells and organs (such as osteoblasts, bone marrow stromal cells, keratinocytes, intestinal epithelial cells, and vascular smooth-muscle cells) suggesting that IL-6 not only plays a critical role in inflammation but also in regulation of endocrine and metabolic functions (Papanicolaou et al., 1998).

The IL-1 family of ligands is associated with acute and chronic inflammation, and plays an essential role in the non-specific innate response to infection. The biological properties of IL-1 family ligands are usually pro-inflammatory. The IL-1 subfamily consists of IL-1 α , IL-1 β , and IL-33, which differ in their length of their precursor and the length of the propeptide for each precursor. Since each are pre-formed in cells, their release is a consequence of injury and is immediate. Therefore, they are termed “alarmins” because they alert the host to initiate the innate response. In the case of IL-1 α , the precursor is fully active; in the case of other members, the precursors are initially weakly active but are converted to more active cytokines upon infiltration of neutrophils.

The IL-1 α precursors reside in primary cells such as keratinocytes, thymic epithelium, hepatocytes, endothelial cells, fibroblasts, and the epithelial cells of mucus membranes (Hacham et al., 2002). Furthermore, precursor IL-1 α can be found on the surface of several cells, particularly on monocytes and B-lymphocytes, referred to as membrane-bound and is considered biologically active (Kurt-Jones et al., 1985). The IL-

1 α plays an important role in sterile inflammation or inflammation without the presence of an infectious agent. The IL-1 α can also be a potent pro-inflammatory cytokine capable of triggering multiple physiological processes including activation of lymphocytes, induction of acute-phase hepatic proteins, fever and anorexia. It also has normal physiological roles in the regulation of cell proliferation and differentiation and is constitutively present in most cells of healthy individuals.

In contrast, IL-1 β is a highly pro-inflammatory cytokine, is not present in healthy individuals, and has been studied more than any other member of the IL-1 family. Both IL-1 β and IL-1 α bind to the same IL-1R1 and trigger a pro-inflammatory signal. The inactive IL-1 β precursor is converted into an active cytokine by the intracellular cysteine protease caspase-1 (Joosten et al., 2013). Unlike IL-1 α , the IL-1 β precursor is not present in normal physiological roles (Joosten et al., 2013). Also unlike IL-1 α , IL-1 β is primarily a product of monocytes, macrophages, and dendritic cells (DC) as well as B-lymphocytes and NK cells (Joosten et al., 2013). Nearly all microbial products induce IL-1 β via TLR activation but other cytokines, such as TNF- α , IL-18, IL-1 α , or IL-1 β can be a stimulus (Dinarello et al., 1987). Pannexin-1, a mammalian protein that functions as a hemichannel for the uptake of dyes, is required for caspase-1 processing and release of IL-1 β via the P2X7 receptor (Pelegrin and Suprenant, 2006). Pannexin-1 can also function for LPS-induced IL-1 β synthesis in the absence of TLR4 (Derks and Beaman, 2004).

Embryonic signaling molecules such as IL-1 have been investigated both in human and rodent embryo implantation processes and is thought to be one of the earliest signals released by embryonic cells (Simón et al., 1994). It is becoming increasingly apparent that in addition to adequate hormonal priming, successful embryonic implantation relies on an appropriate interaction between autocrine-paracrine cytokines produced and received by the blastocyst and endometrium. Among these cytokines, the IL-1 system seems to be relevant to the implantation process. This system is composed of IL-1 α , IL-1 β , and their receptors, IL-1 receptor type I and IL-1 receptor type II, and an inhibitor, IL-1 receptor antagonist (IL-1ra) (Simón et al., 1998). Available information indicates that IL-1 signaling occurs exclusively via type I receptors. The presence and function of the IL-1 system at the endometrial/embryonic/placental interface and the

implications in embryonic implantation have been explored in various mammals, including humans and rodents. In mouse endometrium, IL-1 α and IL-1 β mRNAs have been localized by in situ hybridization and are present during the stages of development preceding implantation (Tackacs et al., 1988; De et al., 1993). Additionally, when the IL-1 receptor type I signal transduction was blocked in the maternal endometrium of the mouse, the blastocysts failed to adhere to the luminal epithelium and resulted in failure of implantation (Simón et al., 1994). The expression of secretory IL-1 β in the pre-implantation embryos recovered from control animals from day 4 of pregnancy until implantation may be required for signaling the uterus for stromal cell proliferation and decidualization (Deb et al., 2005). However, LPS induced early expression of IL-1 β in the embryos that may have disrupted the feto-maternal signaling mechanisms required for successful implantation in the mouse (Deb et al., 2005). Interestingly, Deb and colleagues (2004) showed that high levels of LPS also induced IL-1 α and caused implantation failure in mice. Regardless, the altered pattern of expression of IL-1 in the embryos in response to LPS may have effects that are detrimental to the embryonic development. Although the embryo may not express IL-1 receptor at the pre-implantation stage of pregnancy, the expression of IL-1RI could occur in the uterus of pregnant animals during this stage and could signal the maternal endometrium through the IL-1 receptor. This may initiate a cascade of additional pro-inflammatory substances like TNF- α , IL-6, and IL-8 in the LPS treated animal, which may contribute to infection mediated pregnancy loss. Dudley et al. (1993) observed that IL-1 β and LPS significantly increases the production of PGE₂ and IL-6 from murine decidua. Deb and colleagues (2005) observed an increase in expression of IL- β gene in the uterus of LPS treated animals throughout the pre-implantation period of pregnancy compared to that of the respective controls. The elevated levels of IL-1 β may trigger the production of prostaglandins and other cytokines by the gestational tissues, leading to pregnancy loss.

The mechanism of IL-1 mediated pregnancy loss remains unclear. One possibility is that IL-1 elicits prostaglandin production in gestational tissues, which in turn leads to fetal death. Silver et al. (1997) reported that LPS-induced fetal death is a prostaglandin independent effect. Silver and colleagues (1995) showed that LPS-induced fetal loss is significantly blocked by indomethacin, a non-steroidal anti-inflammatory drug that

inhibits the production of prostaglandins. Many of the biologic effects of IL-1 are mediated by prostaglandins, and IL-1 causes prostaglandin production by gestational tissues in vitro (Romero et al., 1989). Silver and colleagues (1997) showed that IL-1 is an important mediator of LPS-induced fetal death. Pretreatment with anti-IL-1-receptor antibodies significantly reduced the proportion of fetal deaths from the administration of LPS (Silver et al., 1997). Others have demonstrated elevations in amniotic fluid concentrations of IL-1 α in mice treated with LPS, rhesus monkeys infected with group B streptococci, and rabbits inoculated with *Escherichia coli*. The use of cyclooxygenase inhibition, which disrupts the formation of prostanoids by blocking the enzymes COX-1 and COX-2, only minimally decreased fetal death after IL-1 administration (Silver et al., 1997). This may suggest that IL-1 causes negative fetal outcomes by mechanisms other than prostanoid formation. This is further supported by the use of indomethacin, which has prostaglandin-independent anti-inflammatory effects and prevents more IL-1 induced fetal deaths (Silver et al., 1997). It is important to note that both IL-1 and LPS initiate a cascade of additional pro-inflammatory molecules such as TNF- α that may influence a negative fetal outcome. The presence of IL-1 in gestational tissues is normal during pregnancy; however, high levels of IL-1 or disruption in the signaling transduction of the maternal endometrium or embryo may result in fetal loss after administration of LPS.

The inflammatory cytokines produced as a result of TLR signaling, when released in excess, induce serious systemic disorders that are associated with a high mortality rate—such as endotoxic shock, which can be induced by the TLR-4 ligand LPS. Organisms have evolved mechanisms for modulating their TLR-mediated response. The molecules thought to negatively regulate TLR signaling are IRAK-M, SOCS1, MyD88 short, SIGIRR, and ST2, which are presumed to directly down-regulate TLR-signaling pathways.

Several serum proteins change in concentration during the acute phase of a disease or infection and possess pattern recognition capacity. The serum changes are collectively called the acute phase response (APR), and the proteins whose concentrations changed during the acute phase are called acute phase response proteins (APR proteins). The APR proteins include the secretion of fibrinogen, serum amyloid A (SAA), haptoglobin (Hp), α -1 acid glycoprotein, and C-reactive protein, principally by

the liver (Vels et al., 2009). The APR is part of the early-defense or innate immune system and the complement system. The APR has been referred to as the “molecular thermometer” whereby quantitation of individual APP can provide an assessment of the response to the triggering event (Ceron et al., 2005). The liver is one of the major sites of APR production, and the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 are the major signals responsible for induction of the APR. Although APR is highly conserved during evolution, some variations do exist for the APP in terms of animal species. Two ruminant acute phase proteins are Hp and SAA. Ruminants are unique in that Hp is a major APP, and in sheep, as for cattle also, APP production continues in chronic and acute infections, with roles in tissue repair. Generally, major APPs are present at undetectable or very low levels in the serum of healthy animals, but, recently, a study investigating the APP response to scrapie in sheep found large individual animal variation in Hp and SAA prior to onset of clinical disease and it was suggested that this may be due to underlying subclinical conditions (Meling et al., 2012).

The SAA is the first natural pro-inflammatory mediator that can provide signals for the production of IL-1 β and for the activation of the NLRP3 inflammasome, resulting in the secretion of mature IL-1 β (Niemi et al., 2011). LPS is known as a potent inducer of inflammation and the APR. Mastitis caused by *E.coli* is a common disease in lactating dairy cows, and infusion with *E.coli* LPS can be used as a model for studying the bovine APR (Lehtolainen et al., 2004). In vertebrates, the APR is characterized by fever, leukocyte mobilization, and an increased production of various inflammatory cytokines and APP (Gruys et al., 2005). Challenge with LPS stimulated liver macrophages to secrete TNF- α , IL-1, and IL-6, which then induced hepatocytes to synthesize and secrete SAA and Hp (Jacobsen et al., 2004). For example, in dairy cows, LPS administered intramuscularly increased the milk and plasma concentrations of TNF- α , SAA, and Hp, indicating local tissue production in the udder and release of inflammatory cytokines and APP into circulation (Lehtolainen et al., 2004). In addition, Dow et al. (2010) showed that increased concentrations in serum of SAA, TNF- α , and Hp occurred after PG-PS challenge in early pregnant ewes. Generally, Hp has been considered a marker of inflammation only. However, recent data suggest that Hp also can function as an anti-inflammatory agent. For example, Hp has bacteriostatic properties and antagonized TNF-

α to decrease inflammation (Arredouani et al., 2005). In vitro, Hp, one of many proteins secreted by the oviductal epithelium that forms a protective coat around the blastocyst in the rabbit (Herrler et al., 2004), antagonized host immune response to LPS by suppressing production of TNF- α in monocytes. Although the main immunological functions of APP are still not understood, the APP are believed to contribute to host defense by decreasing tissue damage, acting on leukocyte functions, inhibiting bacterial activity through binding of endotoxin, and displaying anti-inflammatory properties (Moore et al., 1997).

Adaptive Acquired Immunity

Unlike the rapid acting innate immune system, the adaptive or acquired immune system develops through encounters with microorganisms over time. The cornerstone of acquired immunity is specificity, the ability to interact selectively only with particular molecular components of microbial pathogens. Acquired immunity is a type of stimulus-response action. Molecules that trigger the mechanisms of acquired immunity are called antigens, and the body must be able to recognize and respond to antigens. The most common antigens are proteins, polysaccharides, glycoproteins, and complexes between these and lipids or nucleic acids. Once the innate immune response has been initiated via pathogen detection by PAMPs and their corresponding TLRs or NODs, and the APP has been initiated, the host body is then able to accurately begin to determine the specifics of the given antigen and correspondingly trigger an adaptive immune response.

Within the adaptive immune response, there are two subcategories: humoral immunity and cell-mediated immunity. Humoral immunity refers to the presence of antibodies in various body fluids where they serve as effectors by identifying antigens and marking them for elimination. Recognition of an immunogen by the surface antibodies of the B cell triggers proliferation and differentiation into memory B cells and plasma cells. The B cells mediate the production of antibodies via activation of the humoral pathway (Goldsby et al., 2003). Only small parts of antigens, called antigenic determinants or epitopes, directly trigger acquired immunity. It is important to note that all immunogens are antigens, but not all antigens are immunogens. Epitopes bind to antigen-specific membrane receptors on lymphocytes or to secreted antibodies. Antibodies are epitope-binding proteins found on B cells or as soluble molecules secreted

by plasma cells. Antibodies can recognize a wide range of epitopes. Structurally, an antibody molecule consists of two identical light chains linked to two identical heavy chains by disulfide bonds. There are 5 antibody classes (IgM, IgG, IgD, IgA, IgE) that have different effector functions, average serum concentrations, and half-lives. Within the amino-terminal variable domain of each heavy and light chain are three complementary-determining regions (CDR). These polypeptide regions contribute the antigen binding site of an antibody, determining its specificity. The association between an antibody and an antigen involves various noncovalent interactions between the epitope of the antigen and the CDR.

One of the most sensitive techniques for detecting antigen or antibody is enzyme-linked immunosorbent assay (ELISA). The principle of ELISA involves binding of an enzyme conjugated with an antibody that reacts with a chromogenic substrate to generate a colored reaction. A number of enzymes have been employed for ELISA, including alkaline phosphatase and horseradish peroxidase. A number of variations of ELISA have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody. A standard curve based on known concentrations of antibody or antigen is prepared, from which the unknown concentration of a sample can be determined. An indirect ELISA can detect antibody quantitatively whereas an antibody coated well sandwich ELISA can detect or measure antigen. A sample containing antigen is added and allowed to react with the immobilized antibody. After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. Another variation for measuring amounts of antigen is competitive ELISA.

While the humoral pathway is mediated by B cells, the cell mediated immunity is carried out by T cells. In contrast to antibodies or B cell receptors, which can recognize antigen alone, T cell receptors only recognize antigen that has been processed and presented in the context of molecules encoded by the major histocompatibility complex (MHC). Furthermore, T cells have the ability to recognize immunogen epitopes and discriminate self from non self via MHC (Huber et al., 1976). The MHC affects the response of an individual to antigens of infectious organisms. The MHC encodes class I and II molecules, which function in antigen presentation to T cells, specifically class I

molecules present to CD8⁺ T_C cells and class II molecules present antigen to CD4⁺ T_H cells. All nucleated cells in the host produce MHC I molecules along with the peptide epitope that recognizes its complementary shaped receptor on the surface of a naïve T-8 lymphocyte. The T cell receptor expressed on the surface membrane of the T cell recognizes only processed antigen fragments that are complexed with MHC molecules. The CD3 is a complex of polypeptide chains involved in signal transduction with the T cell receptor and is required for surface expression of the TCR. This naïve T-8 lymphocyte has the ability to clone the presented peptide epitope and then produce mature T-cells that will have receptors for the specific antigen and bind the pathogen epitope directly. Thus, the initiation of the adaptive immune response via cell-mediated immunity has begun (Marrack and Kappler, 1993).

Unlike MHC I molecules, MHC II molecules are only found in macrophages, dendritic, and B cells and bind peptides derived from extracellular pathogenic proteins through the ingestion of the microorganism and recognition of their foreign molecular pattern. Once bound, MHC II molecules interact exclusively with CD4⁺ T cells (helper T cells). A subset of T helper cells, Th1 and Th2, are identified by their respective cytokine production. The helper T cells then trigger another immune response which may include localized inflammation and swelling due to recruitment of phagocytes (Amilla et al., 1998).

Glucocorticoid Action

The best characterized LPS activation pathway involves the co-factors CD14, MD2, and TLR 4, a specific member of the TLR family expressed on the surface of monocytes, neutrophils and dendritic cells. The LPS-immune response model has been used to demonstrate that the hypothalamic-pituitary-adrenal axis (HPAA) is activated at several points during the APR as well. Since the 1940s, glucocorticoids (GCs) have long been recognized as powerful anti-inflammatory compounds that are one of the most widely prescribed classes of drugs in the world. However, their role in the regulation of innate immunity is not well understood. Adrenocorticotropin hormone (ACTH) released from the anterior pituitary gland is the signal for GCs, like cortisol, released from the adrenal glands in response to stress-induced activation of the HPAA and are responsible for maintaining homeostasis and a number of immunomodulatory properties. The HPAA

is activated during the APR and is part of an elaborate multi-directional communication pathway designed to restore homeostasis, in part, by regulating inflammatory and immune responses. The anti-inflammatory role of GCs has been attributed largely to the ability of the glucocorticoid receptor (GR) to suppress the activities of the pro-inflammatory transcription molecules AP-1 and NF κ B (Smoak et al., 2004). Glucocorticoids are typically perceived to have anti-inflammatory properties by virtue of their ability to suppress the Th1 cytokines (IL-12, IFN- γ , TNF- α , IL-1). Owing to their lipophilic nature, GCs diffuse freely across the plasma membrane and exert their effects through the GR, a member of the nuclear receptor superfamily of ligand-dependent transcription factors (Sapolsky et al., 2000; Evans, 1988). Most if not all, the immunomodulatory effects of GCs appear to be mediated through the cytosolic glucocorticoid receptor (cGR). Following GC-cGR ligation, the cGR dissociates from a protein complex, dimerizes, and translocates to the nucleus to induce or repress the expression of a wide variety of genes. Once secreted the majority of cortisol diffuses across cellular membranes and exerts its biological effects. Originally named for their effects on glucose metabolism, GCs affect nearly every tissue of the body and have a role in regulating numerous processes, including reproductive, cell proliferation, survival, and immune functions (Barnes, 1998). Glucocorticoids target specific cell populations within the immune system to combat hyper-activation or systemic infections. For example, by blocking the expression of Cox-2, GCs target T cells to control hyper-activation in response to excessive TCR binding (Brewer et al., 2003). In contrast, GCs target macrophages to ensure survival in response to LPS induced sepsis (Bhattacharyya et al., 2007).

Activation of HPAA appears to be primarily mediated by the pro-inflammatory cytokines TNF- α , IL-1 α and β , IL-6, leukemia inhibitory factor (LIF) (Chesnokova and Melmed, 2002), the adipokine, leptin (Akirav et al., 2004), and possibly the anti-inflammatory cytokine, IL-10 (Smith et al., 1999). The TNF- α synergistically enhances IL-1-induced ACTH and/or GC secretion (Turnbull and Rivier, 1999), and both amplify and prolong the immune response by activating surrounding cells to release IL-1 and other inflammatory mediators.

Effects of IL-1 on the HPA axis are executed primarily at the level of the hypothalamus by directly, or indirectly, inducing the secretion of corticotropin-releasing hormone (CRH) from neurons within the hypothalamus, which cause the secretion of ACTH from the anterior pituitary. Systemic increases in IL-1 following LPS challenge are largely derived from tissue macrophages, such as the resident hepatic Kupffer cell population (Navarra et al., 2001). Also, IL-1 β drives the production of IL-6, which is essential for HPA axis activation (Turnbull et al., 2003) and the fever responses associated with the APR (Cartmell et al., 2000). Glucocorticoids have been shown to inhibit the induction of IL-1 β in macrophages (Lee et al., 1988) but the inhibition mechanism is controversial. Jeon and colleagues (2000) showed that dexamethasone inhibits LPS-induced expression of IL-1 β gene in LPS activated RAW 264.7 cells and the authors attributed the mechanism behind the inhibition to blocking the actions of the transcription factors NF- κ B and AP-1.

However, not all actions of GCs are immunosuppressive, and emerging data suggest that GCs may also have pro-inflammatory actions. For example, basal levels of GCs are required for the production of epinephrine by the adrenal glands and for proper signaling by norepinephrine both of which mediate stress-induced increases in immune activity (Wurtman and Axelrod, 1966; Joëls and de Kloet, 1989). Traditionally, systemic administration of GCs has been associated with suppression of the immune system, particularly in response to LPS (Coutinho and Chapman, 2011). However, in certain instances, GCs can enhance the secretion of IL-1 β , TNF- α , and IL-6 *in vivo* in cultured cells if GCs are administered prior to the LPS challenge (Johnson et al., 2002). Additionally, GCs have been shown to have a priming effect and can augment the immune response to LPS (Frank et al., 2010). Within the same study, GCs up-regulated the peripheral (liver) and central (hippocampus) pro-inflammatory responses (e.g. TNF- α , IL-1 β , IL-6) to a peripheral immune challenge (LPS) if GCs were administered prior (2 and 24 hours) to challenge. In contrast, when GCs were administered after (1 hour) a peripheral immune challenge, GCs suppressed the pro-inflammatory response to LPS in both liver and hippocampus (Frank et al., 2010). Thus, the temporal relationship between GC treatment and immune challenge may be an important factor determining whether GCs exhibit pro- or anti-inflammatory properties. These pro-inflammatory GC effects

occur at multiple levels, with GCs increasing: (a) migration of microglia, neutrophils, and macrophages to an injury site; (b) and potentiating production and release of pro-inflammatory cytokines; and (c) NF- κ B activity (Madrigal et al., 2002).

Busillo and colleagues (2012) sought to determine the actions of glucocorticoids on regulating both the expression and function of a type of NOD receptor, NLRP3 in human and mouse macrophages. They showed that NLRP3 is a glucocorticoid-responsive gene, specifically in differentiated macrophages, associated with increased levels of NLRP3 intra-cellularly and enhanced maturation and release of IL-1 β in response to an endogenous molecule, extracellular ATP. In addition, GCs also enhanced the secretion of pro-inflammatory cytokines, IL-6 and TNF- α (Busillo et al., 2012). However, GCs acting through GR efficiently suppress cytokine induction downstream of TLR4 and MyD88 activation by LPS (Ogawa et al., 2005). Normally, engagement of TLR4 triggers a cascade of signaling events leading to induction of mitogen-activated protein kinases (e.g. MAPKs), resulting in enhanced transcription of the mRNAs for several pro-inflammatory cytokines including IL-6, IL-1 β , TNF- α , and IL-12 (Yamamoto et al., 2004). A number of levels at which GCs can exert their multiple anti-inflammatory effects have been identified. One pathway states that GCs bind specifically to the intracellular glucocorticoid receptor alpha, thereby promoting dissociation from heat shock protein 90 and subsequent translocation to the nucleus, where the expression of genes with anti-inflammatory effects can be activated (Mogensen et al., 2008). Dexamethasone, a synthetic GC, has been demonstrated to inhibit the activation of the MAPKs extracellular signal, ultimately preventing phosphorylation and activation of MAPKs and the NF κ B pathway (Mogensen et al., 2008). Inflammation induced by LPS has been demonstrated to be inhibited by GCs. For example, Bhattacharyya and colleagues (2007) showed that greater mortality and cytokine production occurred in mice with a conditional deletion of GR than in controls. In addition, the complete absence of GCs renders animals more susceptible to LPS induced systemic inflammation. In humans, timing has proved to be an important issue, since mortality and morbidity were reduced only when dexamethasone was given prior to or concomitant with the initiation of antibiotic treatment (de Gans and van de Beek, 2002).

From a pharmacological standpoint, GCs are the first line of defense to treat chronic and acute inflammatory diseases. However, the actions of GCs are more pleiotropic than previously thought and, therefore, cannot be simply categorized as anti-inflammatory. The ability of molecules to have both a pro- and anti-inflammatory role in both normal physiological and pathological conditions is not unprecedented. For example, both IL-6 and leptin are known to have pro- and anti-inflammatory properties (Hick et al., 2006; Scheller et al., 2011). As reviewed by Busillo and Cidlowski (2013), the shift in the ability of GCs to regulate pro- versus anti-inflammatory gene programs lies in the concomitant signals received by the cell, the duration and magnitude of GC signaling, and the duration and magnitude of the pro-inflammatory stimulus. Busillo and Cidlowski (2013) stated that instead of acting strictly as an anti-inflammatory mediator, GR should be considered as a cellular rheostat meaning that in response to inflammatory stimuli, the output elicited by GCs is fine-tuned by the microenvironment. In conclusion, GCs elicit pro-inflammatory actions by readying and reinforcing the innate immune system but also elicit anti-inflammatory actions to act systemically to repress the adaptive immune response to help restore homeostasis.

Summary

Oocyte maturation, fertilization, and early embryonic development could be disrupted by products of the microorganisms that colonize the mammary gland or by cytokines or other inflammatory mediators produced in response to infection. Among the molecules that could have negative ramifications on the developing embryo are endotoxins (LPS) whose effects on the oocyte and embryo have not been clarified. In one experiment, LPS reduced embryonic development of cultured mouse embryos (Dubin et al., 1995); while, in another experiment, LPS only reduced development if mouse embryos also were exposed to TNF- α (Randall et al., 1991). Other molecules produced during inflammation that might compromise oocyte and embryo function are prostaglandin F_{2 α} (PGF_{2 α}) and nitric oxide (NO). In several tissues, including the endometrium, synthesis of PGF_{2 α} is increased by LPS, by inflammatory cytokines such as TNF- α and IL-1 β and by NO (Davidson et al., 1995; Skarzynski et al., 2000). Because increased concentrations of LPS can have deleterious consequences on oocyte function and embryonic development, identification of the mechanism through which infectious

diseases affect reproductive efficiency may lead to improving fertility in animals experiencing infectious disease or inflammation.

Figure 1. Innate and Acquired/Adaptive Immune Response Summary based on Tina Dow Master of Science Thesis in 2008.

Figure 2. Effects of Acute Gram-Negative Bacterial Immune Challenge on Early Embryonic Survival Summary.

Objectives

The primary null hypothesis for this experiment is injection of 5 and 6 dpc ewes with LPS, TNF- α , or LPS+dexamethasone does not elicit a difference from controls (saline) in concentration of pro-inflammatory cytokines and APPs; specifically, TNF- α , SAA, and Hp in jugular plasma (representative of systemic circulation). The secondary null hypothesis is the treatments will not elicit a difference in rectal temperature, total white blood cell count, mucosal response, and lethargy in ewes. In the event that the primary null hypothesis would be rejected and there will be an increase in TNF- α , SAA, Hp concentrations in blood plasma of 5 and 6 dpc LPS, TNF- α , or LPS+Dex treated ewes, the tertiary hypothesis is that these increased mediators do not relate to early embryonic loss either directly or indirectly. The main objectives of this project were to test the above hypotheses against alternative hypotheses of LPS and TNF- α inducing the inflammatory and acute phase responses leading to pregnancy loss while dexamethasone would reduce response to control levels for all variables, specifically:

1. Characterize the effects of an APR of the innate immune system, via cytokine production, APPs, behavioral changes, and increase in rectal temperature, in day 5 or 6 pc ewes, after intoxication with LPS, on early embryonic loss;
2. To examine if effects of exogenous TNF- α would mimic the effect of exogenous LPS in treated ewes;
3. To examine if dexamethasone given prior to LPS would prevent or decrease the innate immune response and the resulting acute phase response.

Materials and Methods

Thirty-eight mature Dorset x Texel ewes, ~ 50 kg BW, (WVU IACUC #13-0404) were fed free choice native grass hay and had access to fresh water ad libitum. Ewes were assigned randomly into two groups in two experimental replicates: replicate one was conducted in March and replicate two was conducted in April. Ewes were synchronized for estrus (Dixon et al., 2006) using controlled intravaginal drug release (CIDR-G) inserts (Pfizer, New York, NY) for seven days followed by an injection of PGF_{2 α} (4 cc im of Lutalyse[®], Pharmacia and UpJohn, New York, NY) at CIDR removal. Ewes were housed with three fertile rams that were equipped with a marking harness to aid in detection of estrus. Every 12 hours, ewes were observed for signs of standing behavior to

mounting by the rams beginning at 12 hours post CIDR removal and PGF_{2α} injection. Marked ewes were recorded and remained with the fertile rams for approximately twenty-six days.

Day of the first breeding was denoted as 0 dpc. On 5 or 6 dpc, ewes were assigned at random to one of four treatment groups and received via the jugular vein either 2.5 mL of 0.1% BSA/PBS (controls), 2.5 mL of 2.5 µg/kg of LPS (Sigma Altrich, St. Louis, MO), 5 mL of 1 µg/kg of TNF-α in two bolus injections given thirty minutes apart, or 2.5 mL of solution containing 2.5µg/kg of LPS after having received 3.5 mL of solution containing 0.14 mg/kg BW im dexamethasone (Dex) at -12 and 0 hours and LPS at hour 0 (Patterson Veterinary, Devens, MA). The dose of LPS in sheep mimics shipping fever (Elsasser et al., 2004), and LPS acts through the TLR 4 in sheep immune cells (Deng et al., 2012). In preliminary study (Lendt, unpublished), the dosage of LPS evoked a transient, dramatic increase in rectal body temperature accompanied by a transient, dramatic increase in plasma concentrations of TNF-α. Prior to the first replicate, response to a single bolus i.v. injection of 1.0 µg/kg (Wheeler et al., 1990) of human recombinant TNF-α (PeproTech, Rocky Hill, NJ) was monitored in a Suffolk ewe. An increase in rectal temperature and physical signs (muscle trembling, watery nose and eyes, vaginal discharge) were observed.

Jugular samples (6-8 mL) were collected in EDTA treated tubes (from laboratory source) every 30 min post challenge for 180 min, every hour through 12 h, and every 12 h through 48 h. Additional jugular samples were collected on days 9 or 10 and 25 or 26 pcs for determination of concentrations of progesterone for evaluation of luteal function. Changes in body temperature, behavior and/or physical appearance were recorded during the first 12 h of blood sampling. Total white blood cell counts were obtained using a Beckman Coulter Counter (Beckman Coulter, Pasadena, CA) and were processed immediately after collection for total white blood cells counts, blood smears, and aliquots of plasma collection for storage at -80°C. Remaining samples were centrifuged, and plasma was collected and stored at -80°C.

At day 25 or 26pcs, detection for pregnancy was examined via transrectal ultrasonography with a 7.5 MHz transducer and Aloka 500 ultrasound console

(Corometrics Medical Systems, Inc) for visualization of embryonic heartbeat. Number of lambs born from ewes bred from the first service were recorded after lambing.

Assays

A white blood cell differential was determined by thinly spreading 5 μ L of blood over a glass slide, air drying, and staining with Camco Quik Stain (Sigma Altrich, St. Louis, MO). One hundred cells were counted and classified among monocyte, lymphocyte, eosinophil, neutrophil, or basophil cell types. Then the percent of the cell type was multiplied by the total white blood cell to determine the cell concentration (number of cells/ml).

Concentrations of P_4 were assessed in 25 μ L plasma by an Immulite[®] 1000 Progesterone, which is a solid phase, competitive immunoassay using enzyme-labeled chemiluminescent technology¹. Assay standard deviation for low, medium, and high controls were 0.819, 7.95, and 22.9 respectively. The intra-assay coefficient of variation (CV) for low, medium, and high controls were 0.129, 0.212, and 2.81 respectively. The reportable range was 0.20-40 ng/mL and a sensitivity of 0.46 ng/mL. Concentrations of SAA were determined using a multispecies SAA solid phase sandwich ELISA kit (Invitrogen Corporation, Carlsbad, CA). The protocol supplied with each kit was followed to process single plasma samples (1.0 μ L of sample + 499 μ L diluent) in a 1:500 dilution. Concentrations of SAA were reported in μ g/mL with an assay sensitivity of 9.4 μ g/ml and an intra-assay coefficient of covariance of 7.5 % among twenty-four assays. A sheep ELISA kit (Aviva Systems Biology, San Diego, CA) with sensitivity of 5.0465 ng/ml and an intra-assay CV of 4.1% was used to measure Hp concentrations. Plasma samples were diluted 1:2000 (2 μ L of sample + 198 μ L diluent and then 15 μ L of diluted sample + 285 μ L diluent). A non-equilibrium RIA² (Kenison et al., 1990) with an intra-assay coefficient of variance of 9.9% was used to assay concentrations of TNF- α in 100 μ L duplicate plasma samples. The minimal detectable level was 0.045 ng/ml. Concentrations of P_4 , SAA, Hp, and TNF- α are reported in ng/ml.

Statistical Analyses

¹ at the Center for Reproductive Medicine in Morgantown, WV

² at the USDA Growth Biology Laboratory (Beltsville, MD).

Null Hypotheses:

1. The concentration of TNF- α , SAA, and Hp in jugular plasma and total white blood cell concentration, including lymphocyte, monocyte, and neutrophil concentrations, rectal temperature, mucosal response, and lethargy do not differ between pregnant ewes intoxicated on 5 and 6 dpc after injection of LPS or TNF- α or LPS plus dexamethasone or saline (control).
2. The concentration of TNF- α , SAA, and Hp in jugular plasma and total white blood cell concentration, including lymphocyte, monocyte, and neutrophil concentrations, rectal temperature, mucosal response, and lethargy do not differ between ewes intoxicated on 5 and 6 dpc after injection of LPS or TNF- α .
3. The concentration of TNF- α , SAA, and Hp in jugular plasma and total white blood cell concentration, including lymphocyte, monocyte, and neutrophil concentrations, rectal temperature, mucosal response, and lethargy do not differ between ewes intoxicated on 5 and 6 dpc between control ewes and ewes receiving LPS plus dexamethasone *versus* ewes receiving LPS and TNF- α .
4. The concentration of TNF- α , SAA, and Hp in jugular plasma and total white blood cell concentration, including lymphocyte, monocyte, and neutrophil concentrations, rectal temperature, mucosal response, and lethargy collected do not differ between ewes intoxicated on 5 and 6 dpc between control ewes and ewes that received TNF- α *versus* ewes that received LPS and LPS plus dexamethasone.
5. Increases in concentrations of TNF- α , SAA, and Hp in jugular plasma and total white blood cell concentration, including lymphocyte, monocyte, and neutrophil concentrations, rectal temperature, mucosal response, and lethargy on day 5 and 6 after conception do not contribute to early embryonic losses in pregnant ewes.

Data for fever mucosal response, lethargy, concentrations of white blood cells (total, lymphocytes, neutrophils, and monocytes) TNF- α , SAA, and Hp were measured over time and were analyzed by repeated measures using the ANOVA mixed procedure of SAS (v.9.3 SAS Inst., Cary, NC). Main effects of treatment, time, and interaction of treatment by time were evaluated. Additionally, four contrasts were evaluated: [LPS] vs. [TNF- α], [LPS] vs. [LPS+Dex], [TNF- α + LPS] vs. [LPS+Dex + control], [LPS + LPS+Dex] vs [TNF- α + control]. A Bonferroni adjusted p-value was utilized due to

exceeding the allotted degrees of freedom by 1 by adding a contrast. Thus P-values less than 0.0125 were considered significant. Immune response was determined by fever, increases in SAA, Hp, and TNF- α concentrations, as well observed physical changes or signs. All continuous variables were tested for normal distribution before ANOVA; if not normally distributed, data was transformed using $\ln$ or $-1/x$.

Physical changes, pregnancy status, and number of lambs born were analyzed as categorical data using a contingency table with Fisher's exact test and the adjusted Bonferroni p-value of 0.0125 to test for significance.

A logistic fit of all variables was used to predict pregnancy on d 25/26 pc. Since there was a high correlation of pregnancy and progesterone concentration on d 25/26 pc, a linear fit of continuous variables only were used to predict progesterone concentration on d 25/26 pc. In addition, only data from LPS treated ewes or controls were analyzed separately used in a logistic and linear regression models using backward elimination to determine any significant variables in predicting pregnancy and progesterone concentrations, respectively, on d 25/26 pc. An alpha level of 0.05 was used for regression analyses.

Results

Clinical signs:

Ewes treated with LPS and TNF- α exhibited a mucosal response (watery eyes, runny nose, coughing, or vaginal discharge) at ~1-4 hours post intoxication ($p < 0.0001$, Figure 4). When the LPS group was compared to the TNF- α group, there was no difference in mucosal response ($p = 0.0913$). Treatment with dexamethasone inhibited the mucosal response induced by LPS alone ($p < 0.001$). In addition, 66.6% of the ewes treated with LPS became lethargic at ~1-4 hours post treatment, which was the greatest response among all treatment groups ($p = 0.0001$) because no other ewes were lethargic (Figure 4). Like with mucosal response, dexamethasone inhibited action of LPS on lethargy (Figure 4).

Main effects of treatment ($p = 0.0010$), hour ($p < 0.001$), and treatment by hour ($p < 0.0001$) were found for rectal temperatures. Rectal temperatures increased in control, LPS, TNF- α , and LPS+Dex treated groups at 1 hour post treatment (means 39.2°C, 39.8°C, 39.7°C, and 39.6°C respectively) and peaked at 4 hours (means 39.2°C, 40.7°C.

39.4°C, and 39.9°C respectively) (Figure 3). Rectal temperature was highest in LPS and LPS+Dex treated ewes (40.7°C and 39.9°C respectively) at the peak of the response compared to the controls (39.1°C, Figure 3). Rectal temperatures returned to normal by 9 hours post treatment.

Immune system:

Total white blood cell (WBC) count (Figure 5) was different due to the treatment ($p<0.0001$), hour ($p<0.0001$), and treatment by hour ($p<0.0001$). Ewes treated with LPS+Dex had the greatest increase in total WBCs over the sampling period. However, the increase in WBCs in LPS+Dex ewes was sharper, occurring between hour 2 until hour 8 post-treatment, as compared to the increase in LPS treated ewes which gradually increased from hour 2 until hour 9, then sharply declining until hour 11. The total WBC count of control and LPS+Dex treated ewes were different than LPS and TNF- α treated ewes ($p=0.0032$). Ewes treated with LPS were different than LPS + Dex treated ewes (<0.0001). Total WBCs did not differ between LPS and LPS + Dex treated ewes (0.0259). However, LPS+Dex treated ewes maintained a higher total WBC count from hour 2 until hour 10. Ewes treated with TNF- α maintained approximately 10 million cells until hour 6 when they increased to 12 million cells and maintained until hour 12. Control ewes maintained between 8 million and 9 million WBC cells throughout the 12 hours.

Lymphocyte concentrations (Figure 6A) were different due to the treatment ($p<0.0001$) and hour ($p<0.0001$) but not treatment by hour ($p=0.0880$). All groups declined in lymphocyte concentration until hour 5 post treatment when all groups increased with control being the highest (3,000,000 cells/ml) and LPS, TNF- α , and LPS+Dex averaging 1,500,000 cells/ml. Counts in all groups declined at hour 6 post treatment and tapered off to less than 500,000 cells/ml by hour 12 post. In addition, percent of lymphocytes differed with hour ($p<0.0001$) and treatment ($p=0.0023$) in percent of lymphocytes but not treatment by hour ($p=0.2734$) (Figure 7A). Lymphocyte percentage showed a difference between LPS versus LPS+Dex treated ewes ($p=0.0016$). There was no difference between LPS versus TNF- α ($p=0.8218$), control and LPS +Dex versus LPS and TNF- α ($p=0.0375$), control and TNF- α versus LPS and LPS+Dex ($p=0.0276$) in percent of lymphocytes.

Monocyte concentrations were different by treatment ($p=0.0103$) and time ($p<0.0001$) (Figure 6C). However, treatment by time was not different ($p=0.1775$). All groups began at hour 0 with less than 1 million cells/ml. There was a difference in hour ($p<0.0001$) and treatment by hour ($p=0.0001$) when percent of monocytes were analyzed (Figure 7B). In addition, counts in LPS+Dex treated ewes declined to levels below the control (ranging from 300,000 to 1,119,932 cells/ml) until hour 4 (Figure 6C). At hour 4, control ewes increased to 2,137,127 cells/ml, the highest of all treatment groups. The control ewes then decreased at hour 5 but continued to increase in similar concentrations as TNF- α and LPS treated ewes. The greatest increase in neutrophil concentration occurred in LPS+Dex treated ewes between hour 5 and 8 post-treatment with an increase of 3,306,368 cells/ml. Ewes treated with LPS gradually increased also between hour 5 and 11 hours post-treatment with an increase of 3,170,212 cells/ml. Ewes treated with TNF- α increased between hours 5 and 12 post-treatment with a gradual increase of 2,362,099 cells/ml. The percent of monocytes did not differ between treatments ($p=0.2853$). There was no difference between LPS versus LPS+Dex and LPS versus TNF- α treated ewes ($p=0.9382$ and $p=0.7699$ respectively). Similarly, no difference occurred between combined groups: control and LPS+Dex versus LPS and TNF- α ($p=0.3710$) and control and TNF- α versus LPS and LPS+Dex ($p=0.9382$). However, the control ewes maintained the highest percentage of monocytes at hours 6 and 12 post-treatment (Figure 7B). Ewes treated with TNF- α had the second highest percentage of monocytes at hour 6 and 12, 17% and 28% respectively. Ewes treated with LPS+Dex had the highest percentage of monocytes at hour 0 but decreased to the lowest percentage of monocytes at hours 6 and 12 (Figure 7B).

Neutrophil concentrations were not different by treatment ($p=0.0402$) but did differ by hour ($p<0.0001$), and treatment by hour ($p=0.0047$) (Figure 6B). Ewes treated with LPS+Dex began at hour 0 with 3,778,036 cells/ml greater than control ewes. However, at hour 1, control ewes had 776,048 neutrophils/ml greater than LPS+Dex treated ewes. By hour 2, LPS+Dex ewes had a slight increase in neutrophils than control ewes. Beginning at hour 4, LPS+Dex ewes increased by 15,763,963 neutrophils/ml. Ewes treated with TNF- α started at hour 0 with less neutrophils/ml than control ewes but increased by hour 2 to a greater neutrophil concentration than controls and LPS treated

ewes. Ewes treated with TNF- α maintained a higher neutrophil/ml count than LPS treated ewes until hour 10 where LPS increased to the level of LPS+Dex treated ewes. Additionally, TNF- α treated ewes increased in neutrophils at hour 2 and maintained an average of between 6,000,000 and 9,000,000 cell/ml. Ewes treated with LPS stayed below control ewe levels until hour 6 where a gradual increase began to occur. Neutrophil percent differed by treatment ($p<0.0001$), hour ($p<0.0001$), and treatment by hour ($p=0.0002$) (Figure 7C). Neutrophil percentage between the LPS and LPS+Dex treated ewes differed ($p=0.0005$), and percentage for the control and TNF- α combined differed from the combined LPS and LPS+Dex treated ewes ($p<0.0001$). There was no difference in neutrophil percent between LPS and TNF- α treated ewes ($p=0.9363$) and control combined with LPS+Dex versus LPS and TNF- α treated ewes ($p=0.4042$). The highest percentage of neutrophils occurred in the LPS+Dex treated ewes ranging from 70% to 83% between hour 0 and 12 post-treatment. At hour 0, control, TNF- α , and LPS ewes had approximately 40-45% neutrophils as compared to LPS+Dex ewes that had 70% neutrophils. Ewes treated with TNF- α increased to approximately 70% at hours 6 and 12 post-treatment (Figure 7C). By hour 12, LPS treated ewes had approximately the same percentage of neutrophils as LPS+Dex ewes.

There was a difference in tumor necrosis factor-alpha (TNF- α) concentration by treatment ($p<0.0001$), hour ($p<0.0001$), and treatment by hour ($p<0.0001$) (Figure 8A). Additionally, all treatment groups began with approximately 0.2 ng/ml of TNF- α at hour 0; control ewes maintained that level throughout hour 3. Ewes treated with LPS, TNF- α , and LPS+Dex increased in concentration at hour 0.5. However, at hour 1, ewes treated with TNF- α returned to control levels after increasing 0.11 ng/ml between hour 0 and 0.5. Ewes treated with LPS had the greatest increase, 0.67 ng/ml, between hour 0.5 and 1 of all treated groups. These same ewes maintained this level through hour 1.5 and began to gradually decrease in concentration to hour 3. Ewes treated with LPS+Dex displayed a similar pattern as the LPS treated ewes; however, the ewes treated with LPS+Dex did not reach as high of concentration of TNF- α as the LPS treated ewes. Ewes treated with LPS+Dex had a 0.23 ng/ml increase in TNF- α between hour 0.5 and 1 and maintained this level until hour 1.5 where a gradual decrease occurred through hour 3.

There was no difference in haptoglobin (Hp) concentration by treatment ($p=0.0859$), hour ($p=0.4317$), or treatment by hour ($p=0.0996$, Figure 8B). Ewes treated with LPS and LPS+Dex began with the highest Hp levels approximately $0.4 \mu\text{g/ml}$. Ewes treated with LPS showed a steady increase in Hp levels until hour 12. Ewes treated with LPS+Dex decreased to control levels at hour 3, increased at hour 6, decreased at hour 9, and increased to above hour 0 levels at hour 12. Ewes treated with $\text{TNF-}\alpha$ maintained Hp levels similar to control ewes until hour 9 when an increase of $0.17 \mu\text{g/ml}$ occurred. Control ewes maintained Hp levels less than $0.10 \mu\text{g/ml}$. However, there was a difference of hour when change of Hp from 0 to 12 hours post treatment was analyzed ($p<0.0001$) but there was no significant difference of treatment ($p=0.0219$) or treatment by hour ($p=0.0230$) from 0 to 12 hours. The change of Hp from 0 to 12 hours showed a difference between the combined groups: control and LPS + Dex versus LPS and $\text{TNF-}\alpha$ ($p=0.0069$) but no difference occurred between control and $\text{TNF-}\alpha$ versus LPS and LPS+Dex ($p=0.03175$). Similarly, the change of Hp from hour 0 did not differ between LPS and LPS+Dex treated ewes and LPS and $\text{TNF-}\alpha$ treated ewes ($p=0.2349$ and $p=0.1326$ respectively).

There was a difference in serum amyloid A (SAA) concentration by treatment ($p<0.001$), hour ($p<0.001$), and treatment by hour ($p<0.001$) (Figure 8C). Control ewes maintained levels of SAA between $2,000$ and $9,000 \mu\text{g/ml}$ until hour 12 when levels reached $18,103 \mu\text{g/ml}$ (Figure 8C). Ewes treated with LPS+Dex had the highest levels of SAA at hour 0 with a difference of $71,821 \mu\text{g/ml}$ between the control ewes. Levels of SAA decreased at hour 3 in the LPS+Dex ewes but were still higher than other treatment groups. Beginning at hour 3, SAA levels displayed a gradual increase from hour 3 until hour 12 of SAA in the LPS+Dex ewes. Ewes treated with LPS showed a steeper increase at hour 3 until hour 12 reaching levels above LPS+Dex treated ewes. Similarly, ewes treated with $\text{TNF-}\alpha$ increased in a similar pattern to LPS but reaching their peak at hour 9 and decreasing slightly by hour 12. The change of SAA from 0 to 12 hours post intoxication was different by treatment ($p<0.0001$), hour ($p<0.0001$), and treatment by hour ($p<0.0001$). In addition, there was also a difference in change of SAA from hour 0 in the combined group of control and LPS+Dex versus LPS and $\text{TNF-}\alpha$ ($p<0.0001$) and LPS versus LPS + Dex ($p=0.0007$). There was no difference among the combined group

of control and TNF- α versus LPS and LPS + Dex ($p=0.6089$) and LPS versus TNF- α ($p=0.9071$).

Reproductive system:

Pregnancy status was diagnosed by ultrasonography at 25/26 dpc and did not differ among treatment groups ($p=0.298$, Figure 10A) but the greater proportions of pregnant ewes were in control and TNF- α treated ewes, 77.7% and 80.0% respectively, than in LPS and LPS+Dex treated ewes, 44.4% and 50.0% respectively (Figure 10A). Among the treated groups, more control ewes plus ewes treated with TNF- α tended to remain pregnant than treated with LPS or LPS+Dex ($p=0.05$). No other comparisons differed significantly.

Number of lambs born was not different among treatment groups ($p=0.1923$, 10B). The control and TNF- α treated ewes had higher number of lambs born, 77.7% and 60.0% respectively, while LPS and LPS+Dex treated ewes had the lower number of lambs born, 33.3% and 30.0% respectively (Figure 10B).

At 9/10 dpc concentrations of P₄ did not differ among control, LPS, TNF- α , and LPS+Dex treated groups ($p=0.9509$), means 2.08 ng/ml, 2.01 ng/ml, 2.24 ng/ml, and 2.12 ng/ml respectively (Figure 9A). Similarly, ewes diagnosed as pregnant by ultrasonography at 25/26 dpc did not differ in P₄ concentrations by treatment groups at 9/10 dpc ($p=0.8915$), means 2.08 ng/ml, 1.95 ng/ml, 2.25 ng/ml, and 2.29 ng/ml respectively. At 25/26 dpc concentrations of P₄ tended to differ among groups ($p=0.0733$) with means of 2.98 ng/ml, 1.48 ng/ml, 3.06 ng/ml, and 2.13 ng/ml respectively. Concentrations of P₄ in ewes diagnosed as open by ultrasonography at 25/25 dpc did not differ statistically by treatment groups at d 9/10 dpc ($p=0.9941$), means 2.08 ng/ml, 2.06 ng/ml, 2.22 ng/ml, and 1.95 ng/ml respectively. Ewes diagnosed as open by ultrasonography at 25/26 dpc did not differ in P₄ concentrations by treatment groups at 25/26 dpc ($p=0.3444$), means 2.52 ng/ml, 0.763 ng/ml, 1.12 ng/ml, and 0.975 ng/ml respectively. Similarly, ewes diagnosed as pregnant by ultrasonography at 25/26 dpc did not differ in P₄ concentrations by treatment groups at 25/26 dpc ($p=0.4781$), means 3.11 ng/ml, 2.39 ng/ml, 3.54 ng/ml, and 3.28 ng/ml respectively. Concentrations of P₄ displayed a statistical trend when the control and TNF- α treated ewes were combined and

compared to LPS and LPS+Dex treated ewes ($p=0.0136$), which correlates with the high pregnancy rates within the control and TNF- α treated ewes.

The negative reciprocal of Hp and the log of TNF- α depicted a statistical trend ($p=0.09$ and $p=0.08$ respectively) when applied separately to a simple logistic regression fit of all variables to predict pregnancy. In addition, the log of TNF- α was a significant predictor ($p=0.04$) of progesterone concentration on d 25/26 when applied to a linear fit model. In a multiple regression model, all variables were combined to predict progesterone concentration on d 25/26 pc using backward elimination, the model was reduced to mucosal response ($p=0.03$) as a significant predictor and treatment ($p=0.06$) as a trend. No interaction between treatment and mucosal response was evident.

Ewes treated with LPS were analyzed separately in additional analyses. A logistic regression model predicting pregnancy in LPS and LPS+Dex and in control and TNF- α treated ewes did not yield any significant variables in combination. A multiple linear regression model followed by backward elimination predicting progesterone concentration on d 25/26 pc in LPS treated ewes yielded a significant model ($p=0.03$, $R^2=0.5$) with total white blood cells ($p=0.02$), monocyte concentration ($p=0.02$), mucosal response ($p=0.03$), and lethargy ($p=0.01$) included. The model is as follows: $[P_4 \text{ d } 25/26] = -50.91 + 2.63 (\text{Ln_WBC}) + 4.01 (\text{Ln_Mon}) - 2.39 (\text{Mucos_R}) + 4.096 (\text{Lethargy})$. Similarly, in control and TNF- α treated ewes a linear regression model predicting progesterone concentration on d 25/26 pc yielded a significant model ($p=0.04$, $R^2=0.61$) with Hp, lymphocyte, neutrophil, monocyte concentration, and mucosal response included. The model is as follows: $[P_4 \text{ d } 25/26] = 44.28 + 0.47 (-\text{Hp_rec}) - 5.10 (\text{Ln_Lymph2}) - 0.17 (\text{neutr}) - 4.74 (\text{ln_mon}) - 2.29 (\text{mucos_r})$.

Figure 3. Rectal temperatures in Dorset Texel ewes after treatment with either saline (Control n=9), LPS (n=9), TNF- α (n=10), or LPS+ Dex (n=10). Effect of treatment (0.0010), hour (<0.0001), and treatment x hour (<0.0001).

A

B

Figure 4. Contingency analyses of mucosal response (A) and lethargy (B) in Dorset Texel ewes after treatment with either saline (Control n=9), LPS (n=9), TNF- α (n=10), or LPS + Dex (n=10). A) Effect of treatment ($p < 0.0001$) B) Effect of treatment ($p < 0.0001$)

Figure 5. Total white blood cell (WBC) concentration in jugular blood in Dorset Texel ewes after treatment with either saline (Control n=9), LPS (n=9), TNF- α (n=10), or LPS + Dex (n=10). Effect of treatment ($p < 0.0001$), hour ($p < 0.0001$), treatment x hour ($p < 0.0001$). Note: 24 hour time point was included for reference only.

Figure 6. Concentrations of lymphocytes (A), monocytes (B), and neutrophils (C) in jugular blood in Dorset Texel ewes after treatment with either saline (Control $n=9$), LPS ($n=9$), $\text{TNF-}\alpha$ ($n=10$), or LPS + Dex ($n=10$). A) Effect of treatment ($p<0.0001$), hour ($p<0.0001$), treatment $\times$ hour ($p=0.0880$) B) Effect of treatment ($p=0.0402$), hour ($p<0.0001$), and treatment $\times$ hour ($p=0.0047$) C) Effect of treatment ($p=0.0103$), hour ($p<0.0001$), treatment $\times$ hour ($p=0.1775$) Note: 24 hour time point was included for reference only.

Figure 7. Percent of lymphocytes (A), monocytes (B), and neutrophils (C) in jugular blood in Dorset Texel ewes after treatment with either saline (Control n=9), LPS (n=9), TNF- α (n=10), or LPS + Dex (n=10). A) Effect of treatment ($p=0.0023$), hour ($p<0.0001$), treatment by hour ($p=0.2734$) B) Effect of treatment ($p=0.2853$), hour ($p<0.0001$), treatment x hour ($p=0.0001$) C) Effect of treatment ($p<0.0001$), hour ($p<0.0001$), treatment x time ($p=0.0002$)

Figure 8. Concentrations of TNF- α , Hp, and SAA in jugular plasma in Dorset Texel ewes after treatment with either saline (Control n=9), LPS (n=9), TNF- α (n=10), or LPS + Dex (n=10). A) Effect of treatment ($p < 0.0001$), hour (< 0.0001), and treatment x hour ($p < 0.0001$) B) Effect of treatment ($p = 0.0034$), hour ($p < 0.0001$), treatment x hour ($p < 0.0001$) C) Effect of treatment ($p < 0.0001$), hour (< 0.0001), and treatment x hour ($p < 0.0001$)

A

B

Figure 9. One way ANOVA analyses of progesterone concentrations on days 9 and 10 pc (A) and days 25 and 26 pc (B) in Dorset Texel ewes after treatment with either saline (Control n=9), LPS (n=9), TNF- α (n=10), or LPS + Dex (n=10). A) Effect of treatment ($p=0.9509$) B) Effect of treatment ($p=0.0733$)

A

B

Figure 10. Contingency analyses of pregnancy status on d 25/26 pc (A) and lambing percentage (B) in Dorset Texel ewes after treatment with either saline (Control n=9), LPS (n=9), TNF- α (n=10), or LPS + Dex (n=10). A) Effect of treatment (p=0.2986) B) Effect of treatment (p=0.1923)

Discussion

While none of the ewes treated with saline or dexamethasone and LPS showed inflammatory responses, ewes treated with TNF- α or LPS had significant increases in rectal temperature (fever), behavioral changes characteristic of a sick animal, and vaginal discharges. A respiratory and/or vaginal mucosal response was elicited in 88% of LPS and 50% of TNF- α treated ewes. Rectal temperatures increased by 0.5°C within 1 hour post treatment and continued to increase in LPS treated ewes until hour 4 reaching a temperature 1°C higher than controls, while TNF- α treated ewes returned to a temperature similar to controls until hour 9 when the temperature began to rise again. However, LPS+Dex attenuated the increase in temperature seen in LPS treated ewes with ewes treated with LPS+Dex showing an increase in temperature at hour 1 and maintaining an intermediate temperature until hour 5 when it began to decrease to baseline.

Infectious fevers are induced by inflammatory mediators like TNF- α , IL-1 β , and IL-6, which are produced by phagocytes after activation by invading pathogens and/or their products or structural components like LPS. Body core temperature is regulated in the pre-optic-anterior hypothalamic area (POA) and it is generally thought that cytokines are released into the bloodstream and transported to the POA (Turnbull and Rivier, 1995). Prostaglandin (PG) E₂ is formed within the brain and in high concentrations by macrophages in inflamed tissues in response to cytokines and acts in the thermoregulatory pathways to increase the “set-point” around which temperature is regulated. The synthesis of PGE₂ from arachidonic acid is mediated by cyclooxygenase 1 and 2 (COX-1 and COX-2) (Hopkins, 2007). The latter is predominantly expressed in cells involved in inflammatory reactions (Hopkins, 2007). Glucocorticoids have been shown to down-regulate pro-inflammatory genes such as those encoding for COX-2 (Barrios-Rodiles et al., 1999). Possibly, the LPS+Dex treated ewes displayed less fever due to down-regulating the encoding genes for COX-2 and disruption of synthesis of PGE₂.

Traditionally, and in alignment with the hypothesis of the current study, administration of GCs has been associated with suppression of the immune system, particularly in response to LPS (Coutinho and Chapman, 2011). The anti-inflammatory

role of native or synthetic GCs have been attributed largely to suppression of pro-inflammatory transcription molecules for cytokine inhibition through the GR and disrupt certain signal transduction pathways like NF- κ B and consequently TLR-4, which binds LPS. Activation of TLRs induces an intra-cellular signaling cascade that culminates in the activation of the NF- κ B family of transcription factors. In general, GCs act to suppress TLR-mediated signaling through inhibition of NF- κ B and its associated transcription factors.

Glucocorticoids also function as effectors of the HPAA limb of the stress response in which the pituitary gland releases ACTH, which leads to adrenal GC production and release. As a result, GCs inhibit functions of virtually all inflammatory cells; for example reducing circulating numbers of lymphocytes, monocytes, and neutrophils; inhibiting secretion of cytokines; and inducing lympholysis through apoptosis. Typically, the mucosal barrier effectively prevents bacteria and endotoxin from entering the body and the small number of bacteria that penetrate the mucosal barrier are phagocytized. Numerous studies reported that excessive release of inflammatory mediators can damage mucosa throughout the host. Zhang and colleagues (2008) found that dexamethasone can reduce endotoxin levels and inflammatory mediators and down-regulate expression of NF- κ B in expression of ileal mucosa thus serving as a potential model for how GCs might inhibit other types of mucosal responses. Only ewes treated with LPS exhibited lethargy, which provided evidence that dexamethasone reduced the level of endotoxin and provided anti-inflammatory response as expected. The possibility of induction of another signaling pathway besides the NF- κ B pathway was strengthened by the pattern of APPs elicited in each treatment. Despite the possibility of NF- κ B and its corresponding TLR-4 being blocked all ewes, other than controls, showed increased concentrations of Hp and SAA through h 12 indicating that a compensatory pathway was activated. Without induction of cytokines, the APR response would not have been elicited as strongly.

Not all actions of GCs are immunosuppressive. Some data indicate that GCs have pro-inflammatory actions. In certain instances, GCs enhance secretion of pro-inflammatory cytokines and result in a priming effect. Previous studies have demonstrated that rather dexamethasone display of anti- or pro- inflammatory properties

is dependent on the temporal relationship between GC treatment and immune challenge. For example, Frank and colleagues (2010) reported that if GCs are administered prior (2 and 24 hours) to a peripheral LPS challenge, pro-inflammatory mediators are up-regulated. However, within the same study, if GCs were administered 1 hour after a peripheral challenge, GCs suppressed the pro-inflammatory response to LPS. In the present study, dexamethasone was given prior to challenge as an anti-inflammatory mediator and produced high concentrations of Hp and SAA at challenge indicating that the dose had a pro-inflammatory effect and primed the immune system for the bacterial challenge. Additionally, ewes treated with LPS+Dex had the highest total white blood cell count at hour 0. The ability of GCs to suppress the pro-inflammatory response was also demonstrated within the current study. Although the ewes treated with LPS+Dex had a high level of total white blood cells, monocytes, neutrophils, Hp, and SAA prior to challenge, a decrease occurred in all of those immune components until at least hour 3. The half-life of dexamethasone is in the range of 1.8 to 3.5 h and at approximately 3 hours post-treatment, a slow increase occurred in neutrophils, monocytes, Hp, and SAA and peaks at 6 to 12 hours indicating that the dexamethasone had been depleted based on its half-life.

A number of endocrine and metabolic changes, which together are called the APR, occur in mammals at the onset of an inflammatory process. The blood proteins, APPs, are primarily synthesized by hepatocytes as part of the APR but production of SAA has been demonstrated in the gastrointestinal tract, mammary gland, kidneys, and airways (Vreugdenhil et al., 1999). These systemic responders act as part of the innate immune defense system with the goal of re-establishing homeostasis and promoting healing. Generally, major APPs are present at undetectable or very low levels in the serum of healthy animals (Cray et al., 2009), but studies in the sheep APP response have shown that there is a large individual animal variation in Hp and SAA prior to onset of clinical disease and it was suggested that this may be due to underlying subclinical conditions (Meling et al., 2012). Haptoglobin, displays a variety of important biological functions. It has a high binding capacity for hemoglobin and exerts an inhibitory effect on prostaglandin synthesis. Haptoglobin also has immunosuppressive properties: it blocks neutrophil responses to various stimuli, inhibits lectin induced lymphocyte transformation

and decreases antibody production (Oh et al., 1990). Serum amyloid A, another APP, promotes chemotaxis of monocytes and T cells. In addition, SAA has inhibitory effects and is thought to be important in down-regulation of the inflammatory process. In cattle and pigs, SAA increased after exposure to complex stressors like transportation, tie stall housing, slippery floors, and social isolation (Lomborg et al., 2008). In addition, concentrations of Hp in pigs increased after repeated bleeding protocols (Salamano et al., 2008). Previous work has indicated that Hp and SAA are non-specific in terms of inflammatory disease, injury or infection, but highly sensitive, effective markers of inflammation in ruminants (Eckersall and Bell, 2010). In cattle and sheep, elevated blood serum concentrations of Hp and SAA, as two major APPs, have been associated with several diseases. In cattle, Hp is effective in the diagnosis and prognosis of mastitis, peritonitis, and endometritis (Murata et al., 2004). Traditionally, major APPs increase 10- to 100-fold and usually increase rapidly within the first 48 h after the triggering event (Johnson et al., 1999). Dow and colleagues (2010) showed an increase in plasma SAA and Hp in ewes treated with PG-PS. Both SAA and Hp concentrations were observed to be significantly different among treated and controls for this experiment. Interestingly, ewes treated with LPS and LPS+Dex started with the highest concentrations of Hp and SAA at hour 0. The LPS+Dex treated ewes could have been experiencing pro-inflammatory effects and the APR had been initiated. The LPS treated ewes could have had underlying subclinical infections that increased the Hp levels or the stress of handling those animals in the days prior to challenge and the morning of challenge could have also initiated an APR prior to the challenge. The LPS treated ewes continued to increase in Hp levels until 12 hours while the LPS+Dex ewes decreased in Hp levels at hours 3 and 9 and increased at hours 6 and 12 post treatment. The LPS+Dex ewes at hour 3 could have been experiencing the anti-inflammatory effects of dexamethasone as it blocked the NF- κ B pathway in certain immune cells and therefore decreased cytokine production which would have decreased synthesis of APPs from the hepatocytes in the liver. The increase in Hp between hours 3 and 6 in the LPS+Dex treated ewes could have been the effects of dexamethasone decreasing until finally between hour 9 and 12 dexamethasone had been depleted and Hp levels increased to above initial levels. The control ewes all maintained Hp levels below 0.2 μ g/ml throughout the 12 hours indicating that those ewes may have

been healthy and had very low levels in their serum as shown by Cray and colleagues (2009). In addition, TNF- α treated ewes also maintained low levels of Hp until hour 9 similar and had increased concentrations at hour 12 either due to the stress of handling the animals or the initiation of the APR within the first 48 hours after the triggering event as shown by Johnson and colleagues (1999). Although the LPS+Dex treated ewes had the highest SAA concentration at hour 0, SAA decreased at hour 3, possibly due to the anti-inflammatory effects of dexamethasone, but then re-bounded at hour 6 and increased until hour 12. Ewes treated with LPS and TNF- α didn't begin to increase until hour 3 but reached levels higher than the LPS+Dex treated ewes by hour 9 and 12. In conclusion, treatments of LPS, TNF- α , and LPS+Dex elicited an APR. Glucocorticoids are known to mediate upregulatory effects on the APR. Experiments that used the application of exogenous dexamethasone increased APP transcription, IL-6 receptor, and hepatocyte proliferation (Yeager et al., 2004) thus providing an explanation for why ewes treated with LPS+Dex had increased levels of Hp and SAA at hour 0.

White blood cells (WBC) are a heterogenous group of nucleated cells that can be found in circulation for at least a period of their life and they play an important role in phagocytosis and immunity and therefore in defense against infection. Except for the LPS+Dex treated ewes, total white blood cell counts were low at hour 0. It is likely that the injection of dexamethasone 12 hours prior to challenge induced leukocytosis as shown by Anderson and colleagues (1999) in cattle. Lymphocytes are immune cells fundamental in cellular and humoral immunity and comprise 70-80% of WBC in blood of sheep. Lymphocytes belong to the B (bone marrow) or T (thymus) systems; both cell types are morphologically indistinguishable. The B lymphocytes are responsible for synthesis of antibodies while the T lymphocytes differentiate into a type of effector T lymphocyte: cytotoxic, helper, or regulatory. All groups at challenge exhibited a high percentage of lymphocytes. However, their percentage gradually decreased through hour 12; while the percent of monocytes and neutrophils increased with neutrophils being the highest percent by hour 12. The change that occurred from mostly lymphocytes to monocytes and neutrophils is in accordance to the initiation of the innate immune system upon encounter of the bacterial challenge. The innate immune system response is mediated primarily through phagocytic cells and antigen-presenting cells, such as

granulocytes like neutrophils, macrophages, and dendritic cells (Iwasaki and Medzhitov, 2004). Therefore, the change in percentage of the total white blood cells from lymphocytes to phagocytic cells like neutrophils is indication that an innate immune response was initiated by the injection of LPS and TNF- α . Interestingly, ewes treated with LPS+Dex had the highest concentration of monocytes and neutrophils at hour 0 possibly due to the priming effect of dexamethasone resulting in leukocytosis and an increased neutrophil: lymphocyte ratio (Anderson et al., 1999). However, after the challenge and second dose of dexamethasone at the time of challenge, appeared to have an anti-inflammatory effect until approximately hour 4, which coincides with the half-life of dex and the rise in the monocytes and neutrophils could be an indication that dexamethasone was depleted. Neutrophils were the highest percentage of cells in all groups by hour 12. These cells are the most abundant cellular component of the host immune system and primary mediators of the innate immune response. Stress of handling the animals could have caused the production of WBCs in controls. Stress has been shown to influence the immune responses of cattle. For example, total white blood cell counts and differentials were altered following handling and transportation of cattle, specifically neutrophils and monocytes increased, while lymphocytes decreased (Murata et al., 1987).

Lymphocyte concentrations consistently decreased throughout the experiment and did not increase until 24 hours post-challenge. The decrease could have been attributed to the induction of a strong innate immune response or perhaps handling stress has a greater effect on the lymphocyte population than on monocytes or neutrophils. Hopster and colleagues (1998) found that a rise in plasma cortisol concentration was associated with a decrease in number of circulating lymphocytes between 0 and 8 h post injection of endotoxin and did not increase until 21 h later. In addition, the rate of lymphocytes disappearing from the circulation is in accordance with what has been found in rats that were restrained for 2 h (Dhabhar et al., 1995). This could imply that environmental stress may have contributed to a decrease in lymphocyte numbers. Stress caused by handling can affect the numbers of peripheral leukocytes, but leukocyte redistribution can rapidly reverse after cessation of stress (Dhabhar et al., 1995).

Ewes treated with LPS, TNF- α , or LPS+Dex showed increases in TNF- α concentrations when compared to the controls. This is in accordance with the initiation of an early innate immune response induced by LPS. Ewes treated with LPS showed the greatest concentration of TNF- α produced by immune cells as they were activated through the receptor-ligand interaction of PAMPs and PRRs, specifically TLR-4 to produce TNF- α (Ohashi et al., 2008). Also in accordance with the originally stated hypothesis, ewes treated with LPS+Dex showed approximately half the concentration of TNF- α as the ewes treated with LPS. This could be attributed to the ability of dexamethasone to reduce activation of the NF- κ B signaling pathway that is required for synthesis of pro-inflammatory cytokines like TNF- α (Chinenov and Rogatsky, 2007). However, the dosage used for dexamethasone was not sufficient to completely reduce the synthesis and secretion of TNF- α or perhaps TNF- α is being produced via an alternative pathway. Ewes treated with TNF- α did have detectable systemic concentrations of TNF- α . However, concentrations were not much higher than control ewes suggesting that the dosage used was not concentrated enough or the TNF- α injections should have been sustained over time to more accurately mimic the production of TNF- α in LPS treated ewes. Oliver and colleagues (1993) reported that the half-life of free plasma TNF- α is 18.2 minutes. Ewes treated with TNF- α reflected the previously reported half-life. The ewes treated with TNF- α had the highest concentration of TNF- α at hour 0.5; however, TNF- α was given in two bolus injections thirty minutes apart so TNF- α concentration was sustained until hour 1.5 when a decrease occurred as TNF- α should have been metabolized based on the half-life. Interestingly, LPS elicited greater TNF- α concentrations in the jugular vein than in ewes stimulated with PG-PS (Dow et al., 2010). Although a different TLR, TLR 2, plays a role in PG-PS binding, the same NF- κ B cytokine signaling pathway is activated following binding suggesting that a similar response should be elicited between both PG-PS and LPS. Ewes treated with LPS+Dex had the second highest TNF- α concentration at hour 0 of the treatment groups. In mice, enhanced TNF- α responses to LPS have been observed when animals were exposed to dexamethasone 24-48 h before LPS (Fantuzzi et al., 1994).

Interestingly but not in accordance to the stated hypothesis, ewes treated with TNF- α had as high a pregnancy rate as control ewes, 80% and 77% respectively. The

inflammatory cytokine, TNF- α , has been shown to have negative effects on the host as well as the developing embryo (Gendron and colleagues, 1990). They reported that increased production of TNF- α , induced by injection of LPS, was associated with failure of mice to establish pregnancy. Although PG-PS binds to different TLRs on macrophages than LPS, both the PG-PS and LPS binding lead to activation of NF- κ B. Previous studies used mated ewes inoculated with either PG-PS, a gram-positive bacterial cell wall component, or killed *Streptococcus pyogenes* to mimic the effects of mastitis to decrease early embryonic survival (Stewart et al., 2003; Holásková et al., 2004). In addition, Dow and colleagues (2010) showed that ewes treated with PG-PS experienced 100% embryonic loss when compared to controls and had an increase in concentration of TNF- α in both the caudal vena cava and jugular veins. Alternatively, TNF- α has normal physiological functions as well. For example, Terranova and colleagues (1995) identified TNF- α binding sites on the ovaries of several mammalian species. In addition, the localized release of TNF- α in ovine follicles is a prelude to programmed ovarian cell death and follicular rupture (Murdoch et al., 1997). Possibly, the increased concentration of TNF- α may not have been toxic to the embryo or the dosage was not sufficient enough to cause damage to the developing embryo.

Ewes treated with LPS or LPS+Dex had the lowest pregnancy rate, 44% and 50% respectively, with losses occurring between 5 to 6 and 25 to 26 dpc when transrectal ultrasound was performed. Blastocyst formation is initiated shortly after entry of the embryo into the uterus on days 2 to 4. The blastocyst does not hatch from the zona pellucida until days 8 to 9. In 2007, Loureiro and colleagues observed that during times of heat stress or innate immune challenges resulting in the secretion of pro-inflammatory cytokine TNF- α , the zona pellucida alone was unable to protect the embryo from apoptosis. Specifically, based on results using heat shock, capacity for induced apoptosis becomes acquired between the 8- and 16- cell stages (Paula-Lopes and Hansen, 2002). The 8- cell stage occurs approximately 2.5 days after fertilization in sheep. The signal for maternal recognition of pregnancy has not yet occurred and will not until days 12-13 in sheep. Accordingly, Hansen (2007) found that there is a decrease in vulnerability to environmental stresses for the embryo as it develops. The exact mechanism for the decreased susceptibility of the embryo is not fully understood, but it could be that as the

embryonic genome is turned on, the embryo gains the protection of a very early innate immune system.

Cullor (1993) indicated that LPS induced luteolysis and reduced conception rate and early embryonic survival through the release of inflammatory mediators. Dexamethasone should have negated the effects of LPS and its subsequent release of TNF- α that may cause induced embryonic loss by inhibiting NF- κ B signal transduction within TNF- α synthesizing immune cells. However, ewes treated with LPS+Dex had low pregnancy rates and ewes treated with TNF- α had high pregnancy rates, which suggests that early embryonic loss may not be occurring through the NF- κ B pathway. Although all TLRs signal through the conserved signaling cascade of NF- κ B and its associated molecules, the complexity of the TLR- induced cellular responses indicates that there must be additional regulatory mechanisms and signaling pathways downstream of TLRs, which may link TLRs to other signaling pathways. Kawai and colleagues (1999) used MyD88 deficient mice to illustrate the complexity among TLRs. MyD88 deficient mice were unable to activate NF- κ B in response to IL-1 and many TLR ligands, including PG-PS (Kawai et al., 1999). Surprisingly, the TLR-4 ligand LPS could still activate NF- κ B in the absence of MyD88 (Kawai et al., 1999). Therefore, MyD88 is required for all signaling events downstream of some TLRs, but MyD88 is dispensable for some TLR-4 induced signals.

Another pathway through which pregnancy loss might have occurred is a second PRR found on macrophages, neutrophils, dendritic cells, and epithelial cells known as the NOD and their corresponding NLRs. The NLRs are intracellular sensors for microbial products and some sub-families activate the NF- κ B pathway to initiate the same inflammatory responses as TLRs. Two members of the NLR family, NOD-1 and NOD-2, recognize breakdown products of bacterial cell walls that ultimately culminated in activation of the NF- κ B transcription factor. The use of dexamethasone should have interrupted signaling through this type of NLR family and reduced synthesis and secretion of TNF- α . However, another member of the remaining NLR family members could be implicated in early embryonic loss. The NLRP3 is activated upon exposure to whole pathogens, as well as PAMPs, DAMPs, and environmental irritants. Upon activation, the NLRP3 forms a structure known as an inflammasome which mediates

caspase-1 dependent processing of cytokines such as IL-1 β (Martinon et al., 2002). The exact signaling mechanism for the NLRP3 inflammasome is not understood. One mechanism involves NLRP3 oligomerization that leads to PYD domain clustering for interaction with the PYD- and CARD- containing adaptor ASC, which recruits procaspase-1. Procaspase-1 allows formation of the active caspase-1 which then processes cytokine preforms such as IL-1 β to generate active molecules. A conserved mechanism of activation is yet to be formalized; however, both inflammasome activity and pro-IL-1 β availability are influenced by integration with other pro-inflammatory signaling pathways such as those triggered by TLR ligation. In relation to pregnancy, it is becoming increasingly apparent that in addition to adequate hormonal priming, successful embryonic implantation relies on an appropriate interaction between autocrine-paracrine cytokines produced and received by the blastocyst and endometrium. Among these cytokines, the IL-1 system seem to be relevant to the implantation process. Perhaps synergistic release of IL-1 β with TNF- α could be implicated in the early embryonic loss caused by the initiation of the innate immune response during a bacterial challenge. Deb and colleagues (2005) stated that increased expression of IL-1 receptor in the uterus of LPS treated pregnant animals during early stages of development could initiate a cascade of additional pro-inflammatory substances like TNF- α , which may contribute to pregnancy loss.

Ewes that had low to medium levels of plasma TNF- α and Hp were more likely to be pregnant than those that had high levels of TNF- α and Hp. This is different from previous work done by Akers (2014) who found that high producing dairy cows with high plasma Hp levels were more likely to be pregnant than low producing cows with high Hp or cows with low Hp. In addition, since pregnancy and progesterone are highly correlated, ewes that had higher levels of TNF- α and exhibited a mucosal response had lower levels of progesterone by days 25 or 26 post coitus strengthening the possibility that high levels of TNF- α may decrease chances of becoming pregnant. Since a mucosal response can be indicative of activation of the host immune response, including production and release of various pro-inflammatory cytokines other than TNF- α , those ewes that produced a mucosal response had lower progesterone levels by day 25 and 26

indicating that collectively the host immune response may have been detrimental to the developing embryo which may have resulted in a loss of pregnancy.

Ewes treated with LPS showed that progesterone concentration on days 25 or 26 pc can be predicted by the total white blood cell count, monocyte concentration, presence of mucosal response and lethargy. Interestingly, ewes that were not treated with LPS showed that Hp, lymphocyte and neutrophil concentration, and presence of mucosal response were indicative of progesterone levels on day 25 and 26 pc. Microbial pathogens consist of multiple PAMPs, which activate not only TLRs but other PRRs, and possible crosstalk between them might be required for induction of effective innate immune response. As a consequence, TNF- α alone would not be solely responsible for the detrimental effects that occur to the developing embryo during an innate immune response. Therefore, dexamethasone, may have blocked the NF- κ B pathway enough to decrease some aspects of the innate immune response, but it is highly likely that the immune system was still able to compensate for the interruption of one pathway with several more different pathways. Additionally, dexamethasone could have sufficiently blocked the NF- κ B pathway early in the innate immune response to prevent detrimental effects to the embryo; however, the innate immune response lasts hours to days and it is possible that after dexamethasone has been depleted, the treatment was not sustained long enough to maintain the protective, anti-inflammatory effects that were experienced in the first few hours of the challenge.

Therefore, injection of TNF- α alone might not have been enough stimulus to damage the developing embryo. Instead, for maximal detrimental effects, several other pro-inflammatory cytokines along with TNF- α might be required. Regardless of indirect or direct actions, bacterial infections, such as mastitis cost the dairy industry approximately \$2 billion each year (Pyorala, 2002). The mechanism behind early embryonic loss should be elucidated so that improved management and treatment techniques can be utilized to prevent unnecessary bacterial related economic losses in the animal agriculture industry.

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