INTRODUCTION

Infections of the mammary gland (mastitis) or uterus (metritis) are common sources of inflammation in lactating dairy cows, particularly during the periparturient period. Other health disorders common during this period (e.g., milk fever and ketosis) do not arise from infectious organisms, but instead have metabolic origins. Although the etiologies of infectious and metabolic disorders differ, epidemiologists report a significant association between their occurrences. For example, Curtis et al. (1985) reported that cows with milk fever were more than 5 times as likely to contract clinical mastitis as animals without milk fever. These results do not imply cause and effect; however, they suggest an association between the occurrences of one disease with that of a second disorder. Potential causal relationships between periparturient metabolism and immune function have been investigated for about the last 20 years, but this research has intensified recently. More research needs to be performed, but at this time elevated levels of ketones have been the most consistent metabolic variable to negatively impact immunity. Our group is investigating metabolic regulators that may also be important in the regulation of immune function of the transition cow.

ASPECTS OF PERIPARTURIENT METABOLISM

The periparturient dairy cow experiences a dramatic increase in nutrient requirements that cannot be met by feed intake alone as she transitions from pregnancy into lactation (Bell, 1995). Thus, the animal experiences a period of negative energy and nutrient balance requiring the mobilization of body tissue lipid, protein, and calcium in order to sustain productive function (Goff and Horst, 1997). Feeding strategies to optimize these metabolic adaptations have received significant research attention (Overton and Waldron, 2004), but the incidence of periparturient metabolic disorders and infectious diseases in the dairy industry persists (USDA, 2008).

IMMUNOSUPPRESSION: AN INTERACTION BETWEEN METABOLISM AND IMMUNOPHYSIOLOGY?

In addition to the potential metabolic disorders associated with negative energy and calcium balance, periparturient dairy cows also undergo a period of reduced immunological capacity during the weeks around calving. This immune dysfunction is not limited to isolated immune variables; rather it is broad in scope and affects multiple functions of various immune cell types (Sordillo and Streicher, 2002). The combined results of these dysfunctions are that dairy cows may be hyposensitive and hyporesponsive to antigens, and therefore more susceptible to infectious disease such as mastitis during the periparturient period (Mallard et al., 1998). Grommers et al.
(1989) reported that fewer mammary quarters responded to low-dose *E. coli* endotoxin, and maximum somatic cell count also was somewhat later and less pronounced during early lactation than during mid-lactation. Furthermore, when live *E. coli* were administered into the mammary gland, periparturient cows experienced more rapid bacterial growth, higher peak bacterial concentration, higher fever, and equal or greater proinflammatory cytokine concentrations in foremilk than did midlactation cows (Shuster et al., 1996).

Research results from our laboratory are in agreement with this decreased immune function around the time of calving and perhaps give some insights into which mechanisms may be impaired. Neutrophils (PMN) are recognized as being one of the most important cell types in protecting of the mammary gland and uterus from infection (Paape et al., 2002). We isolated PMN from midlactation (220-350 DIM and 100-200 d of gestation, \( n = 9 \)), prepartum (12 d prior to calving, \( n = 8 \)), and postpartum (7 DIM, \( n = 8 \)) cows and studied various functional activities of these cells. The PMN from postpartum cows produced fewer intracellular (data not shown), extracellular (data not shown), and total (Figure 1) reactive oxygen species (ROS). This postpartum decrease in ROS expression is in agreement with other reports (Mehrzad et al., 2001) and could contribute to the attenuated pathogen killing capacity that has been reported after calving (Dosogne et al., 2001). A novel finding from our lab relates to the ability of PMN to produce neutrophil extracellular traps (NETs). These bacteriocidal structures were first reported by Brinkmann et al. (2004) and were subsequently reported to be expressed at similar levels in milk and blood (Lippolis et al., 2006), contrary to other antimicrobial mechanisms. Using the same experimental design as above for the ROS production, we report that PMN NETs expression is increased in PMN incubations isolated from cows 12 d prepartum, compared to PMN from postpartum or midlactation cows (Figure 2). This finding, along with the expression of NETs in milk (Lippolis et al., 2006), suggests that NET expression by PMN is an important protective mechanism for the mammary gland of transition cows.

The cause of periparturient immunosuppression is not known, but is the subject of much research. Research to date suggests that this immune dysfunction appears to be due to a combination of endocrine and metabolic factors. Glucocorticoids (e.g. cortisol), known immunosuppressants, are elevated around the time of calving, and have been postulated to be at least partly responsible for periparturient immunosuppression (Burton et al., 1995). Furthermore, changes in estradiol and progesterone just prior to calving may directly or indirectly affect immunocompetence (Weber et al., 2001). However, changes in any of these steroid hormones do not overlap with the entire period of immunosuppression, suggesting that other causes are at least partially responsible for immune dysfunction.
Figure 1. Effect of stage of lactation on bovine neutrophil total reactive oxygen species production measured by luminol-dependant chemiluminescence. Neutrophils were collected from midlactation (100-200 days pregnant; n = 9), pre-partum (-12 d; n = 8) and post-partum (7 DIM; n = 8) cows. *Day of lactation effect, P < 0.01. a,b Bars with different letters differ (P < 0.01).

Figure 2. Effect of stage of lactation on bovine neutrophil extracellular trap formation. Neutrophils were collected from midlactation (100-200 days pregnant; n = 9), pre-partum (-12 d; n = 8) and post-partum (7 DIM; n = 8) cows. *Day of lactation effect, P < 0.01. a,b Bars with different letters differ (P < 0.01).
Periparturient negative energy balance has been implicated in contributing to immunosuppression. However, negative energy balance alone had little effect on the expression of adhesion molecules on the surface of bovine leukocytes (Perkins et al., 2001). Furthermore, experimental negative energy balance in midlactation cows did not affect the clinical symptoms associated with an intramammary endotoxin infusion (Perkins et al., 2002). These results are contrary to work in periparturient cows where the presence of a mammary gland (vs. mastectomized cows) and its attendant metabolic demands slowed recovery of neutrophil function, suggesting that the metabolic stress of lactation exacerbated periparturient immunosuppression (Kimura et al., 1999). Other work has investigated individual metabolic components associated with negative energy balance, and has concluded that although hypoglycemia alone is not likely to exacerbate periparturient immunosuppression (Nonnecke et al., 1992), hyperketonemia appears to have multiple negative effects on aspects of immune function (Suriyasathaporn et al., 2000). Ketosis may increase the risk of mastitis in periparturient immunosuppressed cattle because many immune cell types are negatively affected by metabolite levels typical of a ketotic environment (i.e., low concentrations of glucose and high concentrations of ketone bodies and NEFA). A ketotic environment suppressed bovine lymphocyte blastogenesis (Sato et al., 1995), decreased the respiratory burst activity of PMN (Hoeben et al., 1997), lowered the chemotactic capacity of leukocytes (Suriyasathaporn et al., 1999), decreased interferon-γ and tumor necrosis factor-α titers from bovine aorta endothelial cells (Zdzisinska et al., 2000), decreased the bactericidal activity of ovine neutrophils (Sartorelli et al., 2000), inhibited human T-cell proliferation in vitro (Gregory et al., 1993), decreased both macrophage and neutrophil functional capacity (Cerone et al., 2007), and decreased NET formation and bacteriocidal activity (Grinberg et al., 2008). Furthermore, experimental mastitis in ketonemic cows was more severe than mastitis in non-ketonemic cows regardless of preinfection chemotactic response (Kremer et al., 1993). As reviewed by Suriyasathaporn et al. (2000), impairment of the udder defense mechanism in cows experiencing negative energy balance seems to be related to hyperketonemia.

NEW THOUGHTS – ROLE OF METABOLIC REGULATORS ON IMMUNITY

The role of insulin in regulating glucose homeostasis and adipose tissue metabolism has received much attention relative to periparturient metabolism. In this role, insulin actions result in decreased lipolysis and therefore, fewer NEFA released in to the blood. Fewer NEFA in circulation results in less NEFA uptake by the liver with subsequent less hepatic fat accumulation and ketone body release. Insulin is involved in the etiology of energy-related metabolic disorders, and increased insulin action could decrease the incidence and severity of the fatty liver and ketosis complex (Bobek et al., 2004). However, insulin also promotes the uptake of glucose in to non-mammary tissues. Thus, although insulin may attenuate plasma NEFA, it would also result in partitioning of glucose away from the mammary gland and potentially lower plasma glucose concentrations via increased glucose utilization in non-mammary tissues.
Several strategies have been investigated in an attempt to manipulate this “energetic axis” of insulin-regulated fat and carbohydrate metabolism. Slow release insulin administration to dairy cows did result in lower plasma NEFA and hepatic fat accumulation, but also resulted in unacceptable concentrations of circulating glucose except at the lowest levels of insulin release tested (Hayirli et al., 2002). Various strategies to manipulate non-structural carbohydrate and dietary energy intake have also sought to enhance metabolic health either via the modulation of insulin concentrations or via affecting insulin sensitivity or responsiveness (Overton and Waldron, 2004). Finally, nutrients such as chromium (Smith et al., 2008) and several therapeutics have also been studied for their potential effects on insulin action. Among the promising therapeutics, Smith et al. (2007) have reported that the PPAR-γ ligand 2,4-thiazolidinedione (TZD) decreased plasma NEFA and BHBA concentrations without affecting glucose concentrations in periparturient cows.

Although regulators of the energetic axis have been studied for their effects on metabolism, the effects of these strategies on the immune function of transition cows has received relatively little attention. One can easily appreciate how manipulation of the energetic axis could indirectly influence immunity. Given the potential negative effects of NEFA and subsequent high ketone body concentrations on leukocytes, a treatment or dietary manipulation that would lower these compounds in blood would likely be beneficial. However, our laboratory is not only interested in these indirect metabolic effects of insulin or its effectiveness, we want to understand how insulin itself (or compounds that result in insulin-like effects) influence leukocyte function and immunity. Insulin concentrations decrease in the weeks around calving and it is generally accepted that insulin resistance also occurs during this period. Is it a coincidence that immune function is decreased during this same timeframe?

Insulin has been shown to be immune supportive in humans and other species. For example, a short term hyperinsulinemic euglycemic clamp resulted in increased concentrations of PMN in blood and increased phagocytic and chemotactic activity of these cells (Walrand et al., 2004; Walrand et al., 2006). Nielsen et al. (2003) reported that bovine PMN possess insulin receptors and preliminary results from our laboratory have demonstrated that PMN from lactating cows not only possess insulin receptors, but that these receptors are active because downstream signaling proteins become phosphorylated within 30 minutes after in vitro incubation of PMN with insulin. Given these findings, we hypothesized that in vitro PMN incubation with insulin would result in increased functional activity of these leukocytes. Furthermore, we hypothesized that insulin might have differential effects on PMN harvested from cows in different physiological states of pregnancy and lactation due to differences in circulating insulin concentrations and insulin resistance in these different classes of animals.

In addition to the effects of insulin itself, we also investigated the potential role for the PPAR-γ ligand TZD to alter immune function. As discussed previously, TZD has been studied for its some of its insulin-like actions influencing the energetic-axis in transition cows. However, in addition to the metabolic effects of TZD which might also
have indirect effects on immunity, TZD has been shown to directly affect PMN function.

Indeed, PMN are PPAR-γ responsive cells, and in addition to the effects of TZD on insulin action and lipid metabolism, binding of TZD to the transcription factor PPAR-γ results in direct effects on inflammatory and immune-function genes (Houseknecht et al., 2002). Therefore, we hypothesized that in vitro PMN incubation with TZD would result in increased functional activity of these leukocytes. Furthermore, we hypothesized that TZD might have differential effects on PMN harvested from cows in different physiological states of pregnancy and lactation due to differences in circulating insulin concentrations and insulin resistance in these different classes of animals.

To test these hypotheses, PMN harvested from prepartum (12 d before calving, n=8), postpartum (7 d after calving, n = 8), and midlactation (100-200 d of gestation, 220-350 DIM, n = 9) multiparous dairy cows were incubated for 120 min in vitro with either insulin, TZD, or a combination of the two compounds. The in vitro doses of insulin and TZD used were 0, 1.5, and 15 ng/ml and 0 or 300 μg/ml for insulin and TZD, respectively. Insulin had no effect on PMN functional capacity overall, nor were there any insulin by stage of lactation effects on PMN function. However, TZD decreased PMN total ROS production (Figure 3) without affecting intracellular ROS production (data not shown), suggesting that extracellular ROS production might be decreased by TZD. Such a scenario might allow for the maintenance of PMN intracellular bacteriocidal activity concomitant with decreased tissue damage from extracellular ROS release. We are currently investigating this possibility. No TZD by stage of lactation effects were evident and incubation of PMN with TZD did not affect NETs formation, phagocytosis, or bacteriocidal activity of these cells (data not shown).

![Area under the curve](image)

**Figure 3.** Effect of insulin and 2,4-thiazolinedione (TZD) on bovine neutrophil total reactive oxygen species production measured by luminol-depandant chemiluminescence.**1,**

1 Neutrophils were collected from cows (n = 25) and incubated for 120 minutes with 0, 1.5 or 15 ng/mL of insulin either alone or in combination with 300 μg/mL of TZD.

*TZD treatment effect, P < 0.01. **a,**b Bars with different letters differ (P < 0.01)
CONCLUSIONS

Research to develop strategies to maximize the metabolic health of transition cows has been emphasized for much of the past 15 years. This line of research has now been extended to determine the role of metabolites, nutrients, and metabolic regulators in supporting immune function. To date, aspects of energy metabolism - especially ketones, have been reported to negatively impact immune function. Although not as well understood, high-levels of circulating NEFA, and calcium metabolism may also contribute to periparturient immunosuppression. Our most recent research results suggest that insulin does not have a role in PMN function; however, more research is warranted to ensure that these early results are not artifacts of experimental design or laboratory methods. The PPAR-γ ligand TZD did result in significant changes in PMN function that may result in minimized inflammatory damage and improved health of animals during an infectious insult. The potential beneficial direct effects of TZD on immune function are particularly exciting when coupled with the potential for indirect metabolic effects of this compound on immunity.

REFERENCES


