University of Veterinary Medicine Hannover
Department of Physiology

Effects of the peripartal energy balance of dairy cows on the functional capacity of monocytes and their differentiation to macrophages

THESIS
Submitted in partial fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY
(PhD)
awarded by the University of Veterinary Medicine Hannover

by
Melanie Eger
Kronach
Hannover, Germany 2016
Supervisor: Prof. Dr. Gerhard Breves

Supervision Group: Prof. Dr. Gerhard Breves
Prof. Dr. Hans-Joachim Schuberth
Prof. Dr. Dr. Sven Dänicke

1st Evaluation: Prof. Dr. Gerhard Breves
University of Veterinary Medicine Hannover
Department of Physiology

Prof. Dr. Hans-Joachim Schuberth
University of Veterinary Medicine Hannover
Immunology Unit

Prof. Dr. Dr. Sven Dänicke
Friedrich-Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig
Institute of Animal Nutrition

2nd Evaluation: Prof. Dr. Bernd Kaspers
Ludwig-Maximilians-Universität München
Institute for Animal Physiology

Date of final exam: 04.04.2016

Sponsorship: This PhD project was supported by the H. Wilhelm Schaumann Foundation
Parts of the thesis have been published previously in:

**Publications:**


**Presentations on Conferences:**


Table of Contents

1 Introduction

1.1 Relevance of infectious diseases in dairy farming

1.2 The metabolic challenges of the peripartal period

1.3 Peripartal alterations of the immune system

1.4 Monocytes and macrophages in cattle

1.5 Immune cell energy metabolism

1.6 Glucose transporters on monocytes and macrophages

1.7 Hypothesis and aim of the PhD project

2 Background information on investigations in peripartal dairy cows

2.1 Experimental setup: The feeding model

2.2 Incidence of clinical mastitis and metritis

2.3 Blood insulin concentrations

3 Manuscript 1

4 Manuscript 2

5 Continuative Investigations

5.1 Adhesion molecule expression in peripartal monocytes

5.1.1 Introduction

5.1.2 Material and Methods

5.1.3 Results and Discussion

5.2 Impact of glucose availability on monocyte polarization and cytokine production

5.2.1 Introduction

5.2.2 Material and Methods

5.2.3 Results

5.2.4 Summary and Discussion
6 Discussion ........................................................................................................................................41

6.1 Effects of energy balance on the number of classical, intermediate and nonclassical monocytes in peripartal dairy cattle .................................................................41

6.2 Glucose uptake and glucose transporter expression in bovine monocyte subsets, subset-derived macrophages and polarized macrophages ..................43

6.3 Effects of the peripartal energy balance on glucose uptake and GLUT transporter expression in bovine monocytes ..................................................................................46

6.4 Outlook: Regulation of the metabolic switch in immune cells .............................................47

6.5 Summary and closing remarks ..................................................................................................48

7 References .......................................................................................................................................49
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCS</td>
<td>Body condition score</td>
</tr>
<tr>
<td>BHB</td>
<td>β-hydroxybutyrate</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine (C-C motif) receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cM</td>
<td>Classical monocytes</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMI</td>
<td>Dry matter intake</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GLUT</td>
<td>Sodium-independent glucose transporter</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>intM</td>
<td>Intermediate monocytes</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Mac-1</td>
<td>Macrophage-1 antigen</td>
</tr>
<tr>
<td>MIF</td>
<td>Membrane immunofluorescence</td>
</tr>
<tr>
<td>M0</td>
<td>Unpolarized macrophages</td>
</tr>
<tr>
<td>M1</td>
<td>Classically activated macrophages</td>
</tr>
<tr>
<td>M2</td>
<td>Alternatively activated macrophages</td>
</tr>
<tr>
<td>ncM</td>
<td>Nonclassical monocytes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NEB</td>
<td>Negative energy balance</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>R-Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SGLT</td>
<td>Sodium-dependent glucose transporter</td>
</tr>
<tr>
<td>SLC2</td>
<td>Solute carrier family 2</td>
</tr>
<tr>
<td>STAT6</td>
<td>Signal transducer and activator of transcription 6</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion protein-1</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
</tbody>
</table>
Figures

Figure 1: Interrelationships between nutrition and disease in the periparturient dairy cow ................................................................. 10
Figure 2: The murine mononuclear phagocyte system ........................................ 11
Figure 3: Schematic structure of GLUT proteins ............................................... 14
Figure 4: Occurrence of postpartal mastitis and metritis in both BCS groups ........ 18
Figure 5: Blood insulin concentrations ............................................................ 19
Figure 6: Adhesion molecule expression on peripartal monocytes .................... 28
Figure 7: Adhesion molecule expression differs between healthy and diseased cows ............................................................................. 29
Figure 8: Effects of glucose availability on monocyte to macrophage differentiation 35
Figure 9: Impact of glucose availability on the phenotype of monocyte-derived macrophages ...................................................................... 36
Figure 10: Impact of glucose availability on the expression density of CD11b and CD163 on monocyte-derived macrophages ........................................... 37
Figure 11: Cytokine production of polarized macrophages generated under different media glucose concentrations ...................................................... 38
Figure 12: Signaling pathways involved in the metabolic switch from OXPHOS to glycolysis in monocytes and macrophages ................................. 48

Tables

Table 1: Macrophage samples for generation of cell culture supernatants ............ 33
Summary

Melanie Eger

Effects of the peripartal energy balance of dairy cows on the functional capacity of monocytes and their differentiation to macrophages

Peripartal mastitis and metritis are common diseases in dairy cattle and impair profitability by reducing milk yield, fertility and lifespan. With the onset of lactation the increase in energy requirements of dairy cows induces a negative energy balance and lipolysis and enhances gluconeogenesis. Moreover, glucose is redistributed towards the mammary gland for the synthesis of lactose. Negative energy balance is often associated with peripartal alterations in the immune system and the increased susceptibility for infectious diseases in early lactation. Glucose deprivation might impair energy supply of monocytes, key innate immune cells which regulate the immune response and link innate and adaptive immunity after their differentiation to macrophages. Glucose is the main energy source of monocytes and is provided by facilitative diffusion via sodium-independent glucose transporters (GLUT). In cattle, three monocyte subsets have been identified which differ in functional properties: classical, intermediate and nonclassical monocytes. This PhD thesis evaluates whether peripartal negative energy balance and the low postpartal glucose availability may alter the immune response of monocytes by studying monocyte subset numbers, glucose uptake capacities and glucose transporter expression in peripartal monocytes, monocyte subsets, subset-derived macrophages and functionally differing macrophage phenotypes.

To investigate the effects of the peripartal energy balance on monocyte numbers and monocyte glucose uptake 27 dairy cows were allocated to two dietary groups according to their body condition score. From day 42 prior to parturition until day 56 of lactation a feeding regime was applied, in which the group with higher BCS received higher amounts of concentrate before parturition and concentrate feeding was more restricted in this group to achieve a more negative energy balance and to enhance lipolysis in the high condition cows after parturition. Monocyte samples were obtained at days -42, -14, +7, +21 and +56 relative to parturition.
Monocyte numbers of all three subsets peaked in both BCS groups at day 7 after parturition. Noticeably, cows suffering from postpartal mastitis or metritis displayed significantly higher monocyte numbers of all three subsets compared to healthy cows at day +7 only in the group with lower BCS and less negative energy balance. The elevation in monocyte numbers was associated with an increase in the expression of the adhesion molecules CD11a, CD49d and CD62L.

To evaluate glucose uptake capacities and glucose transporter expression of the three monocytes subsets and classically and alternatively activated macrophages, blood samples were obtained from non pregnant non lactating cows. Among monocyte subsets lower glucose uptake and GLUT mRNA expression were revealed in nonclassical monocytes. In macrophages differentiated from monocyte subsets in vitro, glucose uptake remained highest in classical monocyte-derived macrophages while GLUT mRNA expression was higher in nonclassical monocyte-derived macrophages, indicating discrepancies between mRNA and protein expression. Alternative activation of macrophages resulted in an increase in GLUT mRNA expression and glucose uptake while classical activation failed to upregulate GLUT mRNA expression. However, a higher medium glucose concentration promoted a proinflammatory macrophage phenotype.

The glucose uptake capacity of peripartal monocytes decreased after parturition and the expression ratio of GLUT3 to GLUT1 mRNA shifted towards the higher affinity GLUT3 transporter, probably to adapt to the lower glucose availability after parturition. Neither the feeding regime nor postpartal mastitis or metritis affected glucose uptake capacities or GLUT expression. A high lactose production was associated with lower GLUT1 and GLUT3 mRNA expression and a lower GLUT3/GLUT1 ratio, indicating that monocyte glucose transporter expression is downregulated when mammary gland glucose requirements increase.

In conclusion, monocytes are also affected by the postpartal redistribution of glucose. As differences in glucose uptake and glucose transporter expression among bovine monocyte and macrophage subsets were observed, postpartal glucose shortage might modulate the peripartal immune response by altering the activation of monocytes or their differentiation into macrophages. However, to evaluate the consequences further studies regarding functional properties of the cells are desirable.
Zusammenfassung

Melanie Eger

Einflüsse der peripartalen Energiebilanz von Milchkühen auf die funktionelle Kapazität von Monozyten und ihre Differenzierung zu Makrophagen


Die Einflüsse der peripartalen Energiebilanz auf die Zahl und die Glukoseaufnahme von Monozyten wurden anhand von 27 Milchkühen untersucht, die aufgrund ihres Body Condition Scores (BCS) in 2 Fütterungsgruppen eingeteilt wurden. Von Tag 42 vor der Geburt bis zum 56. Laktationstag wurden die Tiere nach einem Fütterungsregime gefüttert, in welchem die Gruppe mit höherem BCS vor der Geburt einen höheren Kraftfutteranteil in der Ration erhielt, wohingegen sie nach der Geburt im Kraftfutteranteil stärker begrenzt wurde, um eine negativere Energiebilanz und
verstärkte Lipolyse in dieser Gruppe zu erreichen. Monozyten wurden an den Tagen -42, -14, +7, +21 und +56 relativ zur Geburt gewonnen.

Die Monozytenzahl in allen drei Subpopulationen erreichte in beiden BCS-Gruppen ihren Maximalwert an Tag 7 nach der Geburt. Es fiel auf, dass nur Kühe, die aus der Gruppe mit niedrigerem BCS und positiver Energiebilanz stammten, wenn sie an einer postpartalen Mastitis oder Metritis erkrankten, an Tag +7 signifikant höhere Monozytenzahlen in allen drei Subpopulationen zeigten als gesunde Tiere. Der Anstieg der Monozytenzahlen ging mit einer vermehrten Expression der Adhäsionsmoleküle CD11a, CD49d und CD62L einher.


1 Introduction

1.1 Relevance of infectious diseases in dairy farming

Peripartal health problems of dairy cows cause major financial losses for the farmer and impair animal welfare. Infectious diseases of the udder or the reproductive tract are common problems in dairy farming which might result in lower milk yield and reduced fertility and longevity. A survival study on English dairy farms demonstrated that only 55% of cows followed from an age of 1 month reached their third calving, while 11.3% were culled prior to the first lactation, 19.0% in the first lactation and 23.5% in the second lactation (Brickell and Wathes, 2011). Reproductive problems and udder health problems represent the most frequent reasons for culling with about 20% to 30% each (Ahlman et al., 2011; Chiumia et al., 2013). Thereby mastitis or high somatic cell counts are the most common factors accounted to udder health problems (Ahlman et al., 2011; Brickell and Wathes, 2011; Chiumia et al., 2013; Grohn et al., 1998) while the reasons for infertility are more variable. However, infectious diseases of the uterus such as metritis or endometritis may result in reduced fertility due to disturbed endocrine signaling, endometrial inflammation or reduced oocyte quality (Bromfield et al., 2015; Ribeiro et al., 2013). Mastitis and metritis are not only risk factors for preliminary culling, moreover, they represent the most frequent diseases in dairy cows and occur often in early lactation (Fleischer et al., 2001; Gulay et al., 2007; Heuer et al., 1999; Ribeiro et al., 2013). Comparing results of about 25 epidemiological or genetic studies Ingvartsen et al. (2003) demonstrated that high milk yield increases the risk of a dairy cow to suffer from mastitis and that a future genetic selection for high milk yield will further increase this risk. Further risk factors for mastitis are a high increase in milk yield (Chiumia et al., 2013) and increased parity (Ahlman et al., 2011; Hardeng and Edge, 2001), the latter potentially being linked to the increase in milk yield with parity (Heuer et al., 1999). The high frequency of mastitis and metritis in early lactation and the association with high milk yield indicate that metabolic factors might contribute to the increased susceptibility for infectious diseases in the peripartal dairy cows.
1.2 The metabolic challenges of the peripartal period

Around parturition dairy cows have to cope with massive metabolic changes. During late pregnancy metabolic demands of the fetus increase maternal requirements for glucose and amino acids by about 30% to 50% (Bell, 1995). With the onset of lactation energy requirements of the mammary gland even exceed those of the uterus by three fold. For the production of 30 kg of milk per day the mammary gland requires 1.7 kg glucose, 1.4 kg amino acids and 1.2 kg fatty acids (Bell, 1995). As feed intake is reduced around parturition and the peak in feed intake is delayed in relation to the peak in milk yield (Bauman and Currie, 1980; Ingvartsen and Andersen, 2000), dairy cows are unable to meet the increased energy requirements for lactation and maintenance by dietary energy intake and a negative energy balance (NEB) occurs which may continue up to several weeks (Accorsi et al., 2005; Hammon et al., 2006). Nevertheless, milk production is maintained at the expense of other physiological processes (Bauman and Currie, 1980). Adipose tissue and muscle protein are mobilized to provide energy and substrates for milk production, leading to losses in body condition and increased non-esterified fatty acid (NEFA) concentrations in the blood (Cardoso et al., 2013; Holtenius et al., 2003; Kuhla et al., 2011). An accumulation of ketone bodies might result in subclinical or clinical ketosis (Drackley et al., 2001). As a consequence of the metabolic challenges dairy cows are more susceptible for metabolic diseases such as milk fever, ketosis and displaced abomasum in early lactation (Fleischer et al., 2001). Moreover, several feeding studies provide evidence that an overfeeding in the dry period and a higher body condition score (BCS) at calving enhance the decrease in dry matter intake and the loss of body condition in early lactation, extend the duration of NEB and increase the risk for metabolic diseases such as ketosis (Agenas et al., 2003; Hammon et al., 2009; Mann et al., 2015; Schulz et al., 2014; Vanholder et al., 2015).

One of the main substrates for milk production is glucose, which undergoes a massive redistribution after parturition. To sustain milk production about 80% of the total glucose is transported towards the mammary gland, mainly for the synthesis of lactose (Bauman and Currie, 1980; Zhao, 2014). Although gluconeogenesis is increased after parturition, blood glucose levels decline (Bell, 1995; Holtenius et al., 2003). Mammary gland glucose transporter expression increases with the onset of lactation to meet the higher requirements for glucose, while insulin responsiveness
and glucose transporter expression decrease in peripheral tissues, e.g. adipose tissue (Holtenius et al., 2003; Komatsu et al., 2005; Zachut et al., 2013). The decrease in blood glucose concentrations and the redistribution of glucose towards the udder might impair energy supply to immune cells and thereby promote susceptibility for infectious diseases in dairy cows.

1.3 Peripartal alterations of the immune system

Due to the increased disease frequency after parturition peripartal changes in the immune system of dairy cows have intensively been studied in the last decades. However, it still remains unclear whether immunosuppression or an enhanced inflammatory immune reaction predisposes peripartal dairy cows for the occurrence of diseases. Immunosuppression might be provoked by an impaired production of reactive oxygen species (ROS) in neutrophils after parturition (Mehrzad et al., 2002) or by a decrease in the percentage of total T cells and T helper cells (Kimura et al., 1999). A proinflammatory state might be evoked by elevated counts of monocytes and increased tumor necrosis factor α (TNF-α) production (Rontved et al., 2005; Sordillo et al., 1995) or by changes in the levels of acute phase proteins (Trevisi et al., 2012). In a review Burvenich et al. (2007) have reported that both, decreased ROS production and elevated TNF-α production are correlated to the severity of Escherichia coli (E. coli) mastitis, and that the severe form often occurs in the first weeks of lactation. In general, leukocytosis, neutrophilia, eosinopenia and monocytosis are observed around parturition (Meglia et al., 2005). The humoral immune response might be altered by a decline in immunoglobulin G and M levels, starting already prior to parturition (Detilleux et al., 1995; Herr et al., 2011).

Several studies indicate that peripartal energy balance affects these changes in the immune system. Rontved et al. (2005) observed higher numbers of monocytes in cows with higher dietary energy supply. In addition, a reduction in concentrate supply resulted in lower blood glucose concentrations and was associated with lower numbers of total T cells, T helper cells, MHCII+ cells and CD21+ cells (Ohtsuka et al., 2006). Some of these changes are abrogated when the onset of lactation is prevented by mastectomy (Kimura et al., 2002). In neutrophils, the expression of several proinflammatory genes, the antiinflammatory interleukin- (IL-) 10, IL-1β and
genes associated with adhesion, motility, migration and phagocytosis is elevated in cows fed a higher energy diet compared with a control diet (Zhou et al., 2015). Hammon et al. (2006) have reported that cows developing puerperal metritis and subclinical endometritis showed higher NEFA concentrations and lower dry matter intake (DMI) compared to healthy cows already prior to parturition and that neutrophil myeloperoxidase activity was reduced in cows with higher NEFA and lower DMI, which they classified as markers for NEB. In summary, these studies provide evidence for the link between peripartal metabolic changes and the alterations in immune responsiveness (Figure 1).

Figure 1: Interrelationships between nutrition and disease in the periparturient dairy cow. Factors which are addressed in this study are highlighted by ellipses. Modified from Goff (2006).

1.4 Monocytes and macrophages in cattle

Monocytes and macrophages are part of the mononuclear phagocyte system (Figure 2). Monocytes originate from a myeloid progenitor in the bone marrow, circulate for a few days in the peripheral blood and then migrate into tissues where they
Introduction

differentiate into macrophages or dendritic cells (for review: Gordon and Taylor, 2005). Monocytes regulate the inflammatory response by producing important proinflammatory cytokines such as IL-1β, TNF-α and IL-6 (Gessani et al., 1993; Heumann et al., 1994; Orlinska and Newton, 1993). They are able to phagocytose bacteria and to produce reactive oxygen species (Hussen et al., 2013). Based on phenotypic characteristics several monocyte subsets have been identified which differ in functional properties. In humans and in cattle, monocytes are classified based on their expression of CD14 and CD16 classical monocytes (cM, CD14++CD16-), intermediate monocytes (intM, CD14++CD16+) and nonclassical monocytes (ncM, CD14+CD16++), in other species different molecules are used, e.g. Ly6C in mice (Hussen et al., 2013; Ziegler-Heitbrock et al., 2010). In similarity to human monocytes, bovine cM exhibit the highest phagocytic capacity, while intM are the main producers of ROS and IL-1β (Hussen et al., 2013). However, differences have been revealed concerning the function of ncM and monocyte migration. In contrast to human monocytes, bovine monocytes are not migrating in response to chemokine (C-C motif) ligand (CCL) 2, whereas CCL5 mainly triggers...
bovine cM to migrate (Hussen et al., 2014). Bovine ncM produce only low amounts of ROS or cytokines while in humans the combined CD16+ subset is the major source of TNF-α (Belge et al., 2002; Hussen et al., 2013). From studies with human or mice monocytes it is known that ncM patrol the vessel wall and are able to rapidly invade the tissue upon damage recognition (Auffray et al., 2007; Cros et al., 2010). Subsequently neutrophils are recruited, followed by cM and intM (Soehnlein and Lindbom, 2010). Whether bovine ncM patrol the endothelium as their mice and human counterparts remain to be clarified.

After migration into tissues monocytes may replenish the tissue macrophage pool and may differentiate into various types of macrophages. Initially two different macrophage types have been defined: classically activated (M1) macrophages and alternatively activated (M2) macrophages (Mosser and Edwards, 2008). Classical activation is mediated by interferon-γ (IFN-γ) and TNF-α or Toll-like receptor (TLR) agonists such as bacterial lipopolysaccharide (LPS) and results in proinflammatory, microbicidal macrophages that are able to produce high amounts of proinflammatory cytokines, whereas alternative activation is triggered by IL-4 and IL-13 and results in macrophages mediating tissue repair and humoral immunity (Gordon and Taylor, 2005). Recently, further activation pathways have been described which are either included in the M2 definition or addressed separately such as innate activation (TLR ligands) or deactivation (IL-10 and transforming growth factor β) (Gordon and Taylor, 2005). The activation of immune cells is always accompanied by dramatic increases in their energy requirements and influences their substrate consumption.

1.5 Immune cell energy metabolism

Immune cells rely on glucose, amino acids and fatty acids as fuels, whereas they are not able to utilize ketone bodies such as acetoacetate or β-hydroxybutyrate (BHB) (Newsholme et al., 1987). High rates of amino acids, predominantly glutamine, are needed in proliferating cells such as lymphocytes (Jones and Thompson, 2007), however, in general glucose is utilized at much higher rates (Pithon-Curi et al., 2004). Resting immune cells exhibit low rates of glucose consumption and rely on oxidative phosphorylation (OXPHOS) or fatty acid oxidation for adenosine triphosphate (ATP) production. However, when activated, immune cell energy metabolism switches to
aerobic glycolysis, and thereby increases glucose requirements and glucose uptake. This metabolic switch has been demonstrated in dendritic cells (Krawczyk et al., 2010), T cells (Cham and Gajewski, 2005; Sukumar et al., 2013), monocytes (Cheng et al., 2014; Dietl et al., 2010) and macrophages (Haschemi et al., 2012). Moreover, functional properties of immune cells influence their metabolic pattern. For example two T cell populations have been identified based on their glucose uptake rate. T cells showing higher rates of glucose uptake and glycolysis resembled CD8+ effector T cells, while T cells with low glucose uptake and preferred utilization of OXPHOS resembled memory T cells (Sukumar et al., 2013). In macrophages differences in metabolic patterns between M1 and M2 macrophages have been intensively studied. The classically activated M1 macrophages rely on glycolysis for ATP production (Haschemi et al., 2012), while alternatively activated M2 macrophages fuel their performance mainly by OXPHOS or β-oxidation (Vats et al., 2006). Consequently, M1 activation induces a stronger increase in glucose transporter expression compared to M2 activation (Freemerman et al., 2014). However, compared to naive macrophages energy requirements of both macrophage types increase after activation. In M1 macrophages glucose uptake is strongly enhanced and fatty acid uptake and metabolism are reduced, whereas in M2 macrophages glucose and fatty acids are absorbed to a greater extend and genes for fatty acid metabolism and OXPHOS are induced (Rodriguez-Prados et al., 2010; Vats et al., 2006). It was hypothesized that the switch to glycolysis allows M1 macrophages to maintain energy production while the mitochondrion can be used for ROS production (Palsson-McDermott and O'Neill, 2013). In monocytes it has been demonstrated that an inhibition of glycolysis can be compensated by increased use of OXPHOS, indicating that the metabolic pattern remains flexible (Dietl et al., 2010). The high glucose requirements of immune cells are sustained by a constant influx of glucose via specialized transport proteins in the plasma membrane.

1.6 Glucose transporters on monocytes and macrophages

Glucose transport can be mediated by two mechanisms relying on different transporters: glucose can either be taken up in co-transport with sodium via sodium-dependent glucose transporters (SGLT) or by facilitative diffusion using sodium-independent glucose transporters (GLUT) (for review: Mueckler and Thorens, 2013;
Zhao and Keating, 2007). Immune cell glucose uptake is mediated by GLUT proteins, which are encoded by the genes of the soluble carrier family 2 (SLC2). Proteins of the GLUT family consist of 12 transmembrane domains, a central cytoplasmic domain and a single N-linked glycosylation side, and the N and C terminal ends are located in the cytoplasm (Figure 3). Multiple studies have investigated GLUT isoforms on monocytes and macrophages with differing results, depending on the method used or the origin of the cells. Most commonly expression of GLUT1 (SLC2A1), GLUT3 (SLC2A3) and GLUT4 (SLC2A4) are reported on human peripheral blood monocytes (Kipmen-Korgun et al., 2009; Maratou et al., 2007), while in human monocyte-derived macrophages GLUT1 and GLUT3 are observed (Malide et al., 1998).

GLUT1-4 display a high affinity for glucose transport, while GLUT5 which has been detected in macrophages in a few studies, exhibits a poor affinity for glucose and mainly transports fructose (Fu et al., 2004; Malide et al., 1998; Zhao and Keating, 2007). GLUT1 is a ubiquitously expressed basal glucose transporter, e.g. it is strongly expressed on erythrocytes and mainly mediates mammary gland glucose uptake. GLUT3 is a high affinity glucose transporter mediating glucose uptake in neuronal tissues such as the brain. GLUT4 is an insulin-responsive glucose
transporter and is mainly present in muscle and adipose tissues (for review: Mueckler and Thorens, 2013; Zhao and Keating, 2007). Insulin binding to its receptor increases glucose uptake by translocation of GLUT4 from an intracellular storage to the plasma membrane (Bryant et al., 2002). This effect is also observed in human monocytes (Daneman et al., 1992; Dimitriadis et al., 2005), however not in macrophages which do not express GLUT4 (Fu et al., 2004; Ouro et al., 2013). To fulfill the increased energy demands of activated immune cells, GLUT transporter expression in the plasma membrane is upregulated in response to activation (Freemerman et al., 2014; Gamelli et al., 1996; Maratou et al., 2007; Ouro et al., 2013). Facilitative glucose transport mainly depends on the glucose gradient and transporter expression. Therefore, peripartal alterations of glucose transporter expression on bovine monocytes might modulate glucose availability for the cells and contribute to immune dysfunction.

1.7 Hypothesis and aim of the PhD project

The peripartal period of dairy cows is characterized by postpartal negative energy balance and higher incidences of metabolic and infectious diseases. Several studies indicate that peripartal alterations in the immune system are linked to metabolic changes. Glucose as a main energy source for immune cells is mainly utilized for milk production. Glucose shortage may affect bovine monocytes as important regulatory cells or alter their differentiation into macrophages.

Therefore, the aim of this study was to investigate whether peripartal energy balance contributes to immune dysregulation by impairing the glucose supply to bovine monocytes. As nothing is known until now about peripartal alterations in the numbers of the recently identified bovine monocytes subsets or their glucose requirements, the following questions are addressed in this thesis:

1. Does energy balance affect the number of classical, intermediate and nonclassical monocytes in peripartal dairy cattle?
2. Is there any evidence for differences in glucose uptake and glucose transporter expression among bovine monocyte subsets, subset-derived macrophages or polarized macrophages?

3. Are glucose uptake and GLUT transporter expression in bovine monocytes altered by peripartal energy balance?
2 Background information on investigations in peripartal dairy cows

2.1 Experimental setup: The feeding model

Monocyte samples for investigations during the peripartal period (Manuscript 1 and 2, Chapter 5.1) were obtained from 27 German Holstein cows housed at the Institute of Animal Nutrition, Friedrich Loeffler Institute, Federal Research Institute for Animal Health in Braunschweig. The animals included in the experiments were part of a larger study investigating the effects of feed additives on the occurrence of ketosis in peripartal dairy cows (Drong et al., 2015). The feeding strategy was based on a model to induce subclinical ketosis established by Schulz et al. (2014). This model combines overfeeding in the dry period and a restricted postpartal energy intake to enhance postpartal NEB and lipolysis and to promote the development of ketosis. With regard to this experimental design the model is appropriate for investigating the impact of different degrees of NEB on bovine monocytes. Briefly, the model is based on the following procedure (Drong et al., 2015; Schulz et al., 2014): The BCS of the cows was determined prior to the start of the experiment and according to this the cows were allotted to two groups differing significantly in BCS, one with normal or low BCS (control group), one with high BCS. Prior to parturition the control group received an energetically adequate diet consisting of 80% roughage and 20% concentrate, according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). The high condition group received 40% of the same roughage and 60% concentrate to induce an energy oversupply. After calving the concentrate proportion in the diet was raised from 30% to 50% in 2 weeks for the control and in 3 weeks for the high condition cows, to enhance NEB and lipolysis in the high condition group. This feeding strategy resulted in higher rates of subclinical and clinical ketosis, defined by BHB serum concentrations, in the high condition group (Schulz et al., 2014). Energy balance was significantly higher in the high condition cows prior to parturition and was more negative and lasted longer after parturition in this group.

For the present study detailed information about study design, feed composition, performance and milk parameters are published by Drong et al. (2015). The groups
selected for the present investigations were both control groups of the entire study, the low body condition group (BCS low group, day -42: BCS 2.77 ± 0.14, Mean ± SD) and the high body condition control group (BCS high group, day -42: 3.95 ± 0.08, Mean ± SD). All information regarding the occurrence of diseases, blood parameters, energy balance and milk production data were provided by Caroline Drong, Institute of Animal Nutrition, Friedrich Loeffler Institute, Federal Research Institute for Animal Health in Braunschweig. The feeding regime in the present study also resulted in a higher loss of BCS, higher ketosis rates and higher postpartal NEFA concentrations in the high condition group, while the difference in energy balance was not significant in the first two weeks after parturition between the low condition and the high condition group with -41.14 MJ NEL/d and -52.22 MJ NEL/d, respectively (Drong et al., 2015).

2.2 Incidence of clinical mastitis and metritis

In Manuscript 1 the impact of postpartal infectious diseases on monocyte numbers and monocyte glucose uptake is considered. During the experimental period 7 of 14 BCS low cows and 7 of 13 BCS high cows developed clinical signs of mastitis or metritis or both diseases. A detailed overview on the occurrence of each disease is given in Fig. 4.

![Figure 4: Occurrence of postpartal mastitis and metritis in both BCS groups. Data provided by Caroline Drong.](image-url)
2.3 Blood insulin concentrations

As insulin is an important mediator of glucose uptake into peripheral tissues we collected serum samples parallel to monocyte sampling at days -42, -14, +7, +21 and +56 relative to parturition. Serum insulin concentration was assessed by radioimmunoassay in the Endocrinology Department of the Clinical for Cattle, University of Veterinary Medicine Hannover. Statistical analysis was carried out using Graph Pad Prism 5 (Graph Pad Software, San Diego, CA, USA).

![Insulin](image)

Figure 5: Blood insulin concentrations. Insulin concentrations were measured in serum samples (BCS high n = 13, BCS low n = 14) by radioimmunoassay. Significant time-dependent differences in Bonferroni post-test are indicated by small letters for the BCS low group and capital letters for the BCS high group. Differences between groups are indicated by *** P < 0.001.

Insulin concentrations were significantly affected by the factors time, BCS and by time x BCS interaction (Figure 5). In the BCS high group the insulin concentration was almost three fold higher compared with the BCS low group at day +7 (P < 0.001), probably a result of the high dietary energy intake. Insulin concentrations decreased from day -42 to day +7 and +56 and from day -14 to days +7, +21 and +56 in the BCS high group (at least P < 0.05). In the BCS low group insulin was significantly higher at day -14 compared with day +7 relative to parturition (P < 0.05). Further investigations regarding insulin effects on monocyte glucose uptake or GLUT transporter expression are included in Manuscript 2.
3 Manuscript 1

Impacts of parturition and body condition score on glucose uptake capacity of bovine monocyte subsets


Published in: Veterinary Immunology and Immunopathology 166 (2015): 33-42
doi: 10.1016/j.vetimm.2015.04.007

Contribution to the manuscript:
I participated in the study design. I collected most of the blood samples and performed all experiments regarding glucose uptake. I analyzed the data statistically and wrote the manuscript.
Abstract

The peripartal period of dairy cows is associated with a higher incidence of infectious diseases like mastitis or metritis, particularly in high-yielding animals. The onset of lactation induces a negative energy balance and a shift of glucose distribution towards the udder. Glucose is used as primary fuel by monocytes which give rise to macrophages, key cells in the defense against pathogens. The aim of this study was to analyze whether animals with high or low body condition score (BCS) differ in composition and glucose uptake capacities of bovine monocyte subsets. Blood samples were taken from 27 dairy cows starting 42 days before parturition until day 56 after parturition. The cows were allocated to two groups according to their BCS. A feeding regime was applied, in which the BCS high group received higher amounts of concentrate before parturition and concentrate feeding was more restricted in the BCS high group after parturition compared with the BCS low group, to promote postpartal lipolysis and enhance negative energy balance in the BCS high group. Blood cell counts of classical (cM), intermediate (intM) and nonclassical monocytes (ncM) were increased at day 7 after calving. In the BCS low group intM numbers were significantly higher compared to the BCS high group at day 7 after parturition. Within the BCS low group cows suffering from mastitis or metritis showed significantly higher numbers of cM, intM and ncM at day 7 after parturition. Classical monocytes and intM showed similar glucose uptake capacities while values for ncM were significantly lower. Compared with antepartal capacities and irrespective of BCS and postpartal mastitis or metritis, glucose uptake of all monocyte subsets decreased after parturition. In conclusion, whereas glucose uptake capacity of bovine monocyte subsets is altered by parturition, it is not linked to the energy supply of the animals or to postpartal infectious diseases.
4 Manuscript 2

Glucose transporter expression differs between bovine monocyte and macrophage subsets and is influenced by milk production

M. Eger, J. Hussen, M. Koy, S. Dänicke, H.-J. Schuberth, G. Breves

Published in: Journal of Dairy Science 99 (2016), 2276-2287
doi: 10.3168/jds.2015-10435

Contribution to the manuscript:
I contributed to the study design and planned the analysis of glucose transporters. I collected most of the blood samples, separated the cells and performed the analyses. I analyzed the data statistically and wrote the manuscript.
Abstract

The peripartal period of dairy cows is characterized by negative energy balance and higher incidences of infectious diseases such as mastitis or metritis. With the onset of lactation milk production is prioritized and large amounts of glucose are transported into the mammary gland. Decreased overall energy availability might impair the function of monocytes acting as key innate immune cells, which give rise to macrophages and dendritic cells and link innate and adaptive immunity. Information on glucose requirements of bovine immune cells is rare. Therefore, this study aims to evaluate glucose transporter expression of the three bovine monocyte subsets (classical, intermediate and nonclassical monocytes) and monocyte-derived macrophages and to identify influences of the peripartal period. Blood samples were either collected from nonpregnant healthy cows or from 16 peripartal German Holstein cows at d -14, +7 and +21 relative to parturition. Quantitative real-time PCR was applied to determine mRNA expression of glucose transporters (GLUT) 1, GLUT3 and GLUT4 in monocyte subsets and monocyte-derived macrophages. The low GLUT1 and GLUT3 expression in nonclassical monocytes was unaltered during differentiation into macrophages, whereas in classical and intermediate monocytes GLUT expression was downregulated. Alternatively activated M2 macrophages consumed more glucose compared to classically activated M1 macrophages. The GLUT4 mRNA was only detectable in unstimulated macrophages. Neither monocytes nor macrophages were insulin responsive. In the peripartum, monocyte GLUT1 and GLUT3 expression and the GLUT3/GLUT1 ratio were negatively correlated to lactose production. The high-affinity GLUT3 transporter appears to be the predominant glucose transporter on bovine monocytes and macrophages, especially in the peripartal period when blood glucose levels decline. Glucose transporter expression in monocytes is downregulated as a function of lactose production which might impair monocyte to macrophage differentiation.
5 Continuative Investigations

5.1 Adhesion molecule expression in peripartal monocytes

5.1.1 Introduction

In the feeding experiment monocyte counts of cM, intM and ncM were elevated at day +7 after parturition and we observed higher numbers of all monocyte subsets in cows suffering from postpartal mastitis or metritis compared to healthy cows in the BCS low group, however not in the BCS high group (Manuscript 1). Therefore we conducted further investigations concerning the underlying mechanism. Monocyte numbers in the blood depend on monocyte influx from the bone marrow and monocyte migration into tissues. Monocyte influx from the bone marrow is mainly regulated by chemokines binding to CCR2 (chemokine (C-C motif) receptor 2), e.g. CCL2 (Serbina and Pamer, 2006), while monocyte migration into tissues is triggered by CCL5 and fractalkine (Ancuta et al., 2009; Weber et al., 2001). Migration of monocytes requires expression of adhesion molecules on the cell surface of both, monocytes and vascular endothelial cells. The adhesion cascade can be subdivided into several steps, whereby different adhesion molecules are involved in each step (for review: Gerhardt and Ley, 2015; Herter and Zarbock, 2013). Leukocytes migration starts with their capturing to the vessel wall which is mainly mediated by selectins, of which L-Selectin (CD62L) is expressed on leukocytes. Subsequently, leukocytes roll on the endothelial wall. On monocytes the Very late antigen-4 (VLA-4, α4β1-Integrin) is involved in rolling. For the firmer adhesion and arrest on the endothelium β2-integrins are required, such as Lymphocyte function-associated antigen-1 (LFA-1), a dimer consisting of CD11a and CD18, and Macrophage-1 antigen (Mac-1), consisting of CD11b and CD18. Subsequently, the so-called crawling is performed to reach sides of extravasation. Monocytes crawl on LFA-1 and Mac-1, while the final transmigration is mainly mediated by the Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31). In our study we investigated expression of CD11a, CD11b and CD18 as part of β2-Integrins, CD31, CD62L and CD49d, the latter forms together with CD29 the VLA-4 dimer.
5.1.2 Material and Methods

Monocyte adhesion molecule expression was analyzed in peripartal blood samples at days -42, +7 and +56 relative to parturition. Leukocytes were isolated as described in manuscript 1. Leukocytes were fixed with 4% paraformaldehyde (PFA) for 15 min. After a washing step with phosphate buffered saline (PBS) (600 x g, 5 min) the cells were suspended in PBS containing 10% dimethyl sulfoxide and stored at -80°C until further use. After thawing cells were fixed again with 4% PFA for 30 min. Thereafter cells were washed with PBS containing 5 g/L bovine serum albumin and 0.1 g/L NaN₃ (membrane immunofluorescence buffer, MIF buffer), counted and adjusted to 5 x 10⁵ cells per well in a 96-well plate. Each well was labeled with one of the following antibodies for 20 min at 4°C in the dark: mouse anti-bovine CD11a, mouse anti-bovine CD18 (both Kingfisher Biotech, Saint Paul, MN, USA), mouse anti-bovine CD11b-FITC, mouse anti-sheep CD31, mouse anti-human CD49d and mouse anti-bovine CD62L (all AbDSerotec, Oxford, UK). Thereafter unconjugated antibodies (all except CD11b) were incubated with goat anti-mouse-PE (Jackson ImmunoResearch, West Grove, PA, USA) as secondary antibody for 20 min at 4°C in the dark. Specific binding of the antibodies was confirmed using isotype controls for a representative sample. After labeling cells were washed again in MIF buffer (600 x g, 5 min), suspended in buffer solution and analyzed flow cytometrically (Accuri C6 Flow Cytometer®, Becton Dickinson GmbH, Heidelberg, Germany). Cellular expression of adhesion molecules was determined as median fluorescence intensity of 10000 monocytes per sample. Two animals were excluded from the analysis, one due to unclear health status at the time point of analysis, one due to morphological alterations of the cells after thawing. Statistical analysis was performed using Graph Pad Prism 6.05 (Graph Pad Software, San Diego, CA, USA). Data were analyzed for effects of time (day relative to calving), effects of BCS and interaction of both factors by repeated measurements two-way ANOVA followed by Sidak post-test. In case of interaction time-dependent effects were analyzed within groups, otherwise time-dependent effects were analyzed for both groups.

5.1.3 Results and Discussion

As monocyte counts for all three subsets were elevated at day +7 after parturition (Manuscript 1) we investigated adhesion molecules expression on monocytes of both
BCS groups to determine potential changes in monocyte migration. The expression of CD11a increased from day -42 to day +7 (P < 0.001, Fig. 6A), while the expression densities of CD11b, CD18 and CD31 were not significantly changed during the peripartal period (Fig. 6B-D). The expression densities of CD49d and CD62L increased from day -42 to day +7 and remained high until day +56 (at least P < 0.05, Fig. 6E, F). Moreover, the expression of CD49d was significantly higher in the BCS low group compared to the BCS high group (effect of BCS: P < 0.05, Fig. 6E).

As monocyte subset counts were significantly increased in BCS low animals suffering from postpartal mastitis or metritis compared to healthy animals, while no differences were observed in BCS high animals (Manuscript 1), we compared adhesion molecule expression on monocytes of healthy and diseased cows of both BCS groups. The expression of CD11a was elevated at day +7 in BCS low cows (P < 0.01, Fig. 7A). A tendency for an interaction between time and disease indicated that this effect was more pronounced in diseased cows compared with healthy cows. In contrast, in BCS high cows CD11a expression was not influenced by the factor disease (Fig. 7A). Monocyte CD11b expression tended to be higher in diseased BCS high cows compared to healthy cows, while in the BCS low group CD11b expression was significantly higher in healthy cows at day +7 (P < 0.01) after it increased from an initially lower expression at day -42 (P < 0.05, Fig. 7B). Expression densities of CD18 and CD31 were not altered by the factor disease in either of the BCS groups (data not shown). In the BCS low group CD49d expression was significantly higher in infectious disease cows compared to healthy cows (P < 0.01, Fig. 7C), while in the BCS high group CD49d expression was not altered by the factor disease. Monocyte CD62L expression was significantly higher in diseased BCS low cows compared to healthy cows merely at day +7 (time x disease: P < 0.01) as it increased from day -42 to day +7 and then decreased again at day +56 in diseased animals (at least P < 0.05, Fig. 7D). Time-dependent effects in the BCS high group were merely observed for the expression of CD62L (P = 0.05)
Figure 6: Adhesion molecule expression on peripartal monocytes. Leukocytes from 25 peripartal cows (BCS high n = 12, BCS low n = 13) were isolated from peripheral blood, fixed with paraformaldehyde and labeled with antibodies specific for the surface molecules CD11a, CD11b, CD18, CD31, CD49d and CD62L. The expression density was measured as median fluorescence intensity (MFI) of 10000 monocytes by flow cytometry. Two-way ANOVA revealed effects of time on expression of CD11a, CD49d and CD62L and an effect of BCS on CD49d. Sidak post-test was applied to detect significant differences among time-points or between groups. * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 7: Adhesion molecule expression differs between healthy and diseased cows. Cows suffering from postpartal mastitis or metritis were combined to an infectious disease group and adhesion molecule expression was compared with healthy cows (BCS low healthy n = 7, BCS low infectious disease n = 6, BCS high healthy n = 5, BCS high infectious disease n = 7). Expression of the surface molecules CD11a, CD11b, CD49d and CD62L was measured as median fluorescence intensity (MFI) by flow cytometry for 10000 monocytes. Significant differences in Sidak post-test between time-points or between groups at one timepoint are indicated by * P < 0.05, ** P < 0.01, *** P < 0.001. Significant time-dependent differences within groups are indicated by small letters for healthy and by capital letters for infectious disease cows.
In summary, the peripartal increase in monocyte numbers at day +7 was accompanied by a higher expression of CD11a, CD49d and CD62L and the only molecule affected by the feeding regime was CD49d. In agreement with our study CD11b, CD18 and CD62L expressions were not affected by negative energy balance in a feeding study using steers as a model (Perkins et al., 2001). In contrast, Diez-Fraile et al. (2003) compared expression of CD11a, CD11b and CD18 on monocytes in two periods prior to parturition and two periods after parturition without observing significant time-dependent changes. The difference to our results might be related to the shorter time span of their study from day -14 until day +35 relative to parturition or to lower animal numbers.

Furthermore, we observed a higher expression of CD11a, CD49d and CD62L in BCS low cows suffering from postpartal mastitis or metritis, which also exhibited elevated monocyte numbers. In contrast, the expression of CD11b was lower in diseased cows at day +7 in the BCS low group and higher in the BCS high group. CD11b and CD11a both mediate firm adhesion and crawling of monocytes, thereby one may speculate that the decreased expression of CD11b may be compensated or caused by the increased CD11a expression. An in vitro study with mouse monocytes reported a shift from a LFA-1 dependent crawling in unstimulated venules to a Mac-1 dependent crawling in TNF-α stimulated venules (Sumagin et al., 2010). This is in contrast to the present observations in the BCS low group. In the BCS high group the higher CD11b expression is not related to the time span when the diseases occurred (mainly around day +7), therefore it is unlikely to be based on a disease-related stimulation of the endothelial cells. As Mac-1 also functions as complement receptor and mediates phagocytosis (Weinstein et al., 2015), alterations in CD11b expression might be linked to other functional properties. The study by Sumagin et al. (2010) also indicated that CD49d becomes more important for adhesion in stimulated venules due to their higher endothelial Vascular cell adhesion protein 1 (VCAM-1) expression, which might explain the higher CD49d expression at day +7. In general, the molecules which were either influenced by parturition or disease or both factors (CD11a, CD11b, CD49d and CD62L) mediate rolling, adhesion and crawling of monocytes. Increased expression of the adhesion molecules might either be related to higher migration, then the elevated monocyte counts are based on an additionally increased influx from the bone marrow, or could be an attempt of the cells to migrate.
in spite of a decreased expression of corresponding adhesion molecules on the endothelial cells.

5.2 Impact of glucose availability on monocyte polarization and cytokine production

5.2.1 Introduction

Activation of immune cells increases glucose uptake and glucose utilization due to a metabolic switch from OXPHOS to glycolysis (Krawczyk et al., 2010). In macrophages the metabolic pattern is influenced by the activation pathway. While energy generation in classically activated M1 macrophages primarily relies on glycolysis (Rodriguez-Prados et al., 2010), alternatively activated M2 macrophages use to a great extent OXPHOS or β-oxidation (Vats et al., 2006). In murine macrophages GLUT transporter overexpression even induces a more proinflammatory, M1-like phenotype (Freemerman et al., 2014). However, our experiments revealed higher glucose transporter expression and glucose utilization merely after alternative activation but not after classical activation (Manuscript 2). For a further characterization of the glucose requirements of bovine macrophages we tested the effects of low and high media glucose concentration on the phenotype of monocyte-derived macrophage and on the cytokine production of M1 and M2 macrophages.

5.2.2 Material and Methods

5.2.2.1 Membrane immunofluorescence

For testing the effects of glucose availability on monocyte to macrophage differentiation we collected blood samples from 6 non pregnant non lactating German Holstein cows housed in the Clinic for Cattle, University of Veterinary Medicine Hannover. CD14+ monocytes were isolated as described in Manuscript 2. Cells were suspended in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Darmstadt, Germany) supplemented with 1 g/L, 2 g/L or 4 g/L D-glucose (Sigma-
Aldrich, Munich, Germany). All media were further supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany) and 100 U/mL Penicillin/Streptomycin (Invitrogen, Karlsruhe, Germany). Monocytes were seeded in 24 well plates (5 x 10^5 cells per well) and incubated at 37°C, 5% CO_2 for 4 d. For each glucose concentration two samples were used, one remained as control, one was stimulated with 100 ng/mL LPS (LPS-EB Ultrapure from E. coli strain 0111:B4, InvivoGen, Toulouse, FR) at day 3. Macrophages were harvested at day 4 by addition of 200 µl Accutase solution (Sigma-Aldrich, Munich, Germany) for 20 min at 37°C. Accutase reaction was stopped by addition of culture medium and cells were washed (300 x g, 5 min) and suspended in PBS containing 5 g/L bovine serum albumin and 0.1 g/L NaN_3 (MIF buffer). Macrophage counts were determined by flow cytometry after propidium iodide was added to exclude dead cells from the analysis (2 µg/mL, Calbiochem, Bad Soden, Germany). For each sample 20000 viable macrophages were stained with the following antibodies: mouse anti-pig CD163-PE (cross-reactive with the bovine homologue) and mouse anti-bovine CD11b-FITC (both AbD Serotec, Oxford, UK) for 20 min in the dark at 4°C. Cells were washed with MIF-buffer (300 x g, 5 min) and the expression densities and percentages of positive macrophages for both molecules were assessed by flow cytometry after addition of propidium iodide (2 µg/mL, Calbiochem, Bad Soden, Germany). Statistical analysis was performed using Graph Pad Prism 6.05 (Graph Pad Software, San Diego, CA, USA). Data were checked for Gaussian Distribution using the Kolmogorov-Smirnov test. Subsequently data were analyzed for effects of glucose concentration by repeated measurements ANOVA or Friedman test (expression density of CD11b on single positive cells), followed by Tukey post-test. Data are presented as means ± SEM.

5.2.2.2. Cytokine production

For investigating the impact of glucose availability on cytokine production M0, M1 and M2 macrophages were generated in media with different glucose concentrations. CD14+ monocytes were isolated from 6 cows and suspended in DMEM with 1 g/L or 4 g/L D-glucose (Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany) and 100 U/mL Penicillin/Streptomycin (Invitrogen, Karlsruhe, Germany). Monocytes were seeded in 24 well plates at
Continuative Investigations

5 x 10^5 cells per well. For each animal 12 samples were planned (Table 1), for one animal the monocyte number was only sufficient for the macrophage samples in the upper row. For generation of classically activated M1 macrophages cells were stimulated with 100 ng/mL LPS (LPS-EB Ultrapure from E.coli strain 0111:B4, InvivoGen, Toulouse, FR) and 50 ng/mL IFN-γ (AbD Serotec, Oxford, UK) at day 3 for 24 h. For alternative activation of M2 macrophages cells were stimulated with IL-4 (50 ng/mL) and IL-13 (50 ng/mL, both Kingfisher Biotech, Saint Paul, MN, USA). Unstimulated cells (M0 macrophages) were used as control. At day 4 one sample of each macrophage subset was additionally incubated with heat-inactivated E. coli (inactivated at 61°C for 30 min, E. coli 1303, isolated from a case of clinical mastitis, provided by Dr. Wolfram Petzl, Clinic for Ruminants, LMU Munich) for 6 h (n = 5) at a concentration of 10^7 bacteria/mL. Cell culture supernatants were carefully aspirated, centrifuged at 750 x g for 10 min to remove bacteria and non adherent cells and stored at -20°C.

Table 1: Macrophage samples for generation of cell culture supernatants.

<table>
<thead>
<tr>
<th>1 g Glucose</th>
<th>4 g Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>M1</td>
</tr>
<tr>
<td>M2</td>
<td>M0</td>
</tr>
<tr>
<td>M1 + E.coli</td>
<td>M2 + E.coli</td>
</tr>
<tr>
<td>M0 + E.coli</td>
<td>M1 + E.coli</td>
</tr>
<tr>
<td>M2 + E.coli</td>
<td>M2 + E.coli</td>
</tr>
</tbody>
</table>

Cytokine production was measured using commercially available ELISA kits for IL-6 (ESS0029, ThermoFisher Scientific, Schwerte, Germany) and TNF-α (DY 2279, R&D Systems, Minneapolis, MN, USA). For measurement of IL-10 production microtiter plates (Nunc-Immuno™ MicroWell™ 96-Well Plate, Thermo Scientific, Braunschweig, Germany) were coated with mouse anti-bovine IL-10 antibody (AbD Serotec, Oxford, UK) at a concentration of 4 µg/mL in a buffer containing Na_2CO_3 15 mmol/L and NaHCO_3 35 mmol/L, pH 9.6. Cells were incubated on a lab shaker for 30 min at room temperature and subsequently over night at 4°C. Plates were washed after each incubation step using an automated washing system performing five washing steps per run and were incubated on the lab shaker at room temperature. Washing buffer contained NaH_2PO_4 2.5 mmol/L, Na_2HPO_4 7.5 mmol/L, NaCl 145 mmol/L and 0.1% Tween 20, pH 7.2 (B-buffer). Plates were blocked with 2% fish
gelatin in B-buffer for 1 h. Recombinant bovine IL-10 (Kingfisher Biotech, Saint Paul, MN, USA) was used as standard in a 1:2 dilution series from 3200 pg/mL to 50 pg/mL. The standard series and the samples were applied for 1 h in duplicates. As detection antibody biotin-conjugated mouse anti-bovine IL-10 (AbD Serotec, Oxford, UK) was used at a concentration of 2 µg/mL in B-buffer supplemented with 2% fish gelatin (1 h at room temperature). Thereafter, Streptavidin-HRP (GE Healthcare Life Science, Freiburg, Germany) was applied and plates were incubated for 45 min. The substrate 3, 3', 5, 5'-Tetramethylbenzidin (6.24 mmol/L, Sigma-Aldrich, Karlsruhe, Germany) was added in a buffer containing citrate 33.3 mmol/L and Na₂HPO₄ 66.7 mmol/L, pH 5.0. After an incubation of about 15 min in the dark, color reaction was stopped by addition of 1 mol/L H₂SO₄. The optical density was detected using a plate photometer (Biotek ELx800, Bad Friedrichshall, Germany) at a wave length of 450 nm (test filter) and 630 nm (reference filter). The optical density of the measured samples was compared with the standard curve to calculate sample cytokine concentrations. Statistical analysis was carried out using Graph Pad Prism 6.05 (Graph Pad Software, San Diego, CA, USA). Data were analyzed for Gaussian Distribution using the Kolmogorov-Smirnov test. Effects of glucose concentration and macrophage subset were analyzed by 2way-ANOVA for repeated measurements in both factors. Sidak post-test was applied to detect differences among macrophage subsets. Data are presented as means ± SEM.

5.2.3 Results

5.2.3.1 Membrane immunofluorescence

We investigated whether glucose availability influences the phenotype of macrophages differentiated from monocytes in vitro. The percentage of viable macrophages expressing CD11b (CD11b+CD163-), CD11b and CD163 (CD11b+CD163+) or none of the molecules (CD11b-CD163-, gating and percentages for one animal are shown in Fig. 8) and the mean fluorescence intensity of both molecules were assessed. The percentage of macrophages expressing merely CD163 was below 2%. 

34
In unstimulated macrophages (control) the percentage of CD11b+CD163- macrophages was increased at a glucose concentration of 4 g/L compared to 1 g/L or 2 g/L glucose (P < 0.05, Fig. 9A). In return, the percentage of macrophages expressing both molecules decreased with significantly lower percentages after differentiation in medium containing 4 g/L glucose compared to 1 g/L (P < 0.05, Fig. 9B). The percentage of macrophages expressing neither CD11b, nor CD163 remained unchanged (Fig. 9C). In LPS stimulated macrophages more CD11b+CD163- cells, less CD11b+CD163+ and more double negative cells were present. However, the percentages of LPS stimulated macrophages expressing CD11b or CD163 were not significantly altered by glucose availability (Fig. 9A,B). The percentage of macrophages expressing neither of the molecules tended to increase with higher glucose concentrations (Fig. 9C). Expression densities of CD11b or CD163 were not significantly influenced by media glucose concentration in control, neither in LPS stimulated macrophages (Fig. 10).
Figure 9: Impact of glucose availability on the phenotype of monocyte-derived macrophages. CD14+ monocytes were cultured for 4 d at 37°C and 5% CO₂ in DMEM medium containing different concentrations of glucose. At day 3 one sample per animal and concentration was stimulated with LPS. Percentages of viable macrophages expressing the surface molecules CD163 and CD11b were assessed by membrane immunofluorescence. Single data points and means ± SEM are shown. * P < 0.05 in Tukey post-test.
Figure 10: Impact of glucose availability on the expression density of CD11b and CD163 on monocyte-derived macrophages. CD14+ monocytes were cultured for 4 d at 37°C and 5% CO₂ in DMEM medium containing different glucose concentrations. At day 3 one sample per animal and concentration was stimulated with LPS. Expression densities (MFI: mean immune fluorescence) of CD163 and CD11b on the different macrophage populations were assessed by flow cytometry. Single data points and means ± SEM are shown.

5.2.3.2. Cytokine production

For the assessment of glucose availability on functional properties of macrophages we investigated cytokine production of M0, M1 and M2 macrophages generated in media containing 1 g/L or 4 g/L glucose and after *E. coli* stimulation. Production of IL-6 was not detectable in cell culture supernatants. TNF-α concentration was not
influenced by the factors macrophage subset and glucose concentration in macrophages without *E. coli* addition (Fig. 11A). In *E. coli* stimulated macrophages TNF-α production was increased in M1 macrophages compared to M0 and M2 macrophages while production was not altered by glucose concentration (Fig. 11A). Production of IL-10 was slightly higher in M1 compared to M2 macrophages without *E. coli* stimulation (P < 0.05, Fig. 11B). After addition of *E. coli* M0 and M1 macrophages generated in 4 g/L glucose medium produced slightly higher amounts of IL-10 (glucose: P = 0.036, Fig. 11B).

**Figure 11:** Cytokine production of polarized macrophages generated under different media glucose concentrations. CD14+ monocytes were stimulated at day 3 of cell culture with IFN-γ and LPS for classically activated (M1) macrophages or with IL-4 and IL-13 for 24 h for alternatively activated (M2) macrophages or remained as control (M0). Cell culture supernatants were collected for M0, M1 and M2 macrophages generated in DMEM medium containing 1 g/L or 4 g/L glucose with or without *E. coli* stimulation for 6 h at day 4. Cytokine production of TNF-α (A) and IL-10 (B) was measured by ELISA. Differences between subsets in Sidak post-test are indicated by * (P < 0.05) or *** (P < 0.001).

### 5.2.4 Summary and Discussion

During the differentiation of monocytes to macrophages a higher media glucose concentration resulted in a higher percentage of CD11b+CD163- cells while the percentage of double positive cells decreased, indicating a shift towards a more proinflammatory phenotype. This is in agreement with the previously mentioned study
on murine macrophages which reports the induction of a proinflammatory phenotype by glucose transporter overexpression (Freemerman et al., 2014). Therefore, we might assume that bovine macrophages exhibit some common metabolic properties with their murine counterparts despite observed discrepancies in GLUT mRNA expression and glucose consumption (Manuscript 2). Furthermore, we assessed cytokine production to reveal functional impacts of glucose concentration on bovine macrophages. Glucose availability had no effect on TNF-α production and the biological relevance of the marginal increase in IL-10 production remains questionable. Previous results concerning the impact of glucose on monocyte and macrophage functions are also contradictory. While production of IL-1β in human monocytes and phagocytosis of *Pseudomonas aeruginosa* in human and mouse macrophages are dependent on glucose, phagocytosis of latex particles, zymosan or complement-coated sheep erythrocytes is not affected by glucose availability (Orlinska and Newton, 1993; Speert and Gordon, 1992; Wong et al., 1999). A recent study observed effects of glucose concentration on *in vitro* TNF-α production and phagocytosis of fluorescent beads in bovine neutrophils (Garcia et al., 2015). Whether further monocyte activities are influence by glucose availability and whether the *in vitro* experiments are comparable to the *in vivo* situation remains to be studied, especially considering differences between media glucose concentrations in *in vitro* studies and the low blood glucose concentration in cows.
6 Discussion

The high frequency of postpartal mastitis or metritis is a common problem in dairy farming. Negative energy balance and the redistribution of glucose towards the udder might alter peripartal immune responses by limiting the energy supply to immune cells. Monocytes are key innate immune cells which differentiate into macrophages and link innate and adaptive immunity. Therefore, the aim of this study was to investigate whether peripartal energy balance affects the number and the glucose uptake capacity of bovine monocyte subsets. Three questions were addressed regarding (1) the number of monocytes of each subset, (2) differences in glucose uptake and glucose transporter expression of monocyte and macrophage subsets and (3) impacts of the peripartal period.

6.1 Effects of energy balance on the number of classical, intermediate and nonclassical monocytes in peripartal dairy cattle

In the first manuscript the impact of different dietary energy levels and differences in BCS on the number of cM, intM and ncM in blood were examined. Cows in the group with lower BCS and higher postpartal dietary energy supply (BCS low group) exhibited higher numbers of intM at day +7 after parturition. Moreover cows in this group suffering from postpartal mastitis or metritis displayed higher numbers of all three subsets at this time point compared with healthy animals, while no differences in monocyte subset numbers were observed between healthy and diseased cows in the group with higher BCS and lower postpartal energy supply (BCS high group). As discussed in Manuscript 1 a similar increase in monocyte numbers has been described for several human diseases, the functional consequences, however, are not yet fully understood. Nevertheless, the question aroused why the increase in monocyte numbers in response to disease has not been observed in the BCS high group. The previously discussed studies do not discriminate different body mass indices within the patients. In humans however, obesity is generally linked to higher monocyte numbers and a more proinflammatory profile. Monocyte numbers,
Discussion

especially the number of ncM, correlate with body fat mass and body mass index, respectively (Rogacev et al., 2010; Yoshimura et al., 2015). Additionally, monocytes from obese humans are able to produce higher amounts of chemokines such as CCL2, CCL3 and CCL4 and in peripheral blood mononuclear cells a higher production of proinflammatory cytokines such as TNF-α, IFN-γ and IL1-β was observed (Bories et al., 2012; Neumeier et al., 2011). Moreover, proinflammatory M1 macrophages predominate in the adipose tissue of obese subjects which might be based on a failure of monocytes to differentiate into M2 macrophages (Bories et al., 2012; Kraakman et al., 2014). However, obese humans are not easily comparable to the high condition cows in our study, which were suffering from a postpartal energy deficit. The higher monocyte number in diseased low BCS cows might rather be linked to the lower fat mobilization in this group than directly to BCS. Adipose tissue hormones such as leptin and adiponectin might account for the differing immune responses (Demas and Sakaria, 2005; Kabara et al., 2014; Neumeier et al., 2011).

To ensure that the observed differences are based on the feeding regime and are not linked to different courses of disease, a stricter monitoring of disease symptoms, e.g. body temperature, bacterial cultures and a more frequent blood sampling in the week before and after parturition, when monocyte numbers were particularly elevated, would be desirable for further studies.

To investigate the mechanisms of the changes in monocyte counts we further assessed the expression of the adhesion molecules CD11a, CD11b, CD18, CD31, CD49d and CD62L on peripartal monocytes (Chapter 5.1). In summary, the elevations in monocyte numbers, the time-dependent increase at day +7 as well as disease-associated increase at day +7 in BCS low cows suffering from mastitis or metritis, were linked to a higher expression density of CD11a, CD49d and CD62L, while CD49d was the only molecule expressed with higher density in BCS low compared to BCS high animals and CD11b was the only molecule with differing expression levels between healthy and diseased BCS high cows. CD11a, CD11b, CD49d and CD62L are molecules mediating rolling, adhesion and crawling of monocytes. Whether the differences in their expression densities reflect an impaired or enhanced monocyte migration could not be answered in this study. For future studies, this question could be addressed by migration assays. Moreover, other mechanisms involved in migration must be considered. Each monocyte adhesion molecule binds to its corresponding molecule on the cell surface of endothelial cells,
these are e.g. Intercellular Adhesion Molecule (ICAM) 1 and 2 for LFA-1, ICAM-1 for Mac-1, VCAM-1 for VLA-4, PECAM for PECAM, P-Selectin glycoprotein ligand 1 and CD34 for L-Selectin (Carlos and Harlan, 1994; Gerhardt and Ley, 2015). Therefore, changes in endothelial cell adhesion molecule expression might also affect monocyte migration. Moreover, regulatory factors involved in monocyte migration such as cytokines or chemokines might be altered by parturition and postpartal diseases. Monocyte recruitment from the bone marrow is mainly dependent on CCL2 and other chemokines, e.g. CCL7, binding the CCR2 receptor (Shi et al., 2011; Shi and Pamer, 2011). In response to low TLR ligand concentrations, CCL2 is thought to be released by bone marrow mesenchymal stem cells (Shi et al., 2011). Its receptor, CCR2, is mainly expressed on classical CD14$^{\text{high}}$CD16$^{-}$/ Ly6C$^{\text{high}}$ monocytes (Ancuta et al., 2003; Shi et al., 2011), which emigrate from the bone marrow and may develop to nonclassical CD16$^{+}$/ Ly6C$^{\text{low}}$ monocytes in the blood (Ancuta et al., 2009; Sunderkotter et al., 2004). The migration of monocytes into tissues may then be mediated by CCL5 binding to CCR1 and CCR5 on CD16- monocytes (Weber et al., 2001) or fractalkine and its receptor CX3CR1 on CD16+ monocytes (Ancuta et al., 2003). Moreover, recruited neutrophils may trigger subsequent migration of monocytes into the inflammatory tissue by releasing their intracellularly stored granula (Soehnlein and Lindbom, 2010). In cattle, CCL5 predominantly induces migration of classical monocytes, while neutrophil degranulation products trigger intM to migrate and upregulate their CD11a and CD31 expression (Hussen et al., 2014; Hussen et al., 2015).

### 6.2 Glucose uptake and glucose transporter expression in bovine monocyte subsets, subset-derived macrophages and polarized macrophages

To assess glucose uptake and glucose transporter expression of bovine monocytes and macrophages we performed a functional assay using the glucose fluorescent analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (2-NBDG) and determined the expression of GLUT1, GLUT3 and GLUT4 transporters on mRNA level. The use of a glucose fluorescent probe was adapted from studies with human cells and several pretests were performed to determine the experimental 2-NBDG
concentration and time-span (Manuscript 1). Moreover, the inhibition of 2-NBDG uptake by different sugars and GLUT inhibitors was investigated to ensure that 2-NBDG uptake into monocytes was specific for glucose transporters, observing a 20% inhibition by 32 mmol/L D-Glucose, a 55% inhibition by 100 µmol/L Phloretin and a 35% inhibition by 10 µmol/L Cytochalasin B (data not shown). The relatively moderate effect of D-Glucose is probably based on a higher transporter affinity of 2-NBDG, while the inhibition of 2-NBDG uptake by Phloretin and Cytochalasin B is comparable to their effects on the uptake of radiolabeled 2-Deoxy-D-glucose via bovine GLUT1 (Bentley et al., 2012). As D-Fructose did not affect 2-NBDG uptake into monocytes, GLUT5, which exhibits a high transport affinity for fructose (Zhao and Keating, 2007), was not included in the PCR analysis although it has been described on macrophages (Fu et al., 2004; Malide et al., 1998). Expression levels of GLUT1, GLUT3 and GLUT4 were then investigated using real-time PCR analysis with self-designed primers, their specificity was verified by sequencing the PCR products (Manuscript 2).

Among bovine monocyte subsets cM and intM took up more glucose compared to nonclassical monocytes (Manuscript 1) while only GLUT3 but not GLUT1 expression decreased from cM to ncM with intermediate expression levels in intM (Manuscript 2). After differentiation of monocyte subsets into macrophages glucose uptake capacity remained highest in cM, was intermediate in intM and lowest in ncM. However, expression levels of GLUT1 and GLUT3 were opposing to glucose uptake (Manuscript 2). Regulatory modifications on protein level which can account for these differences such as intracellular storage, protein turnover rate and structural modifications are discussed in Manuscript 2. Considering these mechanisms it would be helpful to investigate expression of GLUT1 and GLUT3 on protein level. The low number of monocyte subsets and subset-derived macrophages hindered detection of glucose transporters by Western Blot analysis and bovine specific antibodies were not available for flow cytometry. For future studies it would be desirable to directly assess the amount of GLUT proteins on the cell surface of monocytes and macrophages and to test whether they may be stored intracellularly as described for human monocyte-derived macrophages (Malide et al., 1998). Moreover, the low affinity GLUT5 transporter, which has been described in macrophages (Fu et al., 2004; Malide et al., 1998), might contribute to 2-NBDG uptake in vitro, however the in
*vivo* importance of GLUT5 is questionable due to its low affinity for glucose transport (Zhao and Keating, 2007).

In manuscript 2 we provided evidence that GLUT4 expression is negligible on bovine monocytes and macrophages and that bovine monocytes and macrophages are not insulin-responsive. Therefore it seems improbable that the low mRNA expression is based on the intracellular storage of GLUT4 in vesicles (Bryant et al., 2002). This is in line with the observation from peripartal monocyte samples, where the higher prepartal insulin concentrations in the BCS high group (Chapter 2.3) are not reflected by differences in glucose uptake capacity between both groups (Manuscript 1).

Furthermore, CD14+ monocytes were used to generate classically and alternatively activated macrophages. Classical activation was performed by IFN-γ and the TLR ligand LPS, while alternative activation was induced by IL-4 and IL-13 (Gordon and Taylor, 2005). Alternative activation resulted in an increase in glucose transporter expression and glucose consumption, while classically activated monocytes failed to upregulate glucose uptake or glucose transporter expression. This was in contrast to previous studies (discussed in Manuscript 2), therefore we additionally tested the effects of different media glucose concentrations on monocyte to macrophage differentiation and the ability of polarized macrophages to produce cytokines (Chapter 5.2). In our experiments a higher media glucose concentration induced higher percentages of CD11b+ positive cells which is in accordance with the increase in proinflammatory markers in studies using murine macrophages (Freemerman et al., 2014). IL-10 production was only slightly influenced by glucose availability. For cytokine production (Chapter 5.2) the cells were stimulated in the same manner as for the assessment of glucose uptake and GLUT transporter expression. In manuscript 2 we wondered whether M1 polarization might have been too weak to initiate metabolic changes despite of the effect on CD11b and CD163 expression, however, the high response of TNF-α production in M1 macrophages after *E. coli* stimulation supports the effectiveness of the proinflammatory activation. In contrast, a higher production of the antiinflammatory IL-10 was anticipated in M2 macrophages which showed a clearer polarization in membrane immunofluorescence (Manuscript 2). However, for a better evaluation of the polarization IL-12 production should be assessed to calculate the IL-12/IL-10 ratio which differs between M1 and M2 macrophages (Mosser and Edwards, 2008). Furthermore, the investigation of cellular
oxygen consumption and extracellular acidification rate as markers for OXPHOS and glycolysis might be helpful to assess whether bovine M1 and M2 macrophages resemble their murine and human counterparts in metabolic properties.

6.3 Effects of the peripartal energy balance on glucose uptake and GLUT transporter expression in bovine monocytes

We studied peripartal glucose uptake and GLUT transporter expression in 27 peripartal dairy cows with different dietary energy supply. However, the differences in BCS and dietary energy supply did neither evoke differences in glucose uptake nor in GLUT expression of monocytes. A decline in glucose uptake was observed for the CD14\text{high} subsets cM and intM after parturition with a minimum at day +21 (Manuscript 1). Numerically glucose uptake was higher in the BCS low group prior to parturition, resulting in a stronger decline, but there was no overall statistical effect of BCS. In the CD14 low subset (ncM) glucose uptake decreased only after day +7 (Manuscript 1). The time-dependent differences in glucose uptake of CD14\text{high} monocytes were not mirrored by changes in GLUT1 or GLUT3 expression between days -14 and +21, in contrast, the ratio of GLUT3/GLUT1 mRNA increased (Manuscript 2). The factor disease had no impact on monocyte glucose uptake (Manuscript 1) or GLUT expression (data not shown) of bovine monocytes. While no correlations were observed between energy balance and glucose transporter expression, a high lactose yield was associated with lower expression of GLUT1 and GLUT3 and a lower GLUT3/GLUT1 ratio (Manuscript 2). Therefore, we assumed that the redistribution of glucose towards the udder also influences monocyte glucose transporter expression. Lactose production is strongly correlated with milk yield, therefore GLUT expression tended to correlate to milk yield (data not shown) and the higher probability of high-yielding dairy cows to suffer from diseases (Fleischer et al., 2001) might be linked to a decrease in monocyte glucose transporter expression.

Mechanisms by which the reduced glucose availability might influence peripartal disease susceptibility remain to be studied. One possibility might be the modulation of monocyte subsets and macrophage phenotypes due to their different glucose requirements. As activation is a major impact factor on monocyte and macrophage energy requirements, it might be impaired by glucose deprivation. Further studies
regarding functional consequences are needed to answer these questions. Moreover, factors mediating activation and regulating cell metabolism are closely linked in monocytes and macrophages and should be considered. One important factor involved in the metabolic switch in M1 macrophages is Hypoxia-inducible factor 1α (HIF-1α) (Palsson-McDermott and O'Neill 2013).

6.4 Outlook: Regulation of the metabolic switch in immune cells

Hypoxia-inducible factor 1α, which is normally activated under hypoxic conditions but may be stabilized by succinate under normoxic conditions, mediates proinflammatory polarization of macrophages and contributes to the metabolic switch from oxidative phosphorylation to glycolysis in myeloid cells (Cheng et al., 2014; Cramer et al., 2003; Fujisaka et al., 2013; Tannahill et al., 2013). Following danger recognition the Akt-PI3K (Akt: Protein kinase B, PI3K: phosphatidylinositol-3-kinase) pathway is activated in immune cells, mTOR (mammalian target of rapamycin) is phosphorylated and induces HIF-1α, resulting in an upregulation of glucose uptake, glucose transporter expression and glycolysis (Cheng et al., 2014; Krawczyk et al., 2010; Ouro et al., 2013) (Fig. 12). The metabolic switch to glycolysis can be inhibited by adenosine monophosphate–activated protein kinase or by antiinflammatory cytokines, e.g. IL-10 (Krawczyk et al., 2010). Both, glycolysis and OXPHOS are promoted by the NOTCH signaling pathway, an important factor in M1 activation, this might explain the increase in both metabolic pathways in M1 macrophages (Xu et al., 2015). In alternatively activated macrophages, oxidative metabolism is promoted by PPARγ-coactivator-1β and the STAT6 (signal transducer and activator of transcription 6) pathway (Vats et al., 2006), and the seduheptulose carbohydrate kinase-like protein is involved in regulating glucose metabolism (Haschemi et al., 2012). As these studies were predominantly conducted in murine macrophages, it remains to be elucidated whether the signaling pathways are transferable to bovine monocytes and macrophages. However, targeting HIF-1α or other involved factors might yield in new understandings regarding the differences in glucose requirements of bovine monocytes and macrophages.
Figure 12: Signaling pathways involved in the metabolic switch from OXPHOS to glycolysis in monocytes and macrophages. Pathogen-recognition receptors (PRR) such as Toll-like or lectin receptors induce the phosphorylation of Akt by PI3K. Thereafter mTOR is phosphorylated and HIF-1α is activated. An increase in glucose transporter expression results in increased glucose uptake and a metabolic switch from mitochondrial respiration (TCA: tricarboxylic acid cycle, OXPHOS: oxidative phosphorylation) to glycolysis leads to an increased lactate production. Adenosine monophosphate–activated protein kinase (AMPK) hinders this switch by inhibition of mTOR.

6.5 Summary and closing remarks

In our study peripartal energy supply in dairy cows did neither predispose a dietary group for disease, nor affected glucose uptake or glucose transporter expression of monocytes. However, both dietary groups did differ in monocyte responses to clinical mastitis or metritis, regarding monocyte numbers and monocyte adhesion molecule expression. Moreover, milk yield affected monocyte glucose transporter expression by lactose production. Considering the different glucose requirements of monocyte subsets and polarized macrophages, postpartal glucose shortage might modulate the peripartal immune response by altering the activation of monocytes and their differentiation into macrophages. Considering the low blood glucose concentration in cattle as well as lower glucose availability in tissues, results from in vitro studies have to be evaluated carefully. Moreover, further factors, e.g. mammary epithelial cells, are involved in the immune response to pathogens. Therefore, the functional consequences of the obtained results remain to be studied.
7 References


<table>
<thead>
<tr>
<th>Reference</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheng, S.C., Quintin, J., Cramer, R.A., Shepardson, K.M., Saeed, S.,</td>
<td></td>
</tr>
<tr>
<td>Kumar, V., Giamarellos-Bourboulis, E.J., Martens, J.H., Rao, N.A.,</td>
<td></td>
</tr>
<tr>
<td>Aghajanirefah, A., Manjeri, G.R., Li, Y., Ifrim, D.C., Arts, R.J.,</td>
<td></td>
</tr>
<tr>
<td>van der Meer, B.M., Deen, P.M., Logie, C., O'Neill, L.A., Willems, P.,</td>
<td></td>
</tr>
<tr>
<td>van de Veerdonk, F.L., van der Meer, J.W., Ng, A., Joosten, L.A.,</td>
<td></td>
</tr>
<tr>
<td>B., Puel, A., Biswas, S.K., Moshous, D., Picard, C., Jais, J.P., D'Cruz,</td>
<td></td>
</tr>
<tr>
<td>Dietl, K., Renner, K., Dettmer, K., Timischl, B., Eberhart, K., Dorn,</td>
<td></td>
</tr>
<tr>
<td>C., Hellerbrand, C., Kastenberger, M., Kunz-Schughart, L.A., Oefner,</td>
<td></td>
</tr>
<tr>
<td>Dimitriadis, G., Maratou, E., Boutati, E., Psarra, K., Papasteriades,</td>
<td></td>
</tr>
</tbody>
</table>


References


References


References

E., Nizet, V., Whyte, M., Taylor, C.T., Lin, H., Masters, S.L., Gottlieb, E., Kelly, V.P., Clish, C.,
Auron, P.E., Xavier, R.J., O’Neill, L.A., 2013. Succinate is an inflammatory signal that induces
IL-1beta through HIF-1alpha. Nature 496, 238-242.

Trevisi, E., Amadori, M., Cogrossi, S., Razzuoli, E., Bertoni, G., 2012. Metabolic stress and

and clinical ketosis and association with production parameters in dairy cows in the

Vats, D., Mukundan, L., Odegaard, J.I., Zhang, L., Smith, K.L., Morel, C.R., Wagner, R.A., Greaves,

the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T(H)1-

Weinstein, J.R., Quan, Y., Hanson, J.F., Colonna, L., Iorga, M., Honda, S., Shibuya, K., Shibuya, A.,
Elkon, K.B., Moller, T., 2015. IgM-Dependent Phagocytosis in Microglia Is Mediated by

Wong, S.Y., Guerdoud, L.M., Cantin, A., Speert, D.P., 1999. Glucose stimulates phagocytosis of
unopsonized Pseudomonas aeruginosa by cultivated human alveolar macrophages. Infect
Immun 67, 16-21.

Xu, J., Chi, F., Guo, T., Punj, V., Lee, W.N., French, S.W., Tsukamoto, H., 2015. NOTCH reprograms
mitochondrial metabolism for proinflammatory macrophage activation. J Clin Invest 125, 1579-
1590.

Yoshimura, A., Ohnishi, S., Orito, C., Kawahara, Y., Takasaki, H., Takeda, H., Sakamoto, N., Hashino,
S., 2015. Association of peripheral total and differential leukocyte counts with obesity-related
complications in young adults. Obes Facts 8, 1-16.

 cows do not exhibit hepatic insulin resistance, yet adipose-specific insulin resistance occurs in

Neoplasia 19, 3-17.

Genomics 8, 113-128.

Prepartal dietary energy level affects peripartal bovine blood neutrophil metabolic, antioxidant,

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J., Liu, Y.J.,
MacPherson, G., Randolph, G.J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S.,
Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled “Effects of the peripartal energy balance of dairy cows on the functional capacity of monocytes and their differentiation to macrophages”.

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institutions: Department of Physiology and Immunology Unit, University of Veterinary Medicine Hannover.

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

X

Melanie Eger
Acknowledgements

Finally I would like to thank all the people who accompanied me during the last three years and/or were directly involved in the development of this thesis.

Special thanks go to:

...my first supervisor Prof. Dr. Gerhard Breves for warmly welcoming me in the working group, for offering me the opportunity to work on this fascinating subject and for his support.

...Prof. Dr. Hans-Joachim Schuberth for providing my "second home" in the Immunology Unit, for the possibility to work in the labs, new inspirations, reflection and critical evaluation of all written drafts.

...Prof. Dr. Dr. Sven Dänicke for offering us the opportunity to obtain blood samples from peripartal dairy cows, new ideas for data evaluation and discussion.

...Dr. Jamal Hussen for the introduction into immunology, MACS, MIF and flow cytometry. For answers on all immunological questions, great ideas and assistance whenever problems occurred.

...Dr. Mirja Koy for her knowledge and her guidance in all parts of the real-time PCR analysis.

...Udo Rabe and Silke Schöneberg for constantly supplying all the materials we needed in the lab, the MIF, and for helping to separate the huge amount of blood samples from Braunschweig.

...Furthermore, I want to thank all members of the Institute for Animal Nutrition of the FLI Braunschweig who were involved in the dairy cow trail, especially Caroline Drong, who collected all the data and provided us with all necessary information about the animals, and Dr. Jana Frahm for forwarding the leukocyte counts.
...Dr. Mirja Wilkens for the support regarding statistical questions and regression analysis with SPSS.

...my colleagues in the immunology unit: Dr. Johanna Rautmann and Dr. Christine Gesterding especially for the trips to Braunschweig, collecting and separating hundreds of blood samples. Dr. Annika Bogusch for the tea breaks, sharing success and disappointment, collecting blood samples together and watching bacteria. Laura Rohmeier for the ELISA assistance and for funny days in Vienna.

...my great colleagues Kristin Elfers, Dr. Gesine Herm, Tanja Krägeloh, Lisa Marholt, Patrick Lange, Dr. Stefanie Klinger and Dr. Susanne Riede for providing essential tips for life, highly intellectual discussions, sharing harm and fun, sports, regularly coffee and tea breaks, for maintaining the "mensa tradition" and leisure activities. It was a wonderful time!

...and last but not least: Snowy, Bacardi, Rosi, Laura, Blacky, Milka and all other voluntary and involuntary blood donors.