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Regulation of bovine corpus luteum expressed galectins and their immunoregulatory potential

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Cesaret insani zafere, kararsizlik tehlikeye,

korkaklik ise ölüme götürür.

Yavuz Sultan Selim

Peter´e

Anneme, Babama ve Pinar´a

To Peter and my family

Content

Table of used abbreviations

20α-HSD	20α-hydroxysteroid dehydrogenase
Ang II	Angiotensin II
Aqua dest.	Aqua destillata
Aqua bidest.	Aqua bidestillata
Aqua tridest.	Aqua tridestillata
bMdM	Blood monocyte derived macrophages
CRDs	Carbohydrate-recognition domains
R^2	Certainty-measure for the computation of the quality of the RT-PCR melt curve
cDNA	Complementary DNA
Con A	Concanavalin A
CL	Corpus luteum, corpora lutea
r	Correlation coefficient
Ct	Cycle threshold
DCs	Dendritic cells
DNA	Desoxyribonucleic acid
DMF	N,N-Dimethyl-Formamide
EDN-1	Endothelin-1
E. coli	Escherichia Coli
EDTA	Ethylenediaminetetraacetic acid
E	Estrogen
ECM	Extra cellular matrix
FCS	Fetal calf serum
Fig.	Figure
FACS	Flourescence-Activated Cell Sorting
FL-1,-2,-3	Measure channels of the flow cytometer for emitted fluorescence FL-1 = Green fluorescence, 530 ± 15 nm; FL-2 = Orange fluorescence, 585 ± 21 nm; FL-3 = Red fluorescence, > 650 nm
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
Gal	Galectin
°C	Grad Celsius
g	Gram
GH	Growth hormone
HF	Holstein-Friesian

h	Hour(s)
hCG	Human chorion gonadotropin
Ig	Immunglobulin
JC-1	Fluorochrome 5,5',6,6'-Tetrachloro-1,1',3,3'-Tetraethylbenzimidazol-Carbocyanine iodide
MIF	Indirect membrane immunofluorescence
IFNγ	Interferon gamma
IFNτ	Interferon Tau
IL	Interleukin
Iono	Ionomycin
kDa	Kilo dalton
LBP	Lipid A binding protein
LPS	Lipopolisaccharide
l	Liter
LH	Luteotrophic hormone
MACS	Magnetic activated cell sorting
MHC-II	Major histocompatibility complex class II molecule
M	Medium
mRNA	Messenger ribonucleic acid
µ	Micro
µl	Micro liter
µmol	Micromol
mmol	Millimol
ml	Milliliter
mm	Millimeter
mM	Millimolar
min	Minute
mol	Mol
MCP-1	Monocyte chemoattractant protein-1
MNCs	Mononuclear cells
ng	Nanogram
NET	Neutrophil extracellular traps
NO	Nitric oxide
OT	Oxytocin
qRT-PCR	Quantitative reverse transcriptase PCR
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

Taq-polymerase	Polymerase of bacteria *Thermophilus aquaticus*
p.c	Post conception
p.ov.	Post ovulation
P_4	Progesterone
PJ	Propidiumjodid
PGF	Prostaglandin
PGFM	13,14-dihydro-15-keto prostaglandin F2 alpha
PMA	Phorbol-12-myristate-13-acetate
RT	Room temperature
RNA	Ribonucleic acid
Tab.	Table
n=	Number of single observations
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor alpha
s	Second
SSC	Side-scatter
SEA	Staphylococcus aureus Enterotoxin A
SEM	Standard error of the mean

1 Introduction

Reproductive inefficiency is one of the main economic problem in dairy production, particularly during the early embryonic development period. The maintenance of early pregnancy requires a fully functional corpus luteum (CL) that is not susceptible to regression following fertilization (NISWENDER et al. 1994). The regression of the CL has clear features of an inflammatory process, which involves major changes in tissue composition (NEUVIANS et al. 2004). After induced luteolysis in the bovine CL, histological signs of luteal cell degeneration (JUENGEL et al. 1993), some vacuolization and necrotic processes were seen and it has also been determined that many cells show the characteristics of apoptotic cell death (SCHAMS et al. 2003). In the ovary, the number of T-lymphocytes and macrophages is significantly increased during CL regression in many species (PATE and LANDIS KEYES 2001). These lymphocytes and macrophages are a known source of cytokines (e.g. Tumor Necrosis Factor α, TNF α) that participate in the regression process. It is not known which regulatory pathways or which factors govern the immigration of immune cells and the release of such CL regression-enhancing cytokines.

In this thesis it is hypothesized that galectins affect the process of luteolysis since these lectins, belonging to a family of mammalian β-galactoside-binding proteins, regulate cell growth, differentiation, apoptosis, and cellular attachment and are involved in pathological events such as inflammation and tumor metastasis. To date, significant expressions of galectin-1, -3, -7 and -15 were detected in the genital system. Galectin-1 and -3 were found in mouse ovary throughout non-pregnant, pregnant, and postpartum conditions (NIO and IWANAGA 2007). Galectin-1 and -3 have also been thought to play an important role in homeostasis of the inflammatory response (RABINOVICH et al. 2002a). Galectin-1, for instance, is known to skew an inflammatory Th1-dominated response towards an anti-inflammatory Th2-type response (TOSCANO et al. 2006) by blocking the secretion of pro-inflammatory cytokines in Th1 cells (RABINOVICH et al. 1999a). Furthermore, galectin-1 controls cellular proliferation and the survival rate of murine effector T-cells (BLASER et al. 1998), antagonizes the T-cell activation (CHUNG et al. 2000).

Galectin-3 may contribute to regulation of the Th1/Th2 response. ZUBERI et al. (2004) noticed that galectin-$3^{-/-}$ mice showed a lower Th2 response but a higher Th1 response compared to galectin-$3^{+/+}$ mice. In addition, galectin-3 promotes T-cell growth and proliferation in human (YANG et al. 1996). It has also been described that galectin-3 modulates tumor progression (LAHM et al. 2001).

Both, galectin-1 and galectin-3 regulate the viability of cells and are involved in the process of apoptosis and necrosis. Galectin (gal)-1 induces apoptosis in human thymocytes

(PERILLO et al. 1997) and activated T-cells (PERILLO et al. 1995) *in vitro*. Gal-3 shows pro- or anti-apoptotic activities (TOSCANO et al. 2006). Exogenous gal-3 directly induces T-cell death in humans (FUKUMORI et al. 2003) while the endogenous gal-3 protects T-cells from apoptosis (MATARRESE et al. 2000).

Until now, there is few information about galectin expression in the bovine species. The objective of this study is to enhance the knowledge about the expression of galectins during estrous cycle and early pregnancy in the bovine corpus luteum. In addition it is intended to characterize the expression of gal-1 and gal-3 in immune cells and to characterize their immuno-modulatory potential for bovine immune cells *in vitro*.

2 Literature Review

2.1 Overview of cyclic changes of the corpus luteum

The ovarian cycle is characterized by repeated events of cellular proliferation, differentiation and transformation. It consists of follicular development, ovulation and establishment and regression of the CL. The CL is a temporary reproductive structure that produces progesterone (P_4), required for the development and the maintenance of pregnancy (SCHAMS and BERISHA 2004). Ovulation and constitution of the corpus luteum are under gonadotropic control. During the follicular phase the release of estrogens (E) from preovulatory follicles induces a release of luteinizing hormone (LH) from the anterior pituitary. A positive feedback mechanism between E and LH leads to an LH surge followed by the rupture of the follicle (MCCRACKEN et al. 1999) and the differentiation of follicular cells into luteal cells (SCHAMS and BERISHA 2004). The luteinizing hormone is necessary for establishment of a functional CL maintenance of P_4 secretion during the luteal phase (PETERS et al. 1994).

The growth phase, which is also known as luteinization begins with ovulation of the dominant follicle (ROSIANSKY-SULTAN et al. 2006). The bovine CL develops within 2-3 days after ovulation, supported by angiogenesis and vascularization and stays functional for 17-18 days in non-pregnant cows (MIYAMOTO and SHIRASUNA 2009). After ovulation the lumen of the CL is filled by coagulated blood. Therefore, it is called corpus haemorrhagicum (SKARZYNSKI et al. 2005). The first 5 days of luteinization are characterized by lack of responsiveness of the luteal cells to luteolytic effective dose of Prostaglandin F2 alpha ($PGF_{2\alpha}$). This lack of $PGF_{2\alpha}$ responsiveness can be due to a inadequacy in number or affinity of $PGF_{2\alpha}$ receptors in the early CL (WILTBANK et al. 1995). The growth phase is followed by the static phase with maximum P_4 secretion. At this time period there is further progress of luteinization, the colour of the CL turns from red to yellow-orange and the vascularization of the tissue increases (IRELAND et al. 1980). In non-pregnant cattle the regression phase starts between days 16 and 18 post ovulation (p.ov.) with regression of luteal cells and vessels (NISWENDER and NETT 1994). The luteolysis is triggered by the pulsatile secretion of $PGF_{2\alpha}$ from the endometrium (BERISHA and SCHAMS 2005). $PGF_{2\alpha}$ induces a decrease in P4 release from the CL as well as a decrease in the CL volume and blood flow to the CL (NISWENDER et al. 1976; ACOSTA et al. 2002).

Histologically, the CL is a heterogeneous tissue, which consists of endothelial cells, large and small luteal cells, fibroblasts, smooth muscle cells and pericytes (O'SHEA et al. 1989) as well as immune cells such as monocytes, macrophages, lymphocytes and neutrophilic granulocytes (neutrophils) (PENNY 2000). The steroidogenic cells, particularly large luteal cells, produce and secrete a large amount of P4 during the estrous cycle in the cow (RODGERS et al. 1988;

MEIDAN et al. 1990). Vascular and luteal endothelial cells secrete several vasoactive substances, such as nitric oxide (NO), endothelin-1 (EDN1), angiotensin II (Ang II) and PGs, which regulate P4 secretion within the CL (MIYAMOTO et al. 1993; GIRSH et al. 1996a; GIRSH et al. 1996b; MIYAMOTO et al. 1997; HAYASHI et al. 2000). Therefore, blood vessels and endothelial cells within the CL play an essential role in luteal function in the cow (MIYAMOTO and SHIRASUNA 2009).

The luteolysis is divided into two phases: during the first phase the functional luteolysis occurs with a drastic decline in P_4 synthesis after the release of $PGF_{2\alpha}$ (STOCCO et al. 2007). This leads to a significant reduction of plasma P_4–concentration within 12 hours (NEUVIANS et al. 2004). The secretion of $PGF_{2\alpha}$ appears to be controlled indirectly by oestradiol-17ß, which is produced in ovarian granulosa cells of developing follicles and luteal oxytocin (OT) which is released synchronously by uterine $PGF_{2\alpha}$ (MCCRACKEN et al. 1999). In the second phase, which is called structural luteolysis, the programmed cell death (apoptosis) occurs in luteal cells causing a distinct loss of luteal tissue (STOCCO et al. 2007; SHIRASUNA et al. 2008). Many immune cells, e.g. CD4+ and CD8+ T-lymphocytes, macrophages and eosinophils can be found even before and during luteolysis (PENNY et al. 1999) and play important roles in the regression via releasing of tumour necrosis factor alpha (TNFα) and many other cytokines (BENYO and PATE 1992). For instance monocyte chemoattractant protein 1 (MCP-1) is one of the main actors in the structural luteolysis by augmenting the migration of monocytes, macrophages and CD4+ and CD8+ T- lymphocytes into the corpus luteum (MCCRACKEN et al. 1999). It is mentioned that lysosomal enzymes might play a degenerative role associated with the structural regression of corpus luteum. Moreover changes in the lipid structure of the plasma membrane of luteal cells were reported (BUHR et al. 1979). Additionally the vessels are replaced by collagen fibers, fibroblasts and macrophages, so that only a scar in the stroma of the ovary called corpus albicans remains visible (STOCCO et al. 2007). BERISHA et al. (2010) noticed that the structural luteolysis occurs 12 hours after the beginning of the functional luteolysis and completes in the subsequent 2-3 days (ALACAM et al. 2005).

If the ovulated oocyte is not fertilized, a new cycle will start and luteolysis will occur again. However, if it is fertilized, the luteolytic process must be blocked to maintain pregnancy. The blockade of luteolysis depends on the strength of antiluteolytic signals (BINELLI et al. 2001). It was shown that E_2 injections stimulate $PGF_{2\alpha}$ secretion (THATCHER et al. 1986). Hence blocking of luteolysis may involve inhibition of E_2 production (PRITCHARD et al. 1994). It is obvious that E_2 plays a central role on the luteolytic process. For this reason, it is important that high E_2 levels are maintained during the assumed time of luteolysis (BINELLI et al.

2001). Additionally, recognition of pregnancy in cows seems to depend on P_4 concentrations. It has been demonstrated that inseminated and pregnant cows have higher P_4 milk concentration compared to inseminated and not pregnant cows (BULMAN and LAMMING 1978; LAMMING et al. 1989; MANN et al. 1999). The most important anti-luteotrophic substance is Interferon tau (IFN-τ). It is produced by trophectoderm cells of blastocysts (ROBERTS et al. 1990) and interacts with the by endometrium blocking $PGF_{2\alpha}$ synthesis (BINELLI et al. 2001) preventing the up-regulation of E receptor-α and oxytocin (OT) receptor (SPENCER and BAZER 1996; SPENCER et al. 2007), activating a cyclooxygenase inhibitor, and shifting from a luteolytic $PGF_{2\alpha}$ to a more luteoprotective affecting PGE_2 within the uterine PG-synthesis (HANSEN et al. 1999). ROBINSON et al. (2006) indicated that IFN-τ has been expressed during the elongation of the conceptus in the bovine trophoblast giant cells. IFN-τ production increased between days 14 and 18 in the cow. IFN-τ stimulated the positive effect of $IL_{1\alpha}$ on PGE_2 secretion and P_4 production (MAJEWSKA et al. 2010).

2.2 The role of leukocytes in the luteolytic process

The cells that constitute the CL are present within the luteal tissue and are thought to influence luteal function and/or integrity (LOBEL and LEVY 1968; MURDOCH 1987; PENNY et al. 1998; PENNY et al. 1999; TOWNSON et al. 2002). Results from recent studies indicate that the chemokine MCP-1, is produced in the CL during luteal regression and might favor the attraction of monocytes which differentiate in the CL to macrophages that in turn, take part in the luteolysis process (BOWEN et al. 1996; TOWNSON et al. 1996; TSAI et al. 1997; PENNY et al. 1998; GOEDE et al. 1999; PENNY 2000).

MCP-1 is of particular interest because this chemokine enhances the attachment and migration of blood cells, monocytes and T-lymphocytes into sites of inflammation (MUKAIDA et al. 1992). Macrophages and T-lymphocytes were demonstrated to accumulate in the regressing CL of many species (HEHNKE et al. 1994; TOWNSON et al. 1996; NAFTALIN et al. 1997; GOEDE et al. 1999; PENNY et al. 1999; TOWNSON et al. 2002). Macrophages are involved in the phagocytosis of dying luteal cells (PAAVOLA 1979), in the degradation of extracellular matrix (ENDO et al. 1993), and the secretion of proinflammatory mediators which affect luteal steroidogenesis (BENYO and PATE 1992; RUEDA et al. 2000).

Leukocytes populate the corpora lutea of several species including humans (WANG et al. 1992; BRÄNNSTRÖM and NORMAN 1994; BEST et al. 1996), cattles (SPANEL-BOROWSKI et al. 1997; LAWLER et al. 1999; PENNY et al. 1999) and pigs (STANDAERT et al. 1991). They are present during the development of the bovine corpus luteum and its regression after $PGF_{2\alpha}$-induced luteolysis (MURDOCH 1987; CAVENDER and MURDOCH

1988). CD4- and CD8-positive T-cells are most abundant in the ovary at the onset of functional luteolysis compared to any other stages of the oestrous cycle (PENNY et al. 1999).

Some authors suggest that luteolysis is similar to inflammation (BAUER et al. 2001; NEUVIANS et al. 2004) but several factors contradict this hypothesis. For example, plenty of apoptotic cells are found on the site of luteolysis. But, two characteristic features of the inflammation (swelling and vasodilatation) are not observed (ACOSTA et al. 2002). Furthermore there is no inflammation in the surrounding tissue (NEUVIANS et al. 2004) due to the protection against inflammatory reactions of dead CL cells by resident immune cells (PATE and LANDIS KEYES 2001).

2.2.1 Lymphocytes

TOWNSON et al. (2002) examined the occurrence of lymphocytes in the bovine CL during days 6, 12 and 18 p. ov. In all three phases the T-lymphocytes could be found, however, during the days 12 and 18 p. ov in significantly higher numbers compared to day 6 p. ov The authors demonstrated that the increase of T-lymphocyte numbers as well as increasing numbers of macrophages correlate with the onset of luteolysis (TOWNSON et al. 2002). T cell- and macrophage derived cytokines like TNFα, IL-1β, and interferon-gamma (IFN-γ) suppress the P_4 synthesis in luteal cell cultures and can stimulate the $PGF_{2\alpha}$ production (SAKUMOTO and OKUDA 2004; SKARZYNSKI et al. 2005). Luteal cells can express MHC class II (MHC-II) molecules. Expression of the MHC II was demonstrated *ex vivo* on days 6, 10, 12 and 18 p.ov in the bovine CL with the highest gene expression measured on day 18 p.ov after administration of a luteolytic $PGF_{2\alpha}$ dose (BENYO et al. 1991). The authors assume that MHC-II molecules participate in the luteolysis in bovines, acting as restriction elements for probably autoreactive T-cells (PETROFF et al. 1997).

2.2.2 Monocytes and Macrophages

Macrophages are phagocytes which differentiate from monocytes after immigration into tissue (WU et al. 2004). During luteinization only a very low number of macrophages exist in the bovine CL (BAUER et al. 2001). In contrast, at day 12 and 18 p.ov monocytes and macrophages are (together with lymphocytes) the dominant leukocyte population in the bovine CL (TOWNSON et al. 2002).

The human CL contains more macrophages in the luteinization phase than in the regression phase (DUNCAN et al. 1998). In mares, the highest number of macrophages in the CL was demonstrated on days 16 and 17 p.ov (LAWLER et al. 1999). Macrophages which efficiently phagocytose cellular debris appear also during the structural luteolysis (PENNY 2000; KATO

et al. 2001; WU et al. 2004). Furthermore, the regressing corpus luteum contains an abundance of macrophages in cows (SPANEL-BOROWSKI et al. 1997). During late regression, 70% of all proliferating cells in the bovine CL are CD-14 positive macrophages (BAUER et al. 2001). In addition, the macrophages secret cytokines and chemokines (TNFα, IL-1β, IL-6 and IL-8) which affect the P_4 secretion and the survival of the luteal cells (BAUER et al. 2001; WU et al. 2004).

2.2.3 Neutrophilic granulocytes

In the early phase of an inflammation, particularly in the course of a bacterial infection and some cancers (DE LARCO et al. 2004; WAUGH and WILSON 2008), neutrophils are among the first cells migrating towards the site of inflammation, following chemotactic signals such as IL-8. They are highly specialized, short-lived phagocytes and their major task is to phagocytose and kill bacteria. In mares and rats, neutrophils participate in the luteolysis (AL-ZI'ABI et al. 2002). In mares, 12 and 36 hours after $PGF_{2\alpha}$ injection on day 12 p.ov neutrophils could be demonstrated in the CL (AL-ZI'ABI et al. 2002). In the rat, immunosuppressive doses of dexamethason blocked the function of neutrophils during luteolysis (WANG et al. 1993). Neutrophils are found during the whole oestrous cycle and the pregnancy in the rat ovary. Together with macrophages they constitute the largest immune cell population in the pregnant (day 10 p.ov) and pseudo pregnant (day 9 p.ov) rat CL (BRÄNNSTRÖM and NORMAN 1994). In women neutrophils, attracted by IL-8, are found in the wall of the dominant follicle during ovulation (ZEINEH et al. 2003).

2.3 Galectins

Lectins are proteins that bind to distinct sugar residues with high affinity and specificity and mediate many biological processes. They serve in a wide variety of cell-cell recognition, signaling, and adhesion processes and in intracellular targeting of newly synthesized proteins. Lectins are found in soluble form and on the outer surface of cells, where they initiate interaction with other cells (DRICKAMER 1995; POWELL and VARKI 1995; RINI 1995). Galectins are β- galactoside-specific lectins that bind to galactose-containing oligosaccharides with high specificity (HIRABAYASHI et al. 2002). They are found in animals, plants, fungi, protists, and prokaryotes and are expressed in a variety of cell types, including fibroblasts, ovary cells, epithelial cells, endothelial cells, dendritic cells, macrophages, bone marrow cells, T and B cells (KLYOSOV et al. 2008). Galectins are synthesized in the cytosol and released from the cell by an unorthodox secretory mechanism that bypasses the endoplasmic reticulum and the Golgi apparatus (COOPER 1997; MEHUL and HUGHES 1997). They function in an autocrine or paracrine manner by binding to and cross-linking selected glycoproteins or

glycolipids present on the cell surfaces. Galectins, as other lectins, do not appear to have specific individual receptors. Instead, they are able to bind to a number of different cell surface glycoproteins and glycolipids that carry suitable galactose-containing oligosaccharides. In mammals, fifteen different galectins were reported to date. All galectins contain conserved carbohydrate-recognition domains (CRDs) of about 130 amino acids responsible for carbohydrate binding (COOPER 2002). Based on their biochemical structure, galectins were classified into different types. Prototype galectins (gal-1, -2, -5, -7, -10, -11, -13 and -14) (HIRABAYASHI and KASAI 1993) exist as monomers or noncovalent bound homodimers of a CRD. The chimera-type gal-3 is composed of a non-lectin domain connected to a CRD. Tandem-repeat-type galectins (gal-4, -6, -8, -9 and -12) consist of two different CRDs in a single polypeptide chain. Galectins lack a signal sequence and reside mostly intracellulary (LIU et al. 2002). Galectins are detected in both the cytoplasm and the nucleus and appear to shuttle between these two compartments (DAVIDSON et al. 2002). In addition, galectins can be secreted by cells, likely through a nonclassical secretory pathway (HUGHES 1999). Extracellularly, galectins are capable of binding to glycoproteins and glycolipids that contain suitable galactose-bearing oligosaccharides. A number of different glycoproteins and glycolipids on the surface of different cell types were identified as ligands of galectins (DAVIDSON et al. 2002). Galectins show major cell/tissue specific expression pattern (NIO and IWANAGA 2007).

Galectins play a relevant role in cell-cell and cell-matrix interactions, neuronal cell differentiation and survival, embryonic development, growth and development, malignant progression, metastasis, angiogenesis, proliferation, apoptosis, pre-RNA splicing, and other crucial functions (explained below) (KLYOSOV et al. 2008). Their obvious importance for inflammation and suggested them as useful targets for the development of new anti-inflammatory and anti-cancer therapeutics (COOPER 1997; MEHUL and HUGHES 1997).

Modulation of cell adhesion

The bivalent or multivalent properties of galectins together with their ability to bind to cell surface glycoproteins make them suitable for modulating cell-cell adhesion (LIU 2005). In addition, galectins are known to bind to extracellular matrix proteins, including laminin (MASSA et al. 1993; ZHOU and CUMMINGS 1993; VAN DEN BRULE et al. 1995; KUWABARA and LIU 1996; HUGHES 2001), fibronectin (SATO and HUGHES 1992), elastin (OCHIENG et al. 1999) and hensin (HIKITA et al. 2000). Therefore, they can affect adhesion between cells and extracellular matrices. Since cell adhesion is prominently involved in the immune and inflammatory responses, it is highly conceivable that galectins

can affect various immune and inflammatory processes through modulation of cell adhesion (LIU 2005).

Activation of cells

The above mentioned multiple binding sites as well as the carbohydrate-binding activity of galectins make these proteins capable of cross-linking cell-surface glycoconjugates. Since cross-linking of cell surface proteins is often the first step in transmembrane signal transduction, galectins are expected to induce cell activation. Gal-1 and -3 act as positive and negative regulator of immune cell activation. Gal-3 can activate mast cells resulting in mediator release (FRIGERI et al. 1993; ZUBERI et al. 1994). Interestingly, while gal-3 was first known to bind to IgE (hence the name IgE binding protein), it was later found out also to bind to the high affinity IgE receptor (FcεRI) (FRIGERI et al. 1993). It is therefore, possible that gal-3 activates mast cells by cross-linking FcεRI-bound IgE, FcεRI, or both (LIU 2005). Gal-3 has also been shown to activate human neutrophils (YAMAOKA et al. 1995; KARLSSON et al. 1998). Treatment of neutrophils with LPS led to the mobilization of granular proteins to the cell surface making the cells responsive to gal-3 (ALMKVIST et al. 2001). The binding of neutrophils surface proteins by gal-1 and -3 has been compared and these two lectins were noted to recognize different proteins (ALMKVIST et al. 2002). Thus, it appears that they activate neutrophils through engaging two different sets of receptors (LIU 2005). Gal-3 can trigger human peripheral blood monocytes to produce superoxide anion (LIU et al. 1995) and potentiate their LPS-induced IL-1 production (JENG et al. 1994).

Chemoattraction

Migration of various immune cells induced by chemoattractants, such as chemokines, is essential in the development of immune and inflammatory responses (LIU 2005). Galectins were shown to be able to contribute to the regulation of immune cells migration. Human recombinant gal-1 was found to inhibit chemotaxis as well as trans-endothelial migration of PMN *in vitro* (LA et al. 2003). Gal-3 on the other hand was found to induce chemotaxis in human monocytes and macrophages *in vivo* and *in vitro* (SANO et al. 2000). Gal-9 has been found to be a potent eosinophil chemoattractant (MATSUMOTO et al. 1998).

Recognition of pathogens

The effect of some infectious processes on galectin expression and the possible roles of galectins in the response of the host to infections were described. Galectins can recognize glycans expressed by certain pathogens (LIU 2005). Gal-3 was found to bind to two sites in LPS from Klebsiella pneumonia (MEY et al. 1996). Another study showed that LPS from Pseudomonas aeruginosa bound to gal-3 apparently by involving the outer core portion of the former (GUPTA et al. 1997). Gal-3 was also found to bind to lipophosphoglycans of Leishmania major, through polygalactose epitopes (PELLETIER and SATO 2002). Gal-3 was found to accumulate in Mycobacterium - containing phagosomes in macrophages during the course of infection (BEATTY et al. 2002). *In vitro*, gal-3 can promote neutrophil adhesion to endothelial cells. Therefore, it was proposed that gal-3 plays an important role in the extravasation of neutrophils in the lung infection by streptococcal pneumonia (SATO et al. 2002b).

Galectins as auto antigens

Galectins can also participate in immune and inflammatory responses by functioning as auto antigens (LIU 2005). Auto antibodies to gal-1 were found in sera from patients with multiple sclerosis and the titers were significantly higher than in sera from healthy controls (LUTOMSKI et al. 1997). Auto antibodies to galectins have also been shown to be associated with neoplasm (LIU 2005). A subject with newly diagnosed adenocarcinoma of the colon was found to have a significantly elevated level of IgG anti-gal-3 (MATHEWS et al. 1995). The galectin family members are summarized in Tab. 1.

Tab. 1 Galectin family members of the mammalian lectins.

Galectin	Localization	Species	Source
Galectin-1	In most organs, lymph nodes, spleen, thymus, placenta, prostate, macrophages, B-cells, T-cells and dendritic cells tumors	Many mammals	RABINOVICH et al. 2002b
Galectin-2	Gastrointestinal tract, tumor cells	Human	STURM et al. 2004; GITT et al. 1992
	Hepatoma	Human	
Galectin-3	Mainly in tumor cells, macrophages, epithelial cells, fibroblasts and activated T cells	Many mammals	RABINOVICH et al. 2002a
Galectin-4	Intestinal epithelium	Rat	BARONDES et al. 1994
Galectin-5	Erythrocytes	Rat	GITT et al. 1995
Galectin-6	Gastrointestinal tract	Mouse	GITT et al. 1998
Galectin-7	Epithel cells	Human, Rat, Mouse	MAGNALDO et al. 1998
	Skin and tumors of epidermal origin	Human	BERNERD et al. 1999
Galectin-8	Liver, prostate, kidney, cardiac muscle, lung and brain	Rat	HADARI et al. 1995
Galectin-9	Thymus, T-cells, kidney	Mouse	WADA 1997
	Hodgkins lymphoma	Human	TURECI et al. 1997
Galectin-10	Eosinophils and basophils	Human	KUBACH et al. 2007
Galectin-11	Stomach	Sheep	DUNPHY et al. 2000
	Lens	Rat	OGDEN et al. 1998
Galectin-12	Adipocytes	Human	HOTTA et al. 2001
Galectin-13	Placenta	Human	THAN et al. 2004
Galectin-14	Eosinophils	Sheep	DUNPHY et al. 2002
Galectin-15	Uterus	Sheep, Goat	FARMER et al. 2008

As can be concluded from this table, galectin family members show a wide distribution in mammals. However, except for gal-1 and -3 up to now galectin gene expression has only been determined only in some distinct organs of specific mammals. Also, they were given other names then galectin, for instance gal-15 was found only in the ovine species and called OVGAL15. Interestingly authors suggested pivotal roles in different events of the inflammatory response under physiological and pathological conditions mainly for gal-1 and -3. It was implied that the opposite functions of these two galectins might balance the homeostasis of the inflammatory response (RABINOVICH et al. 2002a). Therefore the importance of these galectins is explained below.

2.3.1 Galectin-1

Within the immune system, gal-1 has been found in most relevant mammalian organs (lymph nodes, spleen, thymus, placenta, prostate, macrophages, B-cells, T-cells). Furthermore, Nio et al. determined that this type of galectin expresses intensely in the non-pregnant mouse ovary, especially in CL at the stage of regression. But under gestation with active P_4 production, gal-1 signal disappeared completely (NIO and IWANAGA 2007). Additionally, KALTNER et al. (2002) observed gal-1 and -3 expressions in the bovine respiratory and digestive tract of adult cattle and during fetal development.

Gal-1 shows a positive as well as negative effect on cell adhesion to extra cellular matrix (ECM) glycoconjugates. It induced cell adhesion in various cell types, such as human melanoma cell lines (VAN DEN BRULE et al. 1995) and olfactory neurons in rat (MAHANTHAPPA et al. 1994). On the other hand, the presence of gal-1 inhibited myoblast interaction with laminin by blocking laminin receptor (COOPER et al. 1991).

Gal-1 can trigger both proliferation or cell growth inhibition depending on the presence of required signals, cell cycle stages or the expression of its carbohydrate receptors on the cell surface (ADAMS et al. 1996).

Gal-1 induces apoptosis via the extracellular way on human thymocytes and activated peripheral T-cells (only Th1 cells, not in Th2 cells) (PERILLO et al. 1995; PERILLO et al. 1997). It acts as a immuno-suppressive with regard to the T-cell function and skewed the balance from a Th1- toward a Th2-dominated immune response in different experimental models of chronic inflammation, autoimmunity and cancer (RABINOVICH et al. 1999b; SANTUCCI et al. 2000; BAUM et al. 2003; SANTUCCI et al. 2003; RUBINSTEIN et al. 2004; PERONE et al. 2006; TOSCANO et al. 2006). In addition, recombinant gal-1 was able to inhibit the secretion of pro-inflammatory cytokines, such as TNFα, IFN-γ and IL-2 by

doing this, to augment IL-5, -10 and TGFß from activated T-cells (RABINOVICH et al. 2007).

BLOIS et al. (2007). suggested that gal-1 has an important role in fetomaternal tolerance. They showed gal-1 treatment to diminish the rate of stress-induced fetal loss and related it to the effect of gal-1 on Th1/Th2 balance and P_4 production in human

2.3.2 Galectin-3

Up to now gal-3 has been found in tumor cells, macrophages, epithelial cells, fibroblasts and activated T cells. Similar to gal-1, gal-3 is expressed very intensively in non-pregnant mouse ovary but elicits no signal in the ovaries of pregnant animals (NIO and IWANAGA 2007). Also, KIM et al. (2008) proved that gal-3 is expressed in the bovine ovary, oviduct, uterus and cervix. In mice gal-3 was found in high concentration in the cyclic CL. However, it has not been detected in a way in the early pregnancy period.

While gal-3 increases adhesion of neutrophils to laminin (KUWABARA and LIU 1996) in the inflammation site, it showed an inhibitory effect on melanoma cell adhesion to ECM in metastases (OCHIENG et al. 1998).

Gal-3 is the only family member that has both pro- as well as anti-apoptotic activity (RABINOVICH et al. 2002a). Cytoplasmic gal-3 opposes to apoptosis by associating with the mitochondrial membrane via antagonizing the release of cytocrome c (YANG et al. 1996; MATARRESE et al. 2000). Also, gal-3 stimulates DNA synthesis and prevents apoptosis of other cell types, such as human fibroblasts and murine granulocytes (INOHARA et al. 1998; HSU et al. 2000). On the other hand extracellular gal-3 directly induces apoptosis in human leukemia cell lines, human peripheral blood MNCs, activated mouse T-cells (FUKUMORI et al. 2003) and in neutrophils (FERNANDEZ et al. 2005).

It has also been shown to serve like a chemokine in inducing migration of human monocytes and macrophages (SANO et al. 2000) to the site of inflammation. Gal-3 suppresses the production of IL-5 in human eosinophils and T-cells, and of IL-12 in dendritic cells (DCs). Furthermore, it induces the secretion of IL-1 from human monocytes, IL-2 from T-cells and IL-8 from neutrophils (RABINOVICH et al. 2007).

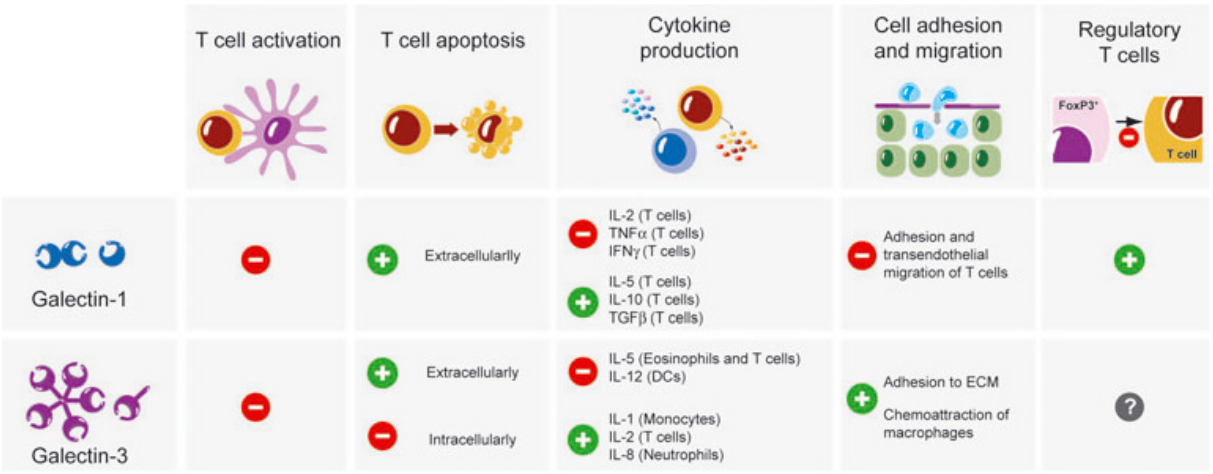

Fig. 1 Role of the gal-1 and -3 in the development and resolution of inflammatory responses (RABINOVICH et al. 2007, modified).

2.3.3 Galectin-15

Gal-15 is expressed specifically by the endometrial luminal epithelium of the ovine uterus and related with blastocyst growth, elongation, and implantation. It regulates blastocyst development, as well as growth, migration, adhesion, and apoptosis of trophoblasts (FARMER et al. 2008). LEWIS et al. (2007) could prove endometrial gal-15 mRNA was higher in pregnant than cyclic goats on Days 17 and 19

2.3.4 The role of galectins for ovary and corpus luteum physiology

Significant expressions of gal-1, -3, -7 and -15 were detected in genital system of several species. The mouse uterus and ovary express gal-1, as shown by Northern and Western blot analyses (CHOE et al. 1997). Although cultured porcine granulosa cells were reported to contain gal-1 (WALZEL et al. 2004), cell types expressing gal-1 in the ovary *in vivo* have not been identified. In cattle, gal-3 was immunolocalized in intersititium-residing macrophages, in cells of the atretic follicles, and in luteal cells of the regressing CL, but not in the growing follicles (KIM et al. 2008). Gal-3 is strongly detected in the regressing corpus luteum of the non-pregnant mouse ovary as well, and disappears completely during gestation with active P_4 production (NIO and IWANAGA 2007). SATO et al. (2002) asuggested, that a ovarian stromal cells constitute a cellular source of gal-7 (epidermal type of galectin), which also act as a growth factor in ovarian follicles (LOHR 2005).

In the CL gal-1 was found more intensely expressed at diestrus than at metestrus, and the highest expression level was detected in the newly formed mouse CL at the starting point of the functional regression stage with an increasing expression of 20α-HSD (NIO and

IWANAGA 2007). When the changes of galectin expression were examined in association with weaning, gal-1 expression always preceded these of gal-3/20α-HSD in CL at the beginning of luteolysis (Nio and Iwanaga, unpublished data). This finding suggests that gal-1 may play a leading role at the initiation of the functional regression in CL (NIO and IWANAGA 2007). CHOE et al. (1997) also describes the expression of gal-1 in the uterus to be regulated by the ovarian steroids, P_4 and E.

At the early stage of CL regression, 20α-HSD is expressed in luteal cells to catalyze P_4 to a biologically inactive form (20α-dihydroxyprogesterone). The a later stage it induces apoptosis of luteal cells, resulting in atrophy and disappearance of murine CL (KOMATSU et al. 2003). Because gal-3 was simultaneously expressed with 20α-HSD in the regressing CL but not expressed in CL with a high activity of P_4 production, gal-3 may be involved in the functional regression of CL.

3 Equipment, material and methods

3.1 Equipment

Absolute plant bank Laminair HL2448	Heraeus-Christ, Hanau
Aqua dest. Processing plant reversion-Osmosis plant, „type RO 50/14SMB type I“	Water and Regeneratestation, Barsbüttel
Aqua tridest. Processing plant „SG Absolute water system type SG-RS90-4 UF“	Water- und Regeneratestation, Barsbüttel
Autoclave type GE406	Getinge AB, Getinge/Schweden
Bio photometer	Eppendorf, Hamburg
Centrifuge„Megafuge 1.0R“	Heraeus Instruments, Osterode
Centrifuge Multifuge 1 S-R	Thermo Scientific, Osterode
Couting chamber to Bürker	Brand, Wertheim
Deep-freeze - 95°C	Kendro, Hanau
Die for Corpus luteum	Hoffmann, Frankreich
Flexible-tube pump to vacuum from fluid	Heidolph 52100, Schwabach
Forceps, circumflex, anatomical	Eickemeyer 170710, Tuttlingen
Heating block „Techne Dir-Block DB.3"	Thermo Dux, Wertheim
Hot air sterilisator „type ST5050“	Heraeus, Hanau
Ice machine type UBE 30-10	Ziegra, Isernhagen
Inkubator with CO_2, Baureihe 5060	Heraeus, Hanau
Laboratory scale„BL310“	Sartorius GmbH, Göttingen
MACS® Multi Stand Metal Column	Miltenyi Biotec, bergisch Gladbach
Magnetic stirrer with heating plate	Janke und Kunkel, Staufen
Microscope "Eclipse 80i"	Nikon Cooperation, Japan
MIDI MACS™ Separation-device	Miltenyi Biotec, bergisch Gladbach
Mini-Sub® Cell GT Gelelektrophorese-chamber	BioRad Laboratories GmbH, München
PCR Thermocycler "T-Gradient"	Biometra, medizinische Analytik GmbH, Göttingen
pH-meter „766 Calimatic“	Knick, Berlin
Photometer "BioPhotometer"	Eppendorf, Hamburg
Pipet animal aid „accu-jet®“	Brand 26404, Wertheim

Pipettes, adjustable „pipetman“ (1-20 µL, 10-100 µl, 20-200 µl, 100-1000 µl)	Gilson, Villers Le Bel/Frankreich
Plastikbox with application lattice	Retail
Plate centrifuge with Plate rotor (UJ II KS)	Heraeus-Christ. Osterode
Special accuracy weighing machine"TE 124 S"	Sartorius AG
StepOnePlus-PCR System	Applied Biosystems, CA, USA
Sterilbank	Kojair Tech Oy, Vilppula, Finnland
Table centrifuge "5415C"	Eppendorf, Hamburg
Transmigration chamber 10 wells; 400 µl/285 µl	Neuro Probe AA10, Gaithersburg, MD/USA
Transport container "Isotherm"	KGW, Deutschland
Vibraxer for Mikrotiterplatten „AM69 Microshaker“	Dynatec, Zug, Switzerland
Vortexer	IKA Werke, Staufen
Water bath with temperature controls „type 1003“	GFL, Hannover
Wet chamber	Retail
Wobble incubator RS 90-4 UF	SG Barsbüttel

3.2 Material

3.2.1 Clinic needs

Accumulation chain to Witte	Eickemeyer, 442015, Tuttlingen
Butterfly-canule „Micro-Flo™“ 21G 0.8mm	Industria Biomedica, AS2102, Milano, Italy
Disinfection solution Vetisept® 2%	Albrecht, Aulendorf
Cannulae 1.80 x 40 mm, sterile, TSK STERIJECT	TSK-Supra, Tochigi/Japan
Vacutainer Brand Luer adapters	Becton Dickinson, 367300, Heidelberg
Vacutainer® System, Holder	Becton Dickinson, Heidelberg
Vacutainer pipe, 10 mL, EDTA-K2 (18 mg)	Becton Dickinson, 367525, Heidelberg

3.2.2 Laboratory needs

24 well cell culture smooth ground plate with cover, sterile	Biochrom, P92965, Berlin
24 well flat top Cell culture plates with cover	Costar, Bethesda, USA
96 well cell culture smooth ground plate with cover, sterile	Biochrom, P92965, Berlin
96 well flat top-micro titre plate, Nunc Immuno Plate	Nunc, 363-401, Wiesbaden
Adhesive object holder "HistoBond®"	Marienfeld, 0810000, Lauda-Königshofen
Adhesive foil to cover PCR 96 well plate "Optical Adhesive Cover"	Applied Biosystems, 4360954, CA, USA
Centrifuge tube, 50 ml from Polypropylene (Falcons, sterile)	Corning, 430829, Wiesbaden
Centrifuge tube, 15 ml from Polypropylene (sterile)	Sarstedt, 62.554.502, Nürmbrecht
Vivaspin 500 µl and 2 ml, filter	Sartorius Stedim biotec, Goettingen
Combitips, 1.25 ml	Eppendorf, 0030069.420, Hamburg
Combitips, 2.5 ml	Eppendorf, 0030069.447, Hamburg
Cryo-Freezing-Container	Nalgen, Rochester, USA
Disposal bag	Brand, 759705, Wertheim
Eppendorf reaction container, 1.5 ml	Greiner, 616201, Frickenhausen
Eppi-Pistille from Polypropylene	Schuett-Biotec, 3200512,Göttingen
Glass bulbs	Assistent, Deutschland
Glass object holder	Marienfeld, Lauda-Königshofen
Holder for PCR 96 well plate "96-Well Support Base"	Applied Biosystems, 4379590, CA, USA
Labor bottle with thread, 500 ml	VWR international, 215L1516, Hannover
MACS Pre-Separation Filters	Miltenyi Biotec, 130-041-401, Bergisch Gladbach
MACS columns, LS	Miltenyi Biotec, 130-041-401, Bergisch Gladbach

Parafilm	American can Company, USA
Pasteur pipette, glass, 22.5 mm	Brand, 747720, Wertheim
PCR-Platte Micro Amp "Fast 96-Well Reaction Plate (0.1 ml)"	Applied Biosystems, 4346907, CA, USA
PCR-Reaction container 0.6 ml	Biozym Scientific, Hess. Oldendorf
PCR-Reaction container 200 µl	Biozym Scientific, Hess. Oldendorf
Petri dish 94 mm diameter	Greiner, 616222, Frickenhausen
Pipe for flow cytometry, 5 ml	Becton Dickinson, 352008, Heidelberg
Pipet tip "Safeseal Tips Premium" 10 µl	Biozym Scientific, 692150,, Hess. Oldendorf
Pipet tip "Safeseal Tips Premium" 100 µl	Biozym Scientific 692066, Hess. Oldendorf
Pipet tip "Safeseal Tips Premium" 1000 µl	Biozym Scientific 691000, Hess. Oldendorf
Pipet tip 200 und 1000 µl	Sarstedt 70/762002, Frickenhausen
Polycarbonate-membran 25 x 80 mm, 3 µm Pore size, PVP Membranes	Cytogen 155812, Ober-Mörlen
Polystyrene pipe	Nerbe Plus 62 161 0000 Winsen/Luhe
Serological pipetes, 10 ml, not pyrogen	Biochrom 94010, Berlin
Single-Bulbs 70 µl "UV-Bulb micro"	Brand 759200, Wertheim
Single-Filter, acetyl cellulose Membrane, 0.2 µm, steril	Renner 26146040, Dannstadt
Single-Pasteur pipettes from Pe-Ld	Merck 612F1767, Darmstadt
Surgical Scalpeles	Aesculap 5518075, Tuttlingen

3.2.3 Reagents

10x TBE, (Tris Boric Acid EDTA)-Puffer	Bio Rad Laboratories 161-0770, München
5x First-Strand-Buffer	Invitrogen, Carlsbad, CA, USA
Acridin-Orange	Sigma-Aldrich A-6014, Steinheim
Agarose	Life Technologies 15510-027, Paisely, Scotland
Albumin Fraction V, bovine, powder, 98%	Roth 8076.2, Karlsruhe

Ammoniumaluminiumsulat, 10%	Roth, Karlsruhe
Ampicillin	Sigma-Aldrich A-9518, Steinheim
Bromphenol blue (3´, 3´, 5´, 5´-Tetrabromhenol-sulfophtalin)	Severin 15375, Heidelberg
BSA (Bovines Serum Albumin)	Serva 11930 Deutschland
$C_6H_5Na_3O_7$ (Tri-Natriumcitrat, 2 x H_2O)	Serotec PBP 005, Oxford, UK
$C_6H_8O_7$ (Citric acid)	Riedel-de-Haen 27109 Seelze
CH_3COOH (Acetic acid)	Riedel de Haen, Seelze
$CH_4N_2O.H_2O_2$ (Urea-hydrogen-peroxide)	Grüssing 12149 Filsum
Concanavalin A (Con A)	Amersham Bioscience 17-0450-01 Freiburg
DAB (3.3`-Diaminobenzidin)	DAKO BioTek Solutions, Ca, USA
DMF (N,N-Dimethyl-Formalide)	Invitrogen, Carlsbad, CA, USA
DMSO (Dimethyl sulfoxide, C_2H_6OS (min. 99.5%))	Sigma-Aldrich D5879, Steinheim
DNA Ladder 1Kb plus	Invitrogen, Carlsbad, CA, USA
dNTP Mix (Deoxynucleotide Triphosphates)	Invitrogen, Carlsbad, CA, USA
DTT 0.1 M (Dithiothriol)	Invitrogen, Carlsbad, CA, USA
E.coli Bakteria to plasmid cloning un-K-12 Wildtype, W-Strain	ATCC No. 9637, S.A. Waksman
EDTA (Ethylendiamine-Tetraacetic Acid)	Sigma-Aldrich ED2SS, Steinheim
EnVision® Detections System	DAKO BioTek Solutions, Ca, USA
Ethanol, absolute to Analysis, undenatureted	Merck 1.00983.1000, Darmstadt
Ethanol, denatureted	Riedel-de-Haen, Seelze
Ethidium bromide	Sigma-Aldrich E87A51, Steinheim
FITC (Fluorescein isothiocyanate)	Sigma-Aldrich F7250, Steinheim
Formaldehyde 37%, p.a., ACS, Rotiuran®	Roth 49794.1, Karlsruhe
Gel Star® Nucleic Acid Gel Stain	Lonza 50535, ME,USA
Glycerol	Merck, Darmstadt
Goat anti-mouse IgG Microbeads	Miltenyi Biotec, 130-048-401, Bergisch Gladbach
	PAA Laboratories E15-819, Linz/

Iscove® DMEM with L- Glutamin	Österreich
IPTG (Isopropyl-β-D-thiogalactopyranosid)	Invitrogen, Carlsbad, CA, USA
KCl (Potassium chloride, crystalline)	Biochrom T046-10, Berlin
Copper(II)-sulfate-5-hydrate	Roth 8174,1, Karlsruhe
LB (Lennox L-broth) Agar	invitrogen 00705091, Karlsruhe
LB (Lennox L-broth) Medium	Invitrogen 00705241, Karlsruhe
Lipopolysaccharide from Escherichia coli Serotype O55:B5	Sigma-Aldrich L-2637, Steinheim
Lipopoly saccharide from Escherichia coli Serotype O111:B4	
Lymphozytenseparationsmedium®	PAA Laboratories J15-004, Linz/Österreich
Medium 199 (M199)	Earle´s Salts, Gibco,USA
Na_2CO_3 Sodium carbonate	Sigma-Aldrich S-2127, Steinheim
Na_2CO_3 x H_2O (Sodium carbonate-10-Hydrate)	Grüssing 12121 Filsum
Na_2HPO_4 (di-Sodium hydrogen phosphate)	AppliChem A2943 Darmstadt
Na_2HPO_4 x $2H_2O$ (Di-sodium hydrogen phosphate-dihydrate)	Grüssing 12149 Filsum
NaCl (Sodium chloride)	Roth 9265.2, Karlsruhe
NaClO (Sodium hypochloride)	Sigma-Aldrich 425044, Steinheim
$NaHCO_3$ (Sodium bicarbonate)	Sigma-Aldrich S-8875, Steinheim
$NaHPO_4$ (Sodium phosphate)	Merck, Darmstadt
NaN_3 (Sodium azide, 10%ig)	Sigma-Aldrich S-2002, Steinheim
$(NH_4)2SO_4$ (75% saturated ammonium sulfate)	Roth 3746.2, Karlsruhe
Oligo-(dt)12-18-Primer	Invitrogen 18418-012, Karlsruhe
Paraffin "Paraplast"	Shandon, Frankfurt
Paraformaldehyde	Sigma Aldrich P6148, Steinheim
PBS (Phosphat Buffert Solution w/o Ca2+, Mg2+ dry subtance)	Biochrom L182.10, Berlin
PCR Rxn Puffer without $MgCl_2$	Invitrogen 401305, Karlsruhe

Penicillin G	Sigma Aldrich 204-038-0, Steinheim
Penicillin-Streptomycin-Glutamine-solution liquid100 box concentrated	Invitrogen 10378-016, Karlsruhe
PercollTM (specific weight: 1.130 g/ml)	Amersham Bioscience 170-89101, Uppsala/Schweden
$PGF_{2\alpha}$ (Prostaglandin $F_{2\alpha}$ Tris Salt)	Sigma-Aldrich 82477-1MG, Steinheim
PJ (Propidiumjodid)	Calbiochem 537059, Bad Soden
Plasmidextraction kit "QIAprep Spin Miniprep Kit"	Qiagen 27104, Hilden
Puffer REact® 2 (10 box concentrated)	Invitrogen, Carlsbad, CA, USA
QIA Gel Extraction Kit	QIAGEN GmbH 28704, Hilden
QIAquick® PCR Purification Kit	QIAGEN GmbH 28104, Hilden
QIAquick® RNeasy Mini Kit	QIAGEN GmbH 74104, Hilden
recombinant bovine TNFα	Serotec PBP005, Oxford, UK
Restriction enzyme Hind III	Invitrogen 15207-012, Karlsruhe
RNaseOUT™ Ribonuclease inhibitor	Invitrogen 10777-019, Karlsruhe
RNAse/DNAse-free Water	Sigma Aldrich W4502, Steinheim
RPMI 1640 Medium (Roswell Park Memorial Institute Medium)	Ambion 7020, Austin, TX
Saponin	
Solution at Stabilization from RNA "RNAlater"	
Streptomycin Sulfate	Sigma Aldrich S6501-256, Steinheim
SuperscriptTM II Reverse transcriptase	Invitrogen 18064-014, Karlsruhe
SYBR Green® PCR Master Mix	Appiled Biosystems 4309155, Darmstadt
Taq DNA-Polymerase	Invitrogen 10342-053, Karlsruhe
TMB(3.3´,5.5´-Tetramethylbenzidin, C16H20N2)	AppliChem A-3840 Darmstadt
TOPO TA Cloning® Kit	Invitrogen K 450641 Karlsruhe
Tris (Trizma Base)	Sigma-Aldrich T8524, Steinheim
X-Gal (5-Brom-4-chlor-3-indolyl-beta-D-galactopyranoside)	Invitrogen 15520-034, Karlsruhe

3.2.4 Antibodies

Monoclonal antibodies were used for the characterization of surface structures on bovine leukocytes, for the separation of bovine monocytes from peripheral blood and for the production of reference cells. All antibodies except Bo1 were from AbD Serotec, Düsseldorf. An overview of the used antibodies and their specificity is shown in below.

Tab. 2 Used antibodies and their specificity.

Name	Specificity	Donor-Isotype	Dilution	Reference / Origin
MCA1653PE	Bovine CD4	Mouse	1:10	BENSAID and HADAM 1991
Bo1	MHC I	Mouse-IgG	1:1	SCHUBERTH et al. 1991
MCA1568PE	Human CD14	Mouse-IgG2a	1:50	JACOBSEN et al. 1993
MCA2226F	Sheep MHCII	Mouse	1:40	PURI and BRANDON 1987
MCA1783F	Bovine IFNγ	Mouse-IgG1	1:10	HASVOLD et al. 2002

For the magnetic separation of monocytes polyclonal Goat anti-mouse IgG (heavy and light chains) coupled to magnetic micro beads was used (SIPKA et al. 2010). The micro beads were used in a ratio of 20 µl per $1x10^7$ cells.

3.2.5 Test animals

Donor animals for blood extraction

The blood samples were taken from 21 female animals of the breed Holstein-Friesian (HF) (1.5 to 7 years old). All animals were clinically healthy and originated from the Clinic for Cattle or the research facilities in Ruthe of the University of Veterinary Medicine Hannover. The blood samples were taken by puncturing the jugular vein under sterile conditions. For the extraction of the blood leukocytes with sodium-heparinized vacutainers were used.

Organ extraction from abattoir derived unknown cows

Ovaries containing CL of HF animals were taken from the abattoir in Bremen. After collection they were placed in medium 199 at 38°C. Generally the origin, the health status and the cycle day of the cattle (n=11) that were killed on the abattoir were unknown. Only CLs determined to be in the luteinisation phase were selected (IRELAND et al. 1980;

JAROSZEWSKI and HANSEL 2000). To identify pregnant animals the uteri were opened and examined.

Ovaries were excluded if the uterine mucous membrane and/or the uterine content showed macroscopic pathological changes. For transportation to the Institute for Immunology, 2 fold penicillin/streptomycin mixtures were added to M199.

3.2.5.1 Culture mediums, buffer and solutions

Washing and dilution buffer for the membrane immunofluorescence (MIF) buffer

Bovine serum albumin	5.0	g
Sodium acid (NaN_3)	0.1	g
PBS ad 1000 ml		
The buffer was stored at 4°C.		

MIF-Saponin Buffer

MIF Buffer	100.0	ml
Saponin	0.5	g

Tissue culture medium

Medium 199	15.0	g
$NaHCO_3$	2.2	g
BSA	1.0	g
Penicillin	100.0	I.U./ml
Streptomycin	100.0	µg/ml
Aqua bidest. ad 1000 ml		
pH-value: 7.2 – 7.4		
Before use the medium was sterile filtrated (pore size: 0.450 µm).		

Material for the separation of cells

Lymphocyte Separation Medium®

The Lymphocyte Separation Medium® is an isotonic, aqueous solution of sodium diatrizoate and a high molecular sugar with the addition of X-ray contrast medium Isopaque. The separation medium has a density of 1.077 g/ml at 10°C. In this study the lymphocyte separation medium was used undiluted.

Sodium chloride solution, 0.9%

NaCl	8.77	g
Aqua tridest. ad 1000 ml		

Phosphate buffered Saline (PBS) without ethylene diamine tetra-acetate (EDTA)

The PBS dry was resolved in aqua tridest. The following components were weighed:

NaCl	8.0	g
KCl	1.24	g
Na_2HPO_4	0.2	g
KH_2PO_4	0.2	g
Aqua tridest. ad 1000 ml		

The buffer has a pH of 7.4 and was stored at 4°C.

Concentrated twice Phosphate buffer (2 x PBS)

The double amount in dry substance PBS was used to the production and added aqua tridest 1000 ml.

Phosphate buffered Saline (PBS) with EDTA (2 mmol/L)

EDTA	292	mg
PBS ad	500	mL

Iscove®-Medium with 10% (v/v) fetal calf serum (I10F+)

Iscove® DMEM with L-Glutamine	500	mL
Fetal calf serum (heat inactivated)	50	mL
Penicillin-Streptomycin	10	mg

RPMI-Medium with 10% (v/v) fetal calf serum (R10F+)

RPMI® liquid with Hepes und L-Glutamine	500	mL
Fetal calf serum (heat inactivated)	50	mL
Penicillin-Streptomycin	10	mg

For cultivation of leukocytes Iscove- and RPMI-medium were used. To inhibit complement activity and killing of possibly existing mycoplasmas, the medium was heat-treated after the addition of fetal calf serum at 56°C for one hour.

Lipopolysaccharide (LPS) stock solution

The LPS stock solution (1 mg/mL PBS) was stored at -20 °C.

Superantigen stock solution

The Superantigen Staphylococcus aureus Enterotoxin A (SEA) was stored in a concentration of 10 µ/ml RPMI10F at -20 °C.

JC-1-Stock solution

The staining has been used for the determination of apoptosis. To produce a stock solution 5.5´, 6.6'-Tetrachloro-1.1', 3.3'-Tetraethylbenzimidazol-Carbocyanine Jodid (JC-1) was dissolved in DMSO to a concentration of 2 mol/l, which was frozen in aliquots of 20 µl at -20°C. For use JC-1 was diluted with PBS to a concentration of 7 µmol/l.

Washing and dilution buffer for the membrane immun fluorescence (MIF)

Bovine serum albumin	5.0	g
Sodium acid (NaN_3)	0.1	g
PBS ad 1000 ml		
Storage at 4°C.		

Paraformaldehyde solution for fixation of reference cells

Para formaldehyde	40	mg
PBS ad 1000 ml		
Storage at 4°C.		

Acridine orange/Ethidium bromide solution for the light microscopy cell counting

Acridine-Orange	250	mg
Ethidium bromide	250	mg
PBS ad 100 ml		
Storage at 4°C.		

Solutions for flow cytometry

To clean the machine, the capillary system was washed after the measurements with sterile filtered aqua tridest and 1% sodium hypochlorite solution.

Carrier Fluid

The carrier fluid for the flow cytometry measurement consisted of sterile filtered PBS and 0.1 mg/ml NaN_3 sodium acid.

Propidium iodide stock solution

The stock solution of 100 g/ml propidium iodide was prepared in carrier liquid and was stored in aliquots at -20°C. For staining of the dead cells, the required volume of stock solution was added to carrier liquid to a reach final concentration of 2 g/ml.

Buffer and solution for DNA analyses

1x Tris Boric Acid EDTA (TBE)-Buffer, pH 8.4

The 10x TBE buffer was diluted with Aqua tridest. and was stored at RT.

TE-Buffer, pH 7.5

Tris	10.0	mmol/l
EDTA	1.0	mmol/l

The substances were dissolved in Aqua tridest. The buffer was autoclaved at 2.8 bar for 20 min at 135°C and the aliquots were stored at RT.

Blue marker

Glycerol	30%
Bromphenol blue	1%

Dissolved in1x buffer, stored at 4°C.

Supplements, medium and culture medium for bacteria cultures

Ampicilline:

Preparation of a stock solution (100 mg/ml) in aqua tridest, stored at -20°C.

Isopropyl-β-D-thiogalactopyranosid (IPTG)

Preparation of a stock solution (100 mg/ml) in aqua tridest, stored at -20°C.

5-Brom-4-chlor-3-indolyl-beta-D-galactopyranosid (x-Gal)

Preparation of a stock solution (100 mg/ml) in DMF, stored at -20°C.

Lennox L-broth (LB)-Medium

20 g broth was dissolved in 1.l aqua dest and autoclaved at 2.8 bar for 20 min at 135°C. After cooling to 55°C the ampicillin (100 g/ml) was added. The medium was stored one month at 4°C.

Lennox L-broth (LB)-Agar

32 g LB Agar was dissolved in 1.1 Aqua dest and was autoclaved at 2.8 bar for 20 min at 135°C. After cooling to 55°C the ampicillin (100 g/ml), 80 µg/ml X gal and 40 g/ml IPTG were added. Then 200 ml of mixture was poured into the petri dishes, which were subsequently allowed to set for 15 min at RT. The agar plates were stored at 4°C for one month.

Plasmid for creation of standard line

The TOPO TA cloning kit containing the plasmid pCR®2.1-TOPO® was used for the cloning of gene sequences into chemically competent E.Coli. (Fig. 2).

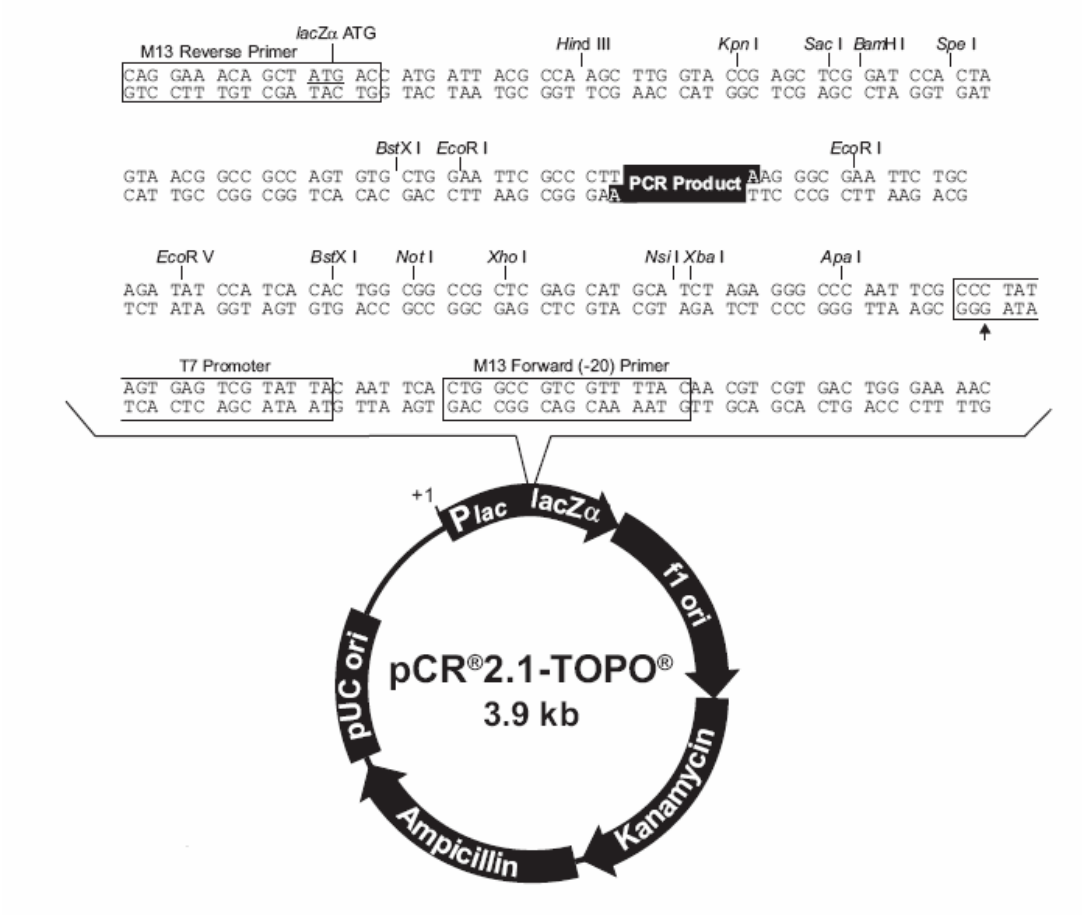

Fig. 2. Plasmid for the creation of an external standard series for the quantitative real time PCR (qRT-PCR).

Plasmid for the cloning of gene sequences in E. coli. Schematically the ligation part for the PCR product, for antibiotic resistance and for the interface diagrams of the restriction enzymes was shown.

The oligo nucleotide primers were selected on the basis of published sequences or newly created. All primers were produced by MWG (Ebersberg, Germany). The optimal concentration of primers was determined using the StepOnePlus PCR system by the manufacturer as described primer optimization. The sequences of primers, the used concentration, the length of the amplicon and the references are presented in Tab. 3.

3.2.5.2 Primer

Tab. 3 Primer sequences and concentrations for the qRT-PCR.

Gene	Forward- (for) and reverse- (rev) primers (5' → 3') (nmol/l)	Amplicon length	Reference
Gal-1	for TCA ACC AGA CGG ACC TAA CC (19) rev GAA GTC ACC ACC TGC AGA CA (20)	113	MOHAN et al. 2004
Gal-3	for CGG ACC ACT GAA TGT GCC TTA T (19,5) rev TGT TCG CAT TGG GCT TAA CTG T (21,1)	98	LAHMERS et al. 2006
Gal-15	for GAA TAA CGA GTC CTT CTG CC (40) rev GCA CAG TAT CTC CCT TCA CC (41,3)	83	Novel design Acc.no. XM_593263

Acc. No: accession numbers of the nucleotide sequence of the NCBI gene database, which was used for the preparation of primers.

3.3 Methods

3.3.1 Acquirement and treatment of Corpora luteal tissue

The ovaries for in vitro experiment, taken from the abattoir were treated immediately under sterile conditions. Every CL was separated from the ovary under the sterile bank. Additionally, CL pieces were separated as central and peripheral parts (SHIRASUNA et al. 2008). These CL pieces were dissected with a stanza (Ø 4 x 6 mm) and incubated for the fixation of mRNA in RNAse-free cryotubes containing 750 µl RNA later® solution for 24 hours at 4°C. After this step, the RNA-later solution was removed, the CL pieces taken to other cryotubes, and frozen by -95°C until extraction of mRNA.

Acquirement of corpora luteal tissue at days 12, 16 and 40 in cyclic and pregnant animals

Twenty-four normal cyclic, German Holstein cows, barn housed at the Clinic for Cattle, University of Veterinary Medicine Hannover, were selected. Ovaries to assessed for normality and the number of follicular waves were detected by transrectal ultrasonography. In this study, only cows with two follicular waves were selected for investigation of the same estrous stage of luteal maturation in each group. Therefore, the condition of the ovary and follicles were checked every two days. Thereafter, the animals were divided into the following four groups: ovariectomy during estrous cycle (not inseminated) on days 12 and 16, with 5 cows on each day and ovariectomy of 5 cows after insemination on day 16. Additionally, a positive control group containing 7 cows at day 40 of pregnancy was included in this study. To determine the exact day of ovulation, all cows received Gonadotropin-Releasing-Hormon (GnRH; 0.01 mg Buserelin, 2.5 ml of Receptal™, Intervet, Germany), PGF2α (0.5 mg Cloprostenol, 2 ml Estrumate™, Essex, Germany) and 7 days later and then GnRH 48 h after PGF2α. Only animals that had a preovulatory follicle after the last GnRH application were included in the study. The inseminated animals in the day 16 and 40 pregnant group were artificially inseminated 12 and 24 hours after GnRH application. The ovaries were collected by laparotomy after administration of epidural anesthesia (4 ml 2% procaine hydrochloride, Procasel™, Selectavet, Germany). Because pregnancy could only be confirmed by ultrasonography from day 25 onwards, the day 16 inseminated cows were slaughtered one day after ovariectomy tocollect the uterus for pregnancy detection (BEINDORFF et al. 2010)

Acquirement of corpora luteal tissue in the animal model on days 10 until 12 post ovulation

Twenty nine HF heifers were selected to scrutinize the luteal regression. All heifers were clinically healthy and originated from the Clinic for Cattle, University of Veterinary Medicine Hannover. The animals were syncronized using the following intramuscular injections: 2.5 ml gonadotropin releasing hormone (GnRH; 10.5 µg Buserelin), seven days later 2 ml PGF2α (150 mg Cloprostenol) and two days later 2.5 ml GnRH. The following work procedures were accomplished in detail: The ovaries of all animals were examined via ultrasonography around the time of the ovulation, in order to specify the exact time of ovulation. Subsequently to the monitoring of the CL-development, the ovaries were examined sonographically on day 5 and 10. Animals with two CL or a cavity in the CL were excluded from the study. On day 10 p.ov the animals were divided in 6 groups, á 5 animals. One group served as negative control where the CL-carrying ovary was taken by surgery without previous PGF2α injection. Ovaryectomy was performed within the animals of the further groups 5 min, 15 min, 30 min, 120 min and 720 min post injectionem with 5 ml (25 mg) Dinoprost (PGF2α analogon). From 4 heifers, ovaries were extirpated 720 min after PGF2α injection. The ovarectomy was performed transvaginally via the dorsal vaginal layers. Before surgery, an epidural anaesthesia with 5 ml 2% procainhydrochloride After disinfection of the vagina with a 2% disinfection solution a 3 cm long cut dorsal the portio vaginalis into the vaginal mucous membrane was performed using a scalpel. Afterwards the entrance to the abdominal cavity was widened bluntly by using the fingers until one could probe the ovaries by hand. To eviscerate the CL-carrying ovaries an effeminator was used, which guaranteed a complete agglutination of all ovary-associated vessels after a 10 minute lasting compression. After evisceration of the ovaries the surgical wounds were not sewn, since the boundaries of the lesions occluded automatically within short time. For pain relief after the surgical intervention 3 mg/kg of a nonsteroidal antiphlogistic (Romefen 10%) was administered intramusculary on two sequential days.

3.3.2 Stimulation of the corpora lutea slices with LPS for evidence of chosen galectins

For LPS stimulation, the ovaries were taken from freshly slaughtered cows. The CL were separated as described above under sterile conditions, laid in 24er well-plate containing 1.5 ml medium 199 per well and incubated for one hour at 37°C. Afterwards the medium was renewed and the in medium 199 prepared test substances were added (LPS, 0.5 and 5 µg/ml). After 30, 60 and 180 minute incubations, the CL pieces were frozen at -95°C until mRNA extraction for detection of gal-1, gal-3 and gal-15.

3.3.3 Separation of leukocyte population from full blood

Blood samples were taken from the Vena jugularis with a sterile canula using heparinised vacutainer tubes. The blood was diluted (1:2 with PBS) and layered on 15 ml Lymphocyte Separation Medium in a 50 ml tube. The tubes were centrifuged (30 min, 4°C, 1000 × g) without brake. This centrifugation serves to separate the leukocyte population from the other cell types. Between blood plasma and lymphocyte separation medium, the interphase contained MNC (mononuclear cells: lymphoid cells and monocytes) and a fraction of thrombocytes. Beneath the separation medium, packed erythrocytes and polymorphonuclear granulocytes (PMN) were located.

3.3.4 Separation of mononuclear cells

The interphase was removed with a wide luminal pipette and transferred in 50 ml centrifuge tubes containing 10 ml PBS. After that step, PBS was filled up to 30 ml and the cells were washed twice (10 min, 4°C; 500 x g and 100 x g) to remove the thrombocytes. After the first washing step the cell solution was exposed to hypotonic conditions to remove erythrocytes: 10 ml aqua tridest was added and shaken for 20 seconds (s). Immediately afterwards 10 ml 2 times concentrated PBS was added to the content and the tubes were centrifugated (10 min, 4°C; 500 x g). Between the separate centrifugation steps the cells were resuspended in 40 ml PBS. With this method between 1.5 and 3 $x10^6$ MNC/ml could be obtained.

3.3.5 Separation of granulocytes

The plasma, the interphase and the Lymphocyte Separation Medium® were removed with a wide luminal pipette and discarded. Erythrocytes were removed from the remaining phase (erythrocytes and granulocytes) using two hypotonic lysis steps. Twenty ml aqua tridest. were added and shaken for 20 seconds (s). Immediately afterwards 20 ml 2 times concentrated PBS was added to the content and the tubes were centrifugated (10 min, 4°C; 500 x g, 250 x g). The supernatant was discarded, and the cell pellet resuspended. Subsequently the cells were washed twice with 20 ml PBS (10 min, 250 x g, 100 x g). The last cell pellet was resuspended into medium ($I10F^+$), where the cells were counted and adjusted to the required concentrations. At the end of this procedure, the PMN populations were 90% to 95% pure. If cell suspensions contained more than 10% MNCs, they were not used for further assays.

3.3.6 Separation of monocytes and lymphocytes

The separation of monocytes and lymphocytes from the MNCs was done by positive selection of monocytes (and negative selection of T-cells) on the basis of their surface marker CD14 in

a magnetic separation system. The MNCs were transferred in 5 ml PBS-EDTA and poured over a 30 µm nylon filter to remove any aggregates. The filters were washed three times with 5 ml PBS-EDTA and the filtered cell suspension was centrifuged (10 min, 4°C, 100 x g). Afterwards the cell suspension was resuspended in 400 µl PBS-EDTA and 8 µl mouse-anti-sheep-MHC II antibody was added. After 30 min incubation at 4°C the cells were washed (10 min, 4°C, 300 x g) to remove unbound antibody. Subsequently PE conjugated 8 µl mouse-anti-human-CD14 antibody was added. After 30 min incubation at 4°C the cells were washed (10 min, 4°C, 300 x g) to remove unbound antibody and transferred to 200 µl PBS-EDTA. An aliquot was diluted 1:50 and checked flow cytometrically for purity, vitality and existence of a positive cell population. The vital cells were quantified flow cytometrically with the reference cell method. Per $1x10^7$ cells 80 µl PBS-EDTA and 20 µl goat anti-mouse micro beads were added. After the incubation (15 min, 4°C) the cells were centrifuged (10 min, 4°C 300 x g) and resuspended in 3 ml PBS-EDTA. For the magnetic cell separation (MACS) a MACS column was used and washed with 3 ml PBS-EDTA. The cell suspension was poured over the filter, which at that point was attached to a magnet block, and the through flow was kept in 15 ml tubes (MHC II positive cells). Afterwards the column was washed three times with 3 ml PBS-EDTA. This fraction was kept in another tube. To obtain the CD14-positive cells the column was removed from the magnet block and eluted with 5 ml PBS-EDTA. The first flow of the cells and the eluate were analyzed flow cytometrically for purity and vitality. The lymphocytes and the monocytes must be pure more than 90% and 96% respectively. The monocytes were centrifuged (10 min, 4°C, 300 x g) and resuspended in medium $I10F^+$. The cell suspension was diluted until it contained $2x10^5$ monocytes/ml. After these steps, the monocytes were either used for the generation of macrophages or were stimulated after a 24 h-resting period with LPS (1 µg/ml). After 3 h incubation (37°C, 5% CO_2), the cells were taken in lyses buffer for mRNA-extraction.

3.3.7 Generation of macrophages from blood monocytes *in vitro*

The macrophages were generated from blood monocytes *in vitro* (bMdM, blood monocyte derived macrophages). The blood monocytes were separated as described before. The cell suspension was placed in 24 well plates so that each well contained $2x10^5$ cells. The maturation of the monocytes to bMdM took seven days (37°C, 5% CO_2). After three days 500 µl of fresh medium ($I10F^+$), which was warmed to 37°C, was added per well. The cells were checked in the inverted microscope for adherence and contamination. Contaminated cell cultures were discarded.

3.3.8 *In vitro* stimulation of monocytes and *in vitro* generated macrophages

MACS-separated monocytes were placed cell culture medium ($2x10^5$/ mL) and the 24 well cell culture plate was incubated (24 h, 37°C, 5% CO_2) (rest phase). Macrophages were stimulated after 7 days in vitro differentiation (see above). LPS (1µg/ml) was added or left for the control group. After a 3 h incubation period (37°C, 5% CO_2) the cells were transferred in lyses buffer for mRNA extraction.

3.3.9 In vitro proliferation assay

Proliferating MNCs increase in size before the division, and, moreover, by the change in the plasma/nucleus relation the complexity of the cells also increases. The blastocytes thereby differ during flow cytometric acquisition from the smaller, quiescent lymphocytes.

Test procedure

The proliferation of MNCs were induced by the superantigen SEA and the lectin Con A. Freshly separated bovine MNCs were added to a 96 well flat-bottom plate ($1x10^5$ cells per well) and stimulated with SEA (1 ng/ml) or Con A (1 µg/ml). Two wells were used as unstimulated controls. The plates were incubated for 4 days (37°C and 5% CO_2). For the FCM measurement, the cells were transferred in FCM tubes containing 100 µl carrier fluid (4 µg/ml PI) and 50 µl reference cells.

Analysis

Dead cells were analyzed on a FL-3/SSC diagram (propidiumiodide negative events). The morphologic characteristics of the cells were analyzed in FSC/SSC diagrams. In the latter a gate was set on smaller, less complex events (resting lymphocytes) and a second gate on bigger and more complex events (blasts). The number of both populations was calculated based on the reference cell method.

3.3.10 Indirect membrane immunofluorescence (MIF)

The indirect membrane immunofluorescence shows the expression of surface structures on cells. Specific antibodies are allowed to react with their target structure. They are recognized by fluorochrome-labeled secondary antibodies.

Cells were stimulated in vitro as described above. After the incubation period the cells were centrifuged (4 min, 4°C, 200 x g) and the supernatants were discarded. The cells were washed in 175 µl MIF buffer. The supernatant was discarded. The cells were resuspended in the

remaining fluid and 25 µl of the diluted specific primary antibody was added (20 min, 4°C). Afterwards the cells were washed twice with 175 µl of MIF buffer and incubated with 25 µl of the secondary FITC-conjugated goat anti-maus-antibody (20 min, 4°C in the dark). Afterwards the cells were washed two more times and then transferred into FCM tubes containing 150 µl of carrier fluid (2 µg/ml PJ) for flow cytometric measurement. To be able to exclude unspecific binding of the primary and secondary antibody, conjugate controls (25 µl MIF buffer) as well as isotope-controls were carried out (25 µl irrelevant monoclonal antibody of the same isotope as the specific primary antibody).

3.3.11 Intra cellular immune fluorescence (ICIF)

Intra cellular mediators (ex. Interferon gamma, IFNγ) were determinated by ICIF. To allow intracellular accumulation of IFNγ and to prevent secretion, cells stimulated for 48 h in the 24 well plate were pretreated with Brefeldin A (0.5 µg/ml) for 24 hours before analysis. The cells were transferred to mini tubes, centrifuged (5 min, 300 × g) and incubated for 30 min with 500 µl 4% paraformaldehyd for fixation. Afterwards the cells were centrifuged (5 min, 300 × g) and washed once with PBS and once with MIF-saponin buffer. The supernatant was discarded and the cells were incubated for 30 min in darkness with 40 µl FITC-labelled IFNγ-specific antibody diluted in MIF-saponin buffer. After incubation time, the cells were washed and resuspended with 500 µl MIF-buffer and incubated for another 30 min in the dark. After the last incubation the cells were washed two more times, resuspended in 200 µl buffer and measured flow cytometrically.

3.3.12 Identification of apoptotic mononuclear and polymorphonuclear cells

Evidence of intrinsic path of apoptosis with JC-1

The determination of the mitochondrial membrane potential (MMP) was carried out with the fluorochrome 5.5 ', 6.6'- Tetrachloro- 1.1', 3.3'-Tetraethylbenzimidazol-Carbocyanine iodide (JC-1) (FOSSATI et al. 2003). JC-1 is a metachromatic fluorochrome, which, due to its lipophilic characteristic, can diffuse through the membranes of vital cells. The cation aggregates in functional mitochondria of vital cells, because these exhibit a MMP from -180 mVs to -200 mVs. After a stimulus with light of a wavelength of 488 nm this stain emits light with a wavelength of 590 nm and therefore can be detected by the flow cytometry in the detector for orange light (FL2-Detektor). If the MMP of the mitochondria decreases below -80 mVs to -100 mVs, in consequence of apoptosis via the intrinsic pathway, the aggregates of JC-1 are able to diffuse into the cytoplasm and change into their monomeric form.

Consequently the wavelength of the emitted light changes to 527 nm and can be seized by the FL-1 detector for green light. For the simplification of the nomenclature, cells with depolarized MMP are called in the following 'apoptotic cells'. It should be pointed out that, on this occasion, the intrinsic pathway of the apoptosis is meant.

Test Procedure

The cells were separated as described before and resuspended in $I0F^{+}$ ($6x10^{6}$ cells pro/ml). Therefore, the final concentration of fetal calf serum in the suspension amounted to 2.5%.50 µl of the cell suspension were put into the wells of the 96-well cell culture plate. As control the cells were incubated in Iscove-medium with 2.5% FCS. After incubation for 24 h at 37°C and 5% CO_2, the JC-1 staining was carried out. At first the cell culture plates were centrifuged (4 min, 20°C, 250 x g) and the supernatant was discarded. The cells were resuspended in 100 µl PBS and centrifuged again (4 min, 20°C, 250 x g) in order to remove the supernatant. After the resuspension in the residual liquid, 100 µl of a 7 µM JC-1 solution was added to the cells and incubated for 15 min at 37°C. After washing twice the cells were taken up by 200 µl of carrier liquid (2 µg/ml PJ), and they were transferred to the FCM tubes and measured by flow cytometry (10.000 events). All samples were made in triplicates.

Analysis

During the data collection a live-gate was set upon MNCs or neutrophils in the forward-scatter/side-scatter plot. A second gate was set upon PI^{high}-negative cells in the FL3/SSC-Plot (Fig. 3, A). By linking both gates non-MNC cells and necrotic cells were excluded from the evaluation. The cells that weren't necrotic were represented in a FL3/FL1 dot plot (green fluorescence = FL1; red fluorescence = FL3) (Fig. 3, B). For the evaluation 3 different conditions for each vital cell were differentiated: vital, not apoptotic cells in the left lower quadrant (Fig. 3, B1), apoptotic cells, whose MMP is depolarised and which appear thus after JC-1-staining in the left upper quadrant (Fig. 3, B2 and intermediate cells in the two right quadrants (Fig. 3, B3). The membranes of intermediate cells are impaired in their integrity. Thus, PI can penetrate into the cells and JC-1-molecules can leak out of the cytosol into the extracellular space. Therefore green fluorescence is lowered in some of these cells. In addition the ratio of necrotic cells within the cultured cell population can be calculated.

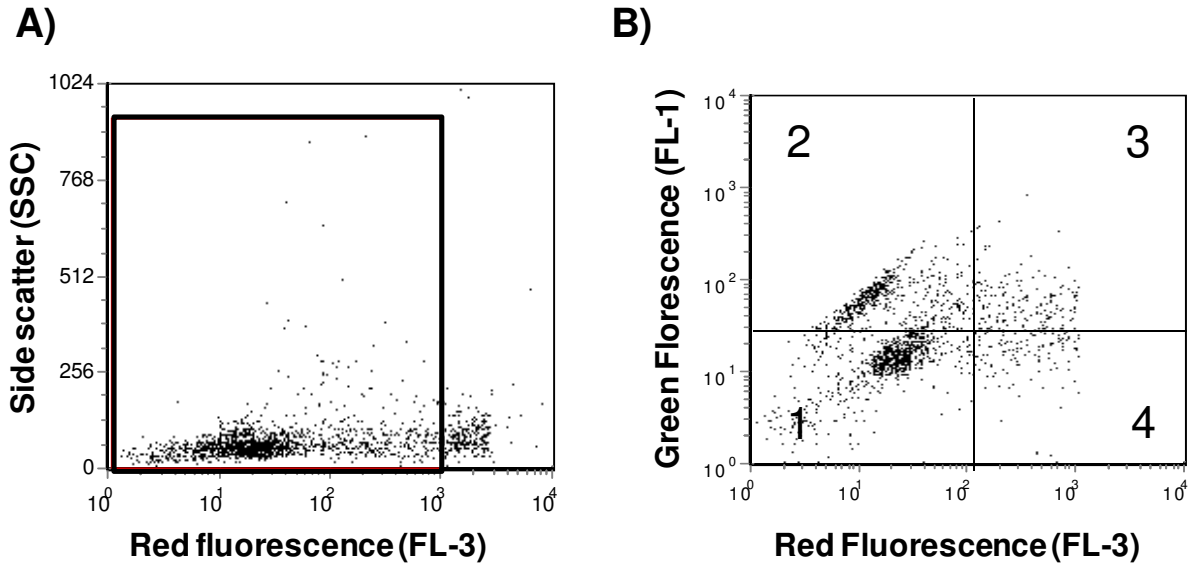

Fig. 3 Flow cytometric analysis of JC-1 stained bovine MNCs.

Bovine MNCs after 24 h in culture were measured by flow cytometry after JC-1 staining. A) Gating on PI-negative, vital MNC in a FL-3/SSC dotplot. B) Vital MNCs are displayed in a FL-3/FL-1 dotplot. (1: non-apoptotic, vital cells; 2: apoptotic cells; 3, 4: intermediate (apoptotic -> necrotic) cells).

3.3.13 Molecular biology procedures

3.3.13.1 mRNA preparation from corpora lutea tissue for quantitative RT-PCR

The gene expression of selected galectins was examined in corpora lutea stimulated with LPS for different for 30, 60 and 180 min *in vitro*

RNA extraction

The RNeasy® Mini Kit was used for extraction of total RNA according to the manufacturer's instructions.

The CL pieces were halved by a sterile scalpel and one part was weighed by mini scale. For RNA extraction pieces of 25-50 mg were used. After transfer to sterile microcentrifuge tubes, the CL pieces were homogenised in RLT buffer containing β-Mercaptoethanol (β-ME) (10µl β-ME per 1ml RLT) using an Eppendorf pistill.

Tab. 4: Volumes of RLT buffer for tissue disruption and homogenization

Amount of starting material (mg)	Volume of buffer RLT (µl)
< 20	350
20-30	600

The lysate was centrifugated for 3 min at full speed. The supernatant was transferred to a new microcentrifuge tube. 70% ethanol was added to clear the lysate. The sample was transferred to an RNeasy spin column and was centrifuged (15 s, ≥10.000 rpm). The flow-through was discarded. Subsequently 350 µl of RW1 buffer was added and centrifuged for 15 s. To avoid contamination with genomic DNA, 80 µl of DNase-I solution (10 µl DNase I + 70 µl buffer RDD) was added to the filter and incubated for 15 min at RT. DNA digestion was followed by an additional washing step with 350 µl buffer RW1 for 15 s and two washing steps with 500 µl of RPE buffer for 15 s and 2 min respectively. The filters were then placed in new microcentrifuge tubes, and 50 µl and 30 µl of RNase free water was added consecutively and centrifuged for 1min. The filters were discarded and the flow-through, containing the total RNA, was placed on ice immediately. The purity and concentration of the total RNA was checked by a photometer. The obtained RNA was used for reverse transcription or stored at -95°C.

Synthesis of cDNA by reverse transcription

The extracted RNA was employed for reverse transcription using the enzyme Superscript™ II Reverse Transcriptase in order to gain complementary DNA (cDNA). To rewrite only mRNA, oligo-(dt) 12-18 Primer were used which were taken up by the Poly-A-end of the mRNA molecules and served as a start region for the reverse-transcriptase. In 200 µl reaction tubes 10 µl RNA, 1 µ l Oligo-$(dt)_{12-18}$-Primer and 1 µl Trinucleotide were mixed. The components were incubated for 5 min at 65°C in the PCR Thermocycler "T-Gradient“in order to unfold secondary RNA structures. Afterwards, the samples were put immediately on ice and 7 µl of master mix were added.

Tab. 5 Mastermix for reverse transcriptase

Reagent	Volume	Final concentration
5 x first strand buffer (250 mmol/l Tris-HCl, 375 mmol/l KCl, 15 mmol/l $MgCl_2$)	4.0 µl	
RNase inhibitor (RNaseOUT™)	1.0 µl	40 IU
Reducer (DTT, dithiothreitol)	2.0 µl	0.1 mol/l

All substances were purchased from Invitrogen.

The second incubation (2 min, 42°C) was followed to reach the temperature optimum for the reverse transcriptase. 1 µl (200 U) Superscript™ II Reverse Transcriptase was added in the reaction and incubated for 50 min at 42°C. In order to stop the reaction and avoid an interaction of the enzymes, the samples were incubated for 15 min at 70°C for enzyme denaturation. The obtained cDNA was stored at 4°C for up to one week or at -20°C for several months.

Examination of the purity and concentration of cDNA and RNA

The purity and the concentration of cDNA and RNA were determined by optical densities at 260 nm and 280 nm using an Eppendorf-Biophotometer. For the measurement in the photometer the samples were diluted 1:50 in TE buffer. The purity of the samples was evaluated by the quotient of optical density at 260 nm and 280 nm. For RNA samples, quotients ranging from 1.9 to 2.1 were acceptableLower values indicated a contamination with genomic DNA. For cDNA samples quotients between 1.6 and 2.0 were acceptable. Lower values indicated contamination with proteins. The concentration (C) in µg/ml was calculated on the bases of the optical density at 260 nm (OD_{260}) after the following formula:

$C\ [\mu g/ml] = CD_{260} \times 50$

3.3.13.2 Production of an external standard line for quantitative RT-PCR

Gene expression was examined by absolute quantification in the RT-qPCR. For that purpose the target sequence was amplified with specific primers and cloned in a vector (plasmid). This recombinant plasmid was transformed to a chemically competent E. coli clone. After extraction from the bacteria, the length and sequence of the cloned nucleotide was verified. A

dilution series (10^6 to 10^2copies) of the linearized plasmid was measured parallel to the samples in the qRT-PCR to calculate the copy number of target genes.

Production of the target sequences

The target sequences for cloning were obtained from cDNA of bovine MNCs and macrophages. The production of cytokines and chemokines was stimulated with Con A (1 µg/ml), LPS (1 µg/ml) and SEA (1 ng/ml). After an incubation period of approx. 24 h the RNA of the MNCs was extracted and written in cDNA.

cDNA amplification by means of conventional RT-PCR

The amplification of target-sequences for the cloning step was conducted in a PCR-Thermocycler "T-Gradient". For the PCR reactions 5 µl cDNA as well as the mastermix (Tab. 6) was placed into a sterile 200 µl PCR tube. Initially cDNA was denatured completely for 3 min with 94°C. Then 30 cycles were run, which consisted of a denaturation step for 45 sec at 94°C, the alignment of the primer pairs for 30 sec at 58°C and an alongation of the product for 90 sec at 72°C. A final extension step was performed for 10 min at 72°C to make sure, that the adenine-superfluity at the 3' end of the amplicon necessary for the following cloning-step was synthesized by the Taq-Polymerase (Polymerase of the bacterium Thermophilus aquaticus). The samples were used for the cloning procedure immediately.

Tab. 6: Mastermix for the PCR thermo cycler „T-Gradient"

Reagent	Volumes	Final concentration
10x PCR rxn Buffer without $MgCl_2$ [1]	5 µl	1x
10 mmol/l dNTP Mix [1]	1 µl	0.2 mmol/l
50 mmol/l $MgCl_2$ [1]	1.5 µl	1.5 mmol/l
Primer Mix (each 10 µmol/l)	2.5 µl	0.5 µmol/l
Taq Polymerase (5 U/µl) [1]	0.25 µl	1 U
RNAse/DNAse-free water [2]	34.75 µl	

The substances were purchased by Invitrogen[1] und Sigma Aldrich[2]

Cloning of the target sequence into the vector

For further amplificationhe target sequence was cloned into a plasmid, which was used for the transformation of bacteria. Plasmids are ring-shaped, double stranded DNA molecules which can be incorporated in the bacterial cell, independent from the chromosomal DNA. In this work the plasmid pCR® 2.1-TOPO® with the length of 3931 nucleotides was used. It provides ampicillin resistence and a lacZ-Gene in the ligation area. For the TOPO® cloning reaction 3 µl fresh PCR product, 1 µl salt solution and 1 µl water (RNase-and DNase free) were incubated with 1 µl TOPO® vector for 15 min at RT. After the incubation period, the product was used immediately for the transformation of chemically competent E. coli bacteria.

Transformation of the chemically competent E. coli bacteria

After the ligation, the plasmids were transformed into chemically competent E. coli. The insertion of the vector was carried out via heat shock transformation. Two µl of fresh ligation samples were mixed with 50 µl of the bacteria and were incubated on ice for 30 min. Afterwards disintegration of the cell wall and admission of the plasmid was provoked by heat shock at 42°C in the heating block for 30 sec. After the addition of 250 µl S.O.C. Medium (Reagent of the TOPO TA Cloning® Kit) the samples were incubated for 1 h at 37°C on a shaker to allow the development of the ampicillin resistence and subsequently plated on LB agar containing x-Gal and IPTG. To control, whether the transformed plasmid was recombinant, i.e. containing the goal sequence or not, the vector possessed a lacZ gene, which codes for the β-Galactosidase enzyme. A successful ligation results in deactivation of the gene. The x-Gal served as chromogene substrate for ß-Galactosidase that becomes blue during conversion while IPTG, a Galactose-derivate, acted as artificial inductor of the Lactose Operons which does not interfere with the metabolism of the bacteria. The bacteria colonies with a successfully ligated plasmid could not convert the x-Gal and appeared white, while the colonies with intact lacZ gene showed a blue colouring because of the activity of the IPTG galactosidase and the resulting metabolization of x-Gal. This allowed for a pre-selection of the desired bacteria colonies, which subsequently were further multiplied in LB-liquid-media.

Extraction of the plasmid

To investigate the presence of recombinant plasmids in the bacteria single colonies were transferred in 3 ml of ampicillin-containing medium and incubated for 12-16 h at 37°C on a shaker. One ml of the suspension was stored at 4°C until the completion of the investigation and, in case of successful cloning, was used for the production of glycerol stocks. The

remaining 2 ml of the bacterial suspension were used for plasmid extraction with the QIAprep Spin Miniprep Kit. The method is based on the lysis of the bacteria under alkaline conditions (BIRNBOIM and DOLY 1979) and subsequent adsorption of DNA on a silica membrane. The bacteria were pelletized for 10 min at 8.400 x g at room temperature and the supernatant was removed. The bacterial pellet was resuspended in 250 µl of buffer P1 and then transferred into an Eppendorf tube. Afterwards 250 µl of buffer P2 were added and mixed by inverting the tube. LyzeBlue was added to buffer P1, so that the cell suspension turned blue after addition of buffer P2 and mixing resulted in a homogeneously colored suspension. After addition of 350 µl of buffer N3, the blue color faded and the suspension looked colorless. After this step the tubes were centrifugated for 10 min (16000 x g) and the supernatant was pipetted into the QIAprep spin column and was centrifuged for 1 min (16000 x g) and the flow-through was discarded. Afterwards the column system was washed once with 500 µl buffer PB (1 min, 16000 x g) and with 750 µl buffer PE (1 min, 16000 x g). The flow through was discarded after each step. For the removal of wash buffer, the empty tube was centrifuged again (1 min 16000 x g). The column was placed into a fresh Eppendorf tube for eluting the DNA and 50 µl of buffer EB was added. After 1 minute of incubation the DNA was eluted (1 min 16000 x g) into the Eppendorf tubes.

The presence of the cloned sequence in the extracted plasmids was checked immediately by conventional PCR followed by agarose gel electrophoresis. Bacterial colonies comprising the recombinant plasmid was used for the construction of glycerol stocks (850 µl bacterial suspension + 50 µl glycerol) in cryo tubes. The bacterial stocks were frozen at -95°C. To verify the integrity of the cloned sequence, 600-700 ng of plasmid DNA of all selected galectins were sent for sequencing (SEQLAB, Göttingen) with 5 pmol M13 forward primer from the TOPO cloning kit. Plasmids were only used if the presence of the target sequence was verified.

Agarose-Gel Electrophoresis

The amplified DNA fragments were separated via agarose-gel electrophoresis and made visible using dye gel star. With the help of an additionally implemented dimensions standard it was checked whether the dimensions of the amplificon agree with those of the cloned target sequences. In the electrical field DNA fragments migrate with different speeds depending on their size, the current, the voltage and also the buffer conditions and the agarose concentration in the gel. The smaller the isolated fragments are, the faster they move in the electric field. The stain added to the gel interacts with double-stranded DNA by and is visible in ultraviolet light. The agarose gel was produced as described before in chapter 3.2.5.1 and poured into the

gel electrophoresis chamber, which contained a fixed comb for the sample pockets. After cooling, the comb was removed. On a piece of parafilm, 10 µl of the DNA samples were mixed with 4 µl loading buffer containing bromphenol blue and placed into the sample pockets of the gel. The loading buffer allowed for control of the running front along the gel during gel electrophoresis, as well as for a better application of the samples into the sample pockets due to the implemented glycerin. Three µl of a 1 Kb DNA length standards were pipetted in one of the sample pockets of the gel for determining the size of the DNA fragments. The agarose gel electrophoresis was performed in 1x TBE buffer at a voltage of 85 volts over 45 minutes. Afterwards the bands were controlled under the ultraviolet light and then photographed by a gel imager.

Linearization of the plasmid

As PCR primer show a weaker binding affinity to circular DNA, the plasmid was linearized. Restriction enzymes were selected regarding their target nucleotide sequence for cleavage to avoid fragmentation of the cloned sequences. For the linearization of the plasmids encoding gal-1, gal-3 and gal-15 the restriction enzyme *Hind* III was used, which cleaves the bases between 5 `-A ↓ AGCTT-3` and 3 `TTCGA ↑ A-5`. *Hind* III was isolated from *Haemophilus influenza.* Together with the 10-fold concentrated buffer „REact ® 2“ the restriction enzymes cleaves 1 µg dsDNA in 1 h at 37°C. After linearization the DNA was purified using the QIAquick ® PCR Purification Kit. The Kit removes the enzymatic residuals and adsorbs the DNA under the alkaline conditions (pH ≥7.5) into a Silica membrane. All centrifugation steps (1 min, 16000 x g) were carried out at RT. All reagents in the following are components of the QIAquick ® Kit PCR Purification. First, the sample was mixed in a ratio of 1:6 with Buffer PBI in an Eppendorf tube. A pH of ≥7.5 was indicated by the yellow colour of the solution. The sample was placed on a column and centrifuged. The column was washed with 750 µl of buffer PE after discarding the flow-through the empty column was centrifuged (1 min, 16000 x g). Subsequently the column was placed on a new eppendorf cup and the DNA was eluted by adding 50 µl buffer EB (1 min 16000 x g).

Calculation of copy numbers

The DNA concentration was determined by optical density at 260 nm (OD_{260}) in an Eppendorf BioPhotometer as explained in part 3.3.13.1. Exact copy numbers per µl were calculated according to the formula below using the DNA concentration, the known length of the target sequence and the average molecular weight of the nucleotides.

$$MW = P \times 660 \text{ g/mol}$$

$$S\,[\text{copy}] = \frac{6\times10^{23}\,[\text{copy/mol}] \times \text{DNA}\,[\text{g/µl}]}{MW\,[\text{g/mol}]}$$

MW = Molecular weight of the PCR product
P = PCR product length of the base pairs
S = Standard concentration
DNA = Measured DNA concentrations in the photometer
660 g/mol = Average molecular weight of the base

A dilution series (10^9–100 copies/µl) was prepared, which was tested subsequently in the RT-qPCR. The dilution series was stored at -20°C. After three freeze-thaw cycles new dilution series was prepared.

3.3.14 Quantitative real time PCR

Principle of quantitative real time PCR

The RT-qPCR provides a quantification of PCR products in "real time" which means simultaneous to the amplification cycle. The master mix contains the stain SYBR Green which fluorescens green after binding to double stranded DNA. The fluorescence is in direct proportion to the amount of the amplified DNA, which is recorded online by the StepOneTM software version 2.0. During RT-qPCR the same three reaction steps are repeated as in a conventional PCR: 1) Melting of the double-stranded DNA, 2) Alignment of the primers and 3) Extension of the product. The three reaction steps are described as a cycle. A complete PCR involves 40 to 50 cycles. In the early cycles of the PCR only a basal fluorescence signal can be detected, which is also called a background signal. Depending on the initial amount of DNA in the reaction the fluorescence of the PCR product begins to rise after a certain cycle number. The cycle, in which the fluorescence changes from the background signal to the exponential phase, is named threshold cycle (Ct) and correlates directly with the original copy number of the samples. Due to inhibitory reaction products and enzyme limitation the exponential phase the PCR, turns first into a linear phase and finally to a plateau phase (Fig. 4). Since SYBR Green binds nonspecific to any double-stranded DNA, a melting curve analysis was performed after the amplification cycles. By raising the temperature, the samples were gradually denatured. Depending on their size the amplification products disintegrate at a specified temperature, which is seen in a sudden decrease of the fluorescence signal shown as a peak in the melt curve diagram. A melt curve analysis showing more than one peak indicates unspecific binding of primers or formation of primer dimers.

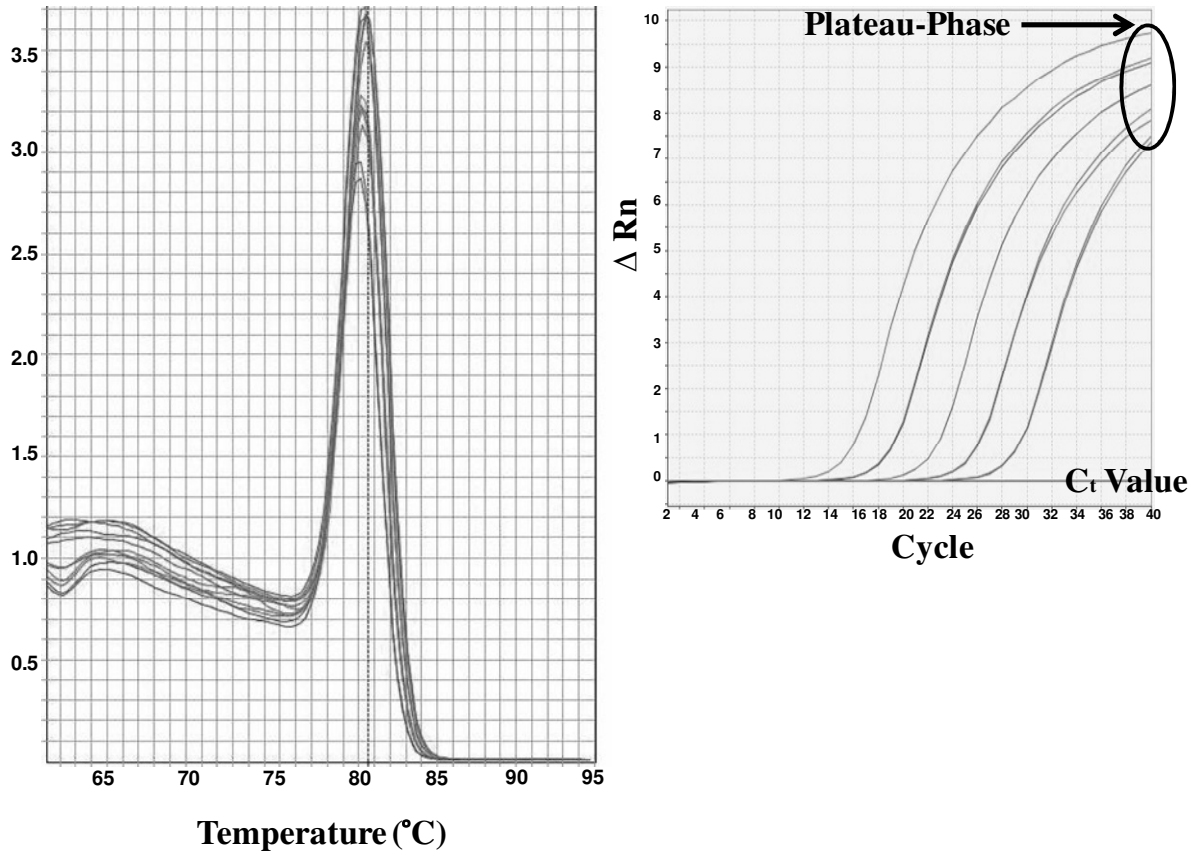

Fig. 4 Melting curve and amplification plot of a qRT-PCR.

A) Melting curve of gal-15 depending on the temperature. B) Increase of the fluorescence signal depending on the PCR cycle number of gal-15 standard dilutions in an amplification plot.

Primer optimization

The optimum primer concentrations for each target gene were determined as recommended by Applied Biosystems using the matrix from Tab. 7. A duplicate from the standard line (1.000 copies/µl) was used as template and a duplicate with DNase/RNase free water served as non template control. The combination of forward- and reverse-primer, showing the highest possible sensitivity together with a low or missing amplification in the negative control was selected for further experiments.

Tab. 7 Matrix for the primer optimization, which showns the combinations of different concentrations of forward and reverse primers (nmol/l).

Reverse-Primer (nmol/l)

Forward-Primer (nmol/l)	50	300	900
300			
900			

Measurement of unknown samples

The RT-qPCR was used to measure expression of gal-1, -3 and -15 mRNA in bovine luteal tissue derived directly after slaughter, as well as after the stimulation with LPS in M199 medium. Total RNA was extracted as described above and 200 ng were converted to cDNA as previously described. For RT-qPCR SYBR Green ® PCR Master Mix was used as described above. Each experiment contained serial dilution (10^2 to 10^6) of the respective cDNA subclone and a negative-control. For one reaction 1 µl cDNA and 24 µl of the master mix were pipetted in a Micro Amp^{TM} Fast 96-well PCR plate (Tab. 8). All reactions were conducted in duplicates.

Afterwards 40 cycles were run, each consisting of a denaturation of the samples at 95°C for 15 seconds, the alignment of the primers and the extension of the products at 60°C for 1 min. After the final amplification cycle a melting curve analysis was performed. Temperature was constantly raised from 60°C to 95°C in intervals of 0.3°C with constant recording of fluorescence.

Tab. 8 Mastermix for Gal-1 and Gal-3 volumes in µl per reaction.

Reagents	Volumes (µl)
SYBR Green® PCR master mix	12.5
Water (RNAse-, DNAse-free)	8.5
Forward primer (5 µmol/L)	1.5
Reverse primer (5 µmol/L)	1.5

Tab. 9 Mastermix for Gal-15 volume in µl per reaction.

Reagents	Volumes (µl)
SYBR Green® PCR master mix	12.5
Water (RNAse-, DNAse-free)	5.5
Forward primer (5 µmol/L)	4.5
Reverse primer (5 µmol/L)	1.5

Analysis

The StepOneTM software noted the fluorescence of by the SYBR Green® stain and specified the Ct-values for each individual sample. The Ct-values of the external standard dilution series served to calculate a standard curve, on which basis the copy numbers of the genes contained in the samples was derived.

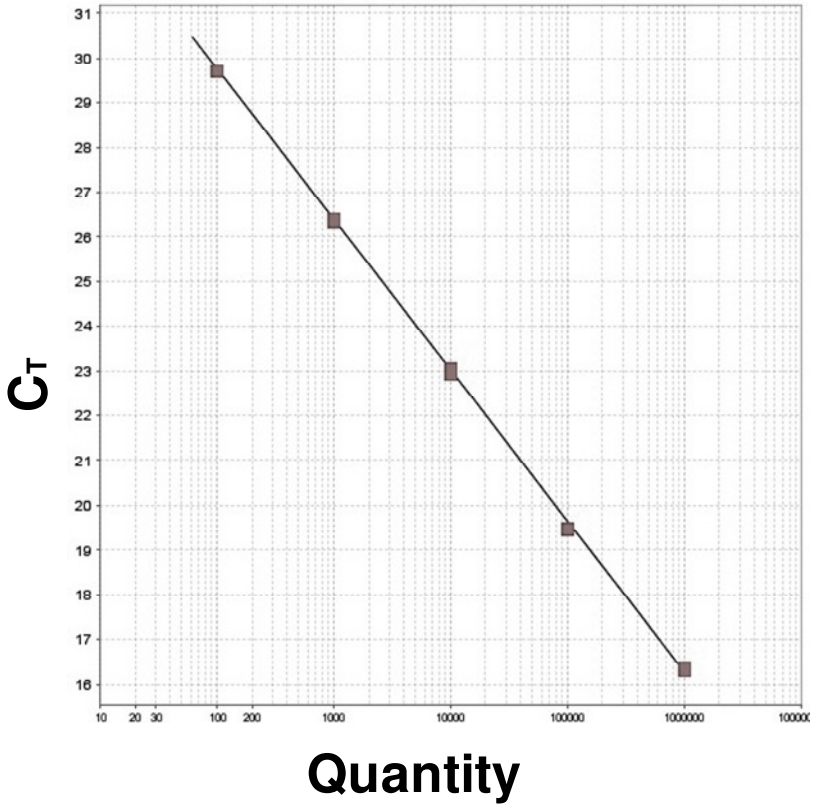

Fig. 5 Regression line of a standard series in the qRT-PCR.
Plasmids of the gal-15 were used from 102 to 106 copies. The Ct values (cycle threshold) are plotted against the copy numbers.

For the validation of the qPCR the parameters efficiency, linearity of the standard curve (R^2 and quality of the melt curve were analyzed. From the slope of the regression line computed by the software the efficiency of amplification can be determined. With slope of -3.32 a 100 percent efficiency of amplification is present; the product doubles itself in each cycle. A slope between -3.1 and -3.6 (corresponds to an efficiency of 90-110%) is considered as evaluable. If amplification is not sufficiently efficient, the output quantities cannot be computed accurately. Additionally R^2 was computed. Thus the linear connection between Ct-values and assigned copy number was examined; the R^2-value should be > 0.985. The melt curve was checked for the appearance of only one defined peak, to secure that only one product was amplified. Only if all parameters mentioned corresponded to the given criteria, the computed copy-numbers of the unknown samples were used for further evaluation.

3.3.15 Flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. It uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5 µm to 40 µm diameter. Cells are hydro-dynamically focused in a sheath of PBS before intercepting an optimally focused light source, normally a laser.

In these studies a FACScan® was used, working with an Argon-laser, which emits light of 488 nm wavelength. The FACScan® is able to detect FSC, SSC and fluorescence emission within three different wavelengths (FL-1 = 515-544 nm green, FL-2 = 564-606 nm orange, FL-3 = >650 nm red). Forward-scattered light (FSC is a measurement of mostly diffracted light and is detected just off the axis of the incident laser beam in the forward direction by a photodiode. Forward-scattered (FSC) light is proportional to cell-surface area or size. Side-scattered (SSC) light is proportional to cell granularity or internal complexity (RADBRUCH 1992). SSC is a measurement of mostly refracted and reflected light that occurs at any interface within the cell where there is a change in refractive index. SSC is collected at approximately 90 degrees to the laser beam by a collection lens and then redirected by a beam splitter to the appropriate detector. Every measurement is characterized via five different parameters (FSC, SSC, FL-1, FL-2, FL-3).

The data obtained with the flow cytometer was analyzed using the „WinMDI Version 2.8“software. Flow-cytometry data can be plotted in a single dimension, to produce a histogram, or in two-dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates." A gate is a numerical or graphical boundary that can be used to define the characteristics of particles to include for further analysis.

3.3.15.1 Quantification of vital cells using the reference cell method

A part of *in vitro* incubated cells die during the incubation under the influence of the cultural conditions and the stimulating signals. The unknown number of vital cells present in the suspension can be determined by adding a known number of cells to the cell suspension. At least one parameter must be different from the target cell suspension which can be measured, with the same device settings. Therefore the vital cell events must be compared with reference cell events in order to calculate absolute vital cells.

3.3.15.2 Production of reference cells

For staining, about $2x10^7$ freshly separated bovine MNCs were placed in a 15 ml tube and centrifuged (5 min, 4°C, 80 x g). After discarding the supernatants, the cell pellet was resuspended in 100 µl PBS and incubated with undiluted monoclonal antibody Bo1 for 15 min at 4°C. Subsequently the cell suspension was washed with 5 ml MIF buffer and centrifuged for 7 min (4°C, 80 x g). Again, the supernatant was removed, the cell pellet resuspended in 100 µl of 1:40 diluted FITC-conjugated goat-anti-mouse antibody and incubated in darkness (20 min, 4°C). Another washing step was performed, where the cells were centrifuged, resuspended in 50 ml PBS and centrifuged again. For fixation, the cells were suspended in 30 ml of a 4% paraformaldehyd-solution. After dark incubation for 24 h at 4°C, the cells were centrifuged for 15 min at RT, decanted and washed again with PBS. The obtained cell pellet was taken up in carrier fluid containing PI (2 µg/ ml) to a final concentration of $4x10^5$ cells/ ml. The concentration was controlled before application and corrected if necessary. In the flow cytometer the reference cells were characterized using their morphology (MNCs) as well as their red or green fluorescence.

3.3.15.3 Quantification of vital cells in the Flow cytometry

The contents of a micro titre plate well were transferred to flow cytometry tubes containing 100 µl of carrier fluid with PI (4 µg/ml). Additionally 50 µl of reference cell suspension (equaling $5x10^4$ cells) were added. The optimal ratio of reference cells to cultural cells is 1:1 to 1:3. During the FACS measurement of the cell mixture (10.000 events) reference cells (Bo 1^+ / PI^+) as well vital cultivated cells ($Bo1^-/PI^-$) were detected. The absolute number of vital cells was calculated with the following formula:

$$\text{Number of the culture cells} = \frac{\text{Measured culture cell events} \times \text{used number of reference cells}}{\text{Measured number of reference cell events}}$$

3.3.16 Statistical methods

The statistical analysis has been performed using the program SAS 9.1 (SAS InstituteInc., Cary, North Carolina, USA, 1988) and StatView version 5.0 (SAS Institute Inc., Cary, North Carolina, USA). All data were tested with the Shapiro-Wilk test for normality. In the descriptive statistics normally distributed data was described by the mean (MW), the standard

error (SEM), the standard deviation (s) as well as the variation coefficient (CV). The Wilcoxon Signed-Rank Test and Friedman Test were used for the paired and not normally distributed data. For impaired data the Mann Whitney U Test was used. Data with a right-slated distribution which were not normally-distributed became normalized by converting them into the 10^{th} logarithmic value. Afterwards the normal distribution of the logarythmitized data was tested again by using the Shapiro Wilk test. In order to examine whether dependent samples from two classes of a qualitative characteristic vary concerning a quantitative characteristic, the average values were compared by means of a T-test.

To detect correlations between quantitative, normally distributed sets of data were the test according to Pearson was performed. The assessment of the significances of the correlation coefficients (r) has been carried out on the bases of the description of PETRIE (2006). Relations between two parameters or differences between parameter classes or dependent samples with an error probability $p<0.05$ were considered significant.

4 Results

In the following, galectin expressions in the bovine corpus luteum, in cellular subsets are described. Additionally, the immuno-modulatory potential of this special subgroup of lectins will be characterized for bovine immune cells *in vitro*.

4.1 Galectin mRNA expression in the corpus luteum

4.1.1 Galectin mRNA expression in the corpus luteum during estrous cycle and early pregnancy in the cow

In order to determine the galectin expression in the corpus luteum during the estrous cycle and early pregnancy, non-inseminated cows were ovariectomized on day 12 and 16 of the estrous cycle. Inseminated and pregnant cows were ovariectomized day 16 and day 40 (BEINDORFF et al. 2010).

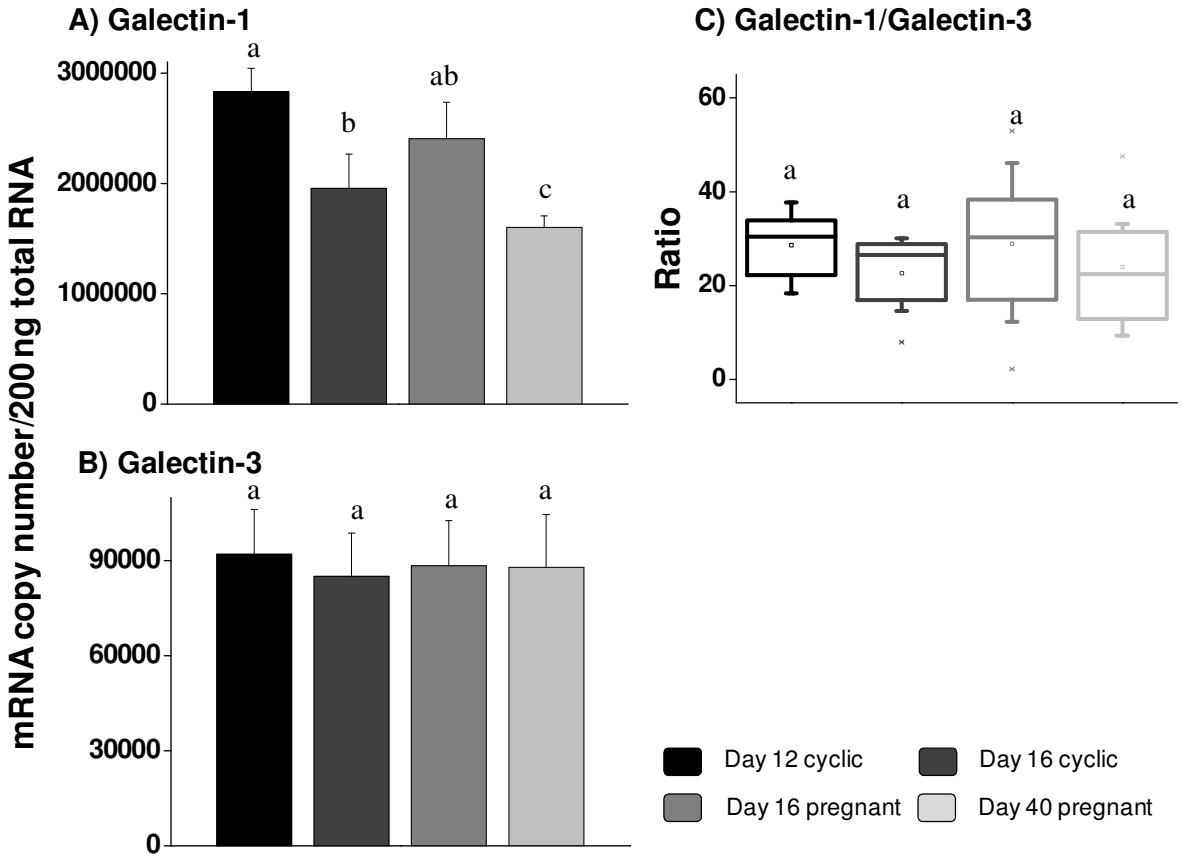

Fig. 6 Gene expression of gal-1 and gal-3 in during estrous cycle and early pregnancy in the cow.

Messenger RNA copy numbers of gal-1 (A) and gal-3 (B) in the bovine corpus luteum on day 12 (n=5) and day 16 (n=5) of the estrous cycle and on day 16 (n=5) and day 40 (n=7) of pregnancy. C) Ratio between copy numbers of gal-1 and gal-3. Gene expression was determined by qRT-PCR (Mean ± SEM). CL centre and CL periphery were analyzed together.

The expression of galectins in the CL tissue during the transition period from the estrous cycle to pregnancy is shown in Fig. 6. Gal-1 was expressed significantly different between days 12 and 16 in cyclic and days 16 and 40 in pregnant cows ($p < 0.01$) whereas gal-3 expression remained constant. The gal-1expression decreased depending on the progression of the cycle or the pregnancy process. The expression of gal-1 and gal-3 did not differ between day 16 cyclic and day 16 pregnant cows. The ratio of gal-1/gal-3 mRNA copy numbers (roughly 30:1) was the same in all analyzed groups (Fig. 6C).

4.1.2 Influence of a luteolytic effective dose of $PGF_{2\alpha}$ on the gene expression of selected galectins in the bovine corpus luteum

To examine the influence of $PGF_{2\alpha}$-induced luteolysis on galectin gene CLs were removed surgically from HF heifers (RADDATZ 2008) before and at different time points (5 min, 15 min, 30 min, 120 min, and 720 min) after injection of $PGF_{2\alpha}$.

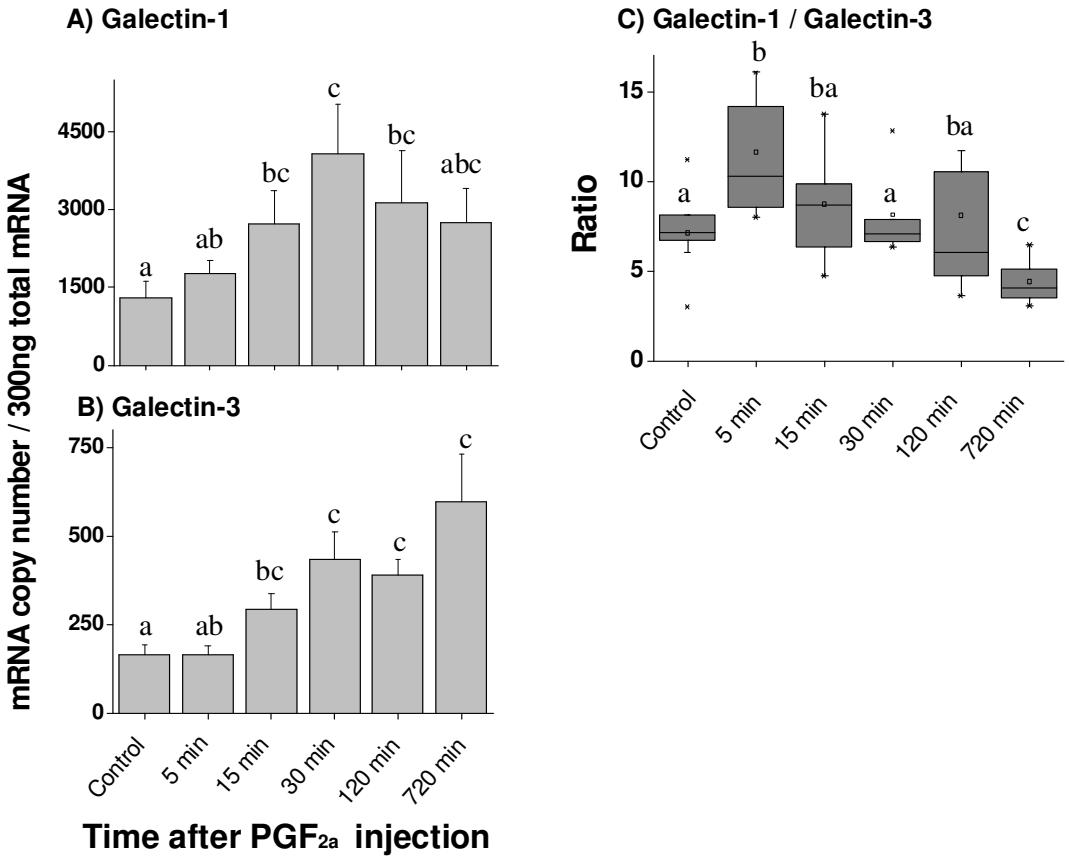

Fig. 7 Gene expression of gal-1 and gal-3 during $PGF_{2\alpha}$-induced luteolysis.

At defined times after intramuscular PGF2α injection the cows (n=5) for each time point and control; n=4 at 720 min) were ovariectomized and the expression of gal-1 (A) and gal-3 (B) was determined by qRT-PCR (means ± SEM). C) ratio between of gal-1 and gal-3 mRNA copy numbers. CL centre and CL periphery were analyzed together.

Gal-1 expression significantly increased at 15 min after $PGF_{2\alpha}$ injection and remained significantly higher expressed till 120 min after $PGF_{2\alpha}$ injection (Fig. 7A). Also, gal-3 expression was significantly higher expressed at 15 min after $PGF_{2\alpha}$ injection. This up-regulation remained constant till 720 min after $PGF_{2\alpha}$ injection (Fig. 7B).

Gal-1 mRNA copy numbers exceeded those of gal-3 with a gal-1/gal-3 ratio of 8:1 before $PGF_{2\alpha}$-induced luteolysis. Five min after $PGF_{2\alpha}$–induced luteolysis the ratio significantly increased. Thereafter the gal-1/gal-3 ratio decreased again reaching a significantly lower level (about 5:1) at 720 min compared to the starting situation.

4.1.3 Influence of lipopolysaccharide on galectin gene expression in the bovine corpus luteum

Whether bacterial products exert an influence on the CL galectin expression was analyzed in vitro by stimulating CL pieces with LPS.

Potential differences between central and peripheral parts of the CL were not observed. All three tested galectins (gal-1, gal-3, and gal-15) showed the same mRNA copy numbers in both CL locations (Fig. 8) with highest copy numbers for gal-1, followed by gal-3 and by gal 15 which expression (about 140 copy numbers per 100ng total RNA) was negligible. Subsequent experiments in which the galectin expression in central CL pieces and peripheral CL pieces were analyzed after LPS stimulation also revealed no differences (data not shown).

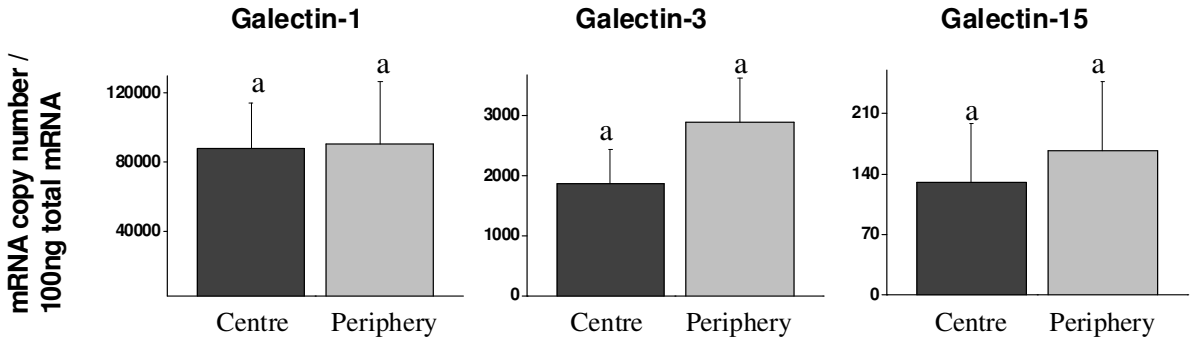

Fig. 8 Galectin gene expression in central CL and peripheral CL (n=5).

Messenger RNA copy numbers of gal-1 gal-3 and gal-15 in the bovine corpus luteum. Gene expression was determined by qRT-PCR (Mean ± SEM).

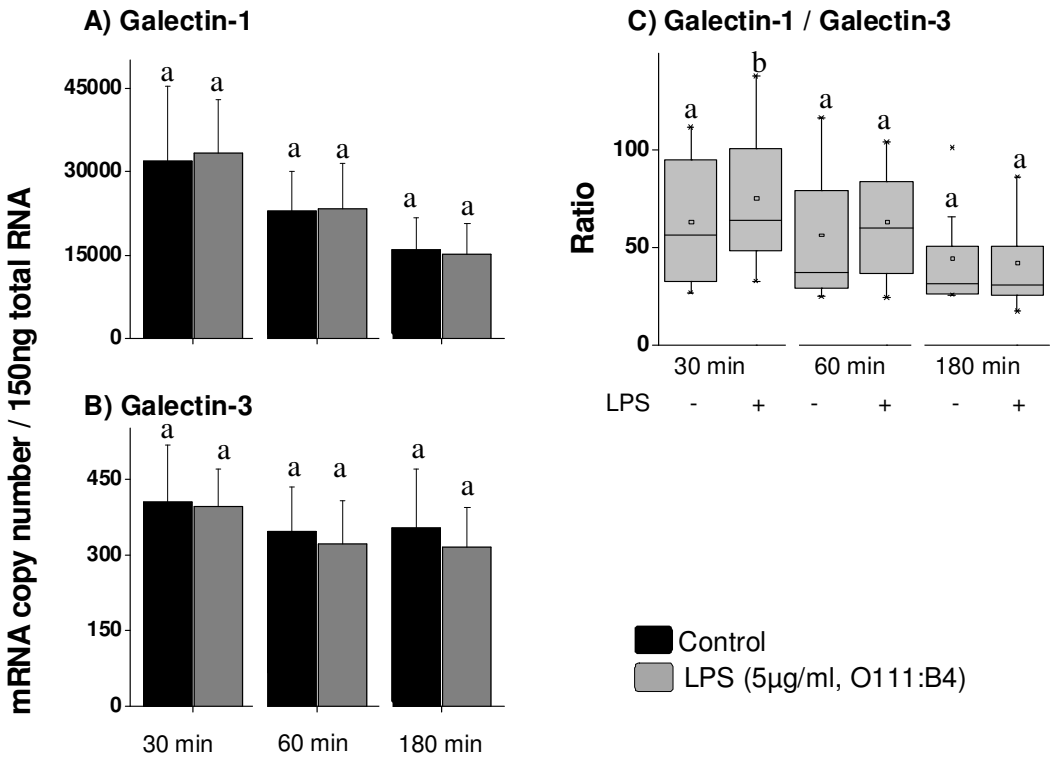

Fig. 9 Galectin gene expression in CL tissue after LPS (serotype O111:B4) stimulation in vitro.

Gene expression of A) gal-1 (A), B) gal-3, and C) ratios between gal-1 and gal-3 mRNA copy numbers. Pieces of bovine CL (from the centre and the periphery) (n=5) were stimulated in vitro with LPS (5 µg/ml) for indicated times. Gene expression was determined by qRT-PCR (Mean ± SEM). CL centre and CL periphery were analyzed together. Small letters indicate significant differences ($p < 0.05$).

Whereas gal-1 expression dropped by one half between 30 min and 180 min in vitro incubation, the expression level of gal-3 remained rather constant (Fig. 9AB). Gal-1 and gal-3 expression was not significantly affected by LPS (O111:B4). Subtle changes in the expression level of gal-1 and gal-3 at 30 min after LPS stimulation resulted in a significant rise of the gal1/gal-3 ratio (Fig. 9,C).

To test whether the lack of LPS induced expression changes was due to the selected LPS, another LPS serotype was tested (LPS =55:B5).

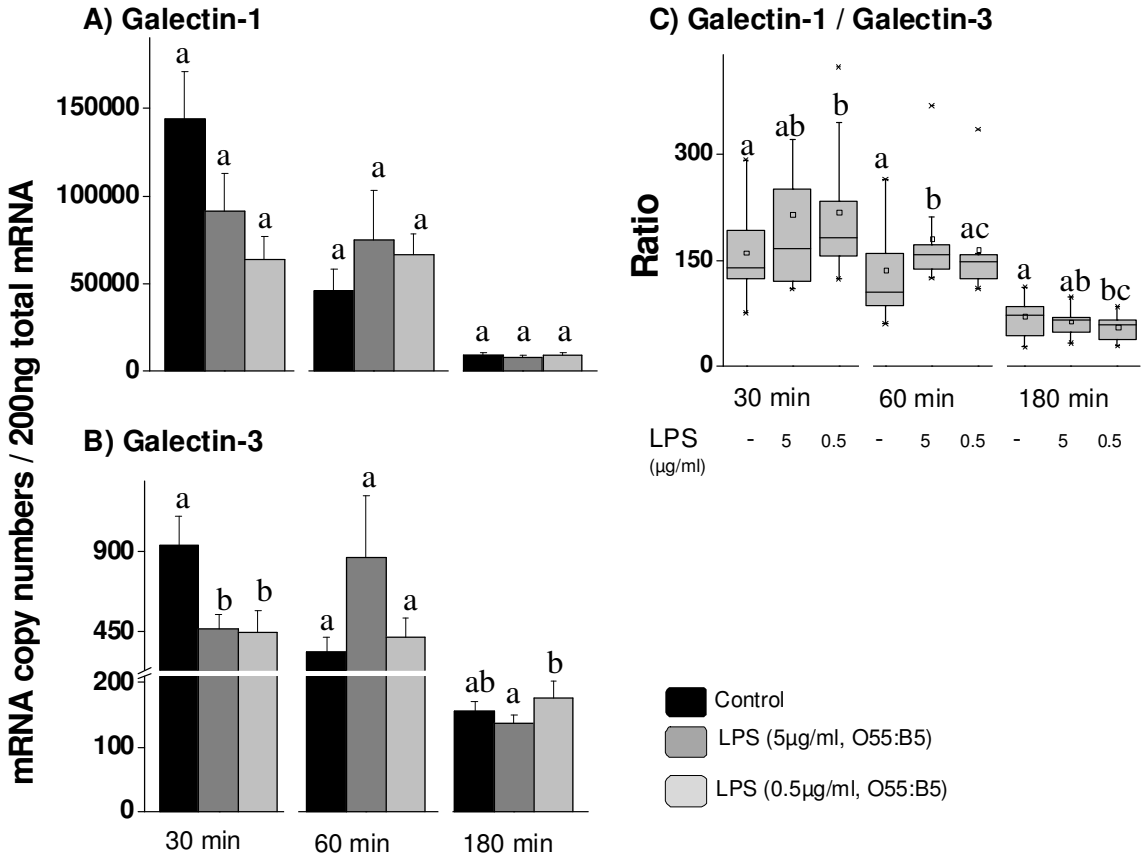

Fig. 10 Galectin gene expression in CL tissue after LPS (serotype O55:B5) stimulation in vitro.

Gene expression of A) gal-1, B) gal-3, and C) ratios between gal-1 and gal-3 mRNA copy numbers. Pieces of bovine CL (from the centre and the periphery) (n=6) were stimulated in vitro with LPS (O55:B5) in two different concentrations for indicated times. Gene expression was determined by qRT-PCR (Mean ± SEM). CL centre and CL periphery were analyzed together.

Again, the expression of both gal-1 and gal-3 decreased with incubation time irrespective of the culture/stimulation condition (Fig. 10AB). At each time point LPS stimulation had no significant effect on the gal-1 expression, whereas 30 min after stimulation with LPS gal-3 expression was significantly lower. After 180 min in vitro the stimulation with LPS in the lower concentration caused a significantly higher gal-3 expression.

When comparing gal-1 and gal-3 gene expression in the bovine CL (Fig. 10C), gal-1 expression was in general 150 x higher than gal-3 expression. The ratio of gal-1/gal-3 decreased with time in all set ups. At 30 min after in vitro culture LPS (0.5 µg/ml) stimulated CL showed a significantly higher gal-1/gal-3 ratio compared to unstimulated controls. The same enhancement could be observed in CL stimulated 60 min with LPS (5 µg/ml). A prolonged incubation for 180 min with 0.5 µg/ml LPS resulted in a significant decrease of the gal-1/gal-3 ratio.

4.2 Galectin mRNA expression in leukocyte subpopulations

To analyze galectin expression in different leukocyte subpopulations, monocytes (CD14-positive cells after MAC-separation), lymphocytes (CD14-negative cells after MAC-separation), and neutrophils were separated from blood. Macrophages were generated from blood monocytes. The different subpopulations were used to determine baseline expression of selected galectins by qRT-PCR.

Gal-3 was found to be expressed in every tested leukocyte population. The highest expression of gal-1 was found in MAC-separated monocytes whereas in vitro generated macrophages expressed highest copy numbers of gal-3. Interestingly, neutrophils appeared the only subpopulation in which no gal-1 expression was exceptionally low (< 100 mRNA copy numbers).

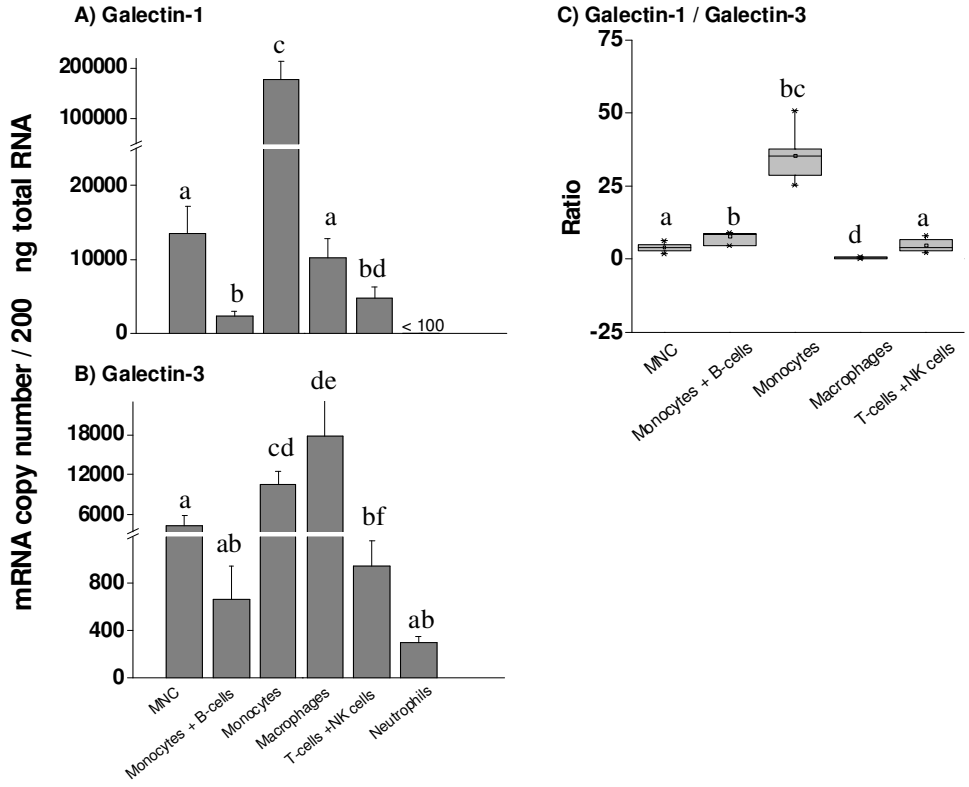

Fig. 11 Gene expression of gal-1 and gal-3 in leukocyte populations.

The mRNA expression (in 200 ng total RNA; mean ± SEM) of gal-1 (A) and gal-3 (B) was determined by qRT-PCR in mononuclear cells, a mixture of monocytes and B-lymphocytes (MHC class II+ cells after MAC-separation), monocytes, macrophages, a mixture of T-lymphocytes and NK cells (MHC class II-negative and CD14-negative cells after MAC separation) and neutrophils (n=5 for each cell subpopulation). C) ratio of gal-1 and gal-3 mRNA copy numbers.

Gal-15 gene expression was restricted to monocytes, B-lymphocytes and neutrophils. Expression has not been tested in macrophages (Tab. 10).

Tab. 10 Expression of gal-15 in non-stimulated MNCs and subpopulations.

Cell type	N	mRNA copy numbers[1]
MNC	6	< 100
Monocytes+B-Lymphocytes[2]	6	488 ± 0
Monocytes[3]	6	887 ± 57
T-Lymphocytes/ NK-cells[4]	6	<100
Neutrophils	5	557 ± 40

1) In 200 ng total-RNA Mean ± SEM: MNC: mononuclear cells; 2) MHC class II+ Monocytes and B-cells after MAC-separation; 3) CD14-positive cells after MAC-Separation.4) MHC class II-negative and CD14-negative cells after MAC-Separation.

4.2.1 Galectin expression in stimulated T-lymphocytes and NK-cells

To test whether a mitogen activation leads to an altered expression of gal-1, gal-3, and gal-15 T-lymphocytes and NK-cell mixture (see in Tab. 10) were stimulated for 3 and 6 hours with the lectin Con A (3 µg/ml) (Fig. 12).

Gal-15 gene expression was consistently below 100 copy numbers (data not shown).

In unstimulated cultures, gal-1 expression significantly dropped between 3 h and 6 h (Fig. 12A, $p < 0.01$), whereas gal-3 expression was significantly higher at 6 h compared to 3 h cultures (Fig. 12B, $p < 0.05$). Stimulation of the cells with Con A for 3 h or 6 h had no significant effect on the gal-1 expression. Compared to control set-ups, gal-3 was significantly up regulated after 3 h stimulation and significantly down regulated after 6 h stimulation. In unstimulated control cultures the gal-1/gal-3 ration significantly dropped between 3 h and 6 h culture. The stimulation with Con A, either for 3 h or 6 h left the gal-1/gal-3 ratio unchanged (Fig. 12, C).

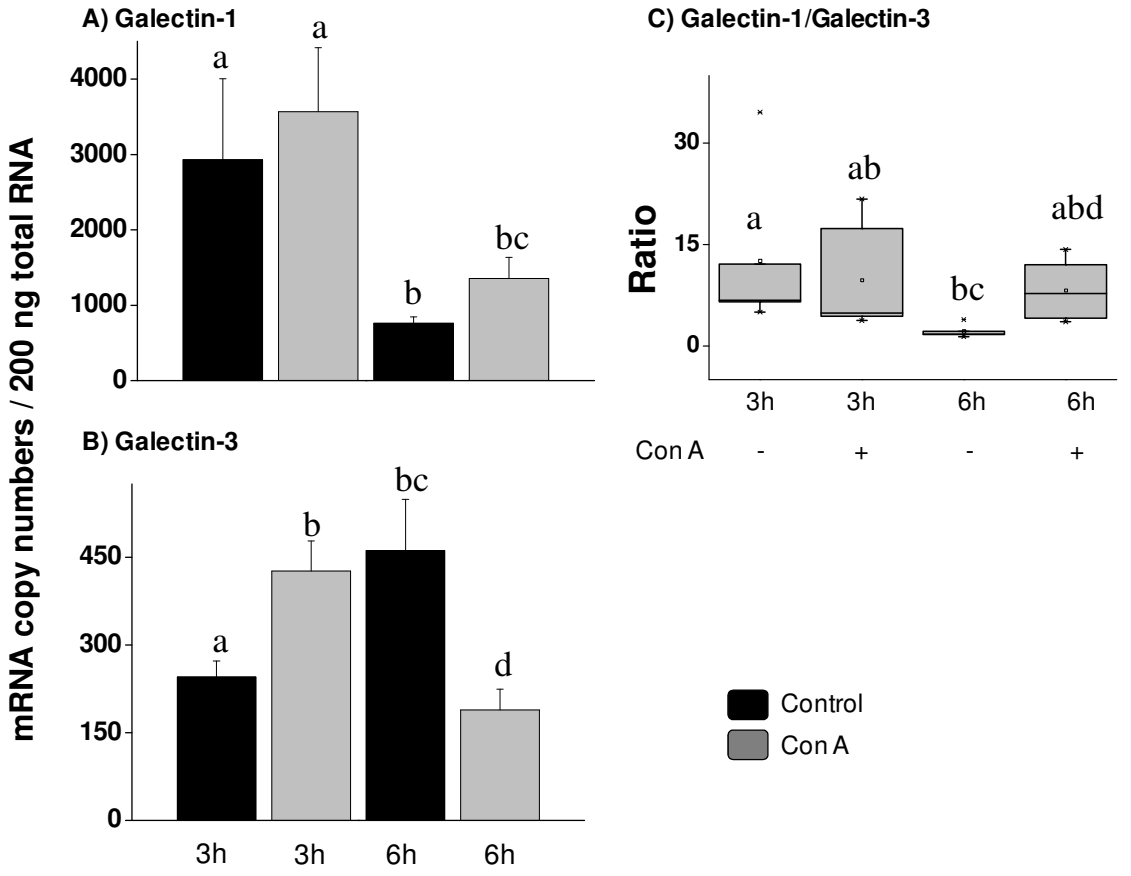

Fig. 12 Gene expression of gal-1 and gal-3 in non-stimulated T-cells-NK-cells mixture and after Con A incubation (n=6).

The mRNA expression (in 200 ng total RNA) of A) gal-1 and B) gal-3 is presented in non-stimulated T-cells or after stimulation with Con A. C) Ratio of gal-1/gal-3 was also shown. Gene expression was determined by qRT-PCR (Mean ± SEM).

4.2.2 Galectin expression in stimulated monocytes and monocyte-derived macrophages

For monocytes and in vitro generated monocyte derived macrophages it was tested whether stimulation with LPS alters the expression of galectins.

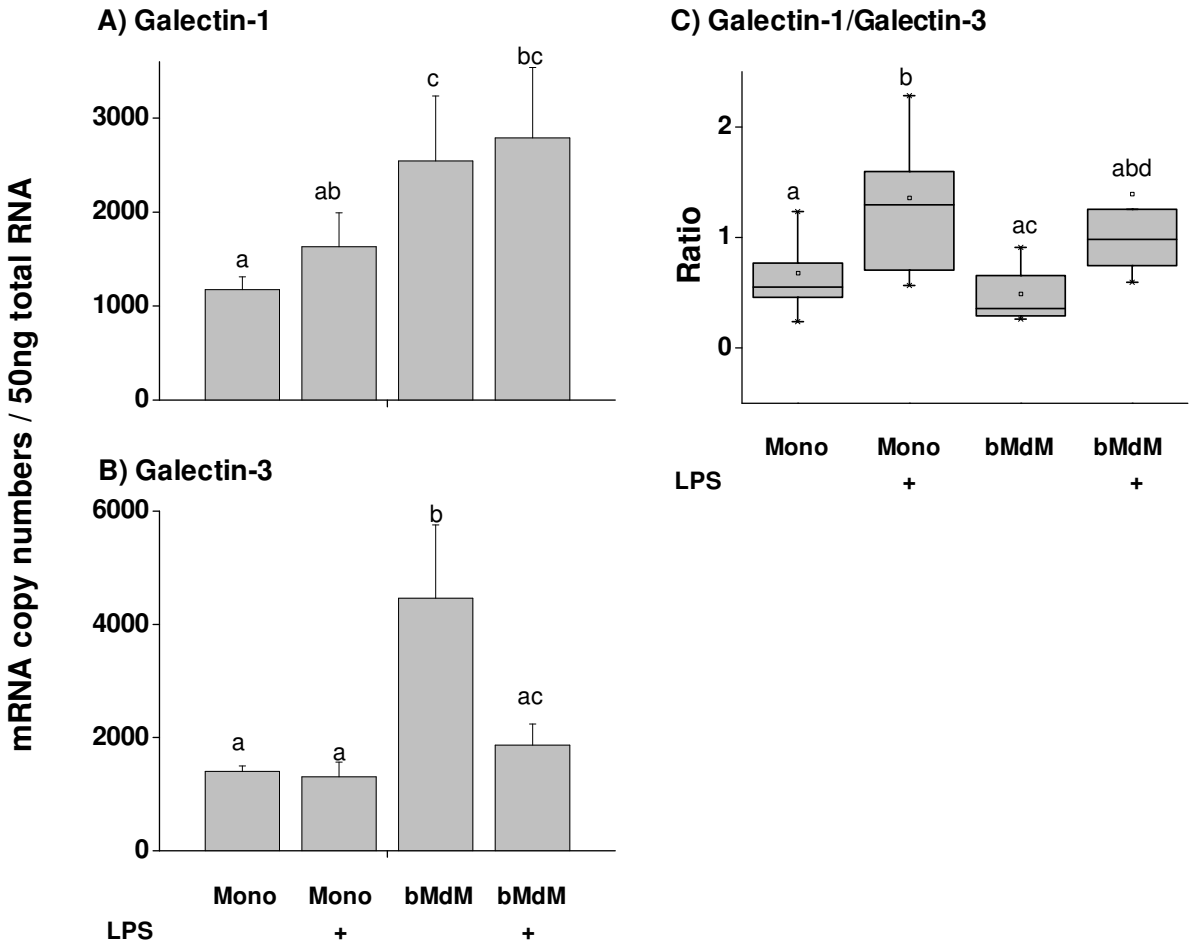

Fig. 13 Gene expression of gal-1 and gal-3 in monocytes and macrophages (bMdM.

The mRNA expression (copy numbers in 50 ng total RNA after RT-PCR; mean ± SEM) of gal-1 (A) and gal-3 (B) in monocytes (n=8) and blood monocyte-derived macrophages (n=8) after 3 h in vitro. Parallel set ups were stimulated with LPS (1 µg/ml) or left untreated for control. (C) Ratio between copy numbers of gal-1 and gal-3.

In monocytes, gal-1 and gal-3 expression remained largely unchanged after LPS stimulation (Fig. 13AB). The slight but insignificant higher expression of gal-1 after LPS-stimulation, however, resulted in a significant higher gal-1/gal-3 ratio in monocytes (Fig. 13C).

In macrophages, the LPS stimulation left the gal-1 expression unchanged but significantly reduced the gal-3 expression (Fig. 13AB). However, the selective changed gal-3 expression had no impact on the gal-1/gal-3 ratio in macrophages (Fig. 13C).

4.2.3 Galectin expression in stimulated neutrophils

Unstimulated neutrophils, cultured for 3 h displayed consistently low copy numbers of gal-1 and gal-3 (Fig. 14AB). The copy numbers of gal-15 measured at 3 h in vitro were about 6 times higher (Fig. 14D).

After 6 h in vitro, copy numbers for all three galectins were insignificantly different from the values obtained after 3 h, except for gal-3. After 6 h of LPS stimulation, both the gal-3 and gal-15 dropped significantly compared to controls (Fig. 14BD), whereas gal-1 expression showed a significantly higher expression (Fig. 14A).

Consistent with the LPS-induced reduced expression of gal-3 and the higher expression of gal-1 after 6 h in vitro, the ratio between gal-1 and gal-3 was significantly higher compared to unstimulated control set-ups (Fig. 14C, $p < 0.05$).

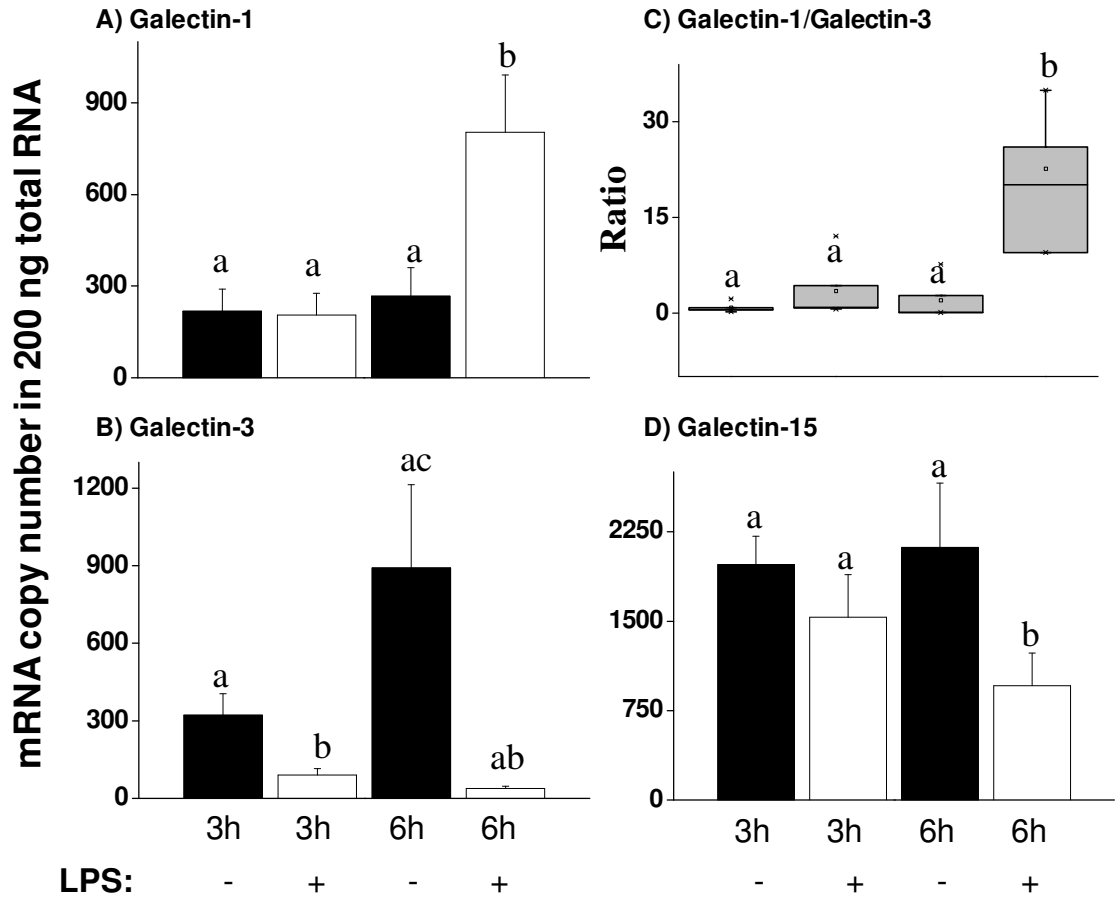

Fig. 14 Gene expression of gal-1, gal-3 and gal-15 in LPS-stimulated neutrophils.
Separated neutrophils (from 6 donors) were stimulated in vitro for 3 h or 6 h with LPS (1 µg/ml). Unstimulated set-ups served as control. Gene expression of gal-1 (A), gal-3 (B) and gal-15 (D) was analyzed by qRT-PCR (copy numbers in 200 ng total RNA, means ± SEM). C) ratios between gal-1 and gal-3 copy numbers.

4.3 Immuno-modulatory potential of galectins *in vitro*

4.3.1 Influence of galectins on the vitality and the blastogenesis of mononuclear cells after *in vitro* stimulation

Aim of the following part of this study was to examine whether galectins have a proliferation-inducing effect and whether they have an effect on the superantigen- or the mitogen-induced proliferation reaction of bovine mononuclear cells.

The stimulation of MNC with the superantigen SEA and the lectin Con A significantly induced a higher number of viable blast cells after 4 days in vitro (Fig. 15A). Notably, gal-1, gal-3 as well as the combination of gal-1 and gal-3 also induced significantly higher numbers

of blast cells compared to unstimulated controls, although significantly less than Con A or SEA (Fig. 15A), (Fig. 16). To test whether the proliferation induction by galectins was lectin-specific, parallel set-ups were made with the addition of 5 mM lactose (Fig. 15B). Addition of lactose completely blocked the blastogenesis-inducing effect of the galectins, whereas it has no effect on the SEA- or Con A-induced proliferative response.

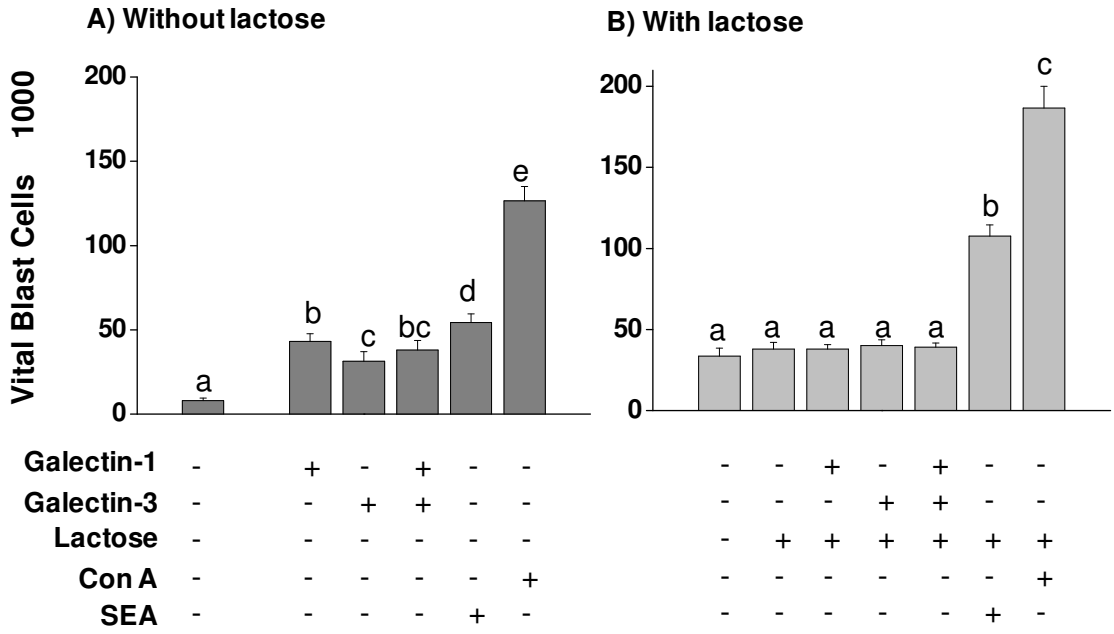

Fig. 15 Galectin-, superantigen-, and mitogen-induced blastogenesis of bovine MNCs *in vitro*.

Freshly separated MNC (from 3 donors) were stimulated with gal-1(2 µg/ml), gal-3 (2 µg/ml), gal-1+gal-3 (1 µg/ml), SEA (1 ng/ml) or Con A (2 µg/ml) without (A) or with (B) addition of lactose (5 mM) *in vitro*. The cells were incubated at 37°C and 5% CO_2 for 4 days. The total numbers of the vital blast cells (means SEM) were determined flow cytometrically.

To test whether gal-1 or gal-3 modulate a superantigen or mitogen-induced proliferative response, the galectins were added to MNC cultures stimulated with SEA or Con A (Fig. 16).

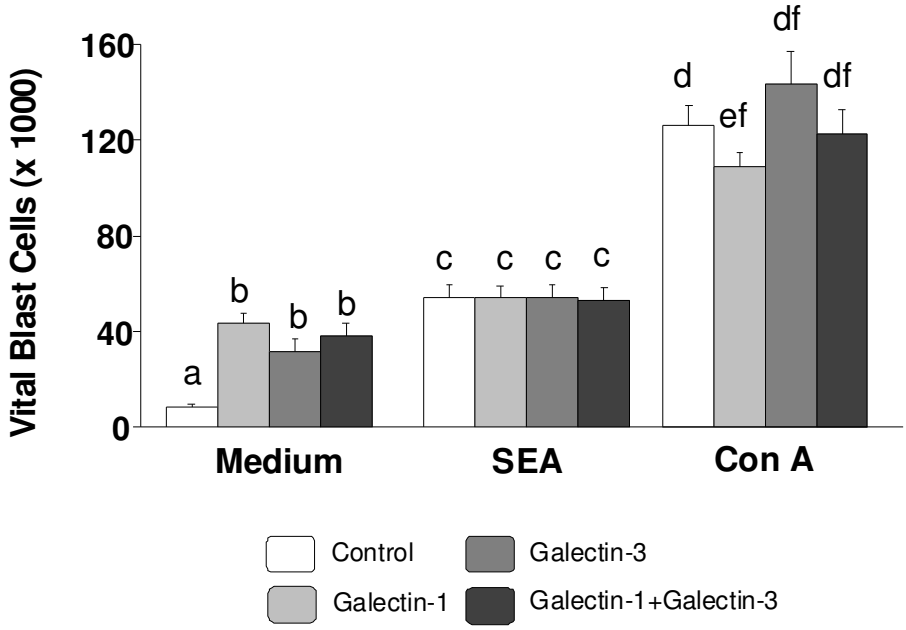

Fig. 16 Influence of galectins on the superantigen or mitogen-induced blastogenesis of bovine MNCs after *in vitro* stimulation.

Freshly separated MNC (from 8 donors) were stimulated in the absence of SEA and Con A (Medium) with gal-1 (2 µg/ml), gal-3 (2 µg/ml) or gal-1+ gal-3 (1 µg/ml). Parallel setups contained SEA (1 ng/ml) or Con A (2 µg/ml) together with galectins (SEA, Con A). The cells were incubated at 37°C and 5% CO_2 for 4 days. The total numbers of the vital blast cells (means SEM) were determined flow cytometrically.

The galectins, although significantly inducing higher numbers of viable blast cells (Fig. 16, Medium) did not change the SEA-induced numbers of blast cells (Fig. 16, SEA). In con A-stimulated cell cultures, gal-1 but not gal-3 or the galectin mixture weakly but significantly reduced numbers of viable cells (Fig. 16, Con A).

4.3.2 Influence of selected galectins on T-cell subpopulations after *in vitro* stimulation

Next it was tested whether gal-1 and gal-3 stimulation of T-cells affects the proliferation of a distinct T-cell subset (CD8+ and/or CD4+ T-cells). After stimulation with SEA or Con A, cells were labeled with anti-CD4 and anti-CD8. The percentages were used to calculate the

CD4/CD8 ratios. Among small lymphoid cells the CD4/CD8 ratio in unstimulated controls ranged at 0.8. The addition of gal-1 and -3 insignificantly raised this value to 1.1 (gal-1) or 1.0 (gal-3). Only among Con A-stimulated blast cells the CD4/CD8 ratio significantly raised to 1.5 (Tab. 11).

Tab. 11 Relation of CD4-positive to CD8-positive small lymphoid cells after in vitro stimulation of bovine mononuclear cells with SEA, Con A, galectin-1 and galectin-3.

	Medium	SEA	Con A	Gal-1	Gal-3
CD4/CD8	0.8 ± 0.1 a	0.9 ± 0.1 a	1.5 ± 0.2 b	1.1 ± 0.1 a	1.0 ± 0.1 a

Bovine mononuclear cells from 7 animals were stimulated in vitro for 4 days with SEA (1 ng/ml), Con A (2 µg/ml), gal-1 (2 µg/ml), or gal-3 (2 µg/ml). Cells were labeled with anti-CD4 and anti-CD8. Given are the ratios between CD4+ and CD8+ small lymphocytes (Means ± SEM). Small letters indicate significant differences ($p < 0.05$).

4.3.3 Modulation of apoptosis in bovine MNC and PMN

In this part of the study the ability of galectins to induce apoptosis in PMN and MNC was examined. The proportion of apoptotic cells was determined after 24 h of in vitro incubation. Gal-3 weakly but significantly raised the fraction of apoptotic PMN. Among MNC, gal- weakly but significantly lowered the fraction of apoptotic cells.

In both MNCs and PMNs, compared to a control group and a SEA stimulated group, there was no effect of gal-1 on apoptosis. However, an apoptotic effect was noted for gal-3 ($p < 0.05$). No differences were determined between galectin groups.

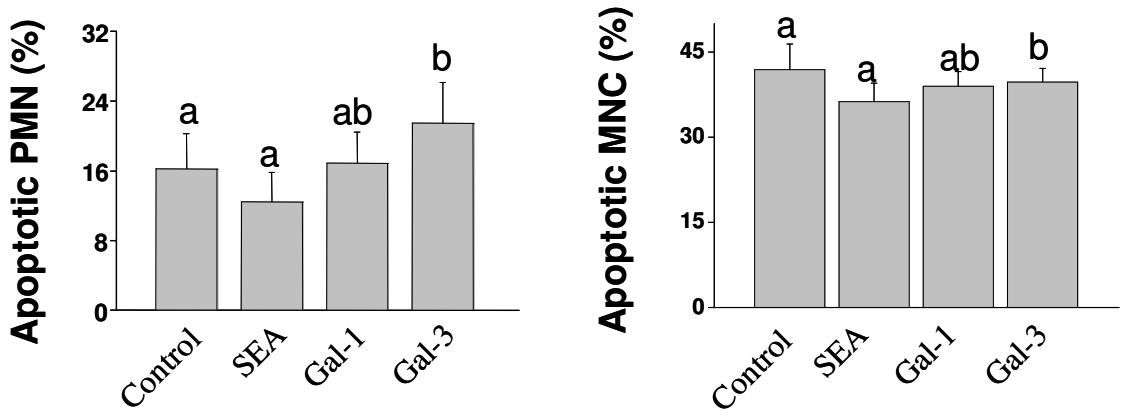

Fig. 17 Proportion of apoptotic PMN and MNC after in vitro stimulation.

PMN and MNC of 5 animals were incubated for 24 h in vitro. Parallel set-ups contained SEA (1 ng/ml), Con A (2 µg/ml), gal-1 (2µg/ml), or gal-3 (2µg/ml). The percentage of apoptotic cells was quantified by flow cytometry (Means ± SEM). Small letters indicate significant differences ($p < 0.05$).

4.3.4 Effect of galectins on the interferon gamma production after *in vitro* stimulation

Whether galectins bias the cytokine production of activated T-cells was analyzed by intra cellular determination of Interferon gamma (IFNγ) after in vitro stimulation. An example is shown in Fig. 18.

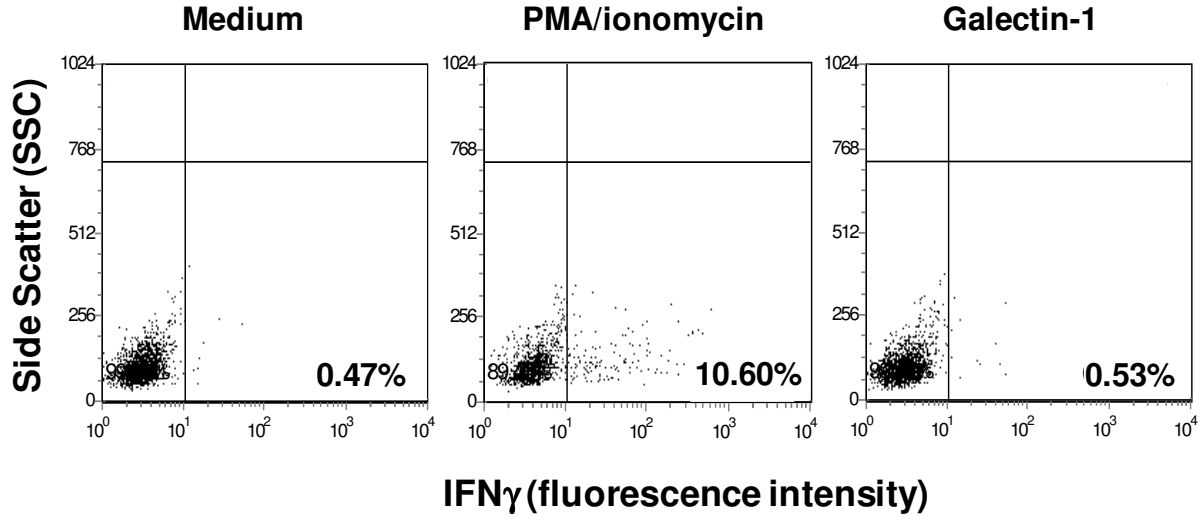

Fig. 18 Interferon gamma-positive mononuclear cells after in vitro stimulation.

IFNγ-positive lymphoid cells were determined by flow cytometry 48 hours after stimulation with PMA/ionomycin or gal-1 compared to the medium control (see Tab. 12 for detail).

Tab. 12 IFNγ-positive lymphoid cells after in vitro stimulation of bovine mononuclear cells MNC.

Medium	PMA/ionomycin	Con A	Galectin-1	Galectin-3
0.6 ± 0.1 a	6.3 ± 1.0 b	6.4 ± 1.3 b	0.5 ± 0.03 a	0.6 ± 0.1a

Isolated MNC of 6 animals were stimulated in vitro for 48 h with Con A, gal-1, or gal-3 (all 2 µg/ml). A mixture of PMA (0.06 nM) and ionomycin (2 nM) served as a positive control. Untreated cultures (medium) served as negative controls. IFNγ-positive cells were determined by intracellular immunofluorescence and flow cytometric evaluation (Means ± SEM of small lymphocytes). Small letters indicate significant differences ($p < 0.05$).

Among Con A or PMA/ionomycin-stimulated cells, a significant higher percentage of IFNγ-positive cells ($p < 0.01$) compared to unstimulated controls (0.6%) was measured. Neither gal-1 nor gal-3 induced IFNγ-positive cells (Tab. 12).

5 Discussion

In recent years, early embryo losses are one of the main economic problems in dairy production. Major reasons for pregnancy failure are ovarian disorders caused by an imbalanced production of chemokines, cytokines, and maybe galectins. The maintenance of early pregnancy requires a fully functional CL that is not susceptible to regression following fertilization (NISWENDER et al. 1994).

The regression of the CL has clear features of an inflammatory process which involves major changes in tissue composition (NEUVIANS et al. 2004). The numbers of T-lymphocytes and macrophages are increased during CL regression (PATE and LANDIS KEYES 2001).

In this thesis it has been hypothesized that galectins affect the process of luteolysis based on the known effects of these lectins on cell growth, differentiation, apoptosis, cellular attachment and their involvement in inflammation and angiogenesis. Especially, since galectins can act as immunologically effective substances like chemokines and cytokines, they are supposed to be involved in CL-regression.

The first aim was to analyze the expression of distinct galectins in bovine corpora lutea during estrus cycle, luteolysis and pregnancy. The second aim was to describe the expression of galectins in bovine immune cell subsets and to analyze some of their potential immuno-modulatory features for bovine immune cells *in vitro*.

5.1 Galectin expression in the bovine corpus luteum

Considering their potential influence on the immune system, it is plausible that galectins are localized in the reproductive organs of the cow. However, to date there is little information on this topic.

The transition of the follicle into the corpus luteum and subsequent luteolysis are controlled by angiogenesis and eventual regression of the blood vessels (PLENDL 2000). Interestingly some authors postulated that galectins function like angiogenic factors, similar to cytokines and chemokines (TOSCANO et al. 2007). Indeed, it was proved that gal-1 (DEMYDENKO and BEREST 2009) and gal-3 (NANGIA-MAKKER et al. 2000,DUMIC et al. 2006) have some roles in angiogenesis. For these important reasons luteal expression and regulation of the galectins were examined in this part of the study.

5.1.1 Expression and regulation of the galectins in the bovine corpus luteum during estrous cycle and early pregnancy *in-vivo*

Corpora lutea taken from healthy HF cows during the transition period from the estrous cycle to pregnancy using invariantly showed gal-1 and -3 expressions after qRT-PCR analysis (Fig. 6). Although initially gal-15 was taken into account, the expression level proved to be very low, close to the detection limit (Fig. 8). Therefore, further studies focused on the two galectins gal-1 and gal-3.

Gal-1 and gal-3 expression was measured separately in peripheral and central corpus luteum regions because of the possibility that gene expression differs between the two compartments. SHIRASUNA et al. (2008) described a differential gene expression due to regional differences in the blood supply. Also, a recent study demonstrated a significantly higher expression of the chemokines CCL5, CCL20 and CXCL8 in central CL parts, whereas TNFα expression was higher expressed in peripheral regions of the CL (RADDATZ 2008). However, in this study no differences between regions were observed after evaluating the gene expression of selected galectins in both parts of the CL (Fig. 8). Therefore, the mRNA copy numbers obtained in central and peripheral parts of the CL were pooled in further analyses.

Depending on the progression of the cycle or pregnancy process it was determined that gal-1 expression decreases significantly ($p < 0.01$), while gal-3 expression stays within similar levels. Furthermore no differences were observed between cyclic and pregnant animals on day 16. In contrast, KIM et al. (2008) found gal-3 to be present at the 3rd month of pregnancy, and in many luteal cells at the pro-estrus stage, but not at the 1st month of pregnancy in cattle. Furthermore, a study using murine ovaries reported that no gal-1 expression was found on day 15.5 and 18.5 of pregnancy, although gal-3 expression was seen weakly on day 18.5 of pregnancy (NIO and IWANAGA 2007). The authors suggested that gal-3 disappeared during gestation with active P_4 production Additionally, it was determined in cattle that on day 16 P_4 levels were similar within the luteal tissue but the plasma P_4 concentration decreased significantly in cyclic animals in comparison with the pregnant animals (BEINDORFF et al. 2010).

It was of high interest to analyze how the gene expression of changes after $PGF_{2\alpha}$-application. Heifers received $PGF_{2\alpha}$ intramuscular to induce luteolysis on day 10 till 12 p.ov. as described in detail by RADDATZ (2008), in order to determine the changes of *in vivo* gene expression in a defined model of an induced luteolysis process.

As soon as 15 min after $PGF_{2\alpha}$ injection, gal-1 and gal-3 expression significantly increased (Fig. 7AB). The time-dependent up regulation differed slightly between gal-1 and gal-3, with gal-1 having maximum expression levels at 30 min and gal-3 at 720 min after $PGF_{2\alpha}$ injection (Fig. 7AB). This fast induction in vivo parallels the rapid up regulated gene expression of CXCL-8, CXCL-1 and TNFα measured previously in the same CL samples (RADDATZ 2008).

Based on mRNA copy numbers, the expression of gal-1 exceeded that of gal-3 (Fig. 7, C). This was true for the whole time period (720 min) after $PGF_{2\alpha}$ injection. However, the gal-1/gal-3 ratio significantly increased at the very beginning of the process (at 5 min) and later significantly decreased. Since gal-1 and gal-3 are described to possess different functions this could reflect different cellular processes in the observed time frame after $PGF_{2\alpha}$ injection.

5.1.2 Expression and regulation of galectins in the bovine corpus luteum tissue during the oestrous cycle *in vitro*

The expression of gal-1 and gal-3 in bovine CL was also analyzed *in vitro* in order to determine the differences in gene expressions between *in vivo* and *in vitro* conditions. Especially it was of interest whether other inflammatory conditions are able to modulate the galectin expression. Here, the LPS stimulation of CL fragments was chosen as an in vitro model. This was based on the hypothesis that circulating LPS (in case of an *E. coli* infection) affects the physiological function of the CL.

In fact, LPS was able to alter the galectin expression *in vitro* (Fig. 10). In contrast to the ex vivo findings (see above) LPS (serotype O55:B5) caused a *decrease* in gene expression of both galectins after 30 min in vitro. The effects of LPS seem to depend on the serotype since LPS (serotype O111:B4) caused no altered galectin expression (Fig. 9). The in vitro generated data have to be interpreted with care since the expression in unstimulated CL fragments rapidly declined over time. Although other mRNA species were not examined, this could indicate a rapid destruction of mRNA by RNAse. The immediate analysis after CL preparation also proved that this expression reduction occurred already within the first 30 min of *in vitro* cultivation (data not shown).

The significant reduction of the gal-3 expression after LPS-stimulation of CL fragments in vitro has not been described in this organ. This LPS effect is at least in contrast to a study of YU et al. (2009) who determined that after stimulation with LPS, the expression of gal-3 was significantly up-regulated compared to the control group in the mouse genital tract, especially in the cervix. It can only be speculated that this differential response is due to signaling in other cells types (e.g. epithelial cells, macrophages) compared to mainly luteal cells.

Since gal-3 directly binds to LPS and is a negative regulator of LPS-induced inflammation, it can also be speculated that the corpus luteum, due to the down-regulation of gal-3 by LPS is rather sensitive and vulnerable to circulating LPS in case of infection with gram-negative bacteria. This is somehow supported by studies with gal-3-deficient macrophages which showed markedly elevated LPS-induced signaling and inflammatory cytokine production compared with wild-type cells (LI et al. 2008).

When gal-1 and gal-3 gene expression were compared to each other, gal-1 expression was higher than gal-3 in the bovine CL *in vitro*. This ratio remained at the same level after 5 µg/ml LPS stimulation, although a lower dose of LPS was able to increase this ratio after 30 min of incubation. In all incubation groups, the ratio of gal-1 to gal-3 decreased depending on the time kinetic. Also under *in vivo* conditions gal-1 was expressed higher than gal-3. During the estrous cycle and early pregnancy, gal-1 expression exceeded gal-3 by 30 fold. The value rose to about 100 fold under *in vitro* conditions. Similarly, during $PGF_{2\alpha}$-induced luteolysis, this ratio was found to be about 5, but after LPS stimulation it increased to 200. Certainly, these differences between groups depend on the experimental conditions and galectin modulations. So it is plausible that gal-1 and -3 regulations were different under *in vivo* and *in vitro* conditions. Whether the higher copy numbers of gal-1 compared to gal-3 translates also in a higher protein ratio between gal-1 and gal-3 has not been addressed in this study.

Another galectin which has been shown to be expressed in the female genital tract of ruminants (gal-15) was also analyzed by RT-PCR in the present study since up to now there is no information about the expression of this galectin in the bovine corpus luteum. However, based on mRNA copy numbers, gal-15 expression was found to be very low (or close to the detection limit of RT-PCR system) in CL pieces after in vitro cultivation, (Fig. 8). Basically, this supports findings of LEWIS et al. (2007) who proved that gal-15 is present in all ruminants, but is expressed only in the uterus of goat and sheep where it has a role in the endometrium during the peri-implantation period of early pregnancy in sheep. The up regulated production of gal-15 mRNA and protein in the uterine endometrium combined with the biological activities of other galectins, make gal-15 a strong candidate mediator of conceptus-endometrial interactions during implantation (GRAY et al. 2002; GRAY et al. 2005). There is also an extracellular role proposed for gal-15 in the uterine lumen as an adhesion molecule stimulating biological responses within the trophoblasts, such as attachment and migration which are critical processes for blastocyst elongation (SPENCER et al. 2004; SPENCER et al. 2007). Although blastocyst elongation occurs in sheep, goats, cattle, and pigs, gal-15 has only been found in sheep (LEWIS et al. 2007). This must not mean that gal-15 has no role in bovine CL function. Given that leukocytes immigrate during the process of luteolysis (SPANEL-BOROWSKI et al. 1997; LAWLER et al. 1999; PENNY

et al. 1999) this could also enhance local gal-15 level, since it has been shown in this work that subsets of bovine leukocytes, especially myeloid cells (monocytes and neutrophils) clearly express this galectin, at least at the mRNA level (Tab. 10).

5.2 Galectin expression in cellular subsets and their roles in immune-modulation

Depending on the age of the CL it can contain up to 20% immune cells. Since the luteolysis process involves the immigration of immune cells, and since an elevated expression of gal-1 and gal-3 was observed after $PGF_{2\alpha}$-induced luteolysis it was analyzed which bovine leukocyte cell types are expressing these galectins and whether this expression can be modulated by activating stimuli. Moreover, since the CL was shown to express gal-1 and gal-3 it was of interest to test defined modulatory effects of these galectins for bovine immune cell functions.

5.2.1 Galectin expression in immune cells

Gal-1, gal-3 and gal-15 gene expression levels were tested whether they were expressed in both inflammatory and non-inflammatory bovine immune cell types including monocytes, T and B lymphocytes, *in vitro* differentiated macrophages and neutrophils.

Gal-3 was found to be expressed in every tested leukocyte population. The highest expression of gal-1 was found in MAC-separated monocytes whereas *in vitro* generated macrophages expressed highest copy numbers of gal-3. This indicates that during differentiation in vitro there is a switch in the preferential expression of galectins. In case of monocytes, which differentiate into macrophages this means that an initial higher expression of gal-1 compared to gal-3 (monocytes, also indicated by the high gal-1/gal-3 ratio) – switches to a dominance of gal-3 over gal-1 in fully differentiated macrophages.

Interestingly, among freshly isolated cells, neutrophils appeared the only subpopulation in which no gal-1 expression could be demonstrated (Fig. 11A). Gal-15 gene expression, for which no data are available for other species so far, was restricted mainly to monocytes and neutrophils. It has not been evaluated whether B-lymphocytes express gal-15. Since the mixture of monocytes and B-lymphocytes displayed less gal-15 mRNA copy numbers than purified monocytes it cannot be excluded that B-lymphocytes do express gal-15. The higher mRNA copy numbers in purified monocytes, however, point to a dominant expression in monocytes (Tab. 10).

Since previously it was shown that CL did not express gal-15 (exceeding 100 mRNA copy numbers) this could either indicate that these pieces also did not contain considerable

numbers of monocytes and/or neutrophils, or that their numbers in the tissue fragments were too low to give a signal above the threshold.

Generally, mononuclear cells apart from monocytes, including T-cells and B-cells expressed gal-1 and gal-3 (Fig. 11A). These data are fairly in line with findings in other species. MULLER et al. (2006) determined that monocytes were the cells among human peripheral blood MNC which dominantly expressed gal-3. Additionally Stewart et al and Fulcher et al. proved that gal-1 was expressed in monocytes and macrophages of the human cardiovascular system (FULCHER et al. 2006; STEWART et al. 2009) and the presence of gal-3 in human monocytes was probed by immunoblot analysis by LIU et al. (1995). They also observed surface expression of galectin-3 to occur on freshly isolated human monocytes.

In T and B-cells gal-1 and gal-3 are coexpressed with high levels in human thymus and lymph nodes (STILLMAN et al. 2006) and gal-3 is lowly expressed in normal human lymphocytes (KONSTANTINOV et al. 1996). It was proven that gal-1 expressed in activated B-cells from T. cruzi-infected mice (ZUNIGA 2001) and gal-3 was expressed in activated murine T-lymphocytes including CD4+ and CD8+ T-cells but not in resting T cells (JOO et al. 2001). However, it was not determined, how many mRNA copies each immune cell contained. Thus, the data are not fully comparable.

Comparing the copy numbers of gal-1 and gal-3, gal-1 expression was 3-25 fold higher than gal-3 expression in peripheral blood MNCs, T-cells and monocytes respectively. Especially in monocytes (Fig. 11C) the gal-1/gal-3 ratio was exceptionally high. Whether these higher ratios reflect an initially anti-inflammatory phenotype the physiologic conditions (in terms of function) remains to proven.

5.2.2 Galectin expression in stimulated T-cells

Since gal-1 and gal-3 have partially opposing functions within the immune system, it was of special interest to analyze whether stimulated cells change expression level of gal-1 and gal-3.

For this purpose, freshly isolated MNC were stimulated with the lectin Con A. Con A is a protein that was originally extracted from the Jack Bean (Canavalia ensiformis) and belongs to lectins. It binds particularly to glycolipids and glycoproteins and has an affinity to bind for terminal α-D-mannosyl and α-D-glucosyl residues (REEKE et al. 1974) distinct from galectins. This type of binding is mitogenic for lymphocytes (PERLMANN 1970; NOVOGRODSKY and KATCHALSKI 1971; RUSCETTI and CHERVENICK 1975).

Interestingly, the *in vitro* cultivation of unstimulated MNC revealed that gal-1 and gal-3 expression was differentially modulated with gal-1 expression being significantly lower after 6 h incubation in vitro (compared to 3 h) and gal-3 mRNA levels being significantly higher

after 6 h in vitro. This indicates that the expression of these two galectins is independently and fast regulated in mononuclear cells in the absence of a stimulus (except the 'unphysiologic' in vitro condition). This is also reflected by the selective modulation of gal-3 expression after Con A stimulation where gal-1 expression remained unaltered and gal-3 expression significantly changed. Gal-3 expression even shows bimodality after Con A stimulation with an up regulation after 3 h stimulation and a down regulation after prolonged 6 h stimulation in vitro. Whether the early changes reflect a protective role of intracellular gal-3 on T-cell apoptosis (STILLMAN et al. 2006) is speculative. A mitogen-induced expression of gal-3 was shown for human CD4+ and CD8+ T-cells (JOO et al. 2001). The authors propose an important role of this galectin in the proliferation of activated T lymphocytes. Based on the down-regulation of gal-3 in bovine cells at 6 h of in vitro culture, this could be relevant for initial events after cells contact with a stimulating agent.

5.2.3 Galectin expression in monocytes, macrophages and neutrophils after LPS incubation

Even under non-stimulating conditions gal-1 and gal-3 were expressed highly in bovine blood monocytes. Since they differentiate in tissues into macrophages which also appeared to express gal-1 and gal-3 it was of interest to analyze whether the expression of these two galectins is altered depending on the stimulation of these cells.

One bacterial product known for its ability to activate monocytes and macrophages during gram negative bacterial infection is LPS. LPS is a glycolipid that designs the outer part of the outer membrane of gram-negative bacteria (RAETZ 1996; TAPPING and TOBIAS 1997). The stimulatory effects of LPS on monocytic cells appear to be elicited by the binding of endotoxin (Lipid A) to specific surface receptors expressed by the immune cells termed CD14 (WRIGHT et al. 1990, FREY et al. 1992; PUGIN et al. 1993; YU et al. 1997). Binding to CD14 is facilitated by a specific protein termed Lipid A binding protein (LBP) produced by hepatocytes in response to cytokines (SCHUMANN et al. 1990; TOBIAS et al. 1992; TAPPING and TOBIAS 1997). During an infection with gram negative bacteria, host immune cells become activated and migrate into affected tissues where they play a major role in the inflammatory response. LPS was shown to trigger the release of many host inflammatory factors such as chemokines, cytokines, and cell adhesion molecules (ROMAN et al. 2004).

During the in vitro differentiation of monocytes into macrophages (bMdM) the bMdM expressed both higher levels of gal-1 and gal-3. Interestingly, as with T-cells (see above), the stimulation (here with LPS) did not significantly alter the gal-1 expression, whereas gal-3 expression decreased significantly in LPS-exposed bMdM compared to the controls (Fig.

13AB). This kind of gal-1 and gal-3 regulation resulted in higher gal-1/gal-3 ratios both in monocytes and macrophages which seems to be solely due to an active down-regulation of gal-3 expression. The observed in vitro effects are difficult to translate into function. In mice, gal-3 is produced by macrophages and directly binds to LPS. And, it was demonstrated that gal-3-deficient macrophages had elevated LPS-induced signaling and inflammatory cytokine production compared to wild-type cells in mice, which was specifically inhibited by the addition of recombinant gal-3 protein. In contrast, blocking gal-3 binding sites by using a neutralizing antibody or its ligand, β-lactose, enhanced LPS-induced inflammatory cytokine expression by wild-type macrophages. (LI et al. 2008). For gal-1 it has been shown that it prevents an LPS induced release of TNFα from macrophages *in vitro*, without affecting their viability (SANTUCCI et al. 2000). Thus, since gal-1 and gal-3 are considered negative regulators of LPS-induced inflammation it should be suspected that LPS-stimulated monocytes/bMdM show an increased expression of these galectins. However, the protective gal-3 even was down regulated in LPS stimulated bovine MdM. Either gal-3 is not LPS-protective in the bovine species or, if galectins play a role in the LPS-protection, the enhanced gal-1/gal-3 ratio indicates a dominant role of gal-1 in this respect.

The effects of LPS on bovine myeloid cells turned out to be differential. In LPS-stimulated neutrophils both gal-1 and gal-3 were regulated. Bovine neutrophils are cells in which gal-1 expression was up regulated after 6 h LPS-stimulation whereas (as with bMdM) gal-3 expression was nearly shut down – either after 3 h or 6 h stimulation (Fig. 14). This resulted in a late (6 h) sharp and significant increase of the gal-1/gal-3 ratio (Fig. 14C).

LPS has been reported to inhibit spontaneous PMN apoptosis (LEE et al. 1993; HACHIYA et al. 1995; SWEENEY et al. 1998; KLEIN et al. 2001) and to protect against TNFα-induced apoptosis in human neutrophils (HACHIYA et al. 1995). The decline of gal-3 and gal-15 gene expression after LPS stimulation can support the apoptosis of neutrophils. LPS was determined as a negative modulator on gal-3 and gal-15 gene expression after *in vitro* incubation.

Apart from the still unproven role of neutrophil-generated gal-1 after LPS-stimulation, the increase of gal-1 expression could be in line with the finding that LPS induces the production of TNF-α, IL-1β, IL-12 and IFN-γ in bovine neutrophils (SOHN et al. 2007). Thus, the finding could reflect that gal-1 has a pro-inflammatory role in the bovine.

Interestingly, neutrophils produced a considerable amount of gal-15 mRNA, although this gene was only very rarely found to be expressed in other cell types or in the bovine CL. Other authors observed that gal-15 plays a role in ovine uterine immune and inflammatory

responses (GRAY et al. 2005). Therefore, it could be possible that neutrophils contribute this inflammatory process by expressing gal-15.

5.3 Modulatory effects of galectins on bovine immune cells

Whereas in the previous work it was analyzed which cells express galectins and how their expression is regulated in vivo and in vitro, it was also of interest how they affect the functions of bovine immune cells.

Unfortunately, bovine gal-1 and gal-3 were not available. Thus the presented work has been performed with recombinant human gal-1 and gal-3. Since these molecules bind sugar residues it was assumed that they can also bind to bovine cells. In order to show this directly gal-1 and gal-3 were FITC-labeled and used for binding studies both with human and bovine cells. However, these pre-studies failed to show significant binding to cells of both species (data not shown). Evidence for a sugar-mediated binding to bovine cells came from proliferation studies (Fig. 15A). Here, both galectins resulted in a significant blastogenesis of bovine MNC. Moreover, this effect could be selectively inhibited by lactose, the competing sugar for the gal-1 and gal-3 binding motif. It was, however, not possible to determine whether the human galectins bind with the same affinities to the bovine ligands on the cells.

One of the major reasons to study galectins effect we hypothesized that galectins may modulate the function of bovine leukocytes (monocytes, *in vitro* differentiated macrophages and neutrophils) during inflammatory conditions.

Compared to the mitogen- and superantigen- induced proliferation, the blastogenesis-inducing potential of gal-1 and gal-3 appeared to be lower (Fig. 15A). At least this parallels findings of others who reported on an involvement of gal-1 and gal-3 in the proliferation of various cell types, including T-cells, fibroblasts, and epithelial cells. As mentioned above, lactose, the competitive inhibitor of galectin binding (LAGANA et al. 2006) completely blocked the galectin-induced blastogenesis which is in line with findings by IGLESIAS et al. (1998).

INOHARA et al. (1998) also determined that gal-3 can act as a mitogen and suggests a possible role of gal-3 in tissue remodeling.

Whether the gal-1- and gal-3-induced blastogenesis acts in a synergistic way to the Con-A- or superantigen-induced proliferation was tested in co-stimulation assays. Whereas the superantigen-induced blastogenesis remained unaffected, only in Con A-stimulated cells, gal-1 was able to reduce significantly the number of induced viable blast cells (Fig. 16). The gal-1 mediated reduction could be due to steric hindrance which allows Con A only insufficiently to bind to its carbohydrate moieties. The lack of gal-3 in mediating this kind of inhibition could indicate that gal-3 binds to other carbohydrate motifs than gal-1.

In summary, gal-1 and gal-3 resulted in the generation of a small population of blast-transformed cells. Whether this was due to the expansion/activation of a selected cellular subset was checked by analyzing the frequency of T-cell subsets (Tab. 11) among small lymphocytic cells after in vitro stimulation. Whereas Con A resulted in a significant change (the CD4/CD8 ratio increased), the superantigen SEA and both tested galectins failed to alter this ratio. Thus, it can be assumed, that both T-cell subsets were equally affected by gal-1 and gal-3 and that there is no preferential action on either T-cell subset. This may not be true for cells in lymphoid organs or cells of other species since it was shown for instance, that the CD4+ CD8+ thymocyte population is very susceptible to gal-1 mediated death (PERILLO et al. 1997; VESPA et al. 1999; GALVAN et al. 2000) and that gal-3 can induce CD8+ T-cell apoptosis in vitro (LI et al. 2010).

Activation of cells can lead to blastogenesis and proliferation but also to cellular death. Since regulation of cell survival as well as cellular death during inflammation is a very important issue to prevent tissue damage and restore homeostasis it was checked whether gal-1 and gal-3 affect the apoptosis of immune cells.

The effects were not very prominent (Fig. 17). Whereas gal-1 had no effect on the apoptosis of PMN, gal-3 slightly but significantly raised the fraction of apoptotic PMN (Fig. 17A). This is supported by findings demonstrating the enhanced spontaneous apoptosis after a transient treatment of resting neutrophils with gal-3 (FERNANDEZ et al. 2005).

Whether the significant but also rather weak reduction in the percentage of apoptotic MNC by gal-3 is of biological relevance remains doubtful (Fig. 17B). The overall lack of an apoptotic effect on bovine lymphocytes is in contrast to findings in other species were gal-1 has been reported to have an apoptotic activity on T-cells and that gal-3, but not gal-1, induces both phosphatidylserine exposure and apoptosis in activated human T cells (STOWELL et al. 2008).

Overall, based on the modulatory actions of gal-1 and gal-3 on bovine immune cells there was no clear indication whether they possess opposing or polarizing functions. One way to analyze this was to look at the induced cytokines after in vitro stimulation of bovine MNC. One antibody specific for bovine IFNγ was successfully used in intracellular immunofluorescence (Fig. 18) but neither gal-1 nor gal-3 (although inducing bovine T-cell blastogenesis) induced measurable IFNγ production in stimulated cells – as opposed to the positive control after stimulation with PMA/ionomycin. Thus, neither gal-1, nor gal-3 seems to polarize bovine T-cells in more pro-inflammatory way (TH1 responses), in which IFNγ induction and release is one of the hallmarks of activated TH1-cells. Whether gal-1 and gal-3 are inducer of TH2-type responses could not be determined. An antibody directed against

bovine IL-4, a typical TH2 cytokine, was not binding in this study after fixation and permeabilization of the cells.

Different members of the galectin family were shown to positively or negatively influence the production of anti- or pro-inflammatory cytokines (RABINOVICH et al. 2007). It was therefore analyzed by qRT-PCR whether there is a bias in the expression of certain cytokines after stimulation of bovine MNC with gal-1 or gal-3. While IL-4, IL-12, IL-17 and IFNγ were found not to be expressed (<100 mRNA coy numbers, data not shown) in any of the galectin stimulated set-ups, IL-10 mRNA expression was observed 17 h after galectin stimulation at comparable levels (data not shown).

Recombinant gal-1 has been documented to inhibit the secretion of pro-inflammatory cytokines, such TNFα and IFNγ (RABINOVICH et al. 1999a) and induce IL-10 production, (STOWELL et al. 2008). In addition, an investigation of the cytokine balance in draining lymph nodes and spleens retrieved from mice treated with recombinant gal-1 exposed decreased amounts of IFNγ and IL-2 and high levels of IL-5, IL-10 and TGFß production (RABINOVICH et al. 2007). Furthermore, LEIJ et al. (2007) reported a significant increase in IL-10 mRNA and protein levels in non-activated and activated CD4+ and CD8+ T cells following exposure to recombinant gal-1. However, with regard to an anti-inflammatory cytokine (IL-10), gal-1 and gal-3 did not appear to differentially induce this mediator in bovine cells.

6 Conclusion

Galectin-1 and -3 appear to be expressed in the whole corpus luteum with no differences between the CL centre and its periphery. Based on the ex vivo and in vitro findings the expression of both galectins is modulated significantly during physiological processes (e.g. luteolysis) or pathological processes (e.g. infection with gram-negative bacteria).

It seems plausible that gal-1 and -3 are regulated differentially under *in vivo* and *in vitro* conditions. Depending on the age of the CL it can contain up to 20% immune cells. It was determined that bovine leukocytes significantly contribute to the galectin expression. Whether they affect the utela cell function by galectin secretion, or whether luteal/endothelial cells derived galectins alter the function of resident immune cells is still unknown. At least the modulatory effects of galectins on cytokine expression, cell proliferation and cell death of bovine leukocytes in vitro was found to be subtle.

Based on these investigations, future studies have to be conducted to explore the precise mechanisms by which galectins regulate the functions of luteal and immune cells in the bovine system.

7 Summary

Deniz Dziallas: Regulation of bovine corpus luteum expressed galectins and their immunoregulatory potential

The decline of successful pregnancy rates in holstein-friesian dairy cattle could be the result of ovarian dysfunctions, which are caused by an imbalanced production of chemokines, cytokines and galectins (gal). The effect of the galectins on the corpus luteum (CL) formation, development and regression is only partially known. Therefore the aim of the present study was to analyze gene expression of gal-1, gal-3 and gal-15 in the CL and in immune cells, which contribute to CL function. Additionally, one aim was to demonstrate immunomodulatory effects of galectins on immune cell functions.

In the first part of this study, CL were obtained from 10 cyclic and 12 pregnant cows via ovariectomy in order to determine the *in vivo* galectin expression. The gene expression of the tested galectins was demonstrated in all CL with no difference between the centre and the periphery. Gal-1 expression decreased depending on the progression of the cycle or the pregnancy process, whereas gal-3 expression showed no alteration between the groups. Notably, no apparent differences existed between day 16 cyclic and day 16 pregnant cows. Based on mRNA copy numbers the ratio between gal-1 and gal-3 was about 30 to 1 in all animal groups.

CL gene expression was determined after $PGF_{2\alpha}$-induced luteolysis in 24 healthy and normal cycling heifers after transvaginal ovariectomy 5 min, 15 min, 30 min, 120 min and 720 min after injection of the $PGF_{2\alpha}$ analogue Dinoprost (25 mg). As soon as 15 min after $PGF_{2\alpha}$ injection, gal-1 and gal-3 expression significantly increased. The time-dependent up-regulation differed slightly between gal-1 and gal-3, with gal-1 having maximum expression levels at 30 min and gal-3 at 720 min after $PGF_{2\alpha}$ injection. This differential expression modulation of gal-1 and gal-3 was reflected by an initial rise in the gal-1/gal-3 ratio, which significantly decreased with time. Since gal-1 and gal-3 are described to possess different functions this could reflect different cellular processes in the observed time frame after $PGF_{2\alpha}$ injection.

In vitro, galectin expression was studied after LPS stimulation of CL fragments obtained from slaughtered cattle. LPS of E. coli (subtypes O111:B4 and O55:B5) was used in different concentration (0.5 µg/ml and 5 µg/ml). LPS (serotype O55:B5) was able to alter the galectin expression causing a decrease in gene expression of both galectins after 30 min in vitro. The

effects of LPS seem to depend on the serotype since LPS (serotype O111:B4) caused no altered galectin expression.

Since the luteolysis process involves the immigration of immune cells, it was analyzed which bovine leukocyte cell types are expressing these galectins and whether this expression can be modulated by activating stimuli. Gal-3 was expressed in every tested leukocyte population. The highest expression of gal-1 was found in monocytes whereas *in vitro* generated macrophages expressed highest copy numbers of gal-3 indicating a switch of preferential galectin expression during monocyte macrophage differentiation. Neutrophils appeared the only subpopulation in which no gal-1 expression could be demonstrated. Gal-15 gene expression was restricted mainly to monocytes and neutrophils. Based on mRNA copy numbers, gal-1 expression was 3-25 fold higher than gal-3 expression in peripheral blood MNCs, T-cells and monocytes. Especially in monocytes the gal-1/gal-3 ratio was exceptionally high. Whether these higher ratios reflect an initially anti-inflammatory phenotype under physiologic conditions (in terms of function) remains to be proven.

Stimulation of mononuclear cells (MNC) with the lectin Con A resulted in a differential modulation of gal-1 and gal-3 gene expression with gal-1 expression being not affected and gal-3 expression being up-regulated after 3 h stimulation and down-regulated after 6 h stimulation in vitro. Whether the early changes reflect a protective role of intracellular gal-3 on T-cell apoptosis is speculative. However, externally administered human gal-3 slightly but significantly reduced the percentage of apoptotic MNC.

The effects of LPS on bovine myeloid cells were differential. In LPS-stimulated neutrophils gal-1 expression was up-regulated after 6 h whereas gal-3 expression was nearly shut down after 3 h or 6 h stimulation with LPS. In monocyte-derived macrophages, LPS caused no alteration of gal-1 expression whereas gal-3 expression decreased significantly. Since gal-1 and gal-3 are both considered negative regulators of LPS-induced inflammation the enhanced gal-1/gal-3 ratio and the down-regulated gal-3 indicates a dominant role of gal-1 in this respect.

In the last part of the study it was analyzed how recombinant human galectins (rhGal-1, rhGal-3) affect the function of immune cells. Evidence for a sugar-mediated binding to bovine cells came from MNC proliferation studies were both galectins resulted in a significant blastogenesis of bovine MNC which could be selectively inhibited by lactose, the competing sugar for the gal-1 and gal-3 binding motif.

Compared to the Con A- and superantigen (SEA)- induced proliferation, the blastogenesis-inducing potential of gal-1 and gal-3 was significantly lower. In co-stimulation assays only rhGal-1 reduced significantly the number of induced viable blast cells after Con A stimulation. Flow cytometric analysis of T cell subsets among small lymphoid cells revealed that neither galectin (as opposed to Con A) altered the ratio of CD4+/CD8+ T-cells. This indicates that both T-cell subsets are equally affected by gal-1 and gal-3 and that there is no preferential action on either T-cell subset. Both galectins did not induce measurable amounts of inflammatory IFNγ (after intracellular immunofluorescence) in stimulated MNC and the mRNA level of anti-inflammatory IL-10 17 h after in vitro stimulation were comparable between rhGal-1 and rhGal-3-stimulated MNC. Thus, neither gal-1, nor gal-3 seem to polarize bovine T-cells in a more pro- or anti-inflammatory way.

The results of the present study described for the first time certain gene expression of distinct galectins in CL cyclicum, CL graviditatis and peripheral blood cells in cattle. Additionally, effects of galectins on bovine immune cells could be demonstrated. Hypothetically, galectins may influence the phases of CL development and regressions by activating and modulating recruited immune cells. Based on these investigations, future studies have to be conducted to explore the precise mechanisms by which galectins regulate the functions of luteal and immune cells in the bovine system.

8 Zusammenfassung

Deniz Dziallas: Die Regulierung des durch den bovinen Gelbkörper exprimierten Galektins und deren immunoregulatorisches Potential

Der Rückgang erfolgreicher Trächtigkeiten bei Holstein-Friesen-Hochleistungskühen könnte das Resultat ovarieller Dysfunktionen sein, welche auf einer Entgleisung der Produktion von Chemokinen, Zytokinen und vielleicht auch Galektinen (gal) zurückgeführt werden können. Die Einflüsse von Galektinen auf das Corpus luteum (CL), seine Anbildung, dessen Entwicklung und Regression sind bisher nur teilweise bekannt. Das Ziel dieser Studie war die Analyse der Expression von gal-1, gal-3 und gal-15 im Gelbkörper und in Immunzellen, welche verschiedene Funktionen in der Gelbkörperphysiologie haben. Ein weiteres Ziel bestand darin mögliche immunmodulatorische Effekte von Galektinen auf Immunzellen zu prüfen.

Für den ersten Teil dieser Arbeit wurden Gelbkörper von 10 zyklischen und 12 trächtigen Kühen mittels Ovariektomie gewonnen und ihre in vivo Galektin-Expression bestimmt. Bei diesen Tieren konnte kein Unterschied der Genexpression in Bezug auf die Lokalisation im CL (Peripherie vs. Zentrum) dargestellt werden. Die Gal-1 Expression zyklus abhängig unterschided. Im Gegensatz hierzu stellten sich in der Expression von Gal-3 keine apparenten Unterschiede zwischen dem 16. Zyklustag und dem 16. Trächtigkeitstag dar. Basierend auf den Zahlen der mRNA Kopien lag das gal-1/gal-3-Verhältnis bei allen untersuchten Tieren und Gruppen bei etwa 30 zu 1.

Die Genexpression im CL gesunder Färsen wurde ex vivo nach $PGF_{2\alpha}$-induzierter Luteolyse und transvaginaler Ovarioektomie (5 min, 15 min, 30 min, 120 min und 720 min nach Injektion von 25 mg Dinoprost) untersucht.

Bereits 15 min nach $PGF_{2\alpha}$ Injektion stieg die Expression von gal-1 und gal-3 signifikant an. Die zeitabhängige Hochregulation unterschied sich zwischen gal-1 und gal-3. Während gal-1 die maximale Expression nach 30 min erreichte, war die gal-3 Expression 720 min nach $PGF_{2\alpha}$ Injektion am höchsten. Die differentielle Expressionsmodulation war gekennzeichnet durch einen initialen Anstieg des gal-1/gal-3 Verhältnisses, welches mit der Zeit signifikant abnahm. Da gal-1 und gal-3 unterschiedliche Funktionen besitzen, könnte dies unterschiedliche zelluläre Prozesse in dem beobachteten Zeitfenster nach der $PGF_{2\alpha}$ Injektion widerspiegeln.

In vitro wurde die Galektin-Expression nach Stimulation von CL-Fragmenten mit LPS analysiert. Gelbkörper wurden von frisch geschlachteten Rindern gewonnen. LPS von E. coli

(Subtypen O111:B4 und O55:B5) wurde in unterschiedlichen Konzentrationen eingesetzt (0,5 µg/ml; 5 µg/ml). Nach Stimulation mit LPS (O55:B5) für 30 min wurde die Expression von gal-1 und gal-3 signifikant gesenkt. Dieser Effekt war Serotyp-spezifisch, da LPS (O111:B4) nicht zu einer veränderten Galektin-Expression führte.

Da die Luteolyse von einer Immigration von Immunzellen begleitet ist wurde analysiert welche Leukozyten-Subpopulationen des Rindes Galektine exprimieren und ob diese Expression durch aktivierende Stimuli beeinflussbar ist. Gal-3 wurde in jeder geprüften zellulären Subpopulation exprimiert. Die höchste Expression von gal-1 fand sich in Monozyten während in vitro generierte Makrophagen die höchsten gal-3 mRNA-Kopienzahlen aufwiesen. Dies deutet auf einen Wechsel der präferenziellen Galektin-Expression während der Monozyten-Makrophagen-Differenzierung hin.

Neutrophile Granulozyten erwiesen sich als die einzige Subpopulation in der keine signifikante Genexpression von gal-1 nachgewiesen werden konnte. Die gal-15 Genexpression war hauptsächlich auf Monozyten und neutrophile Granulozyten beschränkt.

Basierend auf den mRNA-Kopienzahlen der Immunzellen erwies sich die gal-1-Expression als 3 bis 25 fach höher als die Expression von gal-3. Das höchste Verhältnis konnte in Monozyten festgestellt werden. Ob diese hohen Verhältnisse einen initial anti-inflammatorischen Phänotyp unter physiologischen Bedingungen widerspiegeln bleibt zu prüfen.

Eine Stimulation mononukleärer Zellen (MNC) mit dem Lektin Con A führte zu einer differenziellen Expressionsmodulation: die gal-1 Expression wurde debei nicht beeinflusst. Die gal-3 Expression wurde zunächst herauf reguliert (3 h in vitro) und nach 6 h in vitro herunter reguliert. Ob die frühen Änderungen eine schützende Rolle von gal-3 für die Apoptose implizieren bleibt Spekulation. Jedoch konnte der Zusatz von rekombinantem humanen gal-3 zu Con A-stimulierten MNC den Prozentsatz apoptotischer MNC signifikant senken.

Die Effekte einer LPS-Stimulation auf bovine myeloide Zellen erwiesen sich als differenziell. In LPS-stimulierten Neutrophilen wurde die gal-1 Expression nach 6 h in vitro hochreguliert während die gal-3 Expression nach 3 h und 6 h nahezu vollständig abgeschaltet wurde. In Monozyten-stämmigen Makrophagen führte LPS zu keiner Änderung der gal-1 Expression während auch in diesen Zellen die Expression von gal-3 signifikant abnahm. Da gal-1 und gal-3 beide als negative Regulatoren einer LPS-induzierten Entzündung gelten, könnte das LPS-induziert gesteigerte gal-1/gal-3 Verhältnis auf eine dominante Rolle des gal-1 in diesem Zusammenhang hinweisen.

Im letzten Teil der Studie wurde geprüft wie rekombinantes humanes gal-1 und gal-3 (rhGal-1, rhGal-3) die Funktion boviner Immunzellen beeinflusst. Hinweise für eine zucker-vermittelte Bindung an bovine Zellen wurden in MNC Proliferationsassays gewonnen in denen beiden Galektine zu einer signifikant gesteigerten Blastogenese führten, die selektiv durch Laktose – den kompetitierenden Zucker für das gal-1/gal-3-Bindungsmotif - blockiert worden konnten.

Verglichen mit der Con A- und der Superantigen (SEA)-induzierten Blastogenese erwies sich die stimulatorische Kapazität von rhGal-1 und rhGal-3 als signifikant schwächer. In Co-Stimulationsassays konnte nur rhGal-1 die Zahl Con A-induzierter Blasten signifikant senken. Durchflusszytometrische Analysen von T-Zellsubpopulationen unter den kleinen lymphoiden Zellen ergaben, dass keines der beiden Galektine (anders als Con A) das CD4+/CD8+ Verhältnis unter den T-Zellen änderte. Dies deutet darauf hin, dass beide T-Zellpopulationen durch gal-1 und gal-3 gleichsinnig beeinflusst werden. Beide Galektine induzierten keine messbaren Mengen an inflammatorischen IFNγ (nach intrazellulärer Immunfluoreszenz in stimulierten MNC. Überdies war der mRNA-Spiegel des anti-inflammatorischen IL-10 nach 17 h in vitro Stimulation vergleichbar zwischen rhGal-1- und rhGal-3-stimulierten MNC. Dies lässt vermuten, dass weder gal-1 noch gal-3 zu einer Polarisierung von bovinen T-Zell-Immunantworten (pro-, anti-Inflammation) führt.

Die Ergebnisse der vorliegenden Studie beschreiben die Genexpression selektierter Galektine im CL cyclicum, CL graviditatis und in Leukozyten des Rindes. Galektine könnten Phasen der Gelbkörperentwicklung und –regression beeinflussen indem rekrutierte Immunzellen in ihrer Funktion moduliert werden. Basierend auf den Ergebnissen bieten sich weitere Untersuchungen an, welche sich den präzisen Mechanismen widmen durch die Galektine die Funktionen von lutealen und von Immunzellen beim Rind steuern.

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