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Technische Universität München

Lactoferrin: protective role in the bovine mammary gland and newborn calves

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan
für Ernährung, Landnutzung und Umwelt der Technischen Universität München
zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender der Prüfungskommission:	Univ.-Prof. Dr. Dieter Treutter
Prüfer der Dissertation:	1. Univ.-Prof. Dr. Heinrich H. D. Meyer
	2. apl. Prof. Dr. Frieder J. Schwarz

Die Dissertation wurde am 26.01.2006 bei der Technischen Universität München eingereicht
und durch die Fakultät Wissenschaftszentrum Weihenstephan
für Ernährung, Landnutzung und Umwelt am 21.03.2006 angenommen.

CONTENT

ABBREVIATIONS

bp	base pair
CD14	cluster of differentiation antigen 14
CP	crossing point
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
Fig.	figure
GALT	gut-associated lymphoid tissue
GIT	gastrointestinal tract
IκB	inhibitor of kappa B
IEC	intestinal epithelial cells
IL	interleukin
Ig	immunoglobulin
INFγ	interferon gamma
LBP	lipopolysaccharide-binding protein
LF	lactoferrin
LFcin	lactoferricin
LPS	lipopolysaccharide
mCD14	membrane bound CD14
mLN	mesenterial lymph nodes
mRNA	messengerRNA
NF-κB	nuclear transcription factor kappa B
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear neutrophil leukocytes
PP	Peyer´s patches
RNA	ribonnucleic acid
sCD14	soluble form of CD14
SC	somatic cells
RT-PCR	reverse transcription- polymerase chain reaction
SEM	standard error of mean
TNFα	tumor necrosis factor alpha
TLR	Toll-like receptor
WBC	white blood cells

ABSTRACT

Polymorphonuclear neutrophil leukocytes (PMN) are recruited from peripheral blood into milk as part of the inflammatory response. Mobilization of PMN is mediated through cytokines or chemokines synthesized by the mammary tissue and milk somatic cells (SC). Since milk is not a favourable medium for phagocytosis by PMN, the strength of the immune response in the circulatory system is much more prominent than in the milk. The inflammatory response is related to the concentration of SC and the cytokines produced. Its promptness and its magnitude have a profound influence on the severity and the outcome of infections. For a better understanding of the interaction between cytokine synthesis and regulation mechanisms of the immune response during *Escherichia coli (E. coli*) mastitis, host-pathogen interactions were studied in a cell culture model. Therefore, in a first approach, the transcriptional activity of immunologically important factors (lactoferrin (LF), cluster of differentiation antigen 14 (CD14) and various cytokines) were studied in *E. coli* endotoxin (lipopolysaccharide, LPS) activated bovine white blood cells (WBC), monocytes, SC and milk macrophages. Significant cytokine mRNA increases were found in all four cell culture types and genes. In WBC or monocytes higher LPS responses and longer persistence were observed than in corresponding milk cells. This may be ascribed to the role of LF and CD14 on the cytokine production of the investigated cells or may be caused by the blood-to-milk diapedesis. The constitutive transcription of CD14 mRNA in WBC and monocytes was found to be 6- to 15-fold higher than in analogous milk cells. Compared to the other cell culture types, mRNA expression levels for LF in SC were strongly up-regulated (14-fold up to 153–fold) over the entire culture period, independent of LPS treatment. Since SC were derived from subclinical infected mastitic cows, these results emphasizes the protective role of LF in the mammary gland during inflammation.

LF is a cationic iron-binding glycoprotein that is consistently expressed and secreted from glandular epithelial cells. It is a prominent component of the secondary granules of PMN and plays a role in host defense mechanisms via bacteriostatic activity and immunoregulatory properties. To approach the mechanism governing the regulatory functions of LF in the immune system, host protecting effects of oral administered LF were studied in calves. Furthermore, the ability of LF and lactoferricin (LFcin) to

induce mRNA expression changes of immunological important factors in WBC and monocytes and to interfere with the lipopolysaccharide-binding protein (LBP)/CD14 pathway was investigated in an *in vitro* experiment. *In vitro* and *in vivo* studies confirmed the ability of LF to modulate immune response. LF given orally was shown to act as an immunomodulatory agent by enhancing the sizes of Peyer´s patches (PP) in the ileum of calves and increasing IgG levels in the blood serum. The results of this investigation also demonstrate an increase in the number of peripheral blood leucocytes, but also enhanced mRNA expression levels for various cytokines (interleukin (IL)-1ß, IL-8, IL-10) and interferon gamma (INFγ) in response to LF treatment. These findings are in agreement with the *in vitro* observation demonstrating an increase in transcription levels of TNFα, IL-1β, IL-6 and IL-10 in bovine WBC and monocytes following LF or LFcin treatment. The magnitude of increase varied from modest (6-fold) to dramatic (64-fold). Inhibitory effects of LF and LFcin on the LPS induced cytokine mRNA response in those cells could only be confirmed at specific LPS-concentrations.

Together these findings emphasize the ability of LF to modulate the inflammatory process and the overall immune response and reveals host-protecting effects of LF through an improved immune status.

ZUSAMMENFASSUNG

Polymorphkernige neutrophile Granulozyten (PMN) wandern als Teil der Entzündungsreaktion gezielt aus der peripheren Blutbahn in die Milch. Die Mobilisierung von PMN erfolgt über zellvermittelte Immunität mittels Zytokine oder Chemokine, synthetisiert vom Eutergewebe und somatischen Milchzellen (SC). Da die Milch kein geeignetes Medium für phagozytierende PMN Zellen darstellt, ist die Immunantwort im Blutsystem bedeutend stärker als die in der Milch. Die Entzündungsreaktion steht in Zusammenhang mit der Konzentration von SC und den produzierten Zytokinen. Dessen Ausmaß und Geschwindigkeit haben einen gravierenden Einfluss auf den Verlauf und den Ausgang von Infektionen. Für ein besseres Verständnis der Wechselwirkung zwischen Zytokinsynthese und Regulationsmechanismen der Immunantwort während einer *Escherichia coli (E. coli*) Mastitis, wurden Wirt-Phatogen Interaktionen in einem Zellkulturmodell studiert. In einem ersten Ansatz wurde deshalb die Transkriptionsaktivität von immunologisch relevanten Faktoren (Laktoferrin (LF), Zelloberflächenantigen 14 (CD14) und diverse Zytokine) in *E. coli* Endotoxin (Lipopolysaccharide, LPS) aktivierten bovinen weißen Blutzellen (WBC), Monozyten, SC und Milchmakrophagen studiert. Signifikante Zytokin mRNA-Zunahmen konnten in allen vier Zellkulturarten und Genen gemessen werden. In WBC und Monozyten konnte LPS eine höhere Genexpression mit längerer Persistenz auslösen als in entsprechenden Milchzellen. Dieser Unterschied kann der zentralen Rolle von LF und CD14 bei Entzündungsprozessen zugeschrieben oder durch die Blut-Milch Zellmigration begründet werden. In WBC and Monozyten wurde eine 6- bis 15-fach höhere konstitutive Transkription des CD14-Rezeptorgens als in adäquaten Milchzellen gemessen. Unabhängig von einer LPS-Behandlung war die mRNA Expression für LF in SC im Vergleich zu den anderen Zellkulturtypen über den gesamten Versuchszeitraum stark hochreguliert (14-fach bis 153-fach). Da die Milchzellen von Rindern mit subklinischer Mastitis isoliert wurden, unterstreichen diese Ergebnisse die schützende Rolle von LF im Euter während einer Infektion.

LF ist ein kationisches, eisenbindendes Glycoprotein, das im großen Ausmaß von Euterepithelzellen exprimiert und abgesondert wird. Es ist ein prominenter Bestandteil der sekundären Granula in PMN und spielt eine zentrale Rolle in der Immunabwehr durch seine bakteriostatische Aktivität und immunmodulatorischen

Eigenschaften. Um einen besseren Einblick in die regulatorischen Mechanismen von LF zu erhalten, wurde der schützende Effekt von oral verabreichtem LF in Kälbern studiert. Überdies wurde in einem *in vitro* Experiment geprüft, ob LF und Lactoferricin (LFcin) mRNA Expressionsveränderungen von immunologischen wichtigen Faktoren in WBC und in Monozyten induzieren kann und in den Lipopolysaccharid-Binde-Protein (LBP)/CD14 Pfad eingreift. *In-vitro*- und *in vivo* Studien bestätigten die Fähigkeit von LF, die Immunantwort zu modulieren. Es wurde gezeigt, dass oral verabreichtes LF als immunmodulatorisches Agens fungiert, indem es die Flächen der Peyerschen Platten (PP) im Ileum von Kälbern vergrößert und IgG Level im Blutserum erhöht. Die Ergebnisse dieser Untersuchung weisen erhöhte Leukozytenzahlen im peripheren Blut auf, zeigen aber auch gesteigerte mRNA Expressionslevel für diverse Zytokine (Interleukin (IL)-1ß, IL-8, IL-10) und Interferon gamma (INFγ) in Reaktion auf eine LF-Behandlung. Dies deckt sich mit der *in vitro* Studie, die eine Zunahme der Transkriptionslevel von TNFα, IL-1ß, Il-6 und Il-10 in LF- oder LFcin behandelten bovinen WBC und Monozyten vorweist. Das Ausmaß der Zunahme war unterschiedlich und variierte von mäßig (6-fach) bis zu dramatisch (64-fach). Ein hemmender Effekt von LF und LFcin auf die von LPS-induzierte Zytokin mRNA Expression in diesen Zellen konnte nur bei spezifischen LPS-Konzentrationen bestätigt werden.

Zusammengenommen unterstreichen diese Resultate die Fähigkeit von LF den Entzündungsprozess zu modulieren. LF hat Einfluss auf die allgemeine Immunantwort und offenbart einen Wirt-schützenden Effekt durch gesteigerten Immunstatus.

INTRODUCTION

During mastitis polymorphonuclear neutrophils (PMN) are recruited from peripheral blood through the mammary epithelium into milk in response to a variety of inflammatory mediators, such as cytokines, complements or eicosanoids [17; 63]. PMN represent the major line of defense against bacterial infection of the mammary gland due to their phagocytotic activity, presence of bactericidal enzymes and oxy-radicals [38]. The inflammatory response is initiated by macrophages together with epithelial cells by production and release of tumor necrosis factor alpha (TNFα), interferons (INF) and interleukins (IL) as a reaction against invading microorganisms or their components such as lipopolysaccharide (LPS) [12; 15; 79]. LPS, the major components of the outer membrane of gram-negative bacteria cell wall, is a potent stimulator of inflammatory processes in the bovine mammary gland [16; 59; 66]. It is well known that the strength of the immune response in the circulating system is much more prominent than in the mammary gland. Mammary gland neutrophils and macrophages are less effective at phagocytosis and less responsive to stimulating agents than are blood leukocytes because of the indiscriminate ingestion of fat, casein, and other milk components, especially during the periparturient period.

The membrane bound CD14 (mCD14), expressed on the cell surface of monocytes and macrophages and to a lesser degree on neutrophils, binds the LPS-LBP complexes and thus mediates the activation of those phagocytes [1; 100]. lipopolysaccharide-binding protein (LBP) is an acute-phase protein released by the liver during inflammation. Serum LBP enhances the LPS-induced cell activation and cytokine response by binding to the lipid A moiety of LPS and catalysing the transfer of LPS to the CD14 receptor [81]. LBP and CD14 present LPS to the Toll-like receptor (TLR)-4 in association with accessory protein MD-2 and this leads to activation of intracellular signals, including nuclear transcription factor kappa B (NF-κB) and c-Jun N-terminal kinase [83], which initiate the mRNA expression for proinflammatory cytokines. In response to external pro-inflammatory stimuli a signaling cascade is activated leading to phosphorylation and degradation of Inhibitor of kappa B (IκB) and translocation of NF-κB to the nucleus where it initiates gene transcription by binding to DNA [3; 56]. Milk macrophages are beneficial to dairy cows because of their ability to detect invading mastitis-causing pathogens and to

initiate the inflammatory response. They are the predominant cells in secretion of the healthy mammary gland and active mammary gland phagocytic cells [44; 78].

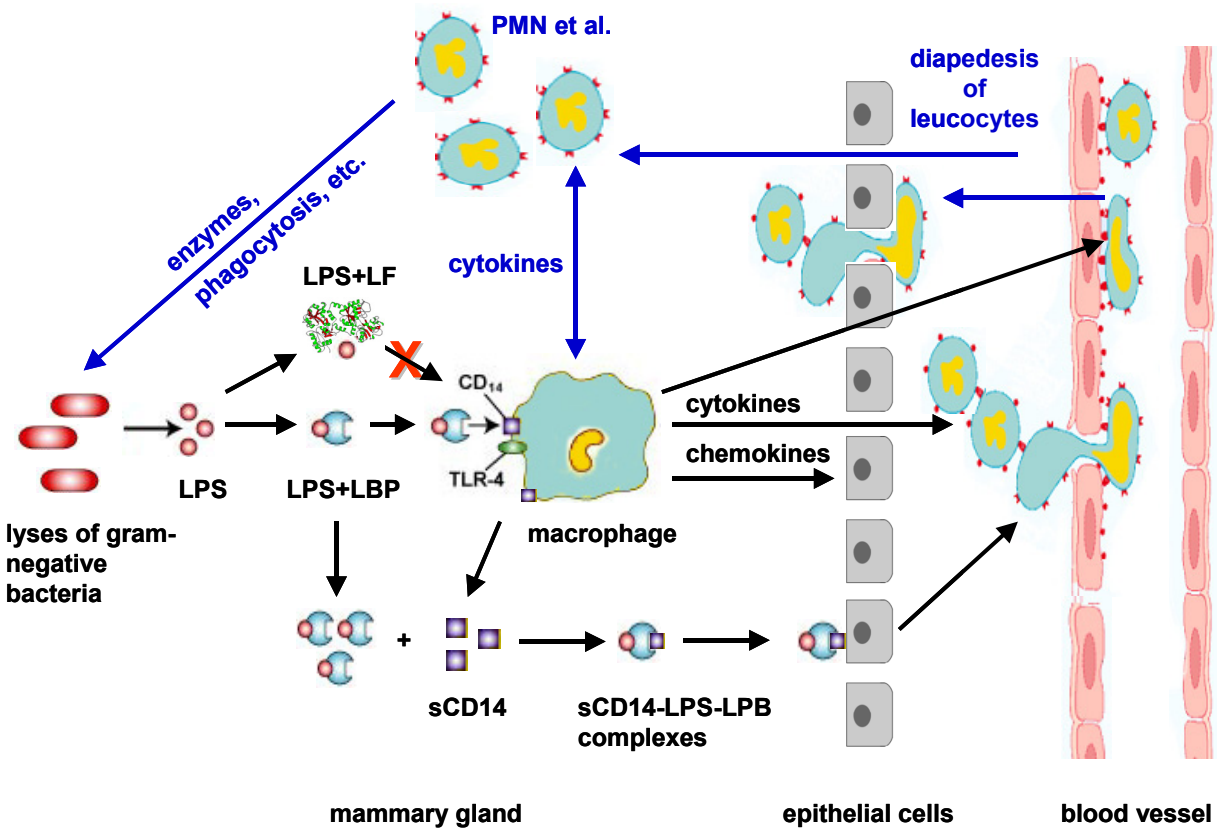

Fig. 1. *Scheme of activation of the immune response via LPS in the mammary gland.*

The soluble form of CD14 (sCD14) results from shedding of mCD14 [6]. Soluble CD14 has been shown to reduce endotoxin-induced activities by competing with mCD14 for LPS binding [100, 101]. In contrast to this attribute sCD14 mediates the LPS-induced activation of cells lacking mCD14, including epithelial and endothelial LPS [92].

Lactoferrin (LF), a member of the transferrin family of iron-binding glycoproteins, was originally discovered in milk [86]. Antimicrobial activity of LF has been previously reported, therefore it has been suggested that milk LF plays an important role in mammary gland defense against mastitic bacteria, particularly towards coliform infection [73]. Subsequently LF has been shown to have a much wider distribution and found to be present at many sites where defence against microbial infections is

of importance. It is present at high concentration in bovine milk and has been found in most body secretions (e.g., tears, saliva, sinovial fluid) as well as in secondary granules of polymorphonuclear leukocytes [50]. During the inflammatory process it is released in infected tissues by epithelial cells and in blood by leucocytes [99; 5; 79]. LF plays a role in host defense mechanisms via its anti-bacterial, anti-viral and anti-fungal activities and the ability to modulate inflammatory processes [5; 27]. Many of the beneficial physiological effects of the intact bovine LF protein do in fact reside in the basic 25-residue lactoferricin (LFcin) peptide [96]. LFcin is released from the N-terminal region via proteolytic cleavage by pepsin at an acidic pH. This reaction has been shown to take place in the stomach releasing LFcin into the intestine, where it is relatively stable. LFcin is described to have a more potent bactericidal and bacteriostatic activity than the intact LF [43; 7].

However the mechanism governing the regulatory functions of LF in the immune system is discussed controversially. It appears that the LFcin containing N-terminal region of LF interacts with certain cell receptors and LPS [26; 46]. The capacity of LF and LFcin to bind free LPS and to interfere with the LBP/CD14 pathway [25] have been suggested to contribute to their anti-inflammatory activities by restraining the cytokine production (TNFα, IL-1β, IL-6) *in vivo* and *in vitro* [49; 18; 51], thus protecting organisms from the harmful effect of sepsis [2]. Haversen et al. [34] demonstrated that LF inhibits the LPS-induced cytokine production at the transcriptional level in two human monocytic cell lines, thus indicating that this bioactive protein may have a direct effect on regulation of cytokines.

In fact, most mechanisms accounting for up-regulation of the immune system by LF involve direct LF interactions with cells [89]. Data on these putative receptors and pathways are not conclusive and sometimes contradictory. Specific receptor binding of LF has been reported for monocytes, macrophages and lymphocytes [8; 10; 13]. LF was shown to regulate lymphocyte maturation and activation [52]. Furthermore release and binding of LF was demonstrated to promote the activation and phagocytosis of PMN and monocytes or macrophages by enhancing motility, superoxide production and release of pro-inflammatory molecules such as nitric oxide (NO), TNFα and IL-8 [29; 84; 87].

Recently it has been recognized that oral administration of LF exerts host-protective effects against infections and cancers in various mammals [94]. Suppressive effects of orally administered bovine LF have been shown against intestinal *E. coli* in milk-

fed mice [93], *Staphylococcus aureus* [9] and rotavirus [88], which are common pathogens causing diarrhoea in calves [95]. Supplementation of LF is able to improve fecal scores and to reduce morbidity in preweaned calves [76].

Intestinal diseases and insufficient function of the immune system in calves are partly responsible for the particularly high mortality and morbidity rates in the neonatal period [4; 77]. Therefore neonatal calves are dependent on passive immunoprotection by the ingestion of diverse substances from colostrum like maternal immunoglobulins (Ig), immune cells, and other substances [4]. Colostrum components can modulate gastrointestinal tract (GIT) development [11; 58] as well as intestinal absorptive capacity [33; 74] and influence the immune systems of the GIT [19; 57]. Among bioactive components, LF is present in high concentration in milk, especially in colostrum and during involution or inflammation [31; 50]. LF that is ingested with colostrum by neonatal calves is absorbed from the digestive tract and then appears in blood plasma [20].

Specific LF receptors were also found to be expressed on the brush border membrane vesicles and the epithelium overlying Peyer´s patches (PP) of the bovine intestinal tract [89; 90]. They are involved in the uptake of LF [91], suggesting that the immunomodulatory properties of LF in the GIT are receptor-mediated [94]. There is also some evidence that LF can influence the development of intestinal epithelia [47; 80] lymphoid tissues in the GIT [23; 82].

AIM OF THE STUDY

The objective of the present study was to examine the unclear mechanisms of the regulatory properties of LF in the immune system through modern molecular biological techniques using immuno-magnetic cell separation, histology, ELISA and one-step real-time RT-PCR. To gain insight into cellular and molecular mechanism of LF, bovine cell culture models were established and stimulated with LPS and LF/LFcin, or with a combination of both. Effects on the cytokine mRNA expression of various immunological relevant factors in blood and milk cells were quantified. A focus was also laid on a feeding trial to determine the effects of LF on the health status of calves. Interactions among LF and the morphological development of the GIT villi and PP as well as mRNA gene-expression levels of pro- and anti-inflammatory markers were investigated to test LF as a preventative supplement in calf nutrition.

MATERIAL AND METHODS

Blood and milk samples

Fresh milk (2 l) was collected from one quarter of each Brown Swiss dairy cow and centrifuged as described by [71]. Blood was taken from a jugular vein of the animals and blood lysis procedure was performed as stated in [70] and [71]. Supernatants of milk and blood samples were discarded and the bovine WBC and SC suspensions were adjusted to $2*10^7$ cell/ml in PBS/0.1% BSA for immuno-magnetic cell separation or $4*10^6$ cells/ml in cell culture media for cell culture. To provide an insight into the composition of milk cell populations a cell differentiation was performed using a panoptic staining method according to Pappenheim [78]. SCC in milk was determined at the Milchprüfring Bayern e. V. (Wolnzach, Germany).

For *in vivo* investigations 15 ml blood was collected from the jugular vein using a EDTA and serum vacutainer tube (Greiner bio-one GmbH, Germany) between day 1 and 2 and afterwards once a week. Lysis of EDTA blood was performed as described above and leukocytes were transferred in 350 µl RNA extraction lysis buffer (Macherey-Nagel, Düren, Germany) for RNA isolation. Serum tubes were centrifuged and the overlaying serum was collected and stored at –20°C until analyses [69, 72]. The bovine IgG content in serum was then measured by sandwich ELISA according to Erhard *et al.* [28]. The serum LF content was measured by ELISA as described earlier [69]. Blood samples were collected for haematological analysis from animals following euthanasia and forwarded in EDTA vacutainer tubes to a veterinary laboratory (Vetmed Labor, Unterhaching, Germany). Hematocrit and haemoglobin concentration, erythrocyte, thrombocyte and white blood cell numbers were determined with the CELL-DYN 3700SL System (Abbott Diagnostika GmbH, Wiesbaden, Germany). Furthermore a blood smear was send to the lab to obtain a differential white cell count.

Immuno-magnetic cell separation of mononuclear cells

Cell separation was carried out as described by [71] using immuno-magnetic beads (Dynabeads M-450, Dynal, Inc., Lake Success, N.Y., USA) coated with anti-mouse IgG. Dynabeads were washed and incubated with antibody BAQ151A (5µg/10^7 beads) (VMRD, Inc., Pullman, Washington, USA, specific for M-M7). To remove

unbound antibodies the suspension was washed using a magnet (Dynal magnet MPC-L). WBC and SC suspensions ($2*10^7$ cells/ml) were incubated with BAQ151A coated biomagnetic beads ($4*10^7$ beads/ ml cell sample). The supernatant was removed gently with a pipette while the mononuclear cells were attached at the tube wall using the magnet. The separated mononuclear cells were washed and finally resuspended at a concentration of $4*10^6$ cells/ml in cell culture medium. Viability of the separated cells was detected by trypan blue staining. To determine the purity of isolated monocytes and macrophages a Pappenheim staining [62] was performed. A schematic representation of the cell separation procedure is shown in Fig. 2.

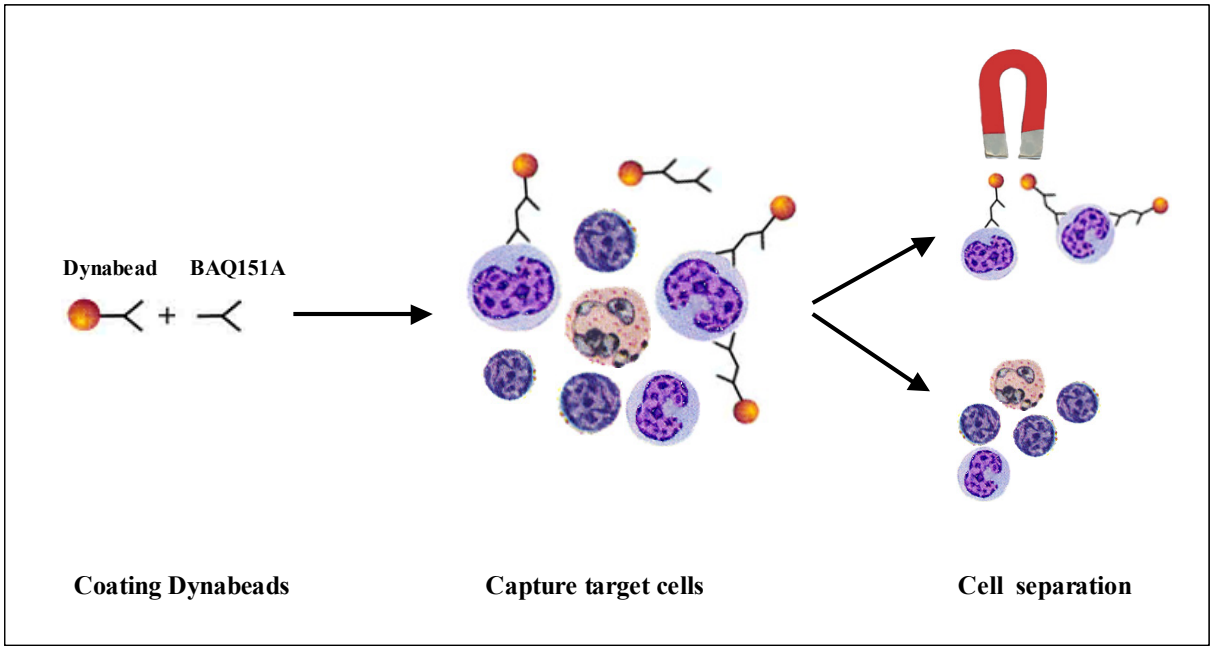

Fig. 2. *Immuno-magnetic cell separation of monocytes and macrophages using BAQ151A coated Dynabeads (Dynal).*

Cell assay

WBC, SC and immuno-magnetic separated monocytes and macrophages of three different cows were cultured at a cell density of $4*10^6$/ml (WBC and SC) and $2*10^6$/ml (monocytes and macrophages) in the first *in vitro* [71] experiment. After a culture period of 24 h the cells were stimulated in duplicates by addition of 10 µg/ml LPS (*E.*

coli O26:B6, Sigma Chemicals, Seelze, Germany) and allowed to incubate for (0, 1, 2, 3, 4 or 8h). A nontreated group served as negative control.

In the second *in vitro* experiment [70] WBC ($4*10^6$/ml medium) or immuno-magnetic separated monocytes ($4*10^6$/ml medium) were treated after a culture period of 24 h with LPS, bovine LF (0-2500 µg/ml, Milei, Leutkirch, Germany), bovine LFcin (0-500 µg/ml, Morinaga Milk Industry, Zama, Kanagawa, Japan) or combinations of these agents (3 µg/ml LPS + 200 µg/ml LF or 3 µg/ml LPS + 500 µg/ml LFcin). Concentration test series were incubated for another 2 h. Time test series (3 µg/ml LPS; 200 µg/ml LF; 500 µg/ml LFcin) were incubated for the increasing length of time (0, 1, 2, 6 and 8 h). Nontreated groups served as negative controls. In both *in vitro* experiments cells were stored in lysis buffer for RNA isolation.

Animals, Husbandry, Feeding and Experimental Procedures

Forty calves, crossbreds of Red-Holstein-Friesian x Fleckvieh, were divided into two groups (LF group and negative control) according to their dates of birth, sex, and weight as described by [69]. The calves were placed into the experiment beginning on the day of their birth, separated immediately after birth from their dam and kept in individual compartments during the colostral phase until the 6^{th} day of live. The newly born calves were given as soon as possible *post natum* 2 up to 3 litres of colostrum per meal (2 times per day) until the 6^{th} (± 1) day. Starting at day 3 the animals in the LF group received colostrum mixed with 0.54 g LF (DSM Nutritional Products Ltd, Basel, Switzerland) at each feeding. After colostral phase (day 6 ± 1) calves received a nonmedicated milk replacer (KALBI MILCH, Schaumann GmbH), water and hay and concentrate. The LF group was given the milk replacer supplemented with 0.16 % LF, the other group served as control. Starting on week 2 to 3 also maize silage was fed. Disease problems that occurred during the experimental periods (respiratory disorders, influenza and diarrhoea) were treated by a veterinarian.

Collection of tissue samples

After slaughtering 10 calves (5 from each group), the GIT was removed and a 1- to 2-cm-long cross-section was taken from the small intestine (mid jejunum, mid ileum), large intestine (caecum, colon), ventral rumen and reticulum, abomasum and omasum. Immediately after collection the tissue pieces were washed twice in physiological 0.9% NaCl solution and the tissue sections were embedded and

transferred in 3.7% buffered formalin (Carl Roth GmbH, Karlsruhe) for 24h as described previously [72]. Tissue samples for RNA extraction collected from the four compartments of the cattle stomach, GIT, liver and mesenterial lymph nodes (mLN) were placed in individually labeled cryotubes and frozen in liquid nitrogen. Cryotubes were stored at -80°C until RNA extraction.

Histology and Histomorphometry

After fixation in formalin the specimen were dehydrated through a graded series of alcohols and embedded in a paraffin. 10 to 15 cuts of 6 µm thickness were made from different regions of each block using a microtom (LEICA RM2145). Paraffin was removed by xylol (Carl Roth GmbH, Karlsruhe), sections were hydrated and stained with hematoxylin and eosin (HE), following the standard protocol [72].

Quantitative measurements were made in at least 30 well-oriented crypt-villus systems for jejunum, ileum, caecum and colon as described earlier [72]. Analysis of villus heights and widths and crypt depths were done on at least 15 well-oriented crypt-villus systems for each intestinal sampling site (Fig. 9), and the ratios of villus heights to crypt depths were calculated. Furthermore, areas of at least 10 Peyer`s patches per slide were evaluated.

Analysis in faeces

Analysis of bacterial communities in chyme samples taken from the colon after slaughtering was made at the Tiergesundheitsdienst Bayern e.V. Grub. The total number of aerobe and anaerobe microbial counts as well as some selected indicator counts of the diverse bacterial strains were analysed [72].

Total RNA extraction and quantification

Total RNA was extracted as described by [71] using the Macherey-Nagel NucleoSpin RNAII kit (Düren, Germany). Tissue were homogenized in the RA1 lysis buffer with the FastPrep extraction system FP120 (Savant Instruments, Holbrook, NY, USA). To quantify the extracted RNA, all spectro-photometric measurements were taken in a photometer using UV-transparent UVette (Eppendorf, Hamburg, Germany). Integrity of the extracted total RNA was verified by optical density OD_{260nm}/OD_{280nm} absorption ratio.

Gene	Primer	Sequence 5' → 3'	EMBL Accession number	Product length
TNFα	Sense Antisense	TAA CAA GCC GGT AGC CCA CG GCA AGG GCT CTT GAT GGC AGA	AF011926	256 bp
IL-1β	Sense Antisense	TTC TCT CCA GCC AAC CTT CAT T ATC TGC AGC TGG ATG TTT CCA T	M37211	198 bp
IL-6	Sense Antisense	GCT GAA TCT TCC AAA AAT GGA GG GCT TCA GGA TCT GGA TCA GTG	NM173923	200 bp
IL-8	Sense Antisense	ATG ACT TCC AAG CTG GCT GTT G TTG ATA AAT TTG GGG TGG AAA G	AF232704	149 bp
IL-10	Sense Antisense	ACT TTA AGG GTT ACC TGG GTT G CTT CTC CAC CGC CTT GCT CTT	U00799	206 bp
INFγ	Sense Antisense	TAA GGG TGG GCC TCT CTT C CCA TGC TCC TTT GAA TGA CC	M29867	143 bp
Caspase 6	Sense Antisense	TGT TCA AAG GAG ACA AGT GTC AG CAG AGT AGC ACA TGA GGA AGT C	BC078785	210 bp
TGFß1	Sense Antisense	AAG GAC CTG GGC TGG AAG TG TCA TGT TGG ACA ACT GCT CCA C	XM592497	239 bp
TGFα	Sense Antisense	TGA CTG CCC AGA TTC CCA CA GCA GCA GTG TAT CAG CAC ACA	M36271	238 bp
IGF-1	Sense Antisense	CGC ATC TCT TCT ATC TGG CC CTG AGC CTT GGG CAT GTC	X15726	311 bp
Cyclin D1	Sense Antisense	TCC TGT GCT GCG AAG TGG A GGT CCA GGT AGT TCA TGG C	BC014078	246 bp
LF	Sense Antisense	GGC CTT TGC CTT GGA ATG TAT C ATT TAG CCA CAG CTC CCT GGA G	AB046664	338 bp
histone 3	Sense Antisense	ACT CGC TAC AAA AGC CGC TC ACT TGC CTC CTG CAA AGC AC	BT020962	232 bp
GAPDH	Sense Antisense	GTC TTC ACT ACC ATG GAG AAG G TCA TGG ATG ACC TTG GCC AG	U85042	197 bp
β-Actin	Sense Antisense	AAC TCC ATC ATG AAG TGT GAC GAT CCA CAT CTG CTG GAA GG	AY141970	202 bp

Table 1. Primers sequences used for one-step real-time RT-PCR.

One-step real-time RT-PCR

Quantitative real-time RT-PCR was performed using the QuantiTect SYBR Green RT-PCR (Qiagen, Hilden, Germany) by a standard protocol RNA in a LightCycler (Roche Diagnostics, Mannheim, Germany) as previously described [71] or in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) [70; 72]. PCR Primers were introduced as depicted in table 1. Crossing point (CP) values were acquired by using the *"second derivate maximum method"* of the LightCycler software 3.5 (Roche Diagnostics). Crossing point (CP) values in the Rotor-Gene 3000 were achieved using the Rotor-Gene software version 5.0 (Corbett Research). Relative mRNA levels were calculated by normalization of the CP (= ΔCP) [48] of the target gene to a single reference gene [71] or the arithmetic mean of the CP of several reference genes [70; 72]. To ensure the stability of standard gene expression for internal standardization of target standardization of target gene expression data, determination of stable reference genes was performed for the *in vivo* investigation [72]. Therefore, a *BestKeeper Index* [65] was used, based on the expression data of at least three reference genes (GAPDH, β-Actin and histone 3). In some cases the cytokine mRNA expression was presented as ΔΔCP increase evaluated in relation to each single time point and compared to un-stimulated control cells.

RESULTS AND DISCUSSION

The present results demonstrate the short term effects of LPS on pro-inflammatory cytokine, LF and CD14 mRNA expression levels in bovine milk and blood cells [71]. Moreover, the influence of bovine LF and LFcin on the cytokine mRNA expression in untreated or LPS stimulated leukocytes and macrophages was shown [70]. During the course of the experiment the separated cells were in vital condition as determined by trypan blue staining. As revealed by Pappenheim staining, the immuno-magnetically isolated blood monocytes and milk macrophages were almost homogeneous, with very little contamination by neutrophils or lymphocytes. Since distribution of SC population was dominated by neutrophils (72%), it can be assumed that the milk samples were taken from cows with infected udders [78]. Next to the *in vitro* investigations the influence of oral administered LF on the morphology of the intestine and on the expression of apoptosis and cell proliferation markers in the GIT was demonstrated in calves. In addition, the results show mRNA expression changes of various cytokines in the GIT and blood in response to LF, as well as altered serum IgG levels and number of peripheral blood leucocytes [72].

In all four described cell culture types significant mRNA increases for the pro-inflammatory cytokines TNFα, IL-1β, IL-6 were found in response to LPS (Fig. 3), with peaks after 1 to 3 h (TNFα>IL-6 >IL-1β). This is in agreement with other studies, showing that mRNAs of these cytokines were immediately transcribed in monocyte-derived macrophages [98] and Kupffer cells [103] after exposure to *E. coli* endotoxin. Intramammary infusion of LPS in bovine led to an increase of TNFα and IL-1 concentration in milk between 2 and 4 h after LPS treatment [85; 64].

Results also indicate that LPS-induced mRNA expression levels of all analysed cytokines were significantly lower ($p<0.001$) in SC and milk macrophages than corresponding blood cells. Besides, a longer persistence of cytokine gene expression could be measured in WBC and monocytes. This is supported by the observation that bovine milk macrophages stimulated by *E. coli* secrete limited amounts of IL-1ß in comparison with adequate blood monocytes [67; 68]. Hawkes *et al*. [35] reported increased cytokine production by approximately 40-50% in human milk cells stimulated with LPS for 24h, whereas peripheral blood mononuclear cells responded to LPS with TNFα, IL-1ß and IL-6 production increased by 350%, 135% and 30%,

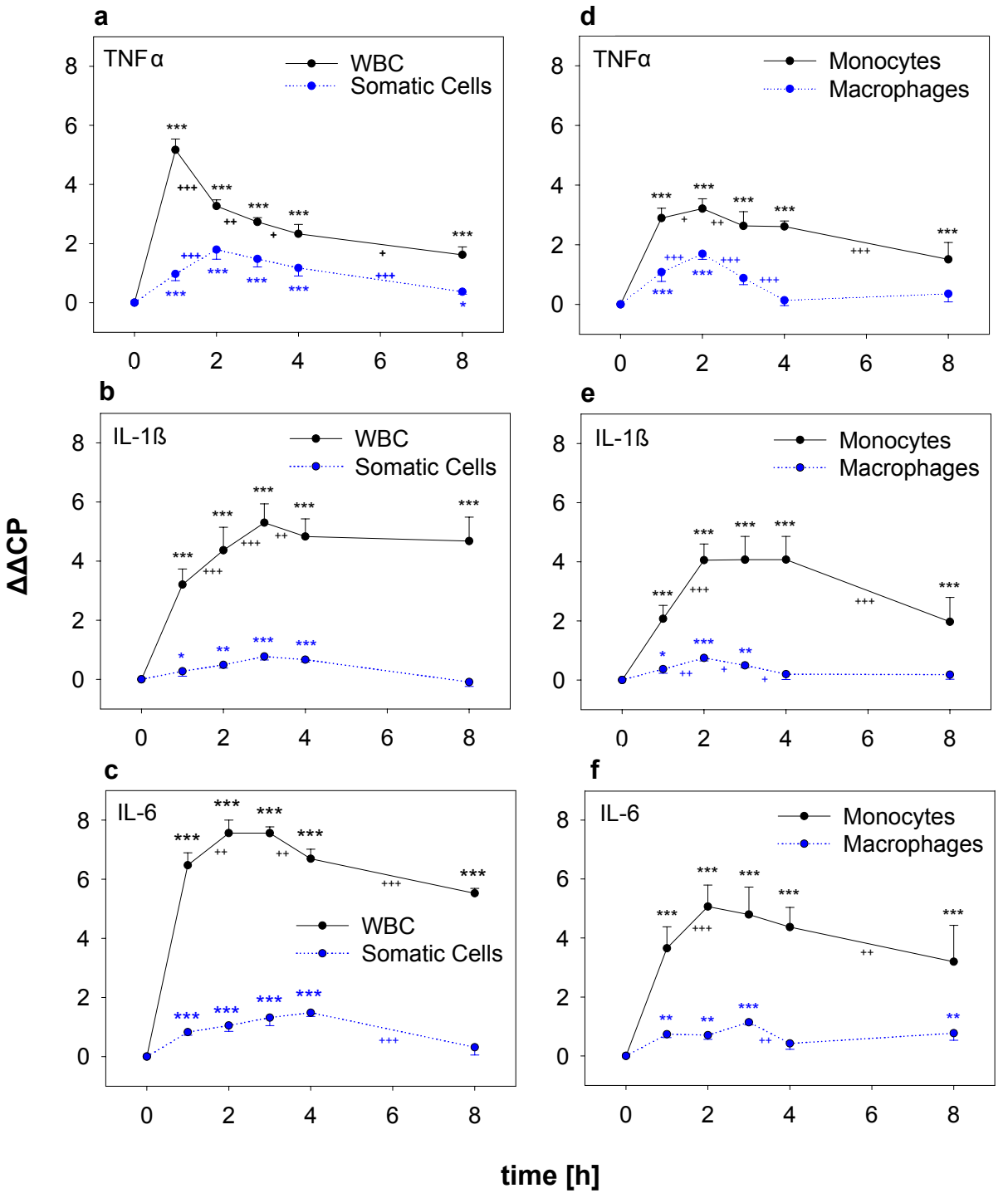

Fig. 3. *Kinetics of cytokine mRNA expression in LPS (10µg/ml) induced WBC and SC (a, b, c) or monocytes and macrophages (d, e, f). The TNFα (a, d), IL-1β (b, e) and IL-6 (c, f). The mean ± SEM from three separate experiments are indicated (n=6). The cytokine mRNA expressions are presented as ΔΔCP increase calculated in relation to time point zero and compared to un-stimulated cells (asterisk), normalised within any single time point [*p<0.05, **p<0.01, ***p<0.001]. Significant changes between the single time points are shown by plus [+p<0.05, ++p<0.01, +++p<0.001].*

respectively. Our hypothesis therefore is that the lower responsiveness of milk cells to stimulating agents is on the one hand caused by the blood-to-milk diapedesis, which utilizes energy reserves of PMN and monocytes [55], and by the interference from milk components [61]. Then again a reduced efficiency may also be attributed to the role of CD14 and LF in mediating initiate immune responses to LPS.

In the present work, transcription of CD14 mRNA was found to be significantly lower in untreated SC and macrophages compared to corresponding blood cells. Paape *et al.* [60] found evidence that differentiation of monocytes into macrophages is accompanied by a decrease in membrane CD14 numbers. This kind of down-regulation of LPS receptors at the surface of immune cells could be important for prevention of an overwhelming release of proinflammatory cytokines thus preventing development of septic shock [24]. The results of this investigation show a significant change in the pattern of CD14 mRNA expression in monocytes and macrophages after *in vitro* incubation with LPS (Fig. 4). In monocytes CD14 mRNA was found to be slightly up-regulated, whereas transcriptional levels for CD14 was down-regulated in LPS treated macrophages. This discrepancy of CD14 gene expression among monocytes and macrophages in response to LPS may be part of an endotoxin tolerance of milk macrophages, caused through previous stimulation of SC with *E. coli* endotoxin in the mastitis infected udders. Furthermore, Lee et al. [45] found evidence for elevated release of sCD14 from CD14-positive cells in bovine milk during intramammary *E. coli* infection. Soluble CD14 is known to bind free LPS and inhibit its interaction with mCD14, thus preventing LPS-induced septic shock and cytokine release [36; 37; 45].

LPS-neutralizing effects were also reported for LF and its peptide [53; 104]. This effects are generated by high affinity binding through the LFcin domain of LF to the lipid-A of bacterial LPS [2]. Additionally, it was demonstrated that LF may down-regulate LPS induced cytokines in mononuclear cells through a mechanism involving LF internalisation, nuclear localization and interference with NF-κB activation [34]. In this study mRNA expression levels for LF in SC were strongly up-regulated compared to the other cell culture types (Fig. 4). These findings are independent of LPS treatment and culture period, suggesting that transcriptional activity of LF in the SC was similar in the infected mammary glands before cell culture. Our results are consistent with those of other investigators, showing that LF is produced and excreted by stimulated PMN in inflamed tissues [25]. Moreover, the hypothesis that

LPS might trigger LF gene expression in neutrophils is supported by the observation that LPS induced slight but significant LF mRNA increases in SC ($p<0.05$). The LPS-neutralizing effect of LF could also have consequences for the sCD14-LPS induced activation of endothelial cells. LF was shown to inhibit the LPS stimulated expression

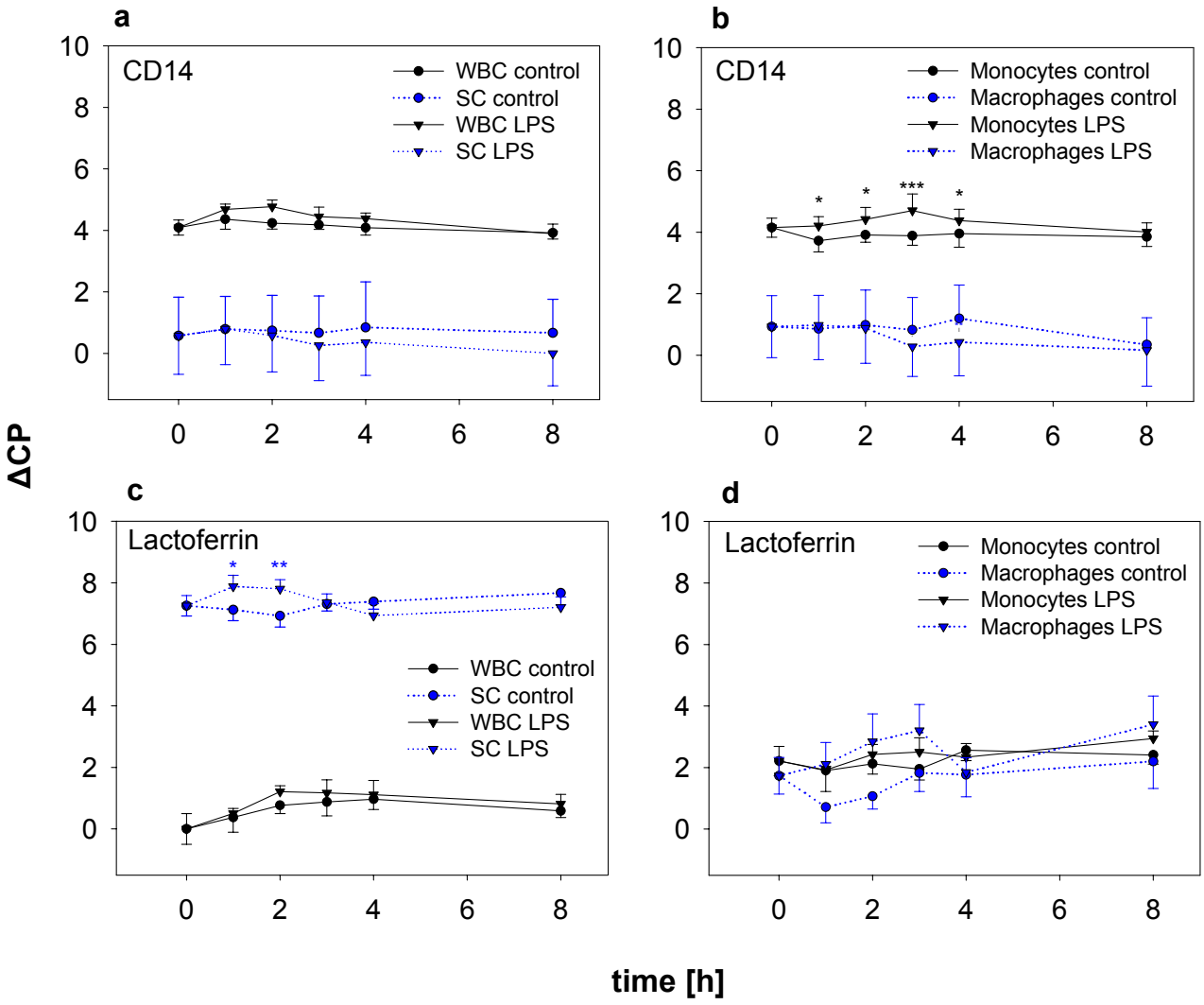

Fig. 4. *Kinetics of CD14 and LF mRNA expression in LPS (10µg/ml) induced WBC and SC (a, c) or monocytes and macrophages (b, d). The CD14 (a, b), LF (c, d) mRNA expression was assessed by real time one-step RT-PCR using GAPDH as a reference gene. To show high expression differences between WBC and SC the LF and CD14 mRNA expressions graphs were only normalised against the GAPDH mRNA expression (=ΔCP).The mean ± SEM from three separate experiments are indicated (n=6). *p<0.05, **p<0.01, ***p<0.001.*

of E-selectins, ICAM-1 and IL-8 necessary for the local recruitment of immune cell at inflammatory sites [5]. It can be concluded that the poor response of milk cells to bacterial endotoxins in the present study was probably initiated by the complex immune regulatory properties of LF following release from neutrophils.

In a second approach therefore the role of LF and LFcin governing the regulatory functions in the immune system was enlightened. LF and LFcin effected a significant up-regulation of TNFα, IL-1β, IL-6 and IL-10 gene expression in WBC after 2h of stimulation ($p<0.001$). The maximum response was attained at a LF concentration of 200 µg/ml, while 200-500 µg/ml LFcin were necessary to obtain similar cytokine mRNA levels. A combined LF/LFcin and LPS (10 µg/ml) treatment showed no significant changes of cytokine mRNA expression levels in those cells, except IL-6 and TNFα, which were shown to be further stimulated at high LFcin concentrations (500 µg/ml). Moreover, the results demonstrated that an addition of LF (200 µg/ml) and LFcin (500 µg/ml) did not affect transcriptional activity of TNFα at various LPS-concentrations (Fig. 5). An increase of TNFα mRNA expression in WBC could only be prevented in the presence of LF in combination with 0.3 µg/ml LPS. This was

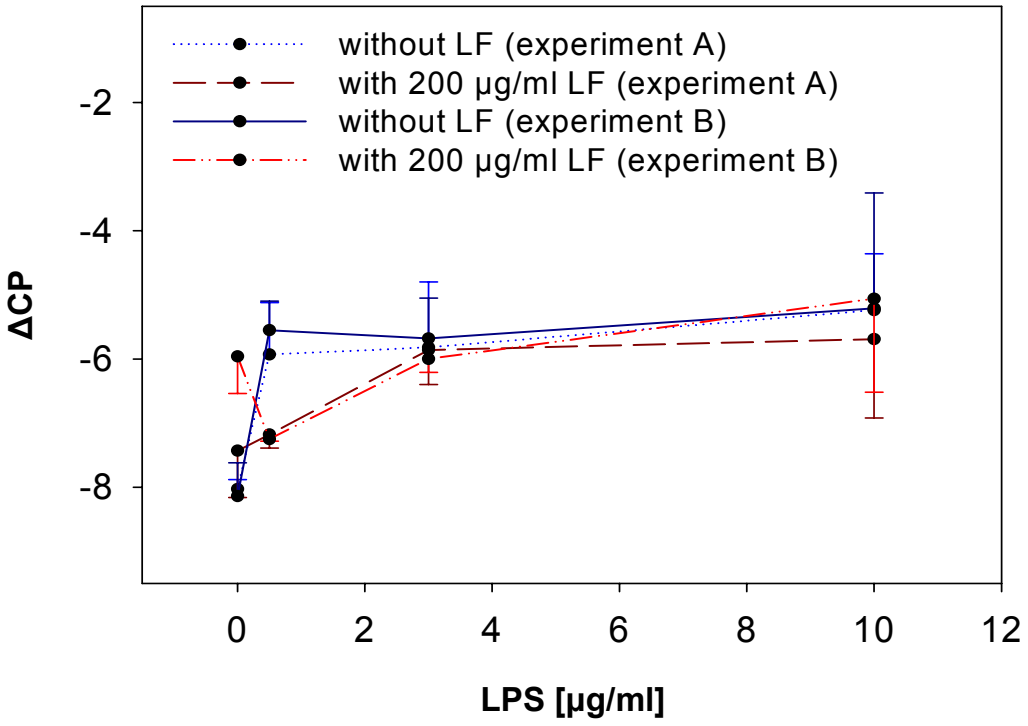

Fig. 5. *TNFα mRNA expression in bovine leukocytes treated with LPS or LPS+LF for 2h. Data are presented as means ± STDV (n=2).*

found to be independent of the different approaches (experiment A: addition of a mixture of LF/LFcin and LPS to the cell culture; experiment B: treatment of cell cultures with LF/LFcin 30 min after LPS stimulation).

Thus, an inhibition of the cytokine gene expression through LPS-neutralizing effects of LF and LFcin seems to be dependent on a defined [LF/LFcin]:[LPS] molar ratio. Since LF and its peptide were able to stimulate cytokine expressions in bovine leukocytes and monocytes in a time-dependent manner, these data suggests that those molecules comprise a dual protective role, one that activates the innate immune response and one that moderates this response from becoming excessive and harmful to the host itself.

Stimulation with LF showed a rapid increase of TNFα (Fig. 6) and IL-10 gene expressions in both cell culture types with maximum accumulation after 1h or 2h of treatment ($p<0.001$). In contrast to LF treatment, stimulation with LFcin induced slighter increases of TNFα and IL-10 mRNA in both cell culture types, which continued to be expressed at a significant higher level to the end of culture period ($p<0.001$). The magnitude of increase of all investigated cytokines varied from modest (6-fold) to dramatic (64-fold). A combination of LF and LPS showed no serious effects on the expression of these cytokines in WBC compared to LPS stimulated cells, whereas in monocytes significant higher levels of IL-10 mRNA expression were noticed after 4h of treatment by the additive use of LF ($p<0.001$). Also a combined addition of LFcin and LPS resulted in elevated TNFα- and IL-10 mRNA expression levels after 1h-2h and 4h of treatment ($p<0.001$). These results suggest that NF-κB might be a limiting factor. This transcriptional factor was found to be uniformly expressed over the entire culture period of 8h, independent of the form of stimulation.

Yamaguchi et al. [102] demonstrated that an injection of LF 8 h before LPS administration of mice suppressed the LPS-induced TNFα production. LF was also reported to down-regulate serum cytokine levels of TNFα, IL-1β, IL-6, induced by lipopolysaccharide in mice, thus providing a natural feedback mechanism and preventing septic shock [42; 49]. Others suggested that LF and LFcin act indirectly by stimulating inhibitors like IL-10 [21]. In comparison to these findings our results couldn't point out remarkable anti-inflammatory activities for LF or LFcin, but showed significant mRNA increases for IL-10 in LF and LFcin treated cells. Likewise, the *in vivo* study demonstrated enhanced cytokine mRNA expressions in WBC for IL-1ß,

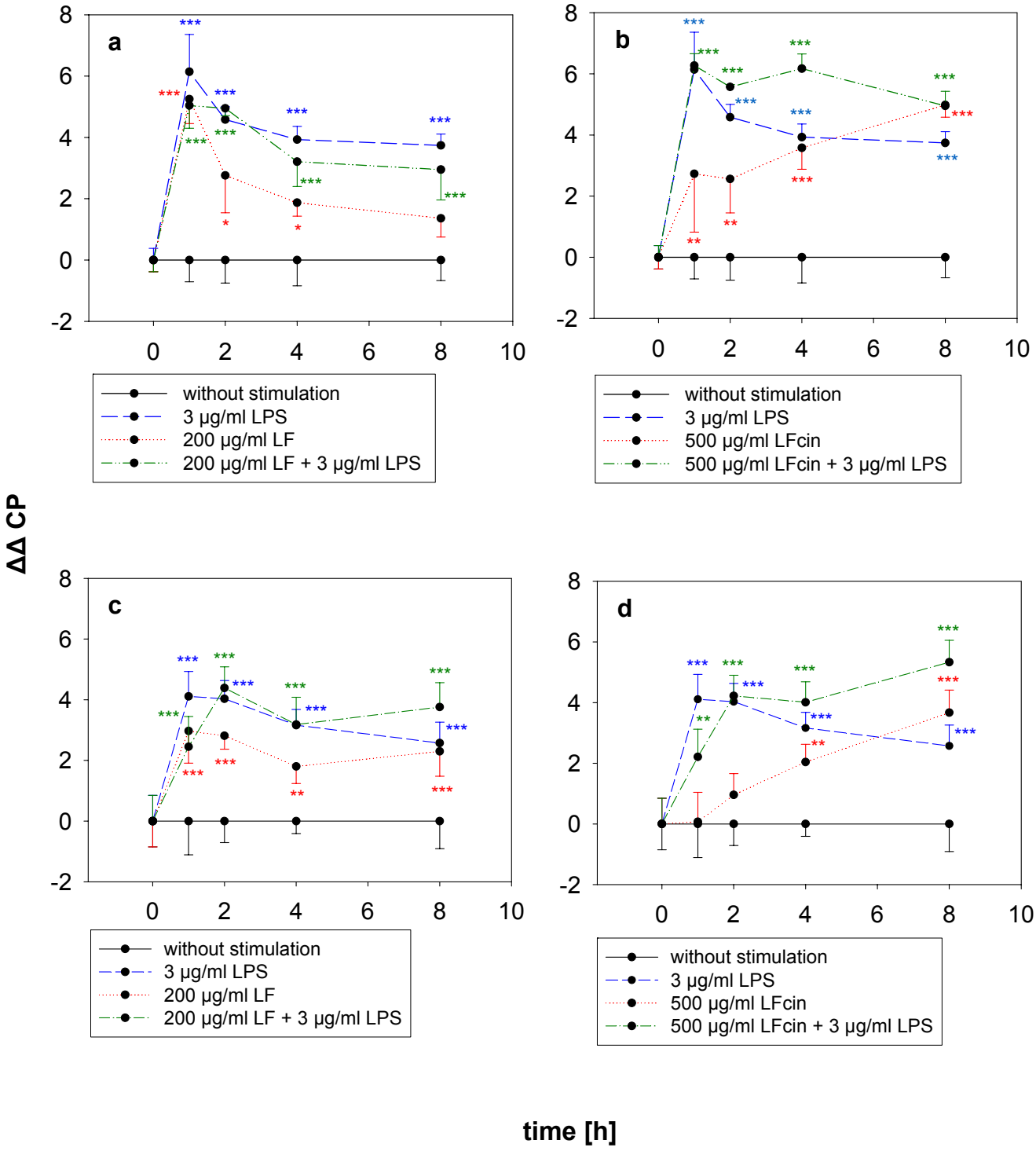

Fig. 6. *Time-dependent TNFα mRNA expression in bovine WBC (6a+6b) and monocytes (6c+6d) treated with LPS, LF, LFcin, LPS+LF and LPS+ LFcin. Data are presented as means ± standard deviation (n=4). The cytokine mRNA expressions are presented as ΔΔCP increase calculated in relation to time point zero and compared to un-stimulated cells (asterisk), normalised within any single time point [*p<0.05, **p<0.01, ***p<0.001].*

IL-10 and INFγ after 1 week of LF administration ($p<0.05$; $p<0.001$; $P<0.05$), while a significant up-regulation of IL-8 expression was observed after 3 weeks ($p<0.05$). Over the entire administration period the trend of mRNA gene expression of the immune markers IL-1ß, Il-8, IL-10 and INFγ decreased significantly, this was justified by applying a Person correlation analysis ($r = 0.46$, $p < 0.001$; $r = 0.46$, $p<0.05$; $r = 0.37$, $p < 0.05$; $r = 0.37$, $p = 0.05$, respectively). IL-10 continued to be expressed at a higher level until week 6 and diminished rapidly until week 7, whereas the expression for IL-1ß, IL-8 and INFγ began to diminish progressively after 1 to 4 weeks respectively (Fig. 7). TNFα and IL-6 mRNA expression levels were not affected by LF. The modest increase of gene expression for the pro-inflammatory cytokines could be explained by the rapid activation of IL-10 mRNA, which was shown to be elevated over a long period of time.

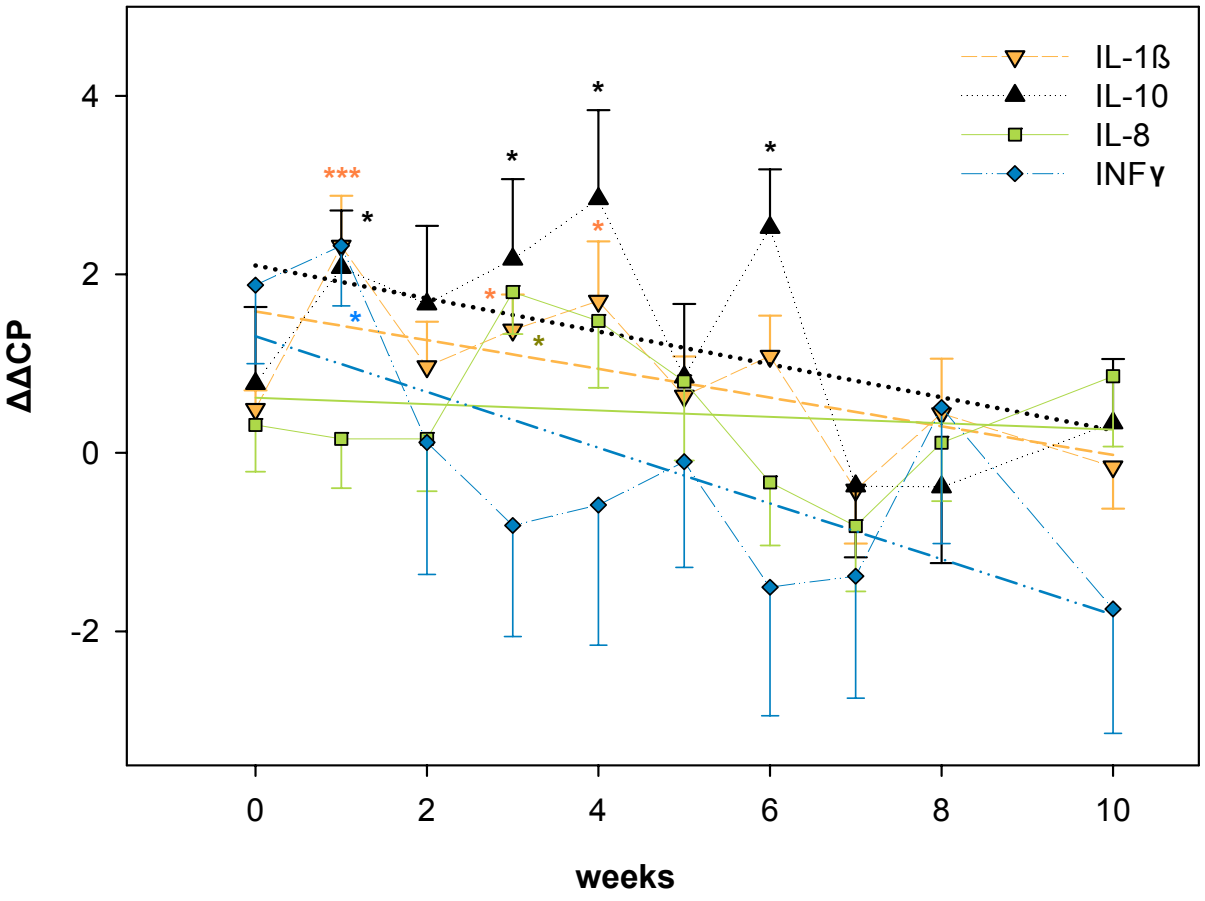

Fig. 7. *Effect of LF on the kinetics of cytokine mRNA expression in WBC. Data are presented as means ± SEM (n=5) [*p<0.05, **p<0.01, ***p<0.001].*

Both *in vitro* and *in vivo* data therefore give evidence that LF or LFcin might induce the up-regulation of IL-10 to inhibit the production of pro-inflammatory cytokines to a later point in time. It is well known that IL-10 provides a negative feedback control of inflammatory processes, strongly inhibits antigen-specific proliferation of T cells and decreases the production of pro-inflammatory cytokines like IL-1 and TNFα [22]. Moreover the results of both studies suggests that LF and LFcin directly stimulate the activity of antigen-presenting cells by specific binding to receptor.

Next to enhanced cytokine mRNA expressions levels the results of the *in vivo* investigation show a significant increase in a number of peripheral blood leukocytes ($p<0.05$), but also significant higher serum IgG levels in LF treated calves (Fig. 7). Furthermore, it could be demonstrated that oral administration of LF enhanced sizes of Peyer´s patches in the ileum of calves ($p<0.05$). The ileal PP are lymphoid follicles, located in the mucosa and extending into the submucosa, that produces immature B-lymphocytes [14; 19]. It is now well established that naturally ingested food components can potentially interact with secondary lymphoid organs along the GIT, developing a specific immune response to the antigens [54]. Ingestion of colostrum rises the proliferation rate of lymphocytes in PP in normal-term calves immediately after birth [19]. LF given orally as an antigen was shown to act as an immunomodulatory agent by increasing the biosynthesis of IgA and IgG in intestinal secretions and in the serum of mice as well as the proliferation of PP cells and splenocytes [23; 82]

In the present study IgG serum concentrations in both groups changed similarly in the course of time, whereas lowest IgG concentrations were already reached around day 21 post natum [69]. Furthermore, a substantial stronger decrease of IgG content was noticed in the control group in contrast to the LF group (Fig. 8). Beginning on the 21^{th} day of life until the 35^{th} day of life, the IgG content of the LF group was significantly higher than that of the control calves ($p<0.05$). As from the 6^{th} week on, the IgG content increased for both groups. This is in agreement with Erhard *et al.* [28], who showed that low point of serum IgG content is reached on day 28 post natum and subsequently accelerates to similar levels of adult animals at the age of 12 weeks.

Serum LF concentrations remained similar, independent of treatment and age of the calves. A possible explanation for the missing significance is the high inter-individual variability in LF-absorption. Since in adult animals very little of an ingested dose of LF

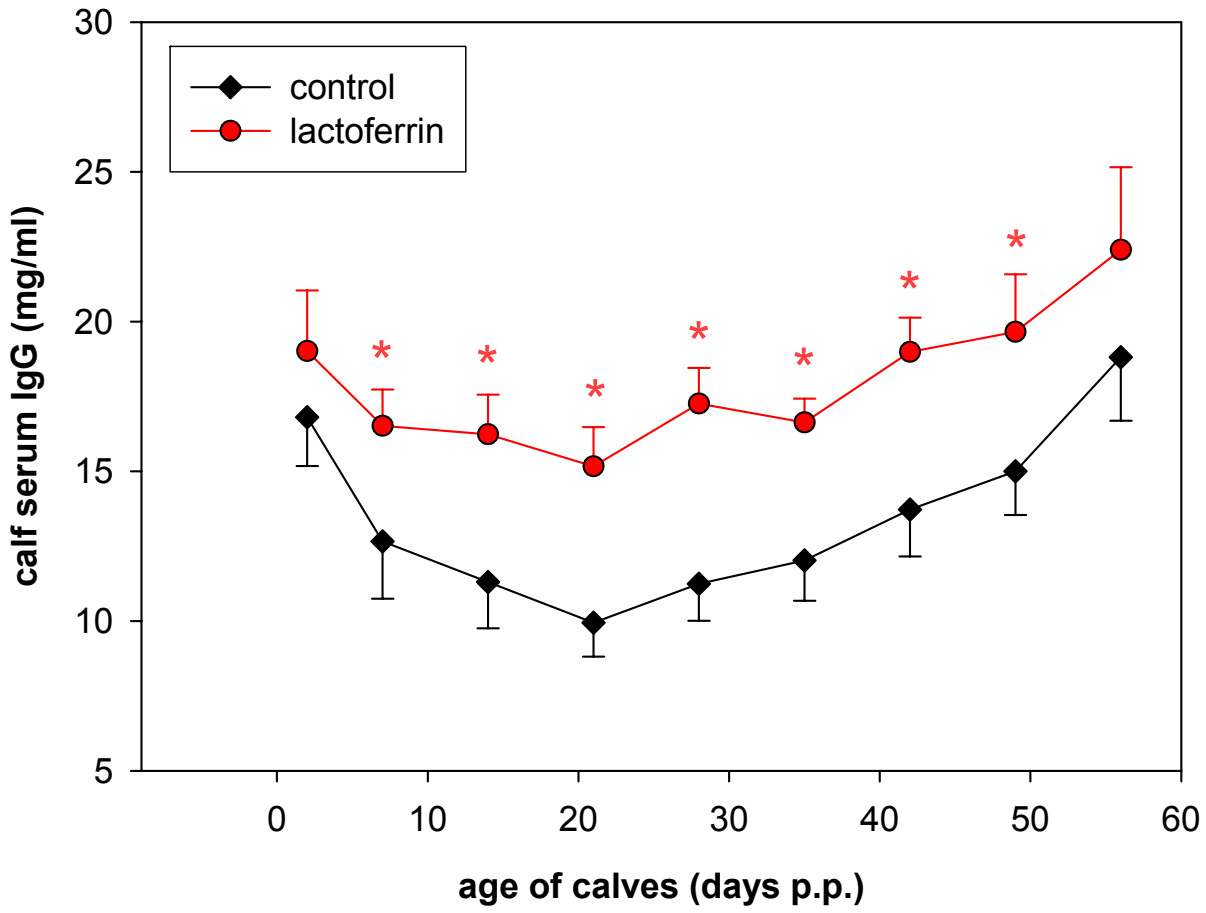

Fig. 8. *Time dependent calf serum IgG contents [69]. Data are presented as means ± SEM (n=20) [*p<0.05].*

is absorbed in the intestinal tract, the immunomodulatory properties of LF in the GIT seems to be predominantly receptor-mediated. Ingested LF is thought to act on intestinal epithelial cells (IEC) and gut-associated lymphoid tissue (GALT) [94; 99] documented as a markedly increase of $CD4^+$ and $CD8^+$ T cells in lymphoid tissues and lamina propria and of IgM^+ and IgA^+ B cells in lamina propria of the small intestine in LF treated mice. This suggests, that LF is first of all able to facilitate the generation of an immune response within the Peyer's Patches. In consequence of activation, lymphocytes pass to the mLN and finally into the blood stream, where they carry out their final effector functions [41; 94]. This is, as observed in the *in vivo* study, accompanied by an increase in leukocyte numbers, serum IgG and cytokine gene expression levels. Moreover cytokines and chemokines produced by IEC or GALT cells in response to LF intake are released into the blood and may directly influence circulating leukocytes [94]. Our results could not give evidence for a LF induced up-regulation of cytokine gene expression in the intestine and mLN.

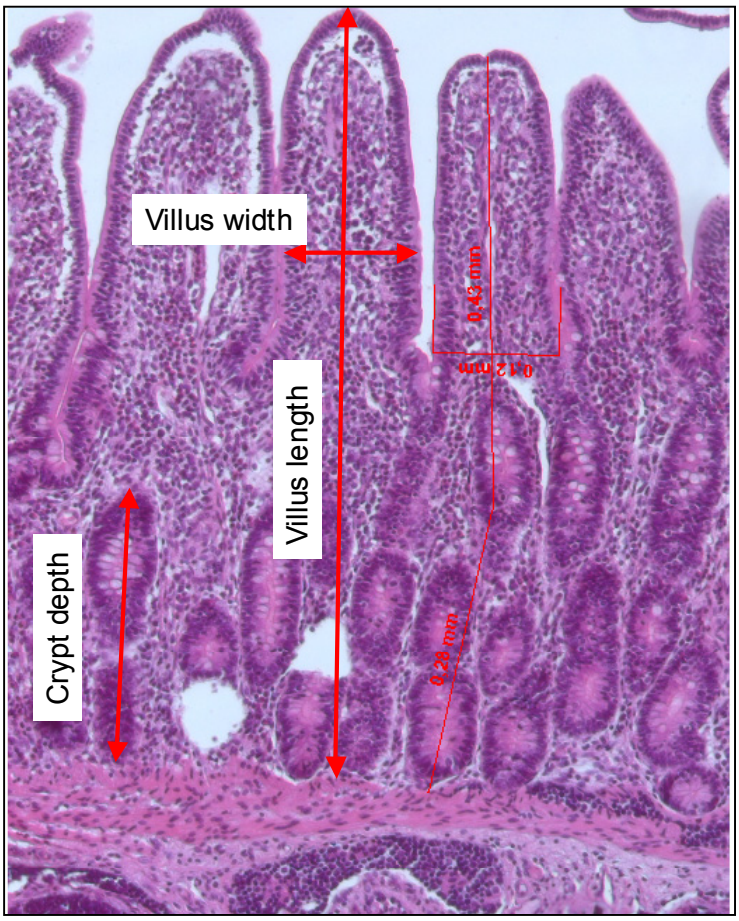

Fig. 9. *Determination of villus length and width, and crypt depth of the jejunum.*

Based on histomorphological analyses, Lf treatment was shown to decrease villus sizes and areas in jejunum ($p<0.05$). In contrast, Schottstedt *et al.* [80] reported that LF supplementation did not influence histomorphometrical parameters of the intestinal epithelium, but negatively affected crypt cell proliferation in the colon of neonatal calves. This may be in part due to the short duration of the experiment. However, our data demonstrate significant caspase-6 mRNA increases ($p<0.05$) in the jejunum of LF treated calves, while diverse cell proliferation markers (TGFα, IGF-1 and cyclin D1) and TGFß1 were not affected by the treatment. Caspases are the final executioners of apoptosis, activated during a signalling cascade in almost all cell types [39]. Apoptosis is especially relevant in the GIT, since it is an important process responsible for maintenance of the cellular balance between proliferation and death and crucial for normal morphology and function [32]. Therefore, an enhanced apoptotic rate seems to be associated with a decrease in villus heights in the jejunum of LF treated calves. Possible mediators for the induction of caspase activity and

epithelial cell apoptosis in the GIT, TNFα and INFγ, were not found to be regulated at the mRNA level in this tissue. These cytokines, which are released in large quantities upon activation of intra-epithelial lymphocytes, have been shown to play a dynamic role in the homeostasis of the villus epithelial barrier [30; 40; 14].

Finally, examination of bacterial communities in chyme was performed to investigate the influence of LF on pathogenic and constitutional germs. The development of the GIT and the immune system is highly influenced by the intestinal flora. Imbalances in micro flora or overgrowth of one bacterial species can alter immunological function [97]. Variation in total number of aerobe and anaerobe microorganisms and *Entero-/Streptococcus* was immense within the groups, whereas microbial counts were numerically lower in the LF treated group. This findings may possibly result from the often described bacteriostatic and bactericidal activities of LF. Additionally, administration of LF tended to reduce the percentage of *E. coli* on aerobe total microbial count. A positive effect of LF on *Lactobacilli*, by enhancing the proportion of those bacteria, could not be demonstrated in this study. Lactic acid bacteria are required for the maintenance of animal health and have been shown to prevent overgrowth and adhesion of various pathogenic bacteria, e.g. *Clostridium perfringens*, to the intestinal wall [75]. Natural individual fluctuation of the intestinal flora and health status of each animal may have covered the effects of LF on the composition of bacterial populations.

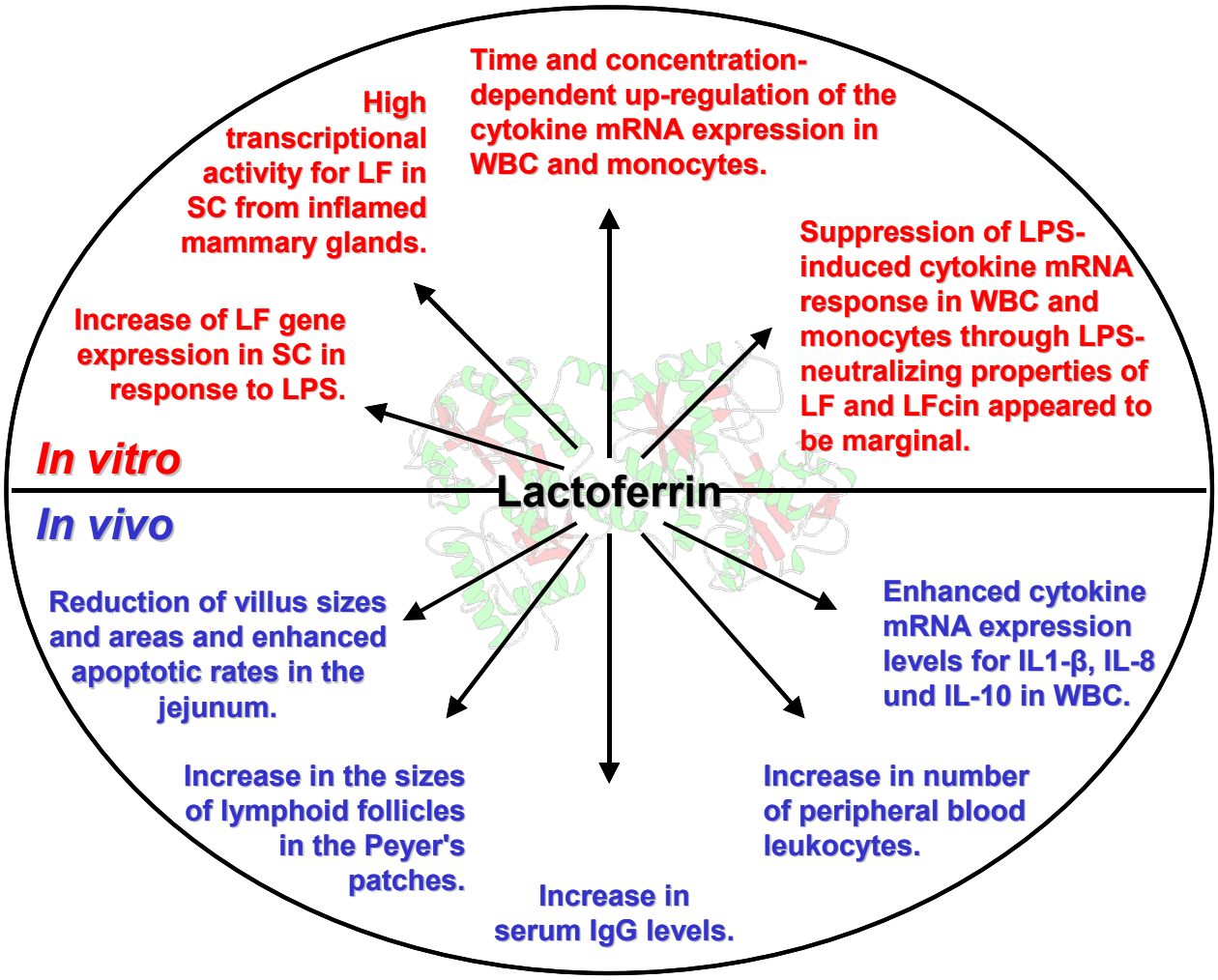

Fig. 10. *In vitro and in vivo effects of LF on the immune system and its influence on the development of the intestinal epithelia.*

CONCLUSIONS

LF was observed to play a key role in host defense of the bovine mammary gland, which acts in a complex mechanism. This includes high transcriptional activity for LF in the inflamed tissues, increase of LF gene expression in SC in response to LPS and intricate immune regulatory properties. Time and concentration-dependent effects for LF and its peptide LFcin on the cytokine mRNA expression in WBC and monocytes were shown *in vitro*. In addition, suppression of LPS-induced cytokine mRNA response through LPS-neutralizing properties of LF and LFcin could not be confirmed in those cells or appeared to be marginal.

The *in vivo* study demonstrates effects of oral administered LF on the systemic immune response as well as mucosal immunity in the intestine of calves. These enhanced immune responses may contribute to the eradication of pathogens, reduction of symptoms, and maintenance of homeostasis during infectious disease.

Both *in vivo* and *in vitro* data therefore provide evidence that beside the iron-binding and antimicrobial properties, the overall immune response is clearly regulated by LF. These findings also suggest that LF receptors are involved in mediating its multifunctional physiological roles in the body.

In conclusion, LF must be considered in general as a polyvalent regulator, which accomplishes its task by interacting with several components involved in infectious or inflammatory processes. Due to the increasing relevance for immunological active food and feed proteins, LF may play an important role in future medication as a preventive supplement and will possibly be used as a replacement for traditional antibiotics against infection and inflammation.

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ACKNOWLEDGEMENTS

First and foremost I would like to thank Prof. Meyer, who enabled me to work at the institute of physiology.

I would especially like to thank Dr. Pfaffl for the the excellent supervision and for providing a lot of freedom preparing this thesis. Throughout my doctoral work he encouraged me to develop independent thinking and research skills.

I want to thank Doreen Tetzlaff, Christine Fochtmann, Angela Sachsenhauser and Inge Celler for their help during the animal experiment and for their exellent technical assistance.

Many thanks to all colleagues, doctorands and employees at the institute for the nice working atmosphere.

I would like to thank my parents who enabled me this education.

I am also greatful to my wife Gordana and our children for their support and patience.

SCIENTIFIC COMMUNICATIONS

Original publications

Prgomet C, Sarikaya H, Bruckmaier RM and Pfaffl MW: Short-term effects on pro-inflammatory cytokine, lactoferrin and CD14 mRNA expression levels in bovine immunoseparated milk and blood cells treated by LPS. *J Vet Med A Physiol Pathol Clin Med* **52**:317-324 (2005).

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Pfaffl MW, Tichopad A., Prgomet C and Neuvians T.P.: Determination of stable houskeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnology Letters* **26**: 509-515 (2004).

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Prgomet C, Sarikaya H, Bruckmaier RM, Pfaffl MW: Isolierung und Charakterisierung von lebenden somatischen Zellsubpopulationen aus Kuhmilch. – In: Abstraktband der Milchkonferenz 2003, 18./19.09.2003, Deutsche Gesellschaft für Milchwissenschaft, Osnabrück, H10.

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Prgomet C *et al.*: Quantification Strategies in Kinetic RT-PCR. – in EMBO Practical Course on Quantification of gene expression by real-time qRT-PCR, 28.05-02.06.2005, EMBL, Heidelberg.

Prgomet C *et al.*: Einfluss einer Laktoferrin-reichen Kälbermilchfütterung auf die Morphologie und die Expression von inflammatorischen Markern im gastro-intestinalen Trakt, 29./30.09.2005 Deutsche Gesellschaft für Milchwissenschaft, Kiel.

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APPENDIX

Appendix 1. Prgomet C, Sarikaya H, Bruckmaier RM and Pfaffl MW: Short-term effects on pro-inflammatory cytokine, lactoferrin and CD14 mRNA expression levels in bovine immunoseparated milk and blood cells treated by LPS. *J Vet Med A Physiol Pathol Clin Med* **52**:317-324 (2005).

Appendix 2. Prgomet C, Peters S and Pfaffl MW : Influence of bovine Lactoferrin and Lactoferricin on the cytokine expression in LPS treated cultivated bovine blood cells. *Journal of the Science of Food and Agriculture* (in press).

Appendix 3. Sarikaya H, Prgomet C, Pfaffl MW and Bruckmaier RM: Differentiation of leucocytes in bovine milk. *Milk Science International* **59**: 581-590 (2004).

Appendix 4. Prgomet C, Prenner ML, Schwarz F and Pfaffl MW: Effect of lactoferrin on the immune-status and the gastro-intestinal morphology in growing calves. *J Anim Physiol Anim Nutr* (submitted).

Appendix 5. Pfaffl MW, Tichopad A., Prgomet C and Neuvians T.P.: Determination of stable houskeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnology Letters* **26**: 509-515 (2004).

Appendix 6. Dzidic A, Prgomet C, Mohr A, Meyer K, Bauer J, Meyer HHD and Michael W Pfaffl: Effects of Mycophenolic acid (MPA) on inosine monophosphate dehydrogenase (IMPDH) I and II mRNA expression in white blood cells and various tissues in sheep. *J Vet Med A Physiol Pathol Clin Med* (in press).

Appendix 1

J. Vet. Med. A **52,** 317–324 (2005)

ISSN 0931–184X

PHYSIOLOGY

Physiology Weihenstephan, Center of Life and Food Science, Technische Universität München, Freising-Weihenstephan, Germany

Short-term Effects on Pro-inflammatory Cytokine, Lactoferrin and CD14 mRNA Expression Levels in Bovine Immunoseparated Milk and Blood Cells Treated by LPS

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With 4 figures and 1 table

Received for publication December 27, 2004

Summary

Leucocytes (WBC) are recruited from peripheral blood into milk as part of the inflammatory response, mediated through cytokines or interleukins (IL) synthesized by mammary tissue and the milk somatic cells (SC). The inflammatory response is related to the concentration of SC and the cytokines produced. To investigate and to compare the kinetics of cytokine production in SC and WBC during inflammation, cell culture models were established, where SC and WBC were cultured in parallel ($n = 3$). In addition, macrophages or monocytes were isolated from milk and blood with antibody-coated magnetic beads and cultivated separately. Isolated cells were pure, unaltered and viable. Cultures were activated with 10 μg/ml lipopolysaccharide (LPS). After 0, 1, 2, 3, 4 and 8 h cells were harvested for RNA isolation. Cytokine [tumour necrosis factor alpha (TNFα), IL-1β, IL-6] mRNA expression responses and transcriptional activity of CD14 and lactoferrin (LF) were quantified via a one-step real-time RT-PCR. Significant cytokine mRNA increases were found in all four cell culture types and genes, with peaks after 1 and 2 h (TNFα > IL-6 > IL-1β). In WBC or monocytes higher LPS responses and longer persistence could be found than in corresponding milk cells (IL-1β > IL-6 > TNFα). SC and macrophages are less responsive to LPS stimulation than WBC or monocytes. The strength of the immune response in the blood system is much more prominent than in the mammary gland. This may be ascribed to the role of CD14 on the cytokine production of the investigated cells, or may be caused by the blood-to-milk diapedesis. The constitutive transcription of CD14 mRNA in WBC and monocytes was found to be 6 to 15 times higher than in adequate milk cells.

Introduction

During mastitis polymorphonuclear neutrophils (PMNs) are recruited from peripheral blood through the mammary epithelium into milk in response to a variety of inflammatory mediators, such as cytokines, complements or eicosanoids (Craven, 1986; Persson et al., 1993). PMNs represent the major line of defence against bacterial infection of the mammary gland because of their phagocytosis activity and their bactericidal enzymes and oxy-radicals (Heyneman et al., 1990). The inflammatory response is initiated by macrophages together with epithelial cells by production and release of tumour necrosis factor alpha (TNFα), interferons (IFN) and interleukins (IL) as a reaction to invading microorganisms or their components such as lipopolysaccharide (LPS) (Boudjellab et al., 1998.; Burvenich et al., 1994; Schmitz et al., 2004). LPS, the major components of the outer membrane of Gram-negative bacteria cell wall, is a potent stimulator of inflammatory processes in the bovine mammary gland (Carroll et al., 1969; Ohtsuka et al., 2001; Pfaffl et al., 2003).

The membrane-bound CD14 (mCD14), expressed on the cell surface of monocytes and macrophages and, to a lesser degree, on neutrophils, binds the LPS–lipopolysaccharide-binding protein (LBP) complexes and thus mediates the activation of those phagocytes (Wright et al., 1990; Antal-Szalmas et al., 1997). Lipopolysaccharide-binding protein is an acute-phase protein released by the liver during inflammation. Serum LBP enhances the LPS-induced cell activation and cytokine response by binding to the lipid A moiety of LPS and catalysing the transfer of LPS to the CD14 receptor (Schumann et al., 1990). Milk macrophages are beneficial to dairy cows because of their ability to detect invading mastitis-causing pathogens and to initiate the inflammatory response. They are the predominant cells in secretion of the healthy mammary gland and active mammary gland phagocytic cells (Lee et al., 1980).

Lactoferrin (LF) is a cationic metal-binding glycoprotein produced by epithelial cells and leucocytes that is present in high concentration in milk, especially in colostrum and during involution or inflammation (Masson et al., 1966; Hagiwara et al., 2003). It plays a role in host defence mechanisms via its bacteriostatic and bactericidal effects and in iron binding (Ellison and Giehl, 1991; Baveye et al., 1999). The ability of LF to bind free LPS and to interfere with the LBP/CD14 pathway (Elass-Rochard et al., 1998) have been suggested to

contribute to the anti-inflammatory activities of LF by restraining the cytokine production (TNFα, IL-1β, IL-6) *in vivo* and *in vitro* (Crouch et al., 1992; Schmitz et al., 2004). It was previously shown that LF inhibits the LPS-induced cytokine production on the transcriptional level in two human monocytic cell lines, thus indicating that LF may have a direct effect on regulation of cytokines (Haversen et al., 2002).

The aim of the present study was to compare the strength of the inflammatory response in the blood system and in the mammary gland in parallel. Therefore the real-time RT-PCR technology was used to investigate the short-term kinetics of LPS-induced cytokine mRNA expression in bovine leucocytes (WBC) and milk somatic cells (SC), as well as in immunomagnetically isolated blood monocytes and milk macrophages. For a better understanding of the interaction of the synthesis of cytokines with the regulation mechanisms of the immune response during *Escherichia coli* mastitis, the transcriptional activity of CD14, LF and various cytokines (TNFα, IL-1β, IL-6) in all four cell culture types was investigated.

Material and Methods

Cell samples

Three Brown Swiss dairy cows were selected for this study based on somatic cell counts (SCC). SCC ranged from 4×10^5 to 1×10^6 cells/ml milk. Fresh milk (2 l) was collected from one-quarter of each cow and centrifuged in 500 ml conical tubes for 30 min and 1500 ***g*** at 4°C. The cream was discarded. The pellet was resuspended in 50 ml phosphate-buffered saline (pH 7.4) and centrifuged for 15 min and 460 ***g*** at 4°C. SCC in milk was determined at the Milchprüfring Bayern e. V. (Wolnzach, Germany). Blood was taken from a jugular vein of the identical cows (at the same time points as the milk was sampled) and coagulation was prevented by ethylenediamine-tetraacetic acid (EDTA). A 25 ml blood was diluted 1:1 (v/v) with lysis buffer (830 mg NH_4Cl; 3.7 mg Na-EDTA, 100 mg KCl in 100 ml H_2O pH 7.4) and centrifuged for 10 min in 50 ml reaction tubes at 220 ***g***. The cell pellet was again suspended in lysis buffer and centrifugation was repeated. Supernatants of milk and blood samples were discarded and the bovine WBC and SC suspensions were adjusted to 2×10^7 cell/ml in PBS/0.1% bovine serum albumin (BSA). To provide an insight into the composition of milk cell populations a cell differentiation was performed using a panoptic staining method according to Pappenheim (Sarikaya et al., 2004).

Immunomagnetic cell separation of monocytes and macrophages

Immunomagnetic beads coated with human antimouse immunoglobulin G (Dynabeads M-450; Dynal, Inc., Lake Success, NY, USA) were washed with PBS/0.1% BSA. Dynabeads were incubated with a monoclonal antibody BAQ151A (5 μg/10^7 beads) (VMRD, Inc., Pullman, WA, USA, specific for M-M7) for 45 min at 25°C with gentle rotation, and washed again to remove unbound antibodies. WBC and SC suspensions were adjusted to 2×10^7 cells/ml in PBS/0.1% BSA and incubated for 30 min at 4°C with BAQ151A-coated biomagnetic beads (4×10^7 beads/ml cell sample) with gentle rotation. The supernatant was discarded gently with a pipette while the rosetted monocytes and macrophages were attached at the tube wall using a Dynal magnet (MPC-L). The separated mononuclear cells were washed with PBS/0.1% BSA. Immunomagnetic selection and washing were repeated three times and finally resuspended at a concentration of 2×10^6 cells/ml in RPMI-1640/10% FBS and 0.1% gentamycin. A schematic representation of the cell separation procedure is shown in Fig. 1. To determine the purity of isolated monocytes and macrophages a Pappenheim staining (Pappenheim, 1912) was performed (Fig. 2). Viability of the separated cells, was determined by trypan blue staining (Lindl and Bauer, 1994).

Cell culture and LPS challenge

The WBC, SC and immunomagnetic separated monocytes and macrophages were incubated as duplicates in RPMI 1640 medium supplemented with 10% FCS (Gibco-BRL, Life Technologies, Gaithersburg, MD, USA) and 0.1% gentamycin (Selectavet, Weyarn-Holzolling, Germany) at a cell density of 4×10^6/ml (WBC and SC) and 2×10^6/ml (monocytes and macrophages) and 200 μl/well were added to 96-well plates. After 24 h culture at 37°C in an atmosphere of 5% CO_2, the cells were stimulated in duplicates by addition of 10 μg/ml LPS (*E. coli* O26:B6; Sigma Chemicals, Seelze, Germany) and allowed to incubate for (0, 1, 2, 3, 4 or 8 h). After LPS stimulation total RNA was extracted from milk- and blood-derived and antibody-treated cells.

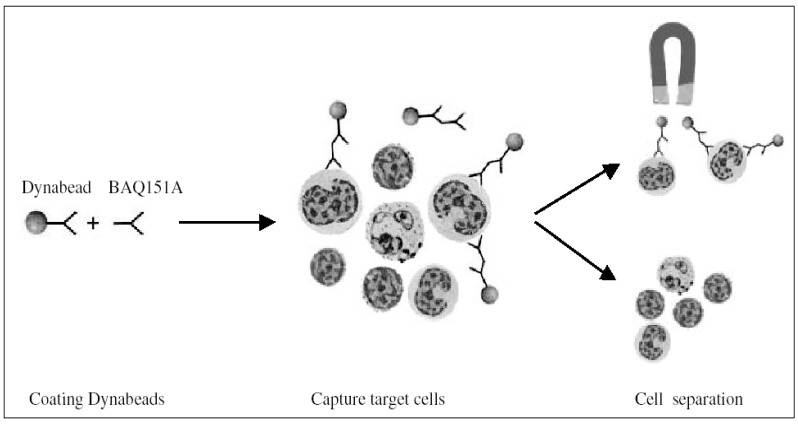

Fig. 1. Immunomagnetic cell separation of monocytes and macrophages using BAQ151A-coated Dynabeads (Dynal).

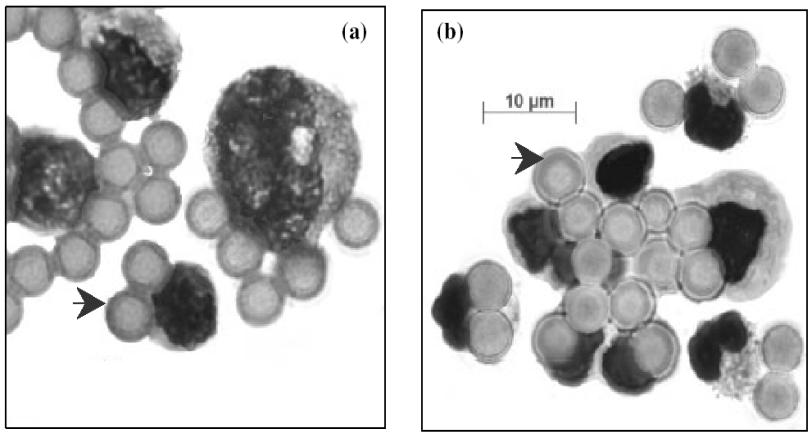

Fig. 2. Pictures of milk macrophages (a) and blood monocytes (b) bound to Dynabeads stained according to Pappenheim. Dynabeads are indicated by the arrowheads.

RNA was stored in 350 µl RA1 lysis buffer (Macherey-Nagel, Düren, Germany). Extractions were performed by using the Macherey-Nagel NucleoSpin RNAII kit. RNA pellets were resuspended in 50 µl of diethyl pyrocarbonate-treated H_2O.

One-step real-time RT-PCR

Real-time RT-PCR was performed using QuantiTect SYBR Green RT-PCR (Qiagen, Hilden, Germany) by a standard protocol recommended by the manufacturer with 5 ng total RNA in a LightCycler (Roche Diagnostics, Mannheim, Germany). Bovine sequence-specific RNA probes for IL-1β, IL-6, TNFα, LF, CD14 and a housekeeping gene, GAPDH were synthesized by MWG Biotech (Ebersberg, Germany) (Table 1). The one-step RT-PCR master-mix was prepared as follows: 5 µl 2*QuantiTect SYBR Green RT-PCR master-mix, 0.5 µl forward primer (1 µM), 0.5 µl reverse primer (1 µM) and 0.1 µl QuantiTect RT Mix. A 6.1 µl of master-mix was filled in glass capillaries and a 3.9 µl volume of total RNA was added as PCR template. Capillaries were closed, centrifuged and placed into a cycling rotor. A five-step experimental run protocol was used: (i) reverse transcription (20 min at 50°C); (ii) denaturation programme (15 min at 95°C); (iii) amplification and quantification programme repeated 35 times (15 s at 94°C; each of optimal annealing temperature for 10 s; 20 s at 72°C; 5 s at 75°C to 82°C with a single fluorescence measurement as shown in Table 1); (iv) melting curve programme (60–99°C with a heating rate of 0.1°C/s and a continuous measurement); (v) cooling programme down to 40°C.

Crossing Point (CP) values were acquired by using the 'second derivate maximum method' of the LightCycler Software 3.5 (Roche Diagnostics). Relative mRNA expression levels are given by the arithmetic formula $2^{-\Delta\Delta CP}$ where the CP of the target gene is normalized to the housekeeping gene GAPDH (ΔCP) (Livak and Schmittgen, 2001). In Fig. 3 the cytokine mRNA expressions are presented as $\Delta\Delta$CP increase calculated in relation to time point zero and compared with unstimulated cells, normalized within any single time point. To show high expression differences between WBC and SC the LF and CD14 mRNA expressions graphs (Fig. 4) were only normalized against the GAPDH mRNA expression (ΔCP).

Statistical evaluation

Two-way ANOVA with length of stimulation as the first factor (0, 1, 2, 3, 4 and 8 h) and cell type (WBC versus SC/monocytes versus macrophages) was applied on normalized mRNA expression results ($\Delta\Delta$CP or ΔCP). Normal distribution was tested and given within the data sets. All statistical analyses were performed by using Sigma Stat 3.0 (SPSS Inc., Chicago, IL, USA).

Results

Efficiency of immunoseparation and viability of the cells

The viability of the freshly separated cells, determined by trypan blue staining ranged from 90% to 98%. As shown by

Table 1. Primers used for one-step RT-PCR

Gene	Primer	Sequence 5′ → 3′	Accession no.	Annealing temperature (°C)	Fluorescence measurement (°C)
IL-1β	Sense	TTCTCTCCAGCCAACCTTCATT	M37211	58	77
	Antisense	ATCTGCAGCTGGATGTTTCCAT			
IL-6	Sense	GCTGAATCTTCCAAAAATGGAGG	NM173923	58	75
	Antisense	GCTTCAGGATCTGGATCAGTG			
TNFα	Sense	TAACAAGCCGGTAGCCCACG	AF011926	60	82
	Antisense	GCAAGGGCTCTTGATGGCAGA			
LF	Sense	ATTTAGCCACAGCTCCCTGGAG	AB046664	60	82
	Antisense	GGCCTTTGCCTTGGAATGTATC			
GAPDH	Sense	GTCTTCACTACCATGGAGAAGG	U85042	60	80
	Antisense	TCATGGATGACCTTGGCCAG			
CD14	Sense	CACCCTAGACCTGTCTGACAA	D84509	59	87
	Antisense	GAGATCAAGCACGCTGAGC			

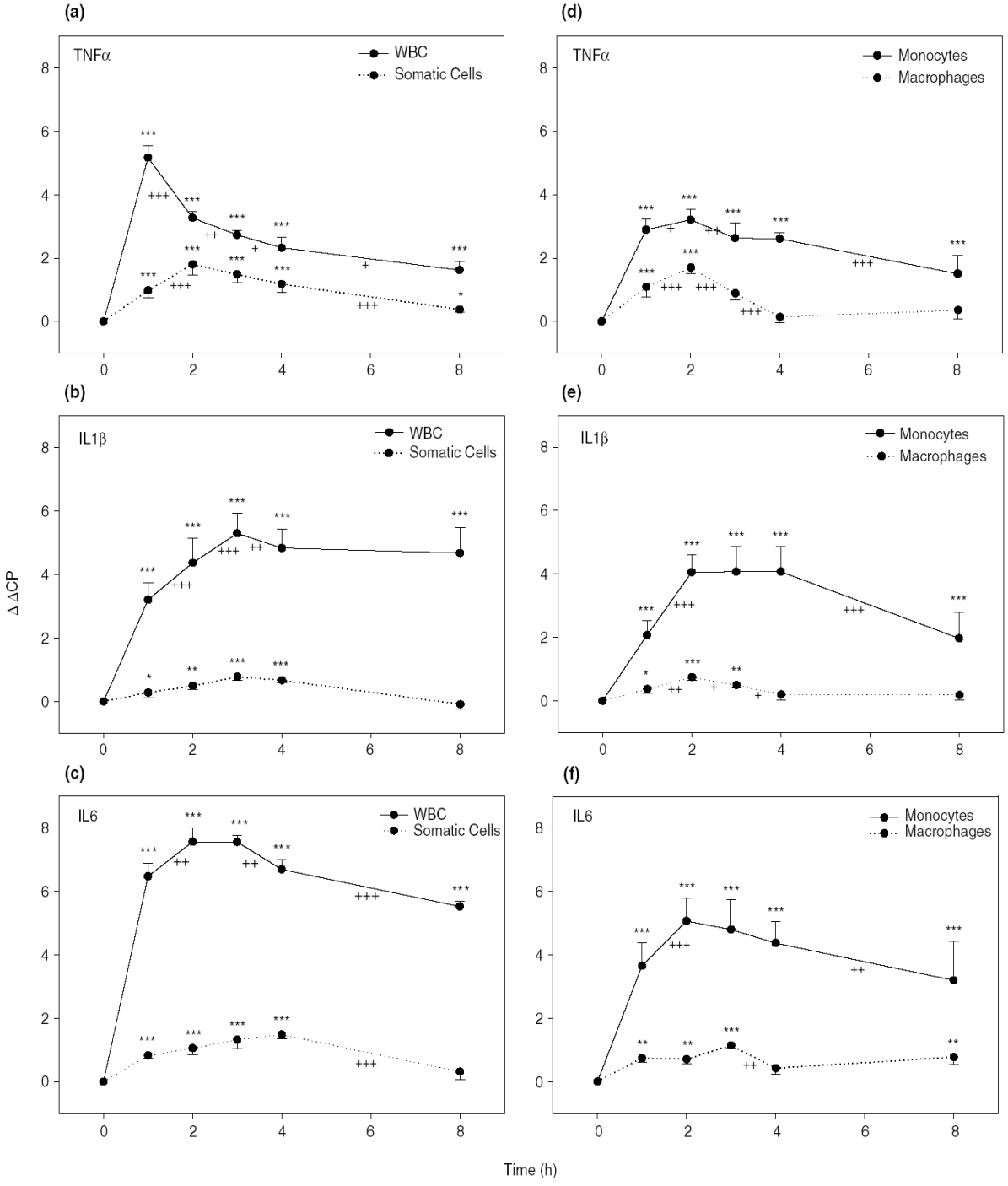

Fig. 3. Kinetics of cytokine mRNA expression in LPS (10 μg/ml) induced WBC and SC (a, b, c) or monocytes and macrophages (d, e, f). The TNFα (a, d), IL-1β (b, e) and IL-6 (c, f) mRNA expression was assessed by real time one-step RT-PCR using GAPDH as house keeping gene. The mean ± SEM from three separate experiments are indicated ($n = 6$). The cytokine mRNA expressions are presented as ΔΔCP increase calculated in relation to time point zero and compared with unstimulated cells (asterisk), normalized within any single time point (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Significant changes between the single time points are shown by plus (+$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$).

Pappenheim staining, the separated monocytes and macrophages prepared with biomagnetic beads were nearly homogeneous with very little contamination by neutrophils or lymphocytes (4–8% contaminating cells), as determined by a leucocyte differential count. The viability of the cells after immunoseparation ranged from 90% to 97%. Even after 8 h incubation with LPS the viability was reduced only down to 88%, compared with time point zero.

Composition of SC populations

Results showed a distribution of lymphocytes, macrophages and neutrophils of 8.2%, 19.8% and 72%.

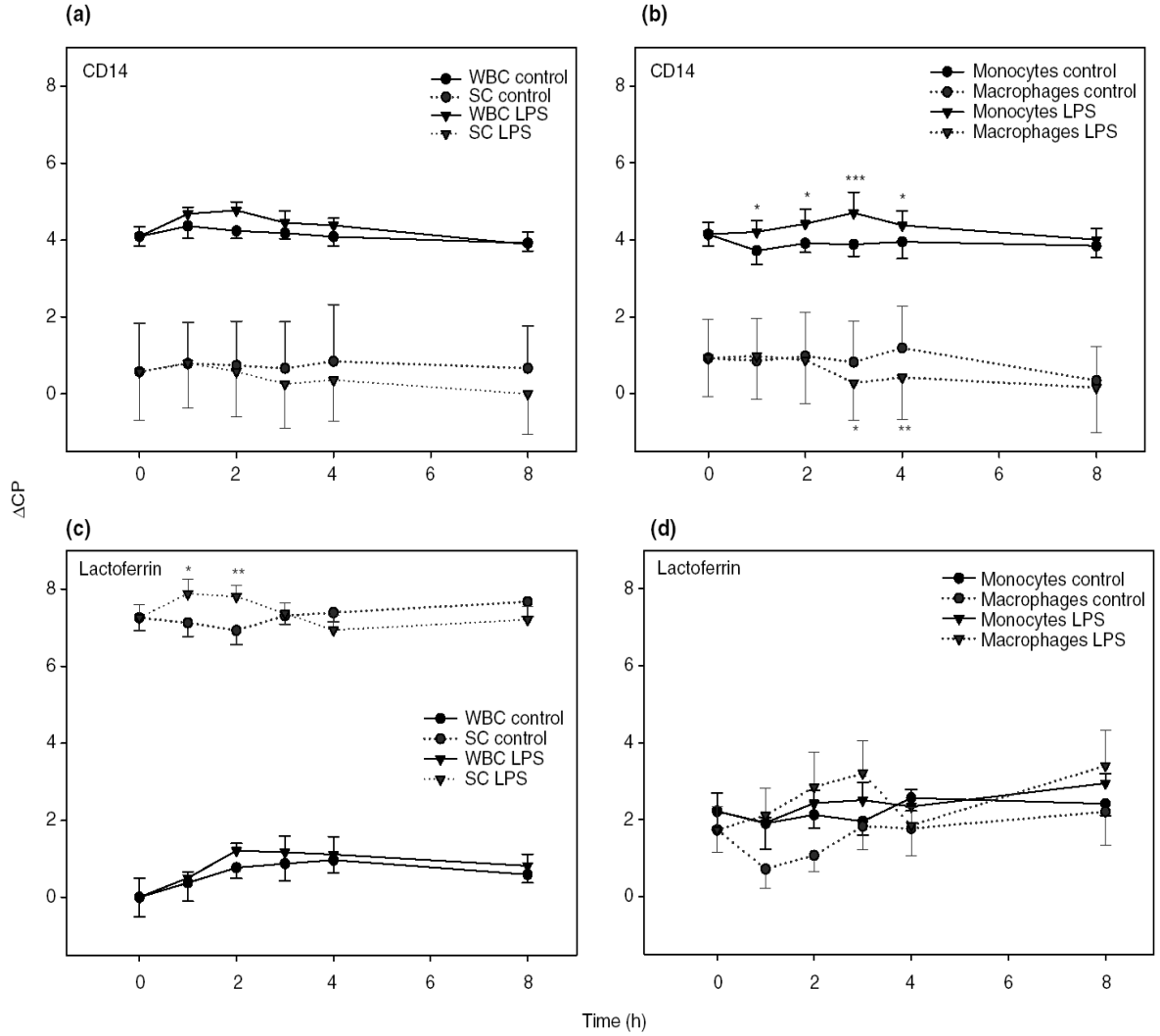

Fig. 4. Kinetics of CD14 and lactoferrin mRNA expression in LPS (10 μg/ml)-induced WBC and SC (a, c) or monocytes and macrophages (b, d). The CD14 (a, b), Lactoferrin (c, d) mRNA expression was assessed by real time one-step RT-PCR using GAPDH as housekeeping gene. To show high expression differences between WBC and SC the LF an CD14 mRNA expressions graphs were only normalized against the GAPDH mRNA expression (ΔCP).The mean ± SEM from three separate experiments are indicated ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Total RNA content and quality

The extractable total RNA was comparable in all samples, with a mean yield of 367 ng RNA/10^6 cells. Purity of the total RNA extracted was determined by optical density measurement at 260 nm and 280 nm. The 260:280 nm ratio reached values between 1.6 and 1.8.

Cytokine mRNA expression in LPS-treated WBC and SC

The WBC and SC spontaneously expressed TNFα, IL-1β and IL-6 mRNA in the absence of stimulation. Transcription levels of those cytokines were found to be similar in the untreated cells. The kinetics of cytokine mRNA expression during culture of LPS-stimulated WBC and SC are shown in Fig. 3a–c. The expression results from all four different types of cell culture from three independent cows were highly repeatable. In WBC and SC the gene expression for TNFα, IL-1β and IL-6 mRNA increased rapidly and the maximum was obtained after 1–3 h (TNFα > IL-6 > IL-1β). In WBC significant higher ($P < 0.001$) LPS responses and longer persistence could be found than in SC (IL-1β > IL-6 > TNFα). The IL-1β and the IL-6 mRNA in WBC continued to be expressed at a higher level to the end of culture period, whereas TNFα gene expression started to decrease progressively after 1 h treatment. Lower but consistent transcription for the different cytokines was obtained at 1–4 h in SC but declined to basal levels at 8 h following LPS challenge.

Cytokine mRNA expression in LPS-treated monocytes and macrophages

Spontaneous expression of TNFα, IL-1β and IL-6 by monocytes and macrophages was measured from unstimulated cultures. No differences in transcription level for those cytokines were measured from unstimulated cultures. LPS-induced cytokine mRNA expression patterns for those cells are shown in Fig. 3d–f. The kinetics of gene expression in LPS-stimulated monocytes and macrophages was found to be similar to that in the corresponding WBC and SC, but at lower

levels. In monocytes cytokine mRNA expression peaked at 2 h and diminished thereafter to approximately 20–30% of its maximal response at 8 h, whereas in macrophages significantly lower ($P < 0.001$) LPS responses could be measured with maximum accumulation after 2–3 h. The expression of TNFα and IL-1β mRNA in macrophages started to decrease rapidly to the basal levels at 2–4 h, whereas significant IL-6 gene expression was detectable until the end of culture period.

Expression of CD14 mRNA in LPS-treated monocytes, macrophages, WBC and SC

To get more insight into the mechanism responsible for the lower response of milk cells to LPS, the CD14 mRNA expression in monocytes, macrophages, WBC and SC was examined (Fig. 4a,b). Differences in transcription level of CD14 could be measured in LPS-treated cells and controls. Untreated WBC and monocytes showed, over the whole treatment period, significant sixfold to 15-fold higher ($P < 0.001$) levels of CD14 transcription than SC and macrophages.

In monocytes CD14 mRNA began to increase at 1 h, peaked at 3 h and began to fall progressively back to the basal level at 8 h following exposure to LPS. In contrast to monocytes, the CD14 gene expression was down-regulated in LPS-treated macrophages after 2 h with a negative peak at 4 h and a return to basal level to the end of the culture period. Similar kinetics could be measured in WBC and SC whereas the increase of CD14 gene expression in WBC and the down-regulation of CD14 mRNA expression in SC was not statistically significant.

Expression of LF mRNA in LPS-treated monocytes, macrophages, WBC and SC

The LF mRNA expression of LPS-treated cells and untreated controls are shown in Fig. 4c,d. Significantly higher ($P < 0.001$) mRNA transcription levels for LF (56-fold up to 153-fold) were observed during the whole cell culture period in SC compared with WBC, independent of LPS treatment. The effects of LPS treatment was a slight but significant increase of LF expression in SC at 1–2 h after induction. No significant changes for LF in response to LPS over the treated 8 h could be measured in monocytes or macrophages culture applications.

Discussion

Monocytes and macrophages isolated with biomagnetic beads were high in purity. As shown by Sarikaya et al. (2004), Pappenheim staining is a suitable method for the classification of blood and milk cells. The viability of the freshly separated monocytes and macrophages ranged from 90% to 97% and in addition, these cells showed a high response to subsequent LPS stimuli.

While macrophages are the predominant cell type found in the milk of healthy, i.e. non-infected, glands, the percentage of neutrophils increases dramatically in mammary secretions in response to inflammation and can constitute >90% of total SC (Lee et al., 1980; Sordillo et al., 1997). The present study shows that the distribution of SC population was dominated by neutrophils (72%). Therefore it can be assumed that the milk samples were taken from cows with infected udders.

In all four described cell culture types significant mRNA increases for the pro-inflammatory cytokines TNFα, IL-1β, IL-6 were found, with peaks after 1–3 h (TNFα > IL-6 > IL-1β). Our results are consistent with those of other investigators, showing that mRNAs of these cytokines were immediately transcribed after exposure to *E. coli* endotoxin. As described earlier in an *in vitro* experiment, the expression of TNFα, IL-1β or IL-8 mRNA by bovine monocyte-derived macrophages was detected as early as 1 h after LPS treatment and mRNA levels were increased over a entire 24-h period (Walter and Morck, 2002). Similarly, the exposure of bovine Kupffer cells to LPS led to an expression increase of TNFα, IL-1β or IL-6 mRNA between 3 and 12 h (Yoshioka et al., 1998).

Tumour necrosis factor α, a major mediator of early inflammatory reaction during per acute coliform mastitis, has been shown to be one of the first and initiator cytokines released by macrophages after exposure to LPS (Fong et al., 1989). As shown by Wittmann et al. (2002), highest mRNA expression of TNFα was found in SC of dairy cows with elevated quarter SCC (>150 000 cells/ml). In an *in vivo* study, TNFα concentration in whey from *E. coli* infected bovine glands increased dramatically from the 10th hour p.i., slightly before IL-1β and IL-8 (Riollet et al., 2000). Intramammary infusion of endotoxin in bovine led to an increase of TNFα and IL-1 concentration in milk between 2 and 4 h after LPS treatment (Shuster and Kehrli, 1995; Persson-Waller et al., 2003). In comparison with our findings these observations did not clearly illustrate any obvious differences in cytokine gene expression over time. Our data clearly demonstrate that mRNA levels of those cytokines were increasing simultaneously, suggesting that IL-1 and IL-6 gene expression was directly induced by LPS. The fact that TNFα is able to induce production of various IL (Fong et al., 1989) may partly explain the relative high expression levels of IL-1β and IL-6 gene in WBC to the end of culture period, whereas TNFα gene expression began to fall progressively after 1 h.

Results also indicate that LPS-induced mRNA expression of all analysed cytokines was significantly lower in SC and macrophages than in corresponding blood cells. Moreover a longer persistence of cytokine gene expression could be measured in WBC and monocytes. These findings are in agreement with two others studies, showing that bovine mammary macrophages stimulated by *E. coli* or *Staphylococcus aureus* secreted limited amounts of IL-1β in comparison with adequate blood monocytes (Politis et al., 1991, 1992). Hawkes et al. (2002) have reported increased cytokine production by approximately 40–50% in human milk cells stimulated with LPS for 24 h, whereas peripheral blood mononuclear cells responded to LPS with TNFα, IL-1β and IL-6 production increased by 350%, 135% and 30% respectively. This may be caused by the blood-to-milk diapedesis, which utilizes energy reserves of PMN and monocytes. PMN found in milk have lost about 38% of the initial glycogen content after diapedesis into the mammary gland (Naidu and Newbould, 1973). Furthermore, mammary gland neutrophils and macrophages tend to be less functional than circulating leucocytes because of the indiscriminate ingestion of fat, casein and other milk components (Paape et al., 1979). They are less effective at phagocytosis than are WBC, especially during the periparturient period the phagocytic and bactericidal activities

of the SC are diminished (Weber et al., 1983; Mehrzad et al., 2002).

Recognition of LPS by the innate immune system is critical for a successful immune response. Differentiation of monocytes into macrophages is accompanied by a change in membrane CD14 numbers. Percentages of bovine large mononuclear cells isolated from bovine blood and normal milk expressing mCD14 averaged 63% and 35% (Paape et al., 1996). Release of soluble CD14 (sCD14) from mononuclear cells and neutrophils is induced by LPS and has been shown to be elevated in bovine milk during intramammary infection induced by *E. coli* (Lee et al., 2003a). Soluble CD14 is known to bind LPS and inhibit its interaction with mCD14 and has been shown to prevent mice against LPS-induced septic shock and cytokine release (Haziot et al., 1994, 1995; Lee et al., 2003b).

In this study, transcription of CD14 mRNA was found to be significantly lower in untreated SC and macrophages compared with WBC and monocytes. As CD14 mRNA levels of the monocytes and macrophages were similar to corresponding WBC and SC, the mononuclear cells appear to be the major source of CD14 mRNA expression. The results of this investigation show a significant change in the pattern of CD14 mRNA expression in monocytes and macrophages after *in vitro* incubation with LPS. In monocytes CD14 mRNA was found to be slightly up-regulated, whereas transcriptional levels for CD14 was down-regulated in LPS-treated macrophages. This is consistent with a previous study showing that incubation of rabbit alveolar macrophages for 24 h with LPS and *E. coli* caused a significant decrease in mCD14 expression (Lin et al., 2004). In contrast, Landmann et al. (1996) reported a weakly reduction in CD14 transcription and mCD14 after 6–15 h of LPS incubation in human monocytes as well as in macrophages, and a twofold increases of CD14 RNA and mCD14 after a 2-day incubation.

The discrepancy of constitutive CD14 gene expression among milk and blood cells may be part of an endotoxin tolerance of milk macrophages, caused through previous stimulation of SC with *E. coli* endotoxin in the mastitis-infected udders. Furthermore sCD14 may have inhibited activation effects of LPS in milk cells by preventing its interaction with mCD14. This kind of down-regulation of LPS receptors at the surface of immune cells could be important for prevention of an overwhelming release of cytokines like TNFα, IL-1β and IL-6 and thus preventing development of septic shock (Dobrovolskaia and Vogel, 2002).

Another potential mechanism for the lower response of milk cells to bacterial endotoxins could be the inhibitory activity of LF on the cytokine production in monocytic cells. LF is found in exocrine secretions of mammals, synthesized by epithelial cells of mammary gland and is a major component of the secondary granules of PMNs (Masson et al., 1969). Mammary tissue was previously identified as the major source of LF mRNA (Pfaffl et al., 2003). A rise of LF mRNA expression was detected in mammary biopsy samples of quarters intramammary infused with LPS (Schmitz et al., 2004). The present study shows strongly up-regulated mRNA expression levels for LF in SC compared with monocytes, macrophages and WBC. These findings are independent of LPS treatment and culture period, suggesting that transcriptional activity of LF in the SC was similar in the infected mammary glands before cell culture. Our results are in agreement with other studies, showing that LF is produced and excreted by stimulated PMN during inflammation (Wang et al., 1995; Baveye et al., 1999).

Lactoferrin has been shown to bind human monocytes cell lines and blood monocytes via receptors and to be taken up intracellularly (Birgens et al., 1983; Britigan et al., 1991). Others demonstrated that LF was transported to the nucleus following uptake by mononuclear cells. They also showed that LPS-induced binding of transcription factor NF-κB to the TNFα promoter was decreased in the presence of LF, suggesting that LF affects intracellular events leading to NF-κB translocation to the nucleus (Haversen et al., 2002). The reduced up-regulation of inflammatory cytokines in milk macrophages cells in response to LPS was probably initiated by the anti-inflammatory properties of LF following release from neutrophils.

In conclusion, the present study demonstrates a time-dependent effect of the cytokine mRNA expression in immunoseparated and LPS-treated milk and blood cells. We also show a lower response of SC and macrophages to bacterial endotoxins than corresponding blood cells. The strength of the immune response in the blood system is much more prominent than in the mammary gland. This may be ascribed to the role of CD14 on the cytokine production of the investigated cells, or may be caused by the blood-to-milk diapedesis.

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Appendix 2

Journal of the Science of Food and Agriculture

J Sci Food Agric (in press)
DOI: 10.1002/jsfa.2377

Influence of bovine lactoferrin and lactoferricin on cytokine expression in LPS-treated cultivated bovine blood cells

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Abstract: Lactoferrin (LF), an iron-binding glycoprotein, and a bactericidal pepsin-derived fragment of LF, lactoferricin (LFcin), are involved in host defense mechanisms via bacteriostatic activity and immunoregulatory properties. The aim of this study was to investigate the effects of bovine LF and LFcin on the synthesis of immunologically important factors in leukocytes [white blood cells (WBC)] and monocytes. Monocytes were isolated from bovine blood using antibody-coated magnetic beads and cultured in parallel to WBC. Cells were treated with LF or LFcin and with *Escherichia coli* lipopolysaccharides (LPS). Various pro-inflammatory [tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-6] and anti-inflammatory (IL-10) cytokine mRNA expression responses were quantified via a one-step real-time reverse transcriptase polymerase chain reaction (RT-PCR). Furthermore, the gene expression for TNFα and IL-10 and the transcriptional activity of nuclear transcription factor kappa B (NF-κB) were measured after 0, 1, 2, 4 and 8 h. LF, LFcin and LPS strongly up-regulated the mRNA expression of all investigated cytokines, with peaks after 1 or 2 h for TNFα and IL-10. The magnitude of increase varied from modest (6-fold) to dramatic (64-fold). In contrast to LF and LPS treatment, LFcin induced a slight increase in TNFα mRNA in both cell culture types, which continued to be expressed at a higher level after 8 h. Compared with LPS-stimulated cells a combination of LF and LPS did not alter the TNFα and IL-10 expression. Simultaneous treatment of LFcin and LPS could increase the TNFα mRNA production, in contrast to LPS treatment alone. The effects of all agents used on cytokine mRNA expression were dose dependent. The NF-κB gene expression in monocytes and leukocytes was not affected by the treatment with LF and LFcin. The ability of LF and LFcin to bind free LPS and to interfere with the LBP/CD14 pathway has been suggested to restrain the activity of this bacterial immuno-stimulatory compound. These studies could not reveal these neutralizing effects because LF and LFcin showed no inhibitory activities on the cytokine production at several LPS concentrations.

Keywords: lactoferrin; lactoferricin; white blood cells; monocytes; lipopolysaccharides; cytokine; real-time reverse transcriptase polymerase chain reaction; mRNA expression

INTRODUCTION

Lactoferrin (LF) is a bovine 80 kDa single-chain, iron-binding glycoprotein.[1] It is present at high concentrations in bovine milk and other exocrine secretions and it is a major component of the secondary granules of polymorphonuclear leukocytes.[2] The biological activities of LF are iron-binding functions as well as non-iron related properties. LF plays a role in host defense mechanisms via its antibacterial, antiviral and antifungal activities and the ability to modulate inflammatory processes.[3,4] Bovine lactoferricin (LFcin) is a 25-amino acid peptide fragment of the intact LF. It is released from the N-terminal region via proteolytic cleavage by pepsin and is described in the literature as having a more potent bactericidal and bacteriostatic activity than the intact LF.[5,6] It appears that the LFcin-containing N-terminal region of LF interacts with certain cell receptors and LPS.[7,8]

LF is known to bind to the lipid A moiety of lipopolysaccharides (LPS) with high affinity. Identical results were reported for bovine and human LFcin.[7,9] LPS, a bacterial endotoxin from the outer membrane of Gram-negative bacteria cell walls, has a high potency to stimulate inflammatory processes.[10,11] Mammals process a cluster of differentiation 14 (CD14) antigen-dependent pathways to detect LPS.[12] The LPS-binding-ability of LF and LFcin has been suggested to inhibit the production of the pro-inflammatory cytokines tumor necrosis factor α

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Contract/grant sponsor: Milei
Contract/grant sponsor: Morinaga Milk Industry
(*Received 17 March 2005; revised version received 15 June 2005; accepted 3 July 2005*)

(TNFα), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) by interfering with the lipopolysaccharide-binding protein (LBP)/CD14 pathway.[13,14] In the mammary gland, the mentioned cytokines are produced by macrophages and epithelial cells in response to invading microorganisms or their components such as LPS.[15–17]

LF has been shown to bind human monocyte cell lines and blood monocytes via receptors and to be taken up intracellularly.[18,19] Haversen *et al.* demonstrated that LF was transported to the nucleus following uptake by mononuclear cells.[20] They also showed that LF down-regulates the LPS-induced cytokine expression (TNFα, IL-1β, IL-6 and IL-10) in two specific monocytic cell lines via the nuclear transcription factor kappa B (NF-κB).[20] Another study pointed out that LF induced TNFα secretion in murine macrophage-like cells and rat macrophages.[21]

The transcription of pro-inflammatory cytokines TNFα, IL-1β and IL-6 mRNA is controlled by NF-κB which, is located in the cytoplasm of most cells. NF-κB is normally found as an inactive heterodimeric complex with the inhibitory protein I-κB. In response to external pro-inflammatory stimuli, a signaling cascade is activated, leading to phosphorylation and degradation of I-κB and translocation of NF-κB to the nucleus, where it initiates gene transcription by binding to DNA.[22,23]

One of the key mediators of inflammatory response is TNFα, one of the first cytokines released by mononuclear cells after exposure to LPS.[24] It induces signaling cascades, leading to necrosis or apoptosis, and plays an important role in resistance to infections and cancers.[25] IL-1β initiates a very efficient and self-amplifying cytokine network that can attract inflammatory cells such as polymorphonuclear neutrophils (PMN). Furthermore, IL-1β is able to activate recruited cells to exert their defense functions and thus enhances the immune response.[26] IL-6 induces the acute-phase reaction and is essential for differentiation and proliferation of B- and T-cells.[27]

IL-10 is known to be an anti-inflammatory cytokine, capable of inhibiting the production of TNFα and IL-1β in activated monocytes/macrophages and plays a role in the development of LPS tolerance.[28–30] It has been reported that IL-10 can inhibit cytokine and chemokine expression by transcriptional suppression via modulation of the NF-κB pathway or by increasing the rate of mRNA decay.[31]

In the present study, one-step real-time reverse transcriptase polymerase chain reaction (RT-PCR) technology was used to show the influence of LF and bovine LFcin on LPS-treated cells. Effects on the mRNA cytokine expression (TNFα, IL-1β, IL-6, IL-10) and NF-κB in cultivated bovine leukocytes and in immuno-magnetically isolated monocytes were quantified. Furthermore, it was investigated whether the formation of LF–and LFcin–LPS complexes may have an effect on cytokine mRNA expression in these cells.

EXPERIMENTAL

Cell samples

To extract the leukocytes, 150 mL of blood from a jugular vein of brown Swiss dairy cows (coagulation prevention with 1 mL of EDTA per 50 mL of total blood) was diluted 1:1 (v/v) with lysis buffer (830 mg NH_4Cl, 3.7 mg Na-EDTA, 100 mg KCl, in 100 mL of H_2O at pH 7.4) and centrifuged for 10 min in 50 mL reaction tubes at 220 $\times$ *g*. The cell pellet was resuspended twice in lysis buffer and centrifugation was repeated. Supernatants of all samples were discarded. Blood cell pellets were resuspended in cell culture media. White blood cells (WBC) were adjusted to 2×10^7 cells mL^{-1} for immuno-magnetic cell separation or 4×10^6 cells mL^{-1} for cell culture.

Immuno-magnetic cell separation of mononuclear cells

Immuno-magnetic beads (Dynabeads M-450; Dynal, Lake Success, NY, USA) coated with anti-mouse immunoglobulin G (IgG) were washed with PBS–0.1% bovine serum albumin (BSA) (pH 7.4). Antibody BAQ151A (5 μg per 10^7 beads) (VMRD, Pullman, WA, USA) was added to the immuno-magnetic beads and gently rotated for 45 min at room temperature. To remove unbound antibodies, the suspension was washed three times with PBS–0.1% BSA using a magnet (Dynal MPC-L magnet) which attached the beads to the tube wall. The WBC suspension (2×10^7 cells mL^{-1}) was incubated with BAQ151A-coated biomagnetic beads (4×10^7 beads mL^{-1} cell sample) for 30 min at 4 °C with gentle rotation. The supernatant was removed gently with a pipette while the mononuclear cells were attached at the tube wall using the magnet. The extracted monocytes were washed with PBS–0.1% BSA three times and finally resuspended at a concentration of 4×10^6 cells mL^{-1} in RPMI-1640 (Sigma-Aldrich Chemie, Munich, Germany) supplemented with 10% (v/v) FBS (Gibco-BRL Life Technologies, Gaithersburg, MD, USA) and 50 mg mL^{-1} gentamicin (Selectavet, Weyarn-Holzolling, Germany). Viability of the separated cells was detected by trypan blue staining.

Cell assay

All isolated cells (bovine leukocytes or immuno-magnetically separated monocytes) were incubated in quadruplicate in RPMI-1640 medium with 10% FBS–0.1% gentamicin at a cell density of 4×10^6 mL^{-1} and 250 μL per well were added to 96-well plates. After a culture period of 24 h at 37 °C in an atmosphere of 5% CO_2, the cells were treated with LPS (0–10 μg mL^{-1}, *Escherichia coli* O26:B6; Sigma Chemical, Seelze, Germany), bovine LF (0–2500 μg mL^{-1}; Milei, Leutkirch, Germany), bovine LFcin (0–500 μg mL^{-1}; Morinaga Milk Industry, Zama, Kanagawa, Japan) or combinations of these agents (3 μg mL^{-1} LPS + 200 μg mL^{-1} LF or 3 μg mL^{-1} LPS + 500 μg mL^{-1} LFcin). Concentration

test series were incubated for another 2 h. Time test series (3 μg mL^{-1} LPS; 200 μg mL^{-1} LF; 500 μg mL^{-1} LFcin) were incubated for increasing lengths of time (0, 1, 2, 4, 8 h). After stimulation, cells were stored in 700 μL of lysis buffers (Macherey–Nagel, Düren, Germany) at −80 °C. Total RNA was extracted using a NucleoSpin® RNA II kit (Macherey–Nagel) and resuspended in 100 μL of diethyl pyrocarbonate (DEPC; Sigma-Aldrich)-treated H_2O. To determine the total RNA concentration, all spectrophotometric measurements were made using a UV-transparent UVette (Eppendorf, Hamburg, Germany). The purity of the extracted total RNA was determined by optical density (OD) measurement at 260 and 280 nm.

One-step real-time RT-PCR

One-step real-time RT-PCR was performed using QuantTect SYBR Green RT-PCR (Qiagen, Hilden, Germany) by a standard protocol in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). The real-time RT-PCR master-mix contained 5 μL of 2 × QuantiTect SYBR Green RT-PCR master-mix, 0.5 μL of forward primer (20 μmol L^{-1}), 0.5 μL of reverse primer (20 μmol L^{-1}) and 0.1 μL of QuantiTect RT Mix. The primers used (bovine specific for IL-1β, IL-6, IL-10, TNF-α, NF-κ-B and housekeeping genes GAPDH and β-actin) were synthesized commercially (MWG Biotech, Ebersberg, Germany). A 6.1 μL volume of master-mix was added to a 3.9 μL total volume of RNA (5 ng μL^{-1}) into Rotor-Gene 100 μL rotor tubes. The tubes were closed and placed in the cycling rotor. The following cycling protocol was used: (1) reverse transcription, 20 min at 50 °C; (2) denaturation program, 15 min at 94 °C; (3) 40 cycles of amplification and quantification, 20 s at 94 °C; each of optimal annealing temperature 59 or 60 °C for 20 s (Table 1); elongation for 20 s at 72 °C; factor-dependent fluorescence measurement at elevated temperatures, 5 s at 77–82 °C (Table 1) with a single measurement; (4) melting curve program from 60 to 99 °C with a heating rate of 0.1 °C s^{-1} and continuous measurements.

Crossing point (CP) values were achieved using the Rotor-Gene software version 5.0 (Corbett Research). Relative mRNA levels were calculated by normalization of the CP (= ΔCP) of the target gene to the arithmetic mean of the CP of the two housekeeping genes GAPDH and β-actin.[32] In some cases the cytokine mRNA expression is presented as ΔΔCP increase evaluated in relation to each single time point and compared with unstimulated control cells.

Statistics

Analysis was done by two-way analysis of variance (ANOVA) and the Student–Newman–Keuls method using treatment (LPS, LF, LFcin, LPS + LF and LPS + LFcin) as the first and length of stimulation (0, 1, 2, 4 and 8 h) as the second factor. It was applied to normalized expression results (ΔCP or ΔΔCP). All statistical analysis was performed with Sigma Stat 3.0 (SPSS, Chicago, IL, USA).

RESULTS

Efficiency of immuno-separation and viability of the cells

The viability of the freshly separated cells, determined by trypan blue staining, ranged between 88 and 96%. As shown by Pappenheim staining, the separated monocytes and macrophages prepared with biomagnetic beads were nearly homogeneous, with very little contamination by neutrophils or lymphocytes. Only 4–8% of contaminating leukocytes were determined by a leukocyte differential count, as described earlier.[33] The viability of the cells after immuno-separation ranged between 89 and 95%. Even after 8 h of incubation with LPS the viability was only reduced to 86%, compared with time point zero or untreated controls.

Total RNA content and quality

The total RNA content was not affected by treatment with different concentrations of LF, LFcin or LPS. The purity of the total RNA extracted was determined

Table 1. Primers used for one-step RT-PCR

Gene	Primer	Sequence 5′ → 3′	Accession No.	Annealing temperature (°C)	Fluorescence measurement (°C)
TNF-α	Sense	TAACAAGCCGGTAGCCCACG	AF011926	59	82
	Antisense	GCAAGGGCTCTTGATGGCAGA			
IL-1β	Sense	TTCTCTCCAGCCAACCTTCATT	M37211	60	77
	Antisense	ATCTGCAGCTGGATGTTTCCAT			
IL-6	Sense	GCTGAATCTTCCAAAAATGGAGG	NM173923	60	77
	Antisense	GCTTCAGGATCTGGATCAGTG			
IL-10	Sense	ACTTTAAGGGTTACCTGGGTTG	U00799	59	82
	Antisense	CTTCTCCACCGCCTTGCTCTT			
NF-κB	Sense	GCTGGACCCAAGGACATG	NM003998	59	80
	Antisense	TGGTCTGCTGCAGAGCTG			
GAPDH	Sense	GTCTTCACTACCATGGAGAAGG	U85042	59	80
	Antisense	TCATGGATGACCTTGGCCAG			
β-Actin	Sense	AACTCCATCATGAAGTGTGAC	AY141970	59	82
	Antisense	GATCCACATCTGCTGGAAGG			

at an OD 260/280 nm ratio with expected values between 1.6 and 1.8. The RNA quality did not differ between the applied treatments (data not shown).

Cytokine mRNA expression in leukocytes and monocytes

Housekeeping genes

Changes of GAPDH and β-actin mRNA expression were marginal and did not approach statistical significance (data not shown).

Determination of efficient LF concentration

The kinetics of cytokine expression during culture of WBC stimulated with LF are shown in Fig. 1(a). LF effected an up-regulation of the gene expression for all analyzed cytokines in leukocytes. The cytokine mRNA expression in these cells increased rapidly up to an LF concentration of 200 $\mu g\,mL^{-1}$ and passed into a plateau with a maximum at 1000 $\mu g\,mL^{-1}$ LF. WBC stimulated with a combination of LF and LPS (10 $\mu g\,mL^{-1}$) showed no significant changes of cytokine mRNA expression compared with LF-treated cells (data not shown). Previously reported LF concentrations in normal milk during lactation were 250–404 $\mu g\,mL^{-1}$.[34] Therefore, we used a concentration of 200 $\mu g\,mL^{-1}$ LF in all further experiments.

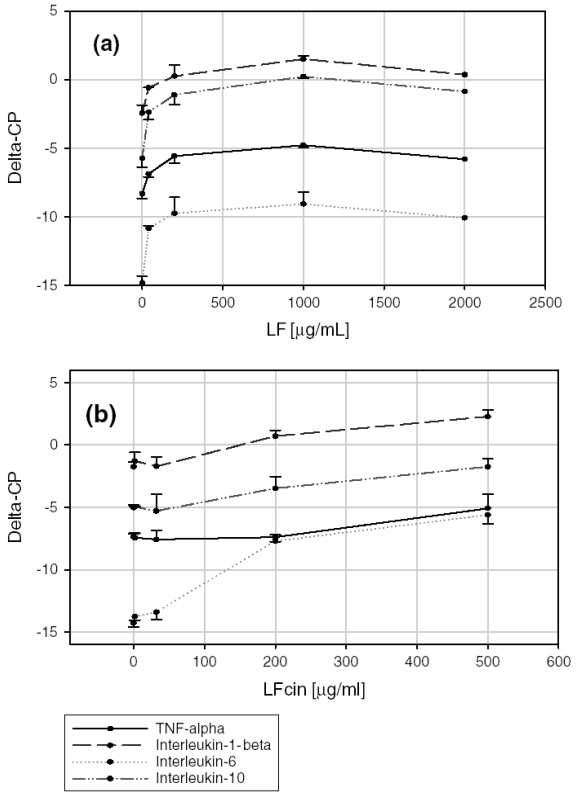

Figure 1. Cytokine mRNA expression in bovine leukocytes treated with LF (a) and with LFcin (b) after 2 h. Data are presented as means ± standard deviation ($n = 4$).

Determination of efficient LFcin concentration

LFcin-induced cytokine mRNA expression for WBC is shown in Fig. 1(b). Transcription levels of those cytokines were found to be similar in all analyzed cytokines. Only IL-6 mRNA expression showed a stronger increase. Also, a combined LFcin–LPS treatment effected no further stimulation of the cytokines except IL-6 with a strong increase and TNFα with a minimal increase after 500 $\mu g\,mL^{-1}$ LFcin treatment (data not shown). The LFcin concentration used of 500 $\mu g\,mL^{-1}$ for all further experiments is proportionally high in comparison with the LF concentration, but the concentration test series showed that the plateau was not reached as it was in the same LF test series. Therefore, it was decided to treat the cells with 500 $\mu g\,mL^{-1}$ LFcin. This represents around 75 times more LFcin molecules per treatment and well in contrast to LFcin molecules located in the N-terminal region of LF.

Determination of efficient LPS concentration

Figure 2 shows the efficient LPS concentration concerning the TNFα expression in LPS- or LPS + LF-treated WBC. The maximum TNFα mRNA expression was almost achieved with the application of 3 $\mu g\,mL^{-1}$ LPS. Higher concentrations did not increase the transcription level significantly. To show the binding abilities of LF and LFcin (data for LFcin not shown) to LPS, variations among treatment A (LF or LFcin was mixed for 30 min with LPS before addition to the cell culture) and treatment B (LF or LFcin was added to the cell culture 30 min after LPS treatment) were analyzed. The experiments could not demonstrate these effects because LF and LFcin stimulated the cells in the same manner as LPS. The LPS dose–response curve in the present study shows that 3 $\mu g\,mL^{-1}$ LPS was easily sufficient to activate bovine leukocytes adequately. Therefore, 3 $\mu g\,mL^{-1}$ LPS was used in subsequent experiments.

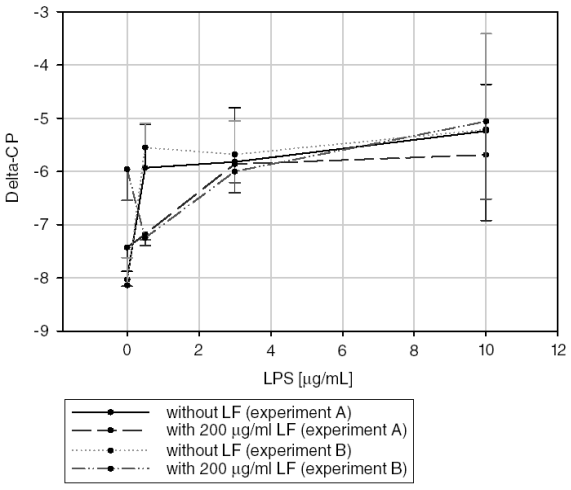

Figure 2. TNFα mRNA expression in bovine leukocytes treated with LPS or LPS + LF. Data are presented as means ± standard deviation ($n = 4$).

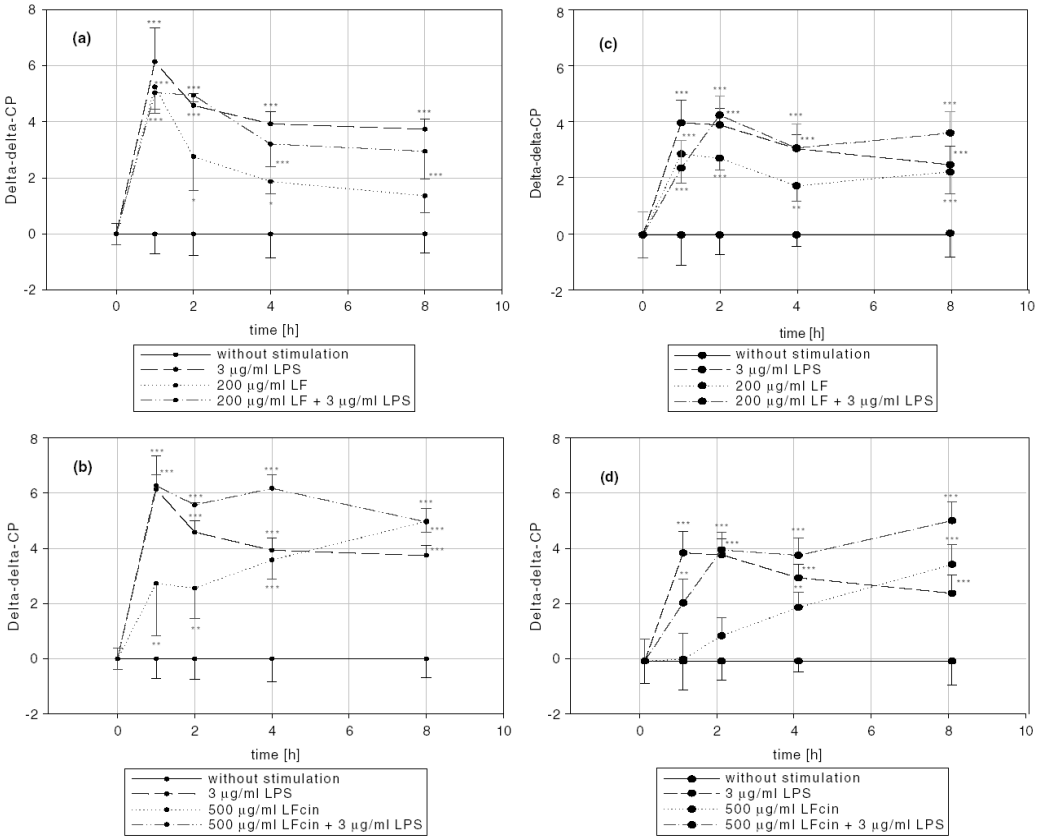

Figure 3. Time-dependent TNFα mRNA expression in (a) and (b) bovine WBC and (c) and (d) bovine monocytes treated with LPS, LF, LFcin, LPS + LF and LPS + LFcin. Data are presented as means ± standard deviation ($n = 4$). The cytokine mRNA expressions are presented as delta-delta-CP increase calculated in relation to time point zero and compared to un-stimulated cells (asterisk), normalised within any single time point [$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$].

Time-dependent cytokine mRNA expression

The kinetics of RNA expression for TNFα and IL-10 during culture of WBC and monocytes stimulated with LPS, LF, LFcin or combinations of these macromolecular molecules are shown in Figs 3 and 4.

The kinetics of TNFα mRNA expression in activated leukocytes [Fig. 3(a) and (b)] and in corresponding monocytes [Fig. 3(c) and (d)] were found to be similar. LPS-induced gene expression in leukocytes (30–60-fold up-regulation, $P < 0.001$) and in monocytes (6–15-fold up-regulation, $P < 0.001$) peaked at 1 h and diminished thereafter to a minimum at 8 h. Stimulation with LF showed a rapid increase in TNFα gene expression in both cell culture types with maximum accumulation after 1 h of treatment. The cytokine mRNA transcription level in WBC began to fall rapidly to nearly basal levels at 2–4 h following LF challenge, whereas in monocytes TNFα continued to be expressed at a higher level to the end of the culture period. LPS-treated WBC showed a longer persistence than LF-activated cells ($P < 0.001$). Combination of LF and LPS effected neither an additional stimulation nor suppression in leukocytes in comparison with LPS-treated WBC. In monocytes, significantly lower TNFα responses could be measured at 1 h when LF was added to LPS ($P < 0.001$).

In contrast to LF and LPS treatment, LFcin induced a lesser increase in cytokine mRNA in both cell culture types and continued to be expressed at a higher level to the end of the culture period with a maximum accumulation at 8 h. The kinetics of TNFα gene expression in LPS-stimulated leukocytes was found to be similar to those for LPS treatment in combination with LFcin. Addition of LFcin to LPS caused elevated TNFα mRNA transcription levels with an ~4-fold higher response at 4 h in WBC ($P < 0.001$). Furthermore, a significantly longer persistence was detectable in comparison with LPS-treated leukocytes. In response to a combined LFcin–LPS treatment, the TNFα gene expression level in monocytes increased rapidly until the end of the culture period and showed a significantly longer persistence than LPS-stimulated mononuclear cells ($P < 0.001$).

The IL-10 mRNA expression levels were found to be similar compared with the TNFα mRNA gene transcription level. LPS-induced gene expression peaked after 1 h in leukocytes and after 2 h in monocytes, with an average 15–60-fold up-regulation ($P < 0.001$). Subsequently a significant decrease in IL-10 transcription was found in both cell culture types. LF achieved a similar response in those cells. However, the LF-stimulated IL-10 mRNA expression

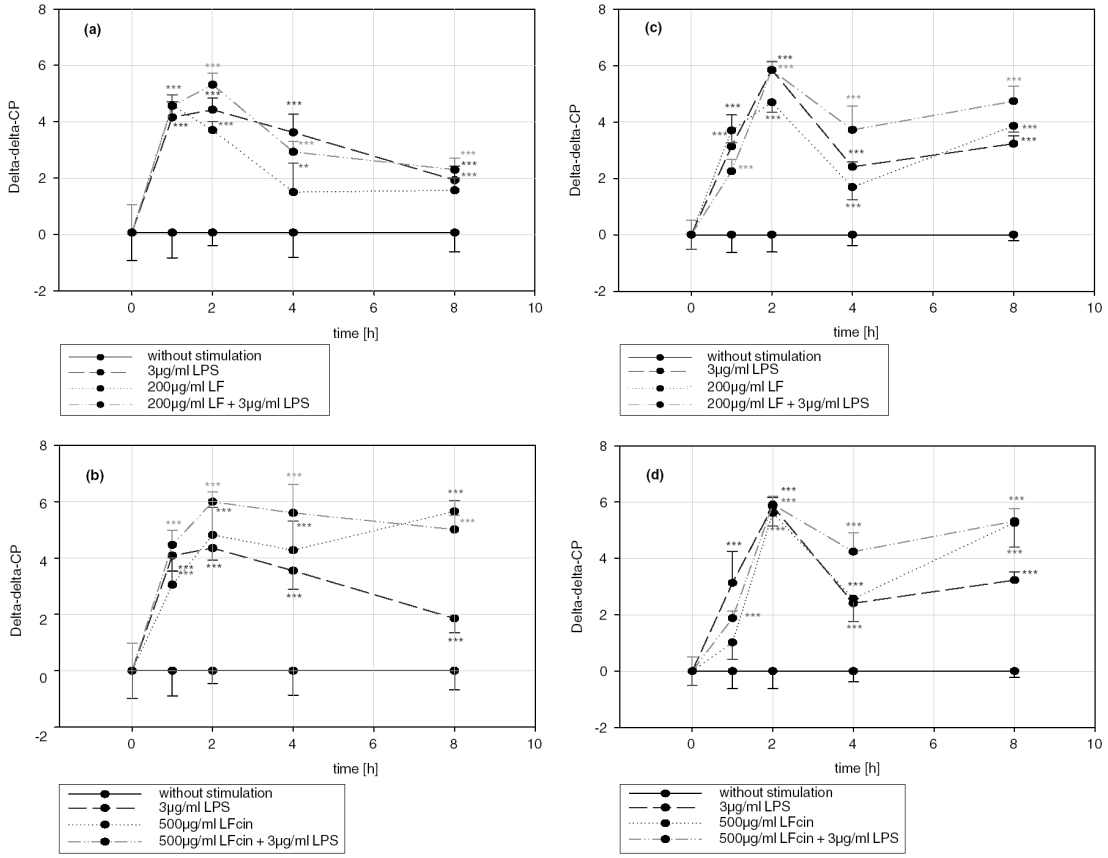

Figure 4. Time-dependent Interleukin-10 mRNA expression in (a) and (b) bovine WBC and (c) and (d) bovine monocytes treated with LPS, LF, LFcin, LPS + LF and LPS + LFcin. Data are presented as means ± standard deviation ($n = 4$). The cytokine mRNA expressions are presented as delta-delta-CP increase calculated in relation to time point zero and compared to un-stimulated cells (asterisk), normalised within any single time point [$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$].

in leukocytes decreased significantly faster than the LPS-induced gene expression, which continued to be expressed at a higher level to the end of the culture period ($P < 0.001$). In monocytes a first significant increase in IL-10 expression was found after 1 h of LF stimulation. Thereafter, it diminished rapidly after 4 h followed by a second significant increase back to the response level at 2 h. Combination of LF and LPS affected neither an additional stimulation nor suppression in leukocytes, whereas in monocytes significantly higher levels of IL-10 mRNA expression were observed after 4 h of treatment by the additive use of LF ($P < 0.001$).

IL-10 mRNA expression in LFcin-induced leukocytes increased rapidly until 2 h with a maximum accumulation after 8 h. A longer persistence than in LPS- and LF-treated WBC could be found. LFcin-induced mRNA expression patterns for monocytes were similar to the response curves for LF-stimulated monocytes. Again, a second increase was found after 4 h following a gene expression decrease after 2 h. The kinetics of gene expression in LPS-stimulated leukocytes and monocytes were found to be similar to those for combined LFcin–LPS treatment. Addition of LFcin to LPS led to an elevated IL-10 mRNA transcription level after 2 h of treatment and continued to be expressed at a significantly higher level towards the end of the culture period ($P < 0.001$).

NF-κB mRNA expressions in activated leukocytes and monocytes

All treatments with different concentrations of LF, LFcin and LPS did not result either in significant increases or in significant suppressions of NF-κB mRNA transcription levels in both cell culture types (data not shown).

DISCUSSION

The multifunctional protein LF has been shown to bind to a number of different molecules such as heparin, bacterial LPS, human lysozyme and DNA.[35] The 90-amino acid N-terminal domain is responsible for this function. This and the N-terminal half lobe as well as intact LF can initiate the IL-1β transcription in transfected K562 cells.[36] The present study shows that LF stimulates the mRNA expression of TNFα, IL-1β, IL-6 and IL-10 in bovine leukocytes and monocytes. LF showed a significant effect on cytokine gene expression beginning at a concentration of 40 µg mL^{-1}. The maximum response was reached at an LF concentration of 1000 µg mL^{-1}. Other studies demonstrate

that 10 μg mL^{-1} LF can activate the TNFα secretion in murine macrophage-like cells.[19] It is well known from the literature that LFcin has more potent bactericidal activity than LF. Bovine LFcin has been reported to inhibit the LPS-induced IL-6 response in monocytic THP-1 cells. In contrast, this study showed evidence for the immune-stimulating activity of LFcin. It was observed that 200 μg mL^{-1} LFcin (for IL-1β and IL-6) and 500 μg mL^{-1} LFcin (for IL-10 and TNFα) were necessary to achieve a significant increase in the mRNA levels of the mentioned cytokines.

A comparison of the two different approaches, LPS and LF/LFcin incubation 30 min before addition to cell culture versus treatment of cell cultures with LF/LFcin 30 min after LPS stimulation, showed no differences in gene expression pattern. Therefore, no influence on prior binding can be confirmed, as shown in earlier *in vitro* studies. It has been demonstrated that LF prevents LBP-mediated binding of LPS to mCD14.[37] Furthermore, LF was shown to decrease the secretion of IL-1β, IL-6 and TNFα from LPS-induced mononuclear cells.[13,14] The present results demonstrated that the LPS-binding abilities of LF and LFcin had no effect on the cytokine production at any LPS concentration. Both were able to stimulate cytokine expressions in bovine leukocytes and monocytes.

Additionally, we tested the time-dependent TNFα, IL-10 and NF-κB expression following LPS, LF, LFcin, LPS + LF and LPS + LFcin treatment in leukocytes and isolated monocytes. All cytokines are regulated via transcriptional, post-transcriptional and translational pathways. NF-κB controls the transcriptional activity of several cytokines (IL-1β, TNFα and IL-6),[22] In this study, TNFα, one of the early cytokines released by macrophages after LPS stimulation,[38] was shown to be immediately transcribed after 1 h of LPS treatment in both cell cultures, whereas induction of IL-10 transcription was slightly delayed. Although IL-10 is known as an anti-inflammatory cytokine, the kinetics of IL-10 gene expression were found to be similar in those cells in response to LPS.

The present data clearly demonstrate that LF increases the TNFα transcription level in leukocytes and monocytes. These results are in agreement with earlier findings[21] showing that LF-induced TNFα secretion in murine macrophage-like cells. They also demonstrated elevated TNFα mRNA level after 1.5 h in LF (10 μg mL^{-1})-treated cells. A decrease was observed after 6 h of incubation. The present studies gave similar results. It was found that TNFα gene expression decreases significantly after 2 h of LF stimulation in leukocytes. In contrast to LF, stimulation with LFcin showed a slower increase but a longer persistence of TNFα gene expression in both cell culture types. A reduction in TNFα expression was not observed with increasing time. The present study shows that a combined LF–LPS treatment has no serious effects on the expression of TNFα and IL-10 in comparison to LPS stimulated cells. These data suggest that NF-κB might be the limiting factor. Addition of LFcin to LPS led to elevated TNFα mRNA expression levels after 1 h of treatment with longer persistence in comparison with LPS treatment.

Yamaguchi *et al.*[39] demonstrated that an injection of LF 8 h before LPS administration to mice suppressed the LPS-induced TNFα production. This might induce some reactions leading to down-regulation of inflammatory cytokines. Others have also suggested that LF and LFcin act indirectly by stimulating inhibitors such as IL-10.[40] In comparison with these findings, our results did not show any anti-inflammatory activities of LF or LFcin, but showed significant mRNA increases for IL-10 in LF- and LFcin-treated cells. This suggests that IL-10 is up-regulated to inhibit the production of pro-inflammatory cytokines to a later point in time. It is known that IL-10 provides negative feedback control of inflammatory processes, strongly inhibits antigen-specific proliferation of T cells and decreases the production of pro-inflammatory cytokines such as IL-1 and TNFα.[41]

The present study showed no significant increase or decrease in the transcriptional factor NF-κB neither with or without stimulation. These findings may be caused by the inhibitory complex of NF-κB and its inhibitory protein, found in most cells. After degradation of this inhibitory protein, NF-κB is translocated to the nucleus, where it can function as a pro-inflammatory cytokine transcription factor.[22] This suggests that sufficient NF-κB is located in leukocytes over the entire culture period of 8 h, which results in constant NF-κB transcription levels.

CONCLUSIONS

This study has shown that LF and LFcin stimulate the cytokine mRNA expression of TNFα, IL-1β, IL-6 and IL-10 in bovine leukocytes and corresponding monocytes. It was also demonstrated that LF and LFcin have no influence on the NF-κB gene expression during a cell culture period of 8 h. Furthermore, a suppressive effect of LF and LFcin on the LPS-induced cytokine mRNA response in those cells could not be confirmed.

ACKNOWLEDGEMENTS

This study was supported by the Milei (Leutkirch, Germany), who provided the bovine LF, and Morinaga Milk Industry (Zama, Kanagawa, Japan), who provided LFcin.

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Appendix 3

Differentiation of leukocytes in bovine milk

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The somatic cell count (SCC) in milk is used as an indicator of udder health status. Elevated SCC are generally considered as an indication of mastitis. In addition, the differential cell count of milk somatic cells can be a useful tool in research because each cell type has its own specific function in the immune response.

The aim of this study was to develop and validate a staining method for milk somatic cells. Therefore the panoptic staining method according to Pappenheim was adapted for the differentiation of milk somatic cell populations. Quarter composite milk samples from 28 German Braunvieh x Brown Swiss cows were separated into 3 groups based on their SCC levels. Group 1 consisted of 12 samples with a mean of 4.57±0.10, group 2 of 8 samples at a mean of 5.39±0.06 and group 3 of 8 samples at a mean of 6.15±0.07 $\log_{10}$ cells/ml, respectively. Results showed a distribution of lymphocytes, macrophages and neutrophils of 20.9, 45.6 and 33.5% in group 1, of 11.4, 25.1 and 63.5% in group 2 and of 3.3, 9.5 and 87.2% in group 3, respectively. In conclusion, the ratio of lymphocytes and macrophages decreases whereas the ratio of neutrophils increases with rising SCC.

Differenzierung von Leukozyten in Kuhmilch

Die somatische Zellzahl der Milch wird als Indikator für den Eutergesundheitsstatus verwendet. Erhöhte Zellzahlen werden im Allgemeinen als Anzeichen für Mastitis betrachtet. Zusätzlich kann die Zusammensetzung der Populationen der somatischen Zellen der Milch eine weitergehende Information liefern, da jeder Zelltyp eine spezifische Funktion in der Immunantwort der Milchdrüse hat.

Ziel dieser Studie war die Entwicklung und Validierung einer Färbemethode für die somatischen Zellen der Milch. Daher wurde die panoptische Färbemethode nach Pappenheim für die Differenzierung von somatischen Zellpopulationen aus der Milch optimiert. Die Viertelgemelke von 28 Deutschen Braunvieh x Brown Swiss Kühen wurden hinsichtlich ihrer Zellzahlniveaus in 3 Gruppen eingeteilt. Gruppe 1 bestand aus 12 Proben mit einem Mittelwert von 4.57±0.10, Gruppe 2 aus 8 Proben mit einem Mittelwert von 5.39±0.06 und Gruppe 3 aus 8 Proben mit einem Mittelwert von 6.15±0.07 $\log_{10}$ Zellen/ml. Die Ergebnisse zeigten eine Verteilung von Lymphozyten, Makrophagen und Neutrophilen von 20.9, 45.6 bzw. 33.5% in Gruppe1, von 11.4, 25.1 bzw. 63.5% in Gruppe 2 und von 3.3, 9.5 bzw. 87.2% in Gruppe 3. Somit konnte gezeigt werden, dass der Anteil von Lymphozyten und Makrophagen mit steigender Zellzahl abnimmt wohingegen der Anteil der Neutrophilen zunimmt.

06 Somatic cells (differentiation in bovine milk) **06 Somatische Zellen** (Differenzierung in Kuhmilch)

1. Introduction

The mammary gland immune response to invading pathogens is predominantly based on cellular reactions mediated mainly via macrophages, neutrophils and lymphocytes (2, 12). While milk from healthy, i.e. noninfected glands is supposed to represent macrophages as the major cell fraction, the percentage of neutrophils increases dramatically in secretions from infected glands in response to inflammation (4, 12, 16). Neutrophils and macrophages are the functional phagocytes of the mammary gland (11) and phagocytic active in milk (6). Macrophages play a central role as alarming cells after the invasion of microorganisms, shown as increased expression of cytokines and inflammatory mediators (1, 17). The synthesis of chemoattractants initiates a rapid influx of neutrophils into the gland (3). Thus both cell populations together have microbicidal activity, whereby neutrophils dominate (10, 15). Specific immune response to bacteria is mediated by lymphocytes that recognize antigens through membrane receptors and produce antibodies against invading pathogens (14). This condition

is primarily intended to be used for vaccination to enhance the neutrophil recruitment in cases of mastitis by the localized antigen-specific lymphoid population (15).

The commonly used parameter for udder health is the somatic cell counts (SCC), however this parameter does not consider any changes of distribution of cell populations. An elevated value is being associated with mastitis. SCC is liable to fluctuations according to the stage and period of lactation even without any infection of the mammary gland (5, 8, 9). The somatic cells consist of lymphocytes, macrophages and neutrophils originating from the bloodstream and of epithelial cells from the tissue. Previous studies using electron microscopy for analysing the SCC show a predominant percentage of immune cells whereas epithelial cells were rarely found (6).

Besides the number of cells also the distribution of cell populations depend markedly on the physiological status of the mammary gland (2, 8, 9). The purpose of the present study was to establish and validate a staining method for differential somatic cell count in secretions to present a potential new technique for precise evaluation of immunological activity of the mammary gland.

2. Materials and methods

2.1 Animals and milking

Twenty-eight cows of the German Braunvieh x Brown Swiss breed were used. Nine animals were in their first, 9 in their second, 5 in their third and 5 in their fourth lactation. Eleven animals were in early (33–127 d), 8 in mid (139–216 d) and 9 in late lactational stage (228–322 d), respectively.

Milking was performed twice daily at 5.00 and 16.00. Quarter composite milk samples (QMS) were collected during morning milking and SCC was measured with Fossomatic™ (FOSS Analytical A/S, Hillerød, Denmark). QMS were classified according to the SCC level into 3 groups: SCC < 100,000/ml, SCC 100,000–400,000/ml and SCC > 400,000/ml. To verify stability of milk cells samples were processed within 2–3 h after milking.

2.2 Cell isolation and viability test

All QMS were gently mixed and 50 ml were centrifuged for 30 min and 1500 x g at 4 °C in conical tubes. The fat layer on top was removed, the supernatant (skim milk) was discarded and the cell pellet was resuspended in 5 ml of ice-cold phosphate buffered saline (PBS, pH 7.5). After refilling to the original volume the suspension was centrifuged for 15 min and 460 x g at 4 °C for washing. The received cell pellet was resuspended in PBS up to 5 ml according to the estimated size of the pellet. The viability of the cells was investigated by the exclusion method with the dye trypan blue (7). Thereby viable cells appear shining white under the microscope while dead cells are blue.

2.3 Panoptic cell staining

Cell differentiation was performed under the light microscope using a panoptic staining method according to Pappenheim, which has formerly been established for haematological analysis (13). Briefly, 25 µl of cell suspension was smeared gently on a slide in wavy lines. After air drying, slides were first stained for 3 min with undiluted and then for 1 min with 1:2 diluted May-Gruenwald solution (Sigma-Aldrich, Steinheim, Germany). As this solution contains methanol, no previous fixation of cells was needed. Slides were further stained for 14 min with a 3% Giemsa solution (Sigma-Aldrich, Steinheim, Germany). Afterwards slides were washed gently with Sörensen buffer (0.01 M, pH 7). To achieve durably stained cells the slides were covered after drying with Eukitt® (Plano GmbH, Wetzlar, Germany).

2.4 Differential cell counting

Two hundred cells were counted under a light microscope with x 1000-fold magnification using immersion oil. Photographs of the detected cell types are shown in Fig. 1. For evaluation cells were classified into lymphocytes, macrophages and neutrophils according to their morphology and diameter. Epithelial cells, eosinophils and basophils appeared in a neglectable number and were not considered for differential cell count calculation.

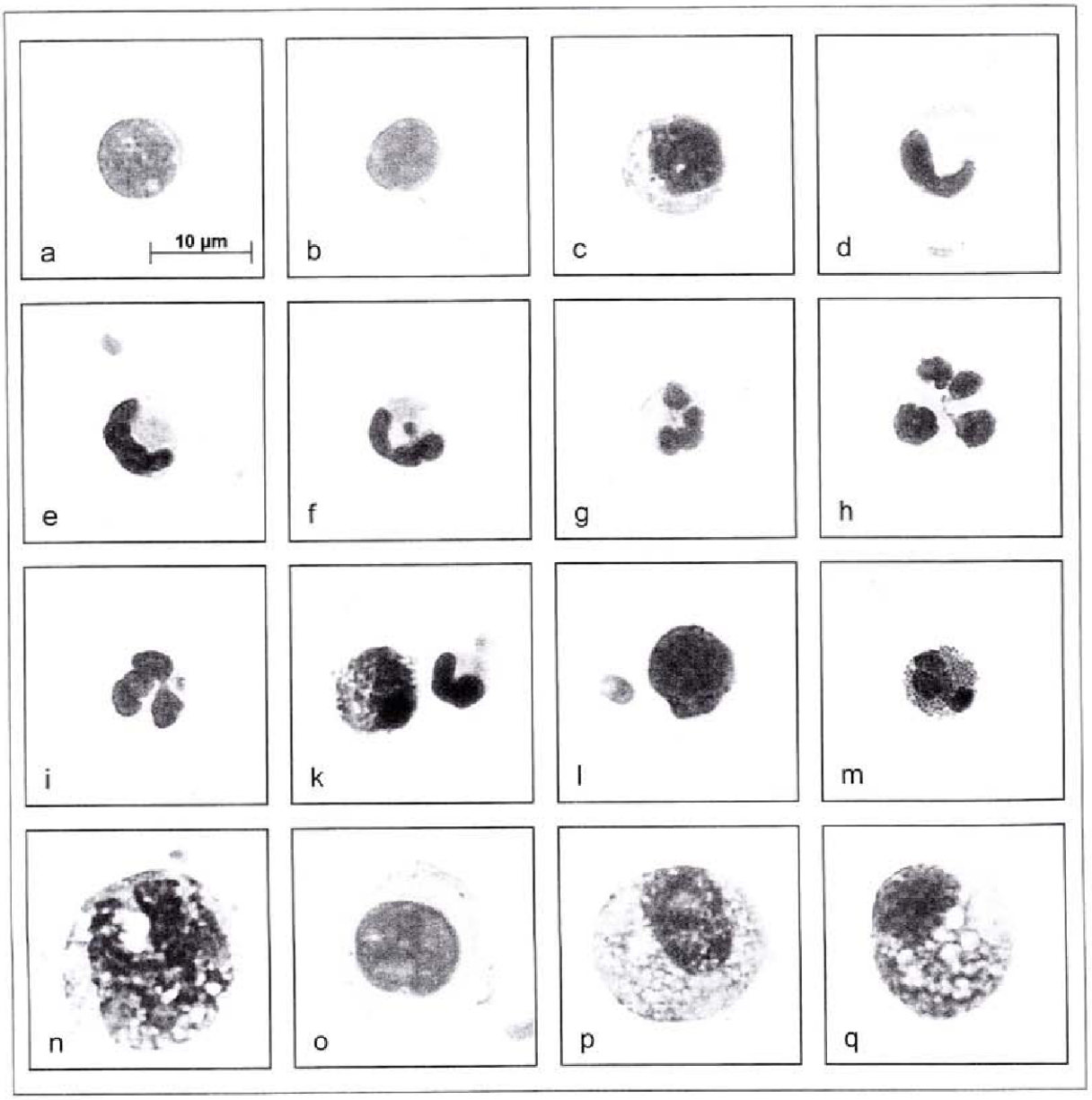

Fig. 1: Photographs of cells in bovine milk stained according to method of Pappenheim; a-b = small lymphocytes; c = large lymphocyte; d–f = band neutrophils; g–i = segmented neutrophils; k = basophil (left) and band (right) neutrophil; l = basophil; m = eosinophil; n–q = macrophages; magnification x 1000-fold

Table1: Cell distribution quarter milk samples of different SCC levels (mean ± SEM)

	Group 1 SCC < 100,000/ml n=12	Group 2 SCC 100,000– 400,000/ml n=8	Group3 SCC >400,000/ml n=8
SCC (log_{10} cells/ml)	4.57 ± 0.10	5.39 ± 0.06	6.15 ± 0.07
Lymphocytes [%]	20.9 ± 1.2[a]	11.4 ± 1.4[b]	3.3 ± 0.3[c]
Macrophages [%]	45.6 ± 1.9[a]	25.1 ± 3.5[b]	9.5 ± 1.9[c]
Neutrophils [%]	33.5 ± 1.7[c]	63.5 ± 3.8[b]	87.2 ± 1.8[a]

[a,b,c] Means within line are significantly different ($p<0.05$)

Lymphocytes are characterized by a round nucleus of blue-red colour and a grey-blue cytoplasm. The volume of the latter distinguishes this cell type into small and large lymphocytes. Thus cell size varies between 8–16 µm. Band neutrophils are circular and present a light-grey to pink cytoplasm. The nucleus in blue-red colour is bent and oblong. These characteristics serve as differentiation parameters against the segmented neutrophils. According to the rule of filament these cells are counted as the latter cell type as soon as the nucleus is threadlike and constricted. The diameter of neutrophils varies from 10 to 15 µm. The subpopulations basophils and eosinophils can be easily distinguished. Their densely packed granules appear dark blue and orange-red, respectively. The macrophages show a diameter of 15–25 µm and are the largest cells in milk. Their shape is diverse with a bluish-grey cytoplasm. Vacuoles and phagocytized fat globules are observed frequently (see Fig. 1).

2.5 Statistical evaluation

The effect of groups on the distribution of cell populations was calculated by 1-way ANOVA and Bonferroni's t-test. Level of significance was determined at $p < 0.05$.

3. Results and discussion

The 28 investigated samples were divided into 3 groups according to their SCC. Group 1 consisted of 12 samples at a mean SCC of 4.57 ± 0.10 log_{10} cells/ml, which is assigned with healthy quarters and the macrophages as dominant cell type (4, 6). As expected due to comparable literature the predominant cell type in group 1 was the macrophages followed by neutrophils and lymphocytes. Group 2 with a mean SCC of 5.39 ± 0.06 log_{10} cells/ml (n=8) showed a shift towards the population of neutrophils. The percentage of lymphocytes and macrophages decreased. The distribution of the cell population in group 3 (n=8) was dominated by neutrophils. At a mean SCC of 6.15 ± 0.07 log_{10} cells/ml lymphocytes and macrophages played a minor role (see Table 1). According to several studies the cell population of neutrophils in group 2 and 3 increased with rising SCC levels (4, 6, 8, 9). The viability of the separated cells, determined by trypan blue staining, ranged from 94 to 98%.

4. Conclusions

The used technique allows cell differentiation in milk samples and hence additional information on the immunological status of the mammary gland can be achieved. To preserve cellular morphological features it is necessary to remove the milk fat completely before preparation of smears. Cell diameter and morphology are an important tool in identification of cell populations.

Our results show that the content of macrophages decreased markedly and that of lymphocytes slightly while the content of neutrophils increased with increasing SCC. The distribution of milk cell populations corresponded with previous findings based on other methods. Therefore, it can be assumed that the cell differentiation based on Pappenheim staining provides reliable results at all SCC levels.

The application of this procedure provides additional information about the distribution of cell types for the understanding of the udder health status. It is conceivable that not only the quantity of cells but also their functionality should be taken into account.

Acknowledgement

This study was supported by the Bayerisches Staatsministerium für Landwirtschaft und Forsten, Germany.

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Appendix 4

Effect of lactoferrin on the immune-status and the gastro-intestinal morphology in growing calves

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Summary

Lactoferrin (LF) is a cationic iron-binding glycoprotein that is abundantly expressed and secreted from glandular epithelial cells and a prominent component of the secondary granules of polymorph nuclear neutrophils (PMNs). Various *in vitro* and *in vivo* experiments demonstrate anti-microbial, anti-viral, anti-mycotic, and anti-inflammatory effects of LF, as well as its role in the modulation of the immune system. To approach the mechanism governing the regulatory functions of LF in the immune system in growing ruminants, host protecting effects of oral administered LF were studied in calves. Five calves were fed LF beginning on their 3rd day of life with colostral milk and starting on their 6th life day only with milk replacer enriched with 0.16 % LF. The average daily intake of LF per calf was 1.5 – 1.6 g/d. Further five calves severed as control group with identical treatment except for the LF supplementation. At the end of the study (61th day of life) all calves were slaughtered and various tissues were sampled for histological and gene-expression studies. LF given orally was shown to act as an immuno modulatory agent by enhancing the sizes of Peyer´s patches in the ileum and increasing IgG levels in the blood serum. The results of this investigation further demonstrate an increase in the number of peripheral blood leucocytes, but also enhanced mRNA expression levels of various Interleukins (IL), like IL-1ß, IL-8, IL-10 and Interferon gamma (INFγ) in those cells in response to LF treatment. Additionally LF feeding decreased villus sizes in the jejunum. Together these findings emphasizes the ability of LF to modulate the immune response and reveals host-protecting effects.

Introduction

Intestinal diseases and insufficient function of the immune system in calves are partly responsible for the particularly high mortality and morbidity rates in the neonatal period (Sangild et al. 2000). Therefore neonatal calves are dependent on passive immunoprotection by the ingestion of diverse substances from colostrum like maternal immunoglobulins, immune cells, and other substances (Barrington and Parish 2001). Colostrum components can modulate gastrointestinal tract (GIT) development (Odle et al. 1996, Xu et al. 2000, Blum et al. 2002), intestinal absorptive capacity (Hammon and Blum 1997, Rauprich et al. 2000), and influence the immune systems of the GIT (David et al. 2003, Norrman et al. 2003). Among bioactive components, LF is present in high concentration in milk, especially in colostrum and during involution or inflammation (Masson et al. 1966, Hagiwara et al. 2003, Prenner et al. 2006) and it is a major component of the secondary granules of polymorphonuclear leukocytes (Masson et al. 1969). LF is a cationic metal binding glycoprotein and plays a role in host defense mechanisms via bacteriostatic activity and immuno-regulatory properties (Baveye et al. 1999). It has been reported that oral administration of LF exerts host protective effects against various diseases in human and animals (Tomita et al. 2002). Suppressive effects of orally administered bovine LF have been shown against intestinal *Escherichia coli* in milk-fed mice (Teraguchi et al. 1995) , *Staphylococcus aureus* (Bhimani et al. 1999) and rotavirus (Superti et al. 1997), which are common pathogens causing diarrhoea in calves (Tromp 1990). Supplementation of LF is able to improve fecal scores and to reduce morbidity in preweaned calves (Robblee et al. 2003). LF that is ingested with colostrum by neonatal calves is absorbed from the digestive tract and then appears in blood plasma (Dawes et al. 2004). LF receptors are expressed on the brush border membrane of the intestine and involved in the uptake of LF (Talukder et al. 2003), as well as for monocytes, macrophages and lymphocytes (Bennett and Davis 1981, Birgens et al. 1983, Britigan et al. 1991). There is also some evidence that LF can influence the development of intestinal epithelia (van Leeuwen et al. 2000 , Schottstedt et al. 2005) and exerts effects on lymphoid tissues in the GIT (Debbabi et al. 1998, Sfeir et al. 2004). In neonates LF is absorbed in the intestine via endocytosis, appears in blood followed by excretion via the

urine and the bile (Masson et al. 1966, Hutchens et al. 1991).

The intestinal epithelium is a continually renewing single layer of cells that covers the entire surface. Cells in the intestinal mucosa are found at various stages of differentiation from the immature crypt cells to terminally differentiated villus cells. This maturation process is compensated by cell shedding and apoptosis and is especially relevant in the GIT for the maintenance of normal gut epithelial function. Proliferation is regulated by feedback signals from the villus to crypt cells mediated by growth factors, such as epidermal growth factor (EGF) and transforming growth factor-alpha and -beta (TGFα, TGFβ), mainly in the crypts (Hutchens et al. 1997). While TGFß inhibits proliferation, TGFα has proliferative effects in epithelial cells and appears to act through the same receptor as EGF. Insulin-like growth factor-1 (IGF-1) exerts its effects by stimulating the proliferation of epithelial and non-epithelial and promotes intestinal wound healing (Dignass and Sturm 2001). Enhanced villus heights are often resulting from an increase in proliferation of crypt cells coupled with a decrease in apoptosis.

Aim of this study was to conduct a feeding trial to determine the effects or modulation of LF on the health status of calves. Interactions among LF and the morphological development of the GIT villi as well as mRNA gene-expression levels of pro- and anti-inflammatory markers were investigated to test LF as a preventative supplement in calf nutrition.

Material and Methods

Animals, Husbandry, Feeding and Experimental Procedures

Male calves from the dairy cattle herd (Agricultural Experimental Station Hirschau, TU München, Freising-Weihenstephan) were available for this experiment. The cows were crossbreds Red-Holstein-Friesian x Fleckvieh. Calves were separated immediately after birth from the cows and kept in individual compartments during the colostral phase until the 6th day of live. The calves in this study were divided into two groups, each 5 animals, according to their dates of birth and weight, as homogenously as possible. Special care was taken to guarantee that dates of birth were fairly uniform between the two groups. The newly born calves were given as soon as possible post natum 2 up to 3 liter colostrum per meal (2 times per day) until the 6th (± 1) day. Starting at day 3 the animals in the LF group received colostrum mixed with 0.54 g LF (DSM Nutritional Products Ltd, Basel, Switzerland) at each feeding. After colostral phase (day 6 ± 1) calves received a nonmedicated milk replacer (KALBI MILCH, Schaumann GmbH), water and hay and concentrate. The LF group was given the milk replacer supplemented with 0.16 % LF, the other group served as control. Starting on week 2 to 3 also maize silage was fed. Further details for feeding are presented in Prenner et al. (2006). The live weight of the calves was determined at first day of life. Live weights were further monitored on the 2nd, 4th, 6th, and 8th week of life, as well as on the day of slaughter. The weighing was done with an electronic scale. An average weight of 47.6 ± 1.5 kg for the control group and the LF treated group was noted at the beginning of the study. At the end of the study (61 ± 2.9 th day of life) the control and LF treated calves weighed 80.4 ± 3.1 kg on average. Over the entire experiment no significant differences were found in weight increase among the control calves (552.5 ± 31.1 g) and the LF group (545.6 ± 21.2 g).

Blood samples

15 ml blood was collected from the jugular vein using a EDTA and serum vacutainer tube (Greiner bio-one GmbH, Frickenhausen, Germany) between day 1 and 2 and afterwards once a week. Tubes were kept on crushed ice. EDTA blood was diluted 1/1(v/v) with lysis buffer (830 mg NH_4CL, 3.7 mg Na-EDTA, 100 mg KCL in 100 ml H_2O pH 7.4) and centrifuged for 10 min at 220 g. The cell pellet was again suspended in lysis buffer and centrifugation was repeated. Supernatants of blood samples were discarded and leukocytes were transferred in 350 µl RNA extraction lysis buffer (Macherey-Nagel, Düren, Germany).

Determination of immunoglobulin in colostrum and blood

The samples of colostrum were taken from the milking number 1, 3, 6, and 12 post partum. Samples were centrifuged at 4500 g. All colostrum samples were stored at -20° C. The immunoglobulin G (IgG) content in the colostrum samples from the first milking was determined, using the ELISA sandwich method (Erhard et al., 1999). Serum tubes were centrifuged for 15 min at 1800 g and the overlaying serum was collected and stored at –20°C until analyses. The bovine IgG content in serum was then measured by sandwich ELISA according to Erhard *et al.* (1999) at the LMU Institute for Animal Health.

Analysis in blood and faeces

Blood samples were collected for haematological analysis from animals following euthanasia and forwarded in EDTA vacutainer tubes to a veterinary laboratory (Vetmed Labor, Unterhaching, Germany). Hematocrit and haemoglobin concentration, erythrocyte, thrombocyte and white blood cell numbers were determined with the CELL-DYN 3700SL System (Abbott Diagnostika GmbH, Wiesbaden, Germany).

Furthermore a blood smear was send to the lab to obtain a differential white cell count. Blood smears were stained with May Grünwald-Giemsa according to the method described by Micu & Manolescu (1978). The percentage of basophilic granulocytes, eosinophilic granulocytes, segmented and non segmented neutrophilic granulocytes, lymphocytes, monocytes and atypical cells were determined.

Analysis of bacterial communities in chyme samples taken from the colon after slaughtering was made at the Tiergesundheitsdienst Bayern e.V. Grub. The total number of aerobe and anaerobe microbial counts as well as some selected indicator counts of the following strains were analysed: *Streptococcus/Enterococcus, Lactobacillus, Escherichia coli and Clostridium perfringens*. 1 g chyme was mixed with 9 ml PBS-solution and mixture was diluted from 10^1 to 10^{10}. Out of every dilution 0.1 ml was pipette on top an agar plate. Agar plates with 3 to 300 bacteria were figured out and the mean calculated.

Histology and histomorphometry

After slaughtering the calves, the GIT was removed and a 1- to 2-cm-long cross-section were taken from the small intestine (mid jejunum, mid ileum), large intestine (mid caecum, mid colon), ventral rumen, reticulum, abomasum and omasum. Immediately after collection the tissue pieces were washed twice in physiological 0.9% NaCl solution. The tissue sections were embedded and transferred in 3.7% buffered formalin (Carl Roth GmbH, Karlsruhe, germany) for 24 h. Tissue samples for RNA extraction collected from the four compartments of the cattle stomach, GIT, liver and mesenterial lymph nodes (mLN) were placed in individually labeled cryotubes and frozen in liquid nitrogen. Cryotubes were removed from liquid nitrogen in the laboratory and stored at –80 °C until analysis.

After fixation in formalin the specimen were dehydrated through a graded series of alcohols and embedded in a paraffin. 10 to 15 cuts of 6 µm thickness were made from different regions of each block using a Microtom (LEICA RM2145, Wetzlar, Germany). Paraffin was removed by xylol (Carl Roth GmbH), sections were hydrated and stained with hematoxylin and eosin (HE), following the standard protocol according to Mayer (1960).

Microphotographs were taken using a digital camera (AxioCam MRc, Zeiss, Jena, Germany) connected with a Zeiss Axioscope 2 microscope and evaluated with a graphics program (Axio Vision 3.1, Zeiss). Quantitative measurements were made in at least 30 well-oriented crypt-villus systems for jejunum, ileum, caecum and colon. Analysis of villus heights and widths of the villi and crypt depths were done on at least 15 well-oriented crypt-villus systems for each intestinal sampling site, and the ratios of villus heights to crypt depths were calculated. The villus height was measured between the tip of the villus and the beginning of the lamina muscularis, the villus width was measured perpendicular to the height. Furthermore, areas of at least 10 Peyer`s patches per slide were evaluated.

Total RNA extraction and quantification

Total RNA from blood samples and different tissues was isolated by using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Tissue were homogenized in the first lysis buffer with the FastPrep extraction system FP120 (Savant Instruments, Holbrook, NY, USA). Tissue was homogenized two times by reciprocating shaking at 6.0 m/sec for 30 sec in extraction tubes containing 200 mg of Matrix Green lysing beads (Bio 101 Inc., Vista, CA). Between the homogenisation steps the samples were stored on ice. RNA pellets were resuspended in 50 µl of diethyl pyrocarbonate (DEPC) treated H_2O. To quantify the extracted RNA, all spectro-photometric measurements were taken in a photometer using UV-transparent UVette (Eppendorf, Hamburg, Germany). Integrity of the extracted total RNA was verified by optical density OD_{260nm}/OD_{280nm} absorption ratio.

One-step real-time RT-PCR

One-step real-time RT-PCR was performed using QuantiTect SYBR Green RT-PCR (Qiagen, Hilden, Germany) by a standard protocol in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). The real-time RT-PCR master-mix contained 5 µl 2*QuantiTect SYBR Green RT-PCR master-mix, 0.5 µl forward primer (20 µM), 0.5 µl reverse primer (20 µM) and 0.1 µl QuantiTect RT Mix. The used primers (bovine specific for TNF-α, IL-1β, IL-6, IL-8, IL-10, INFγ, Caspase-6, TGF-β1, TGFα , IGF-1, Cyclin D1, and housekeeping genes GAPDH, β-Actin and histone 3 are summarized in table 1) were synthesized commercially (MWG Biotech, Ebersberg, Germany). 6.1 µl of master-mix was added to a 3.9 µl total volume of RNA (5 ng/µl) into Rotor-Gene 100 µl rotor reaction tubes. The tubes were closed and placed into the cycling rotor. Following cycling protocol was used: (**1**) reverse transcription: 20 min at 50°C; (**2**) denaturation program: 15 min at 94°C; (**3**) 40 cycles of amplification and quantification: 20 sec at 94°C; annealing for 30 sec at 60°C; elongation for 20 sec at 72°C; fluorescence measurement at 77°C with a single fluorescence measurement, (**4**) melting curve program from 60-99°C with a heating rate of 0.1°C per sec and continuous fluorescence measurements.

Crossing Point (CP) values were achieved using the Rotor-Gene software version 5.0 (Corbett Research). Relative mRNA levels were calculated by normalization of the CP (= ΔCP) of the target gene to the arithmetic mean of the CP of the three housekeeping genes GAPDH, β-Actin and histone 3 (Livak and Schmittgen 2001). Cytokine mRNA expression is presented as ΔΔCP increase evaluated in relation to each single time point and compared to un-stimulated control animals.

Statistics

Values were expressed as means ± SEM. Analysis for the cytokine expression in the blood samples was done by Two-Way-ANOVA and Student-Newman-Keuls method using treatment (LF, control) as first and length of stimulation (0, 1, 2, 4, 6, 8 and 10 weeks) as second factor. It was applied on normalized expression results (ΔCP or ΔΔCP). For the evaluation of LF effects in histomorphology of intestinal mucosal classical t-test was performed. Statistics of IgG values in blood were preformed with Two-Way-ANOVA and Student-Newman-Keuls method using treatment (LF, control) as first and length of stimulation (2, 7, 14, 21, 28, 35, 42, 49 and 56 days) as second factor. All statistical analysis were performed with Sigma Stat 3.0 (SPSS Inc, Chicago, IL, USA).

Results

Histomorphometrical analyses in jejunum, ileum, colon, caecum and Peyer's patches

Results of histomorphometrical analyses are shown in table 2. In the control group, villus heights were lower in the ileum than in the jejunum ($p < 0.01$). These findings were not affected by LF treatment . Villus heights and areas in jejunum were affected by LF treatment and smaller in LF treated group than in control group ($p < 0.05$). No changes of villus height/width ratios was investigated in the jejunum and ileum after LF treatment. In ileum the ratio tended to be lower ($p < 0.1$) than in the jejunum. LF had no effect on the crypt depths and areas in colon and caecum. In the ileum, areas of Peyer´s patches of LF treated calves were enlarged compared to the non-treated group ($p < 0.05$).

Table 1. Primers sequences used for one-step real-time RT-PCR.

Gene	Primer	Sequence 5' → 3'	EMBL Accession number	Product length
TNFα	Sense Antisense	TAA CAA GCC GGT AGC CCA CG GCA AGG GCT CTT GAT GGC AGA	AF011926	256 bp
IL-1β	Sense Antisense	TTC TCT CCA GCC AAC CTT CAT T ATC TGC AGC TGG ATG TTT CCA T	M37211	198 bp
IL-6	Sense Antisense	GCT GAA TCT TCC AAA AAT GGA GG GCT TCA GGA TCT GGA TCA GTG	NM173923	200 bp
IL-8	Sense Antisense	ATG ACT TCC AAG CTG GCT GTT G TTG ATA AAT TTG GGG TGG AAA G	AF232704	149 bp
IL-10	Sense Antisense	ACT TTA AGG GTT ACC TGG GTT G CTT CTC CAC CGC CTT GCT CTT	U00799	206 bp
INFγ	Sense Antisense	TAA GGG TGG GCC TCT CTT C CCA TGC TCC TTT GAA TGA CC	M29867	143 bp
Caspase-6	Sense Antisense	TGT TCA AAG GAG ACA AGT GTC AG CAG AGT AGC ACA TGA GGA AGT C	BC078785	210 bp
TGFß1	Sense Antisense	AAG GAC CTG GGC TGG AAG TG TCA TGT TGG ACA ACT GCT CCA C	XM592497	239 bp
TGFα	Sense Antisense	TGA CTG CCC AGA TTC CCA CA GCA GCA GTG TAT CAG CAC ACA	M36271	238 bp
IGF-1	Sense Antisense	CGC ATC TCT TCT ATC TGG CC CTG AGC CTT GGG CAT GTC	X15726	311 bp
Cyclin D1	Sense Antisense	TCC TGT GCT GCG AAG TGG A GGT CCA GGT AGT TCA TGG C	BC014078	246 bp
Histone 3	Sense Antisense	ACT CGC TAC AAA AGC CGC TC ACT TGC CTC CTG CAA AGC AC	BT020962	232 bp
GAPDH	Sense Antisense	GTC TTC ACT ACC ATG GAG AAG G TCA TGG ATG ACC TTG GCC AG	U85042	197 bp
β-Actin	Sense Antisense	AAC TCC ATC ATG AAG TGT GAC GAT CCA CAT CTG CTG GAA GG	AY141970	202 bp

Table 2. Histomorphometrical parameters (villus height, villus width, crypt depth, area of villus, and size of Peyer´s patches) of jejunum, ileum, colon and caecum in control group (C) and LF treated group (LF). Significant effects of LF are shown in bold with asterisk (* $p < 0.05$, ** $p < 0.01$, NS = not significant).

	Experimental group		SEM		ANOVA [p values]
	C	LF	C	LF	LF effect
Jejunum					
Villus area [100 µm²]	**1078**	**869**	**36**	**34**	**
Villus height [µm]	**970**	**870**	**37**	**30**	*
Villus width [µm]	447	441	31	25	NS
Villus height/width ratio	2.17	1.97	0.13	0.13	NS
Ileum					
Villus area [100 µm²]	1010	837	85	88	NS
Villus height [µm]	788	690	34	47	NS
Villus width [µm]	447	350	46	45	NS
Villus height/width ratio	1.77	1.98	0.18	0.14	NS
Peyer´s patches					
Area [100 µm²]	**4187**	**5859**	**384**	**614**	*
Colon					
Crypt area [100 µm²]	255	219	15	11	NS
Crypt depth [µm]	483	469	9	5	NS
Caecum					
Crypt area [100 µm²]	258	238	12	9	NS
Crypt depth [µm]	506	492	15	4	NS

Haematology

No differences in hematocrit, hemoglobin concentration, and erythrocyte and thrombocyte numbers were found among treatments. In both groups hematologic values were in a normal range, according to Monke et al. (1998). The number of leukocytes were affected by treatment ($p < 0.05$) and increased from 5.9 ± 0.96 in the control group to 9.38 ± 0.85 G/l in the LF group. There were no significant differences among the groups in differential white blood cell count (detailed blood count data not shown).

Native contents IgG in colostrum and serum

For the control group calves, the average was 71.8 ± 37.9 mg/ml, in the LF group, the average was 102.8 ± 37.8 mg/ml. There was a close relationship between the total intake of the IgG through the first drink/formula and the IgG content of the calf serum from the 24th through the 48th hour between the groups. Mean IgG antibody concentration of the first blood sample (24-48 h) were similar for the control (20.3 ± 7.5 mg/ml) and LF (21.3 ± 5 mg/ml) group. In both groups IgG contents decreased from the first sample until week 3 and started to increase up to week 8 approximately to the level of the initial value in the control group (table 3). Comparing the trend of both groups over the entire feeding experiment, significant higher IgG levels were observed in the LF treated group ($p < 0.05$). In comparison to the control LF supplementation resulted in significant higher ($p < 0.05$) IgG contents from day 7 until day 21, whereas from that point differences in IgG concentration could not be measured, except for week 7.

Time dependent cytokine mRNA expression in leukocytes

The kinetics of mRNA expression for IL-1ß, IL-8, IL-10, and INFγ after oral LF administration are shown in figure 1. Over the entire administration period the trend of mRNA gene expression of the immune markers IL-1ß, Il-8, IL-10 and INFγ decreased significantly, applying a Person correlation analysis ($r = 0.46$, $p < 0.001$; $r = 0.46$, $p<0.05$; $r = 0.37$, $p < 0.05$; $r = 0.37$, $p = 0.05$, respectively). The LF-induced cytokine gene expression for IL-1ß ($p < 0.05$), IL-10 ($p < 0.001$) and INFγ ($p < 0.05$), increased rapidly after 1 week of administration. While a significant up-regulation of IL-8 expression was observed after 3 weeks ($p < 0.05$). IL-10 continued to be expressed at a higher level until week 6

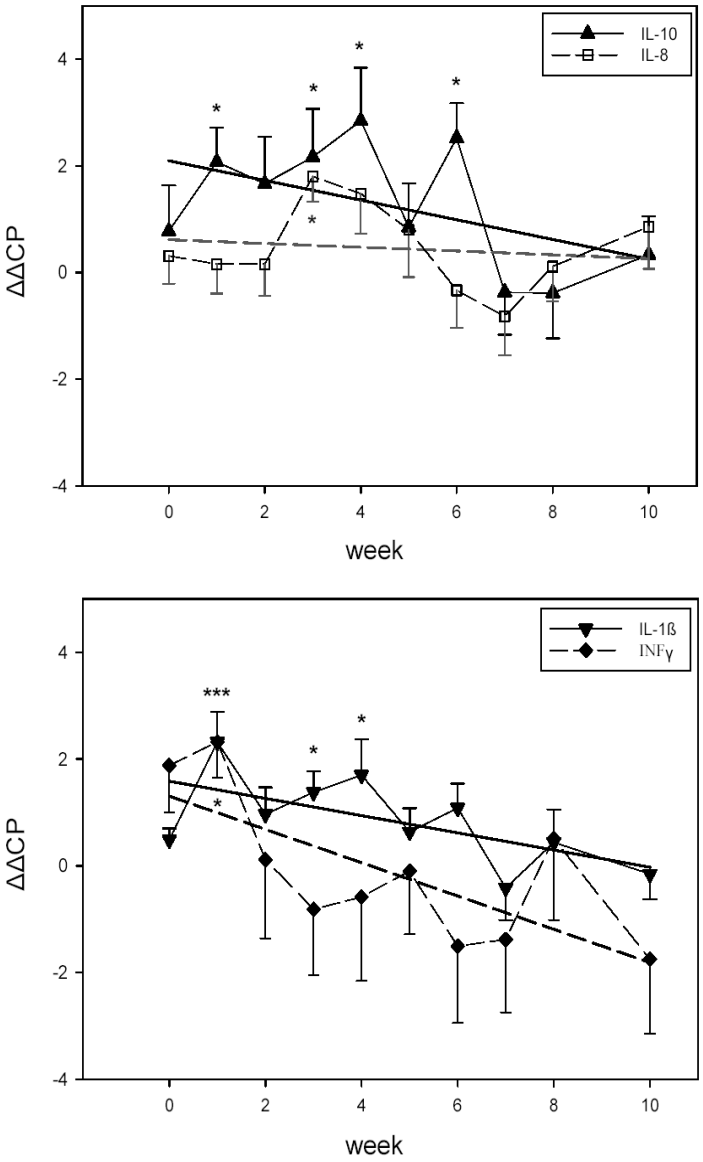

Fig. 1. Effect of LF on the kinetics of cytokine mRNA expression in WBC. Data are presented as means ± SEM (n=5). Significant effects of LF are shown with asterisk (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

and diminished rapidly until week 7, whereas IL-8 and IL-1ß gene expression started to decrease progressively after 3 and 4 weeks respectively and reached basal levels at week 7. INFγ mRNA expression diminished already after week 1 of LF administration. LF did not affect mRNA expression levels for TNFα and IL-6 (data not shown).

Gene expression of cytokines, growth and apoptotic factors in GIT, mLN, stomach and liver

LF induced mRNA expression changes for the diverse growth- (IGF-1, TGFα), apoptotic- (Caspase-6) and proliferation- (TGFα, IGF-1 and Cyclin D1) markers in various tissues are shown in table 4. Oral administration of LF effected a slight down-regulation of the gene expression for IL-6 and IL-10 in the omasum ($p < 0.05$) and a down-regulation of Il-6 mRNA expression in the ileum ($p < 0.05$). In liver a 7-fold decrease ($p < 0.05$) and in abomasum a 6.6-fold increase of INFγ expression was found among LF treatment. LF induced significant Caspase-6 mRNA increases ($p < 0.05$) in the jejunum of LF treated calves, while diverse cell proliferation markers (TGFα, IGF-1 and Cyclin D1) and TGFß1 were not affected by the treatment.

Determination of bacterial communities in chyme of colon

The content of different bacteria in the chyme of colon are shown in table 5. The experiment demonstrates that the quantity of aerobe and anaerobe micro organisms is lower in the LF treated group, but these result are not statistical significant. *Streptococcus/Enterococcus* were observed to represent the largest proportion of all investigated aerobic cells, with an average of 42% (±16) for the control and 46% (±15) for the LF treated animals. The percentage of *E. coli* on aerobe total microbial count seemed to be reduced from 20% (±14) to 13% (±11) due to LF administration. Lactobacilli have been shown to be the largest fraction of all anaerobic cells in both groups (control 61% ± 15 and LF 60 % ± 11). *Clostridium perfringens* germs could be detected in all analysed samples at low concentrations (≤ 3.4 log/g).

	control [mg/ml]	LF group [mg/ml]
1st to 2nd day	20.30 ± 3.33	21.34 ± 2.22
day 6	13.08 ± 2.22	**21.35*** ± 2.54
week 2	10.30 ± 0.46	**18.41*** ± 3.99
week 3	9.66 ± 0.73	**15.39*** ± 1.55
week 4	13.38 ± 1.98	18.53 ± 2.71
week 5	11.67 ± 0.67	15.05 ± 1.14
week 6	13.65 ± 2.98	**20.59*** ± 2.12
week 7	14.70 ± 2.40	15.64 ± 1.51
week 8	19.35 ± 5.42	15.69 ± 0.67

Table 3. Serum IgG contents [mg/ml] in LF treated calves. Data are presented as means ± SEM (n = 5). Significant effects of LF are shown in bold with asterisk [* $p < 0.05$].

Discussion

In the present investigation, we have shown that oral administration of LF enhanced sizes of Peyer´s patches (PP) in the ileum of calves. The ileal PP are lymphoid follicles, located in the mucosa and extending into the sub-mucosa, that produces immature B-lymphocytes (Bruewer et al. 2003, David et al. 2003). It is well established that naturally ingested food components can potentially interact with secondary lymphoid organs along the GIT, developing a specific immune response to the antigens (Mowat et al. 1997). Ingestion of colostrum rises the proliferation rate of lymphocytes in PP in normal-term calves immediately after birth (David et al. 2003). LF given orally as an antigen was shown to act as a immuno modulatory agent by increasing the biosynthesis of IgA and IgG in the intestinal secretions and serum of mice as well as the proliferation of PP cells and splenocytes (Debbabi et al. 1998, Sfeir et al. 2004). Significant higher IgG levels in LF treated calves could also be observed in this and a further study (Prenner *et al* 2006), particularly from week 1 to 7. According to Erhard *et al.* (1999) low point of serum IgG content are reached at day 28 post natum and subsequently accelerates to similar levels of adult animals at the age of 12 weeks. In the present study IgG serum concentrations in both groups changed similarly in the course of time, whereas lowest IgG concentrations were already reached around day 21 post natum. Furthermore a substantial stronger decrease of IgG content was noticed in the control group in contrast to the LF group. Since in this study LF showed no effects on the live weight development and very little of an ingested dose of LF is probably absorbed by the intestinal tract in adult animals (Tomita et al. 2002), the immuno modulatory properties of LF in the GIT seems to be receptor-mediated. Specific LF receptors have been found on the brush-border membrane vesicles and the epithelium overlying PP of the bovine intestinal tract (Talukder et al. 2003). Oral administration of bovine LF has been shown to induce IL-18 and INFγ secretion in the mucosa of small intestine in mice. Moreover this study documented a markedly increase of $CD4^+$ and $CD8^+$ T cells in lymphoid tissues and lamina propria and of IgM^+ and IgA^+ B cells in lamina propria by bovine LF treatment (Wang et al. 2000).

Specific binding of LF has also been reported for B-lymphocytes and macrophages or monocytes, suggesting that this glycoprotein may directly stimulate the activity of antigen-presenting cells. The results of this investigation show a significant increase in leukocyte number of peripheral blood ($p < 0.05$, data not shown), but also enhanced cytokine mRNA expressions of IL-1ß, IL-8, IL-10, and INFγ in the first weeks in response to LF treatment. These results are in agreement with those of Togawa *et al.* (2002), who found up-regulation of IL-10 production in the colonic mucosa after oral LF administration in rats with colitis. Whereas the long term trend of the IL-1ß, IL-8, IL-10, and INFγ mRNA showed a clear decrease in the expression values (figure 1). Further *in vitro* studies reported LF as a promoter of superoxide production, release of pro-inflammatory molecules such as TNFα and IL-8 in murine macrophage-like cells (Sorimachi et al. 1997) and IL-8 from human polymorphnuclear leukocytes (Shinoda et al. 1996). We previously showed that LF and LFcin, a bactericidal pepsin-derived fragment of LF, stimulate the cytokine mRNA expression of TNFα, IL-1β, IL-6 and IL-10 in bovine leukocytes and monocytes (Prgomet et al. 2005). LF was also reported to down-regulate serum cytokine levels of TNFα, IL-1β, IL-6, induced by lipopolysaccharide (LPS) in mice, thus providing a natural feedback mechanism and preventing septic shock (Kruzel et al. 2002, Machnicki et al. 1993). The rapid activation and the elevated expression level of IL-10 mRNA over a long period could explain the modest increase of gene expression for the pro-inflammatory cytokines Il-1ß , IL-8 and INFγ in our study. Il-10 is known to be an anti-inflammatory cytokine capable of inhibiting the production of TNFα and IL-1β in activated monocytes/macrophages and plays a role in the development of LPS tolerance (Fiorentino et al. 1991, Brandtzaeg et al. 1996). Due to the immuno modulatory properties of bovine LF, these data suggest that LF is potentially attractive as a preventative supplement for bowel disease.

As seen in previous studies, villus heights and villus-height/crypt-depth ratios were the greatest in the jejunum among the small intestine (Attaix and Meslin 1991, Sauter et al. 2003), possibly due to enhanced differentiation from crypt cells to villus epithelial cells. This gut segment is thought to play a major role in absorption of the digestion products, because the intestinal surface is expected to be positively associated with absorptive capacity (Johnson et al. 1994). Based on histomorphological analyses, our study indicates that LF treatment decreased the villus sizes and areas in jejunum. These results are in contrast to Schottstedt *et al.* (2005), who reported that LF supplementation among a milk-based formula did not affect histomorphometrical parameters of the intestinal epithelium of neonatal calves. This might have been due to short-term feeding experiment. However, they also showed that LF administration negatively affected crypt cell proliferation in the colon. In

Table 4. Effect of LF on cytokine mRNA expression in various calf tissue. Data are presented as delta-delta CP values (= ΔΔCP) in means ± SEM (n = 5). Up-regulated gene are shown as positive values and down-regulated genes are shown as negative values. Significant effects of LF are shown in bold with asterisk (* $p < 0.05$; ND = not determined).

Effect of lactoferrin on cytokine mRNA expression																				
	TNFα	SEM	IL-1ß	SEM	IL-6	SEM	IL-10	SEM	INFγ	SEM	Casp-6	SEM	Cyc-D1	SEM	TGFß1	SEM	TGFα	SEM	IGF-1	SEM
rumen	0.08	0.29	-1.05	0.44	-0.74	0.20	0.18	0.24	-1.5	0.52	0.39	0.23	0.01	0.09	ND	ND	ND	ND	ND	ND
reticulum	0.06	0.22	1.08	0.65	0.22	0.24	0.26	0.30	-0.18	1.14	-0.02	0.3	0.18	0.22	ND	ND	ND	ND	ND	ND
omasum	-0.08	0.36	0.72	0.56	**0.86***	0.29	**0.74***	0.25	-2.14	0.83	-0.53	0.53	-0.46	0.25	ND	ND	ND	ND	ND	ND
abomasum	0.09	0.16	-0.68	0.56	-0.64	0.50	-0.54	0.48	**2.72***	0.62	-0.22	0.53	0.16	0.22	ND	ND	ND	ND	ND	ND
jejunum	0.04	0.16	0.34	0.39	0.12	0.23	0.08	0.2	0.46	0.39	**-1.18***	0.25	-0.46	0.43	-0.24	0.19	-0.12	0.25	-0.1	0.27
ileum	0.21	0.08	-0.77	0.26	**-0.91***	0.18	0.17	0.11	0.43	0.40	-0.3	0.47	0.54	0.2	-0.09	0.10	-0.03	0.37	-0.49	0.23
mes. lymph.	-0.02	0.19	0.24	0.36	-0.24	0.32	-0.08	0.13	0.26	0.65	-0.16	0.27	-0.06	0.19	ND	ND	ND	ND	ND	ND
caecum	0.23	0.31	-0.47	0.16	0.15	0.31	0.23	0.26	-0.09	0.76	-0.03	0.47	0.03	0.49	-0.06	0.34	0.02	0.17	0.12	0.33
colon	0.12	0.21	-0.50	0.25	-0.72	0.19	-0.44	0.21	1.98	1.40	0.27	0.27	0.62	0.36	-0.20	0.19	-0.30	0.21	-0.62	0.39
liver	-0.48	0.15	0.02	0.19	0.46	0.35	-0.22	0.17	**-2.8***	0.69	1.08	0.47	0.34	0.58	ND	ND	ND	ND	ND	ND

this study, no differences in mRNA expression of TGFα, IGF-1 and Cyclin D1 in the small intestine, colon and caecum were found among LF-fed calves and control group. Therefore other mechanisms than reduced cell proliferation rates are contributed to decreased villus heights of the jejunum in the LF treated group, such as enhanced apoptotic rates in the intestinal epithelium. Apoptosis is especially relevant in the GIT because it is an important process responsible for maintenance of the cellular balance between proliferation and death and crucial for normal morphology and function (Hall et al. 1994). Our data demonstrate significant caspase-6 mRNA increases ($p < 0.05$) in the jejunum of LF treated calves. The members of the Caspase family are the final executioners of apoptosis activated during signalling cascade in almost all cell types (Kidd 1998). Possible mediators for the induction of caspase activity and epithelial cell apoptosis in the GIT are TNFα and IFNγ. Upon activation intraepithelial lymphocytes, which are found in large numbers in the villus epithelium, release large quantities of TNFα and IFNγ. These cytokines has been shown to play a dynamic role in the homeostasis of the villus epithelial barrier by deleting infected and damaged epithelial cells and to restore epithelial cell growth regulation and epithelial integrity (Guy-Grand 1998, Kim et al. 1998, Bruewer et al. 2003). Our results can not support this hypothesis because LF effected no significant up-regulation of those mediators at the mRNA level in the jejunum.

	control	LF group
TMC aerobic	7.10 ± 6.71	6.55 ± 5.70
Streptococcus/ Enterococcus	6.73 ± 6.32	6.15 ± 5.49
Escherichia coli	5.05 ± 4.44	5.78 ± 5.41
TMC anaerobic	7.10 ± 6.47	6.42 ± 5.35
Lactobacillus	7.03 ± 6.43	6.24 ± 5.38
Clostridium perfringens	2.26 ± 1.34	2.08 ± 0.95

Table 5. The content of different bacteria strains in the chyme of colon [log/g chyme]. Data are presented as means ± SEM (n = 5).

The intestine is the pivotal immune organ of the body and the development of the GIT and the immune system is highly influenced by the intestinal flora. Imbalances in micro flora or overgrowth of one bacterial species can alter immunological function (Walker 2000). Beneficial bacteria, such as *Lactobacillus*, are required for the maintenance of animal health. Lactic acid bacteria have been shown to prevent overgrowth and adhesion of various pathogenic bacteria, e.g. *Clostridium perfringens*, to the intestinal wall (Rinkinen et al. 2003). Examination of bacterial communities in chyme was performed to investigate the influence of LF on pathogenic and constitutional germs. Variation in total microbial count (TMC) of aerobe and anaerobe micro organisms and *Entero-/Streptococcus* was immense within the groups, whereas microbial counts were lower (numerically) in the LF treated group. This findings may possibly result from the often described bacteriostatic and bactericidal activities of LF. Additionally, administration of LF tended to reduce the percentage of *E. coli* on aerobe total microbial count. A beneficial effect of LF on *Lactobacilli*, by enhancing the proportion of those bacteria, could not be demonstrated in this study. *Lactobacilli*, which are indicators for improving intestinal health status, were observed to represent the largest proportion of all anaerobic cells. Natural individual fluctuation of the intestinal flora and health status of each animal may have covered the effects of LF in GIT.

Conclusion

The *in vivo* study demonstrates effects of oral administered LF on the systemic immune response as well as mucosal immunity in the intestine of calves. These enhanced immune responses may be conducive to eliminate pathogens, lower incidence of diseases and maintenance of the homeostasis during infectious diseases. Due to the increasing relevance for immunological active food proteins, LF may play an important role in future medicine as a preventive supplement and can possibly used as a replacement for traditional antibiotics against infection and inflammation.

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Appendix 5

Biotechnology Letters **26:** 509–515, 2004.

Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations

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Received 3 December 2003; Revisions requested 23 December 2003; Revisions received 20 January 2004; Accepted 22 January 2004

Key words: β-actin, housekeeping gene, PCR normalisation, RT-PCR, somatotropic axis, ubiquitin

Abstract

The stability of standard gene expression is an elementary prerequisite for internal standardisation of target gene expression data and many so called housekeeping genes with assumed stable expression can exhibit either up- or down-regulation under some experimental conditions. The developed, and herein presented, software called *BestKeeper* determines the best suited standards, out of ten candidates, and combines them into an index. The index can be compared with further ten target genes to decide, whether they are differentially expressed under an applied treatment. All data processing is based on crossing points. The *BestKeeper* software tool was validated on four housekeeping genes and 10 members of the somatotropic axis differentially expressed in bovine *corpora lutea* total RNA. The *BestKeeper* application and necessary information about data processing and handling can be downloaded on http://www.wzw.tum.de/gene-quantification/bestkeeper.html

Introduction

Reporting of the amount of target mRNA requires an accurate template preparation and relevant standardisation (Pfaffl 2001). This affects more advanced methods of gene expression study such as real-time PCR (Pfaffl 2001) or microarrays (Schuchhardt *et al.* 2000), as well as the traditional blotting methods. Since several parameters of the quantification procedure (e.g. inhibitory factors of the tissue, integrity of the RNA, loading error, enzyme or primer performance, etc.) must be controlled, numerous standardisation methods have been proposed (Suzuki *et al.* 2000, Thellin *et al.* 1999, Vandesompele *et al.* 2002). In most of them, just a distinct part of the whole real-time RT-PCR quantification procedure is reflected. For example, if the raw expression data is standardised to the amount of biological material, then the inhibitory, tissue-born residua present in sample will be disregarded. Similarly, if the quantification data is expressed per amount of total RNA extracted, then the predominant ribosomal RNAs (5S, 18S and 28S), known to vary in their proportion in the total RNA, can cause significant shifts in the results. This means that a 'full procedure control' is necessary.

In the relative quantification (Serazin-Leroy *et al.* 1998), the standardisation with another gene, whose expression is believed to be constant, is the method of choice (Suzuki *et al.* 2000, Thellin *et al.* 1999). The sequence of the standard and the target template are present in the sample during the whole assay. Therefore, the standard mimics all disturbances of the target sequence. A myriad of housekeeping genes (HKG), such as tubulins, actins, glyceraldehyde-3-phosphate dehydrogenase (GAPD), albumins, cyclophilin, micro-globulins, ribosomal units (18S or 28S rRNA), ubiquitin (UBQ) have been described. On the other hand, some of these genes has been reported to be regulated occasionally (Foss *et al.* 1998, Schmittgen & Zakrajsek 2000). Taking the above-mentioned arguments into account, one must con-

clude that there is no absolutely ideal way to control disturbances in the quantification procedure.

Before any gene is chosen as a standard, an exhaustive search is needed to ensure that no significant regulation occurs. This can, however, be a circular problem, as the expression data of the tested standard, as well, has to be standardised. A possible solution might be a use of more than just one HKG in a form of weighted expression index. To address this problem, an Excel based spreadsheet software application named *BestKeeper* was established and tested on biological material.

Materials and methods

Collection of bovine Corpora lutea

Thirty-one cows at the mid-luteal phase (days 8–12) were injected intra muscularly with 500 μg prostaglandin (PG) F2α analogue, *Cloprostenol* (Estrumate, Intervet, Germany). *Corpora lutea* (4–5 per group) were collected by trans-vaginal ovariectomy at six intervals after PGF2α-injection. Five control *corpora lutea* were randomly collected from untreated cows at the mid-luteal phase. All *corpora lutea* were aliquoted, immediately frozen in liquid N_2 and than stored at −80 °C until RNA extraction.

Total RNA extraction

The total RNA was extracted from 100 mg slices of deep frozen tissue with the peqGOLD TriFast™ (PeqLab, Erlangen, Germany), utilising the single step modified liquid separation procedure (Chomczynski 1993). The integrity of the total RNA was determined by electrophoresis on 2% (w/v) agarose gels. Nucleic acid concentrations were measured at 260 nm. Purity of the total RNA extracted was determined as the 260 nm/280 nm ratio with expected values between 1.8 and 2.

Two step RT real-time PCR

One μg total RNA was reverse-transcribed to cDNA in 40 μl volume in the Mastercycler Gradient (Eppendorf, Hamburg, Germany) thermal cycler. Following reaction mix was set: RT buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT and 300 μM dNTPs. The RNA was first denaturated at 65 °C for 5 min. For the subsequent RT reaction, 100 μM random hexamer primers (MBI Fermentas, St. Leon-Rot, Germany), 200 units M-MLV H^-, Reverse Transcriptase (Promega, Madison, USA), and 12.5 U RNase inhibitor (Roche Diagnostics, Mannheim, Germany) were added and the reaction incubated at 42 °C for 60 min. Eventually, samples were heated for 1 min at 99 °C to terminate the RT reaction.

Primer sequences of UBQ, GAPD, β-actin, 18S rRNA, IGF-1 (insulin-like growth factors type 1), IGF-2, IGFR-1 (insulin-like growth factor receptor type 1), IGFR-2, IGFBP-1 (insulin-like growth factor binding protein type 1) – IGFBP-6 were designed to span at least one intron (Pfaffl *et al.* 2002). Primers were synthesized commercially (MWG Biotech, Ebersberg, Germany). PCR conditions were optimised on the gradient thermal cycler and on the LightCycler (Roche Diagnostic). Real-time PCR using SYBR Green I technology on the LightCycler was then performed. Master-mix for each PCR run was prepared as follows: 6.4 μl water, 1.2 μl $MgCl_2$ (4 mM), 0.2 μl of each primer (4 pmol), 1 μl Fast Start DNA Master SYBR Green I mix (Roche Diagnostics). Finally, 9 μl master-mix and 25 ng reverse transcribed total RNA in 1 μl water were transferred into capillaries, reaching end volume 10 μl. The following amplification program was used: after 10 min of denaturation at 95 °C, 40 cycles of real-time PCR with 3-segment amplification were performed consisting of 15 s at 95 °C for denaturation, 10 s at 60 °C for annealing and 20 s at 72 °C for polymerase elongation. The melting step was then performed with slow heating starting at 60 °C with a rate of 0.1 °C per second up to 99 °C with continuous measurement of fluorescence. The expressions of the UBQ, GAPD, β-actin and 18S rRNA were quantified separately. Further on, 10 target genes (TG) of interest were amplified: IGF-1, IGF-2, IGFR-1, IGFR-2, IGFBP-1 to IGFBP-6. These factors, all members of the somatotropic axis, were supposed to vary during the *Estrumate* treatment. In each biological sample all 14 mRNA transcripts were quantified.

Data acquisition

Data on the expression levels of studied factors were obtained in the form of crossing points (CP) as described earlier (Rasmussen 2001). The data acquisition was done employing the '*second derivative maximum*' method (Rasmussen 2001) as computed by the LightCycler Software 3.5 (Roche Diagnostics). For further data analysis the Excel based application *BestKeeper* was programmed to accelerate the computing procedure.

Table 1. Descriptive statistics of four candidate housekeeping genes (HKG) based on their crossing point (CP) values. In the two last columns the *BestKeeper* index is computed together with the same descriptive parameters, either for four genes (UBQ, GAPD, β-actin and 18S) or for three genes after removal of 18S (UBQ, GAPD and β-actin).

Data of candidate housekeeping genes ($n = 4$)						
Factor	UBQ	GAPD	β-actin	18S	*BestKeeper* ($n = 4$)	*BestKeeper* ($n = 3$)
N	31	31	31	31	31	31
GM [CP]	20.83	21.48	18.26	12.83	17.99	20.14
AM [CP]	20.86	21.5	18.29	12.97	18.03	20.16
Min [CP]	19.22	19.65	16.71	9.87	16.44	18.65
Max [CP]	23.19	24.3	20.8	16.58	20.86	22.65
SD [± CP]	0.76	0.74	0.79	1.5	0.9	0.69
CV [% CP]	3.66	3.45	4.34	11.57	4.98	3.43
Min [x-fold]	−3.06	−3.56	−2.93	−7.81	2.93	2.8
Max [x-fold]	5.13	7.05	5.82	13.44	7.31	5.7
SD [± x-fold]	±1.7	±1.67	±1.73	±2.83	±1.86	±1.61

Abbreviations: N: number of samples; GM [CP]: the geometric mean of CP; AM [CP]: the arithmetic mean of CP; Min [CP] and Max [CP]: the extreme values of CP; SD [± CP]: the standard deviation of the CP; CV [% CP]: the coefficient of variance expressed as a percentage on the CP level; Min [x-fold] and Max [x-fold]: the extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient; SD [± x-fold]: standard deviation of the absolute regulation coefficients.

Analysis of expression stability of housekeeping genes

Descriptive statistics of the derived crossing points were computed for each HKG: the geometric mean (GM), arithmetic mean (AM), minimal (Min) and maximal (Max) value, standard deviation (SD), and coefficient of variance (CV). All CP data are compared over the entire study, including control and all treatment groups. Herein, four genes, each of $n = 31$, were investigated. The x-fold over- or under-expression of individual samples towards the geometric mean CP are calculated and the multiple factor of their minimal and maximal values, expressed as the x-fold ratio and its standard deviation, are presented [Equations (1) and (2), Table 1]. These x-fold regulation results are corrected via the factor specific real-time PCR efficiency, calculated according Equation (3).

$$Min[x\_fold] = \boldsymbol{E}^{\min[CP]-GM[CP]}, \quad (1)$$

$$Max[x\_fold] = \boldsymbol{E}^{\max[CP]-GM[CP]}. \quad (2)$$

The corresponding real-time PCR efficiency **(E)** can be obtained in two ways. It can be computed either as sample specific (Tichopad *et al.* 2003, Liu & Saint 2002), or as factor specific (Rasmussen 2000) according to Equation (3). The slope of linear regression model fitted over log-transformed data of serially diluted input DNA concentrations plotted against their CPs (Rasmussen 2000, Pfaffl 2001). The maximal efficiency of PCR is **E = 2** where every single template is replicated in each cycle and the minimal value is **E = 1**, corresponding to no replication.

$$\boldsymbol{E = 10^{-1/\text{slope}}}. \quad (3)$$

After the descriptive statistics for the individual candidate, HKG expression levels have been calculated, the first estimation of HKG expression stability can already be done, based on the inspection of calculated variations (SD and CV values). According to the variability observed, HKGs can be ordered from the most stably expressed, exhibiting the lowest variation, to the least stable one, exhibiting the highest variation. Any studied gene with the SD higher than 1 (= starting template variation by the factor 2) can be considered inconsistent (Table 1).

From the genes considered stably expressed, the *BestKeeper Index* specific for the respective sample is calculated as the geometric mean (3) of its candidate HKGs CP values [Equation (4)], where z is the total number of HKGs included.

$$BestKeeper\ Index = \sqrt[z]{CP_1 \times CP_2 \times CP_3 \times ..... \times CP_z}. \quad (4)$$

Table 2. Repeated pair-wise correlation analysis and correlation analysis of candidate housekeeping genes (HKG). A: Genes are pair-wise correlated one with another and then with the *BestKeeper* index ($n = 4$); B: results of the correlation analysis HKG versus *BestKeeper* index is shown ($n = 3$).

2A: Repeated pair-wise correlation analysis ($n = 4$)				
vs.	HKG 1 UBQ	HKG 2 GAPD	HKG 3 β-actin	HKG 4 18S
HKG 2	0.771	–	–	–
p-Value	0.001	–	–	–
HKG 3	0.728	0.803	–	–
p-Value	0.001	0.001	–	–
HKG 4	0.486	0.554	0.576	–
p-Value	0.006	0.001	0.001	–
BestKeeper vs.	UBQ	GAPD	β-actin	18S
Coeff. of corr. [r]	0.766	0.823	0.832	0.902
p-Value	0.001	0.001	0.001	0.001
Repeated pair-wise correlation analysis ($n = 4$) HKG vs. *BestKeeper* index out of 4				
HKG	HKG 1 UBQ	HKG 2 GAPD	HKG 3 β-actin	HKG 4 18S
Coeff. of corr. [r]	0.766	0.823	0.832	0.902
Coeff. of det. [r^2]	0.587	0.677	0.692	0.814
p-Value	0.001	0.001	0.001	0.001
2B: Repeated pair-wise correlation analysis ($n = 3$)				
vs.	HKG 1 UBQ	HKG 2 GAPD	HKG 3 $\beta-$actin	HKG 4
HKG 2	0.771	–	–	–
p-Value	0.001	–	–	–
HKG 3	0.728	0.803	–	–
p-Value	0.001	0.001	–	–
HKG 4	–	–	–	–
p-Value	–	–	–	–
BestKeeper vs.	UBQ	GAPD	β-actin	
Coeff. of corr. [r]	0.903	0.929	0.926	
p-Value	0.001	0.001	0.001	
Repeated pair-wise correlation analysis ($n = 3$) HKG vs. *BestKeeper* index out of 3				
HKG	HKG 1 UBQ	HKG 2 GAPD	HKG 3 β-actin	HKG 4
Coeff. of corr. [r]	0.903	0.929	0.926	–
Coeff. of det. [r^2]	0.815	0.863	0.857	–
p-Value	0.001	0.001	0.001	–

Analysis of the inter-HKG relations

To estimate inter-gene relations of all possible HKG pairs, numerous *pair-wise correlation analyses* are performed. Within each such correlation the *Pearson correlation coefficient* (r) and the probability p value are calculated (Tables 2A and 2B). All those highly correlated HKGs are combined into an index. Then, correlation between each candidate HKG and the index is calculated, describing the relation between the index and the contributing candidate HKG by the Pearson correlation coefficient (r), coefficient of determination (r^2) and the p-value (Tables 2A and 2B).

Analysis of target genes

Target gene (TG) expression data are statistically processed in the same way like those of HKGs, e.g., their GM, AM, SD, CV, Min. and Max. values (Table 4). Also here the *pair-wise correlation analyses* are performed to see any relation between pairs of TGs (Table 3).

To consider if a TG exhibits an expression pattern comparable or different from another TG, they are inspected in the same way as described for the HKGs and finally also correlated with the calculated index. Then, the same parameters of the correlation analysis as for HKG are calculated (Tables 4 and 5). Where a high correlation of TG to the index occurs, an expression pattern comparable to the HKG can be assumed. TGs expressed differentially from the index show no significance and sometimes even inverse correlation coefficients.

Analysis of sample integrity and expression stability within HKGs

Since the occurrence of outliers among prepared samples can obscure the accuracy of the estimation, individual samples are tested (herein $n = 31$) for their integrity (e.g. mRNA respectively cDNA quantity and quality) as well as their expression stability. An intrinsic variance (InVar) of expression for a single sample is calculated as a mean value square difference of single sample's CP value for one factor from a mean CP value of the same factor [Equation (5)].

$$\boldsymbol{InVar}_m[\pm\mathrm{CP}] = \frac{1}{n-1}\sum_{i=1}^{n}\left(C\,P_n^m - meanCP_n\right)^2, \quad (5)$$

Table 3. Descriptive statistics of target genes. Ten genes are analysed based on their CP values in the same way like HKGs (legend in Table 1).

Data of target genes ($n = 10$)										
Factor	TG 1 IGF-1	TG 2 IGF-2	TG 3 IGF-R-1	TG 4 IGF-R-2	TG 5 BP-1	TG 6 BP-2	TG 7 BP-3	TG 8 BP-4	TG 9 BP-5	TG 10 BP-6
N	31	31	31	31	31	31	31	31	31	31
GM [CP]	29.29	23.12	24.56	37.88	29.23	30.51	29.95	31.09	26.7	30.32
AM [CP]	29.31	23.14	24.59	37.89	29.38	30.53	30	31.13	26.74	30.36
Min [CP]	27.59	21.54	23.17	36.54	24.59	28.47	27.13	28.88	23.52	27
Max [CP]	31.42	25.52	27.68	39.92	35.33	33.09	36.47	34.41	29.66	33.52
SD [± CP]	0.79	0.86	0.88	0.66	2.49	0.77	1.32	1.12	1.25	1.1
CV [% CP]	2.71	3.71	3.59	1.74	8.47	2.51	4.41	3.59	4.68	3.64
Min [x-fold]	−3.26	−2.99	−2.63	−2.54	−24.92	−4.12	−7.06	−4.64	−9.06	−10.02
Max [x-fold]	4.37	5.29	8.67	4.1	68.62	5.96	91.86	9.96	7.78	9.16
SD [± x-fold]	1.73	1.81	1.84	1.58	5.61	1.7	2.5	2.17	2.38	2.15

Table 4. Pair-wise correlation analysis of the ten target genes. Target genes are pair-wise correlated among each other. Pearson correlation coefficient (r) and the value of probability p are shown.

Repeated pair-wise correlation analysis [Pearson correlation coefficient (r)]										
vs.	IGF-1 TG 1	IGF-2 TG 2	IGF-R-1 TG 3	IGF-R-2 TG 4	BP-1 TG 5	BP-2 TG 6	BP-3 TG 7	BP-4 TG 8	BP-5 TG 9	BP-6 TG 10
TG 2	0.367	–	–	–	–	–	–	–	–	–
p-Value	0.043	–	–	–	–	–	–	–	–	–
TG 3	0.43	0.586	–	–	–	–	–	–	–	–
p-Value	0.016	0.001	–	–	–	–	–	–	–	–
TG 4	0.073	−0.03	−0.068	–	–	–	–	–	–	–
p-Value	0.699	0.874	0.714	–	–	–	–	–	–	–
TG 5	−0.003	−0.176	0.345	0.064	–	–	–	–	–	–
p-Value	0.984	0.345	0.057	0.729	–	–	–	–	–	–
TG 6	0.257	0.331	0.309	0.102	−0.019	–	–	–	–	–
p-Value	0.163	0.069	0.091	0.587	0.921	–	–	–	–	–
TG 7	0.252	0.612	0.81	−0.006	0.377	0.189	–	–	–	–
p-Value	0.172	0.001	0.001	0.976	0.037	0.307	–	–	–	–
TG 8	0.257	0.832	0.711	0.109	0.057	0.291	0.738	–	–	–
p-Value	0.163	0.001	0.001	0.56	0.759	0.112	0.001	–	–	–
TG 9	0.044	−0.232	0.054	0.269	0.139	0.321	−0.056	0.016	–	–
p-Value	0.812	0.211	0.774	0.144	0.453	0.078	0.766	0.929	–	–
TG 10	0.335	0.379	0.283	0.174	−0.123	0.563	0.116	0.425	0.441	–
p-Value	0.066	0.035	0.123	0.35	0.508	0.001	0.534	0.017	0.013	–
BestKeeper vs.	TG 1	TG 2	TG 3	TG 4	TG 5	TG 6	TG 7	TG 8	TG 9	TG 10
Coeff. of corr. [r]	0.402	0.775	0.665	0.192	−0.041	0.18	0.696	0.811	−0.132	0.266
p-Value	0.025	0.001	0.001	0.302	0.827	0.33	0.001	0.001	0.477	0.147

Table 5. Results of pair-wise correlation analysis of target gene vs. *BestKeeper* index.

Repeated pair-wise correlation analysis: TG vs. *BestKeeper* ($n = 3$ HKG)										
	TG 1 IGF-1	TG 2 IGF-2	TG 3 IGF-R-1	TG 4 IGF-R-2	TG 5 BP-1	TG 6 BP-2	TG 7 BP-3	TG 8 BP-4	TG 9 BP-5	TG 10 BP-6
Coeff. of corr. [r]	0.4	0.78	0.67	0.19	−0.04	0.18	0.7	0.81	−0.13	0.27
Coeff. of det. [r^2]	0.16	0.6	0.44	0.04	0	0.03	0.48	0.66	0.02	0.07
p-Value	0.025	0.001	0.001	0.302	0.827	0.33	0.001	0.001	0.477	0.147

where the term in brackets denotes a difference of respective CP observation (n) of respective HKG (m) from the average CP value of the same HKG. Results are expressed in CP units [± CP] or as percentage of the mean [± %CP]. Further, it is expressed as an efficiency corrected intrinsic variation of x-fold, over- or under-expression of studied factor in the respective sample towards the mean CP of the same factor [± x-fold] [Equation (6)].

$$\boldsymbol{InVar}_m[\pm x\_fold] = \boldsymbol{E}_m^{InVar[\pm CP]}. \quad (6)$$

If justified, strongly deviating samples, due to inefficient sample preparation, incomplete reverse transcription or sample degradation, can be removed from the *BestKeeper* index calculation and its consistence and reliability thus be increased. A removal is recommended over a 3-fold over- or under-expression.

Results and discussion

In this paper, the Excel based tool *BestKeeper,* is presented and was tested in biological materials. The software is able to compare expression levels of up to ten HKGs together with ten TGs, each in up to hundred biological samples. Raw data input in the *BestKeeper* software are on Excel tables, separate for HKGs and TGs. Calculation proceeds in the background and results obtained can be easily printed out. All CP data are plotted in Excel table attached figures. It determines the 'optimal' HKGs employing the *pair-wise correlation analysis* of all pairs of candidate genes and calculates the geometric mean of the 'best' suited ones. The weighted index is correlated with up to ten target genes using the same pair-wise correlation analysis. Data observations are in form of raw CP (Rasmussen 2001) or threshold cycles (Ct) (Livak 2001) generated by a real-time PCR platform. The raw CPs seem to be best estimators of the expression levels as they are (in most cases) normally distributed and a parametric test can thus be performed. Expression data phrased in CP units is comparable with a logarithmic data transformation to the basis of two. This also gives the CP datasets the *Gaussian* distribution justifying usage of parametric methods.

Heterogeneous variance between groups of differently expressed genes, however, invalidates the use of *Pearson correlation coefficient*. Low expressed genes where CPs were obtained somewhere around cycles 30–35 surely show different variance compared to high expressed genes with CPs around 15 or even less. Such two samples cannot be correlated parametrically but on their ranks only. New version of the *BestKeeper* tool is, being prepared, employing also non-parametric methods such as the *Spearman* and *Kendall Tau correlation coefficient*. These methods are useful where genes with very different expression levels are compared.

Herein the software tool was tested on experimental data obtained from total RNA samples extracted from bovine *corpora lutea* under the *Estrumate* treatment. Compared to UBQ, GAPD and β-actin, in 18S, high CP variation in the expression was observed – a reason to exclude 18S from index calculation. On the other hand, all four HKG correlated very well one with another – a reason to retain 18S in the index. Both alternatives were tested and the correlation matrix for four candidate genes are shown in the Tables 2A and 2B. The expressions of UBQ, GAPD and β-actin showed CP variations around 0.75 CP (0.74 CP $<$ SD $<$ 0.79 CP), whereas the 18S expression showed high CP variation (SD = 1.5 CP) as well as up-/down-regulation (± 2.83-fold). Therefore the weighted index, calculated out of 4 candidates, showed a SD = 0.90 cycles. After the exclusion of 18S from index its variation decreased (SD = 0.69 cycles). The analysis showed a strong correlation ($0.766 < r < 0.902$) for all candidates.

Good consistence of the index was proved as its contributing housekeeping genes were tightly correlated with it. In both trials (with and without 18S) a good correlation with high significance level ($p < 0.001$) was observed, but after 18S removal, the significance increased (only rounded data are shown) and the correlation between the remaining HKGs and the index increased ($0.903 < r < 0.929$).

In above-shown way, a robust standardising index based on three HKGs was defined for a gene expression studies on bovine *corpora lutea*. Three genes represent a realistic calculation basis in a common laboratory and the minimal necessary number for a good performance of the analysis.

Correlation analyses of target genes showed (Table 3) that there were some significantly correlated genes (e.g. IGFBP-3 vs. IGFBP-4 and IGF-R-1 vs. IGFBP-4). Similarly, some target genes such as IGF-2, IGF-R-1, IGFBP-3 and BP-4 showed high correlation with the *BestKeeper* index. Tight correlation between applied internal standard and target gene shows regulation stability similar to the standard. Such a target gene can possibly be incorporated into the index.

Numerous genes were differentially expressed in this study, as they were not significantly correlated with the index (e.g. IGF-1, IGF-R-2, IGFBP-1, IGFBP-2, IGFBP-5, IGFBP-6). Some genes exhibited even totally inverse regulation of the expression, e.g. IGFBP-1 and IGFBP-5 as reflected by the negative correlation index (Tables 4 and 5).

Sample integrity was investigated using all four HKGs (no data shown). The InVar of the investigated 31 samples had low CP variation as well as on x-fold level. Three of the investigated samples showed higher variations in the expression stability of the HKGs, but still in the range of acceptance within a 3-fold regulation.

The earlier presented *GeNorm* software (Vandesompele *et al.* 2002) is restricted to the HKG analysis only, whereas, in *BestKeeper* software, additionally up to ten TGs can be analysed. Once a robust *BestKeeper* index was constructed, it can be applied as an expression standard in the same way like any single housekeeping gene. For a subsequent data processing, the CP datasets can be imported into analysis software tools such as *REST* (Pfaffl *et al.* 2002), *GeNorm* (Vandesompele *et al.* 2002) or *Q-Gene* (Muller *et al.* 2002). The *BestKeeper* application and necessary information about data processing and handling can be downloaded on http://www.wzw.tum.de/gene-quantification/bestkeeper.html

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Appendix 6

J. Vet. Med. A (re-submitted)
Blackwell Verlag, Berlin

PHYSIOLOGY

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Effects of Mycophenolic acid (MPA) on inosine monophosphate dehydrogenase (IMPDH) I and II mRNA expression in white blood cells and various tissues in sheep

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with 4 figures and 4 tables

Summary

Mycophenolic acid (MPA) is a mycotoxin and commonly found as *Penicillium* genus secondary metabolite in feedstuffs and silages. Feeding with MPA contaminated silages may modulate the immune system in the farm animals and can cause appetite lost, ketosis, paralysis and abortion. The aim of the present study was to characterize the long term MPA effect on the both IMPDH isoforms I and II mRNA expression in WBC and various tissue of healthy sheep. In treated animals 300 mg MPA/day/sheep were applied. In all investigated tissues the IMPDH I and II mRNA was abundant: white blood cells (WBC), spleen, thymus, ileum, jejunum, kidney liver, pharyngeal and mesenterial lymph node. A efficiency corrected relative-quantification of the IMPDH type I and II isoforms mRNA were performed by normalizing with the constant housekeeping gene expression of ß-actin. High IMPDH I mRNA expression levels were examined in kidney > mesenterial lymph node > jejunum > spleen > pharyngeal lymph node. Medium and low abundance was found in ileum > WBC > liver > thymus. Type II mRNA was highly expressed in liver > thymus > jejunum. In pharyngeal lymph node > spleen > ileum > mesenterial lymph node> kidney >WBC medium to low IMPDH II mRNA concentrations were detected. Under MPA treatment the IMPDH I mRNA expression was not significantly regulated in WBC, only trends of down- and up-regulation were observed. Surprisingly in jejunum an up-regulation could be observed ($p<0.05$). In pharyngeal lymph node a tendency of down-regulation was shown. This may caused from frequent ruminant activities and frequent exposition of MPA to the pharyngeal lymph nodes. In contrast to type I mRNA expression, IMPDH II mRNA was significantly down-regulated in ileum (3.4-fold, $p<0.01$) and tendencies in down-regulation could be seen in jejunum (5.1-fold, $p = 0.14$). In addition significant down-regulation of IMPDH II gene expression over the entire feeding experiment could be shown in WBC of MPA treated animals compared to untreated ($p<0.05$). In conclusion, the recent study demonstrates that feeding sheep with MPA contaminated silage did not induce IMPDH I mRNA expression in various tissues and blood, except in jejunum, but has suppressive effects on IMPDH II mRNA expression in WBC and ileum.

Introduction

The main issue of feed quality and storage is the problem of mould spoilage (Auerbach *et al.,* 1998), where fungal growth, production of mycotoxins and allergenic spores reduces the nutritional value of feed. *Penicillium roqueforti* is present in most silages (Auerbach *et al.,* 1998; Boysen *et al.,* 2000) and produces mycotoxins, e.g. mycophenolic acid (MPA) (Schneweis *et al.,* 2000). Feeding with MPA contaminated silages may modulate the immune system in the farm animals and can cause appetite lost, ketosis, paralysis and abortion.

Inosine monophosphate dehydrogenase (IMPDH) has been identified to be an affected key enzyme in the *de novo* synthesis of the guanine nucleotides (Bentley, 2000). Guanine nucleotides are the essential precursor for RNA and DNA biosynthesis and therefore, the central role in purine metabolism (Weber *et al.,* 1996). Inhibition of IMPDH through MPA is associated with changes in nucleic acid synthesis, gene expression, signalling and ultimately, cell proliferation and differentiation, especially in blood leucocytes (Zhang *et al.,* 1999). Mammalian IMPDH is very sensitive to MPA inhibition, by uncompetitive inhibition of both mammalian IMPDH isoforms (Sintchak & Nimmesgern, 2000; Digits & Hedstrom, 2000). In human medicine the ester pro-drug of MPA is used as an immunosuppressive agent for the prevention of acute graft

rejection in kidney, heart and liver transplantation (Mele and Halloran, 2000). IMPDH enzyme exists in two encoded isoforms, IMPDH isoforms I and II. Each protein consists of 514 amino acids with 84% sequence identity (Weber *et al.,* 1996). Both isoforms were shown to be expressed in most tissues (Jain *et al., 2004*). Whereas IMPDH I is predominantly expressed in resting human lymphocytes, IMPDH II is the inducible enzyme responsible for cell differentiation and is mainly found in activated lymphocytes (Allison & Eugui, 2000) as well as in leukemic and tumour cells (Konno *et al*., 1991; Nagai *et al*., 1992). MPA is a fivefold more potent inhibitor of IMPDH II than of IMPDH I mRNA expression (Carr *et al*., 1993) and is capable of inducing apoptosis in human T-lymphocytes (Cohn *et al.,* 1996). The aim of the present study was to characterize the MPA effect on the IMPDH I and II mRNA expression in WBC and various immunological tissue of healthy sheep over a long term MPA treatment over nine weeks.

Material and Methods

Animal experiment and tissue sampling

Eighteen six-month old male cross-breed sheep (Merino Landschaf x Schwarzkopfschaf), with no clinical signs of any infectious diseases were investigated over nine weeks. The animals were kept in a pen of 15 m^2 with a 12 hour light cycle and a temperature of 15°C to 20°C. The sheep were fed with maize silage (2 kg/animal daily) and hay (semi ad libitum) according to Kirchgeßner (1987). Water was supplied ad libitum. The general health status, including the rectal temperature, was examined daily. The body weight was measured at the beginning, in the middle and at the end of the trial (day 0, 22, 40). Blood samples were collected twice per week via the *vena jugularis* before and during treatment in week 1, 2, 5 and 9. Miscellaneous hematological and biochemical parameters were examined: number of erythrocytes, thrombocytes, leukocytes, differential blood picture, the packed cell volume, the concentrations of haemoglobin, glucose, bilirubin, activity of alanine amino transferase, aspartate amino transferase, and glutamate dehydrogenase. After treatment period the sheep were slaughtered at the slaughterhouse in Grub (EU official slaughterhouse of the Bayerische Landesanstalt für Tierzucht, Poing, Germany) tissue samples were collected 10-15 minutes *post mortem* and stored in liquid nitrogen until total RNA preparation.

Reverse-transcription (RT)

Total RNA was extracted from tissue and WBC according to earlier publications (Pfaffl *et al.,* 1998). Synthesis of first strand complementary DNA (cDNA) from one µg total RNA was performed in 50 µl with MMLV RNAse H-reverse transcriptase (Promega, Madison, WI, USA) using random hexamer primers (MBI Ferments, St. Leon-Rot, Germany), according to the manufactures instructions in a conventional thermocycler (Personal Cycler, Biometric, Göttingen, Germany).

Oligonucleotide primer and identification of ovine IMPDH isoforms

The ovine IMPDH I and IMPDH II sequences was unpublished. Primer pairs were designed, according to available human and mouse IMPDH 1 sequences (NCBI Acc. No. XM-093044, BG276942) and human, mouse, rat, chimpanzee and zebrafish (NCCI Acc. No. BC006124, BC052671, BC060585, XM-516452, BC046905) using HUSAR Analysis Package software version 4.0 (http://genome.dkfz-heidelberg.de/biounit/) for a multiple sequence alignment and *clustal* primer design (HUSAR, DKFZ, Heidelberg, Germany). IMPDH I (sense primer 5'-CCT CGA TTC TCC CAG GAT TCA TAG-3'; anti-sense primer 5'-ATG CCG CAT CTT GGC CTC CA-3'; RT-PCR product length 307 bp) and IMPDH II primers (sense primer 5'-CCT GAA TTC CAG GCC AAT GAA G-3'; anti-sense primer 5'-GAG GAG ATG ATG CCC ACC A-3'; RT-PCR product length 191 bp) were manufactured by MWG Biotech (Ebersberg, Germany) and optimised in real-time RT-PCR. To confirm constant housekeeping gene expression levels in the studied total RNA extractions derived from leucocytes and targeted tissues, β-actin was used as housekeeping gene in real-time RT-PCR. β-actin primers (sense-primer 5'-AAC TCC ATC ATG AAG TGT GAC G-3'; and anti-sense primer 5'-GAT CCA CAT CTG GAA GG-3'; RT-PCR product length 234 bp) were designed using a published ovine nucleic acid sequence (NCBI Acc. No. U39357).

Quantification by real-time PCR

Quantitative analysis of PCR products was carried out in the LightCycler 1.0 (Roche Diagnostics, Mannheim, Germany). One µl reverse transcribed total RNA (20 ng/µg) was used as PCR template. Further reaction components for each reaction were: 1.2 µl $MgCl_2$ (3 mM), 0.2 µl forward primer (4 pmol), 0.2 µl reverse primer (4 pmol), 1.0 µl LightCycler - Fast Start Master SYBR Green I (Roche Diagnostics) finishing with PCR water up to an end volume of 10 µl. Prior to amplification an initial denaturation step (10 min at 95°C) was performed to ensure a complete cDNA denaturation and polymerase activation. Every real-time RT-PCR was performed with 40 cycles. A forth segment was introduced in the cycle procedure at the end of each amplification cycle, with an elevated temperature acquisition point (IMPDH I and II 86°C and β-actin 87°C). Due to the fourth segment and SYBR Green I quantification we achieved only specific RT-PCR product for an exact quantification (Pfaffl, 2001). Amplified RT-PCR products underwent a melting curve analysis to specify the integrity of amplification and finally a cooling step was performed.

Relative quantification model

As exact quantification model we performed an efficiency-corrected relative-quantification (Pfaffl *et al.*, 2002). The crossing point (CP) was determined using the *"second Derivate Maximum Method"* determination by the LightCycler Software version 3.5 (Roche Molecular Biochemicals). In the applied quantification model, according to the shown equation, the IMPDH mRNA

expression level was normalized via the β-actin expression level (see displayed equation). The individual CP means and corresponding real-time PCR efficiencies were calculated with *REST-XL* software (Pfaffl *et al.*, 2002).

$$ratio = \frac{(E_{IMPDH\ 1})^{\Delta CP_{IMPDH\ 1}\ (mean\ Control\ -\ mean\ MPA)}}{(E_{beta\text{-}actin})^{\Delta CP_{beta\text{-}actin}\ (mean\ Control\ -\ mean\ MPA)}}$$

Determination of real-time PCR efficiency

The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation (Rasmussen, 2001): $\mathbf{E = 10^{[-1/slope]}}$, using a pooled total RNA dilution row (each tissue n = 18 animals) ranging from 0.40 pg to 50 ng cDNA input, performed in triplicates.

Statistical evaluation

Statistical analysis of relative expression levels in real-time PCR was calculated by the relative expression software *REST-XL* using *Pair wise Fixed Reallocation Randomisation Test* (Pfaffl *et al.,* 2002). The used mathematical model is based on the PCR efficiency and the mean CPs deviation between the MPA treatment group and control group, normalized via the housekeeping gene expression (herein β-actin) as a reference. Statistical evaluation of the linear regression of the β-actin reference gene CP over nine week in WBC was performed using SAS (SAS Institute, 1999).

Results

Extracted total RNA contents showed no significant variations during MPA treatment period and compared to control groups (data not shown). In all investigated tissues (WBC, spleen, thymus, liver, kidney, jejunum, ileum, pharyngeal- and mesenterial lymph node) the IMPDH I, IMPDH II and β-actin mRNA was abundant. Real-time RT-PCR products showed the expected length of 307 bp, 191 bp and 234 bp, respectively and a single peak in the melting curve analysis. Sequences were additionally verified by sequence analysis (Medigenomics, Martinsried, Germany). Newly elucidated ovine IMPDH I sequence was published (NCBI Acc. No. AJ535327) in sequence databases EMBL (European Molecular Biology Library; http://www.ebi.ac.uk/) and GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.html). Homology on mRNA level resulted in 92.8% and 79.8%; homology on protein level 91.2% and 75.4%, compared to the published human and mouse sequences (XM-093044, BG276942), respectively. All investigated tissues and WBC showed tissue specific real-time PCR efficiencies for the mRNA quantification assays (Table 1).

β-actin housekeeping gene expression

β-actin mRNA expression data showed constancy in the regulation in WBC (Table 2) as well as in the investigated tissues (Table 3). β-actin was justified as a proper choice for a non-regulated housekeeping gene and optimal reference gene. A linear regression (Figure 1) over nine weeks of MPA treatment was plotted for each experimental group (n = 66/67). The mean CP value was 18.5 ± 1.8 for control (n = 67) and 19.1 ± 2.6 for MPA group (n = 66). Linear and non significant regressions reflected the constant and not regulated β-actin expression and showed no significances between the groups and treatment duration.

Table 1. Tissue specific real-time PCR efficiencies of β-actin (reference gene), IMPDH I and IMPDH II (target genes) assays in ovine tissues (each n = 18).

Target	β-actin PCR efficiency	IMPDH I PCR efficiency	IMPDH II PCR efficiency
White blood cells (WBC)	1.66	2.14	1.77
Mesenterial lymph node	1.55	1.97	1.66
Pharyngeal lymph node	1.56	1.81	1.81
Spleen	1.74	1.94	1.81
Thymus	1.56	1.99	1.66
Liver	1.80	1.96	1.72
Kidney	1.63	2.05	1.87
Jejunum	1.63	2.08	1.68
Ileum	1.70	1.95	1.66

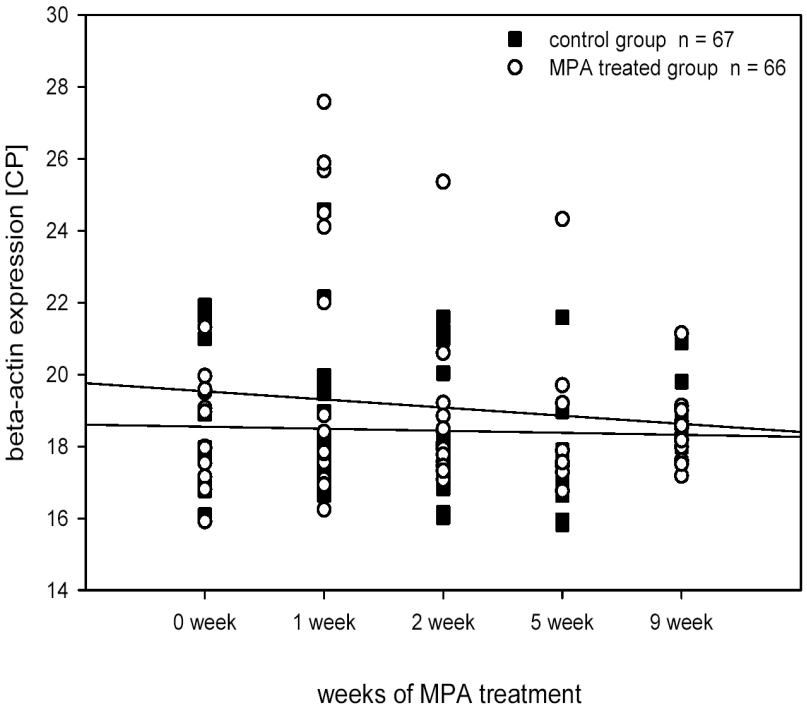

Fig. 1. Linear regression plotted from housekeeping gene (β-actin) mRNA expression data based on crossing points (CP) in control group (0 mg MPA/sheep/day; n = 67) and MPA treated group (300 mg MPA/sheep/day; n = 66).

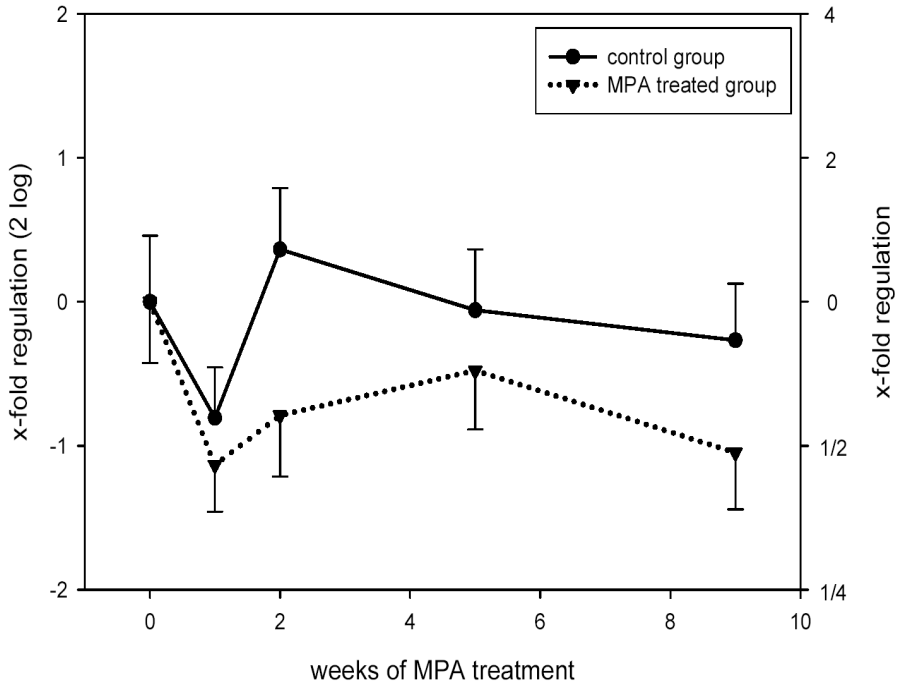

Fig. 2. Effect of MPA treatment time on IMPDH II mRNA gene expression in white blood cells over 9 weeks. Expression is displayed as mean ± std. dev. (2 log scale) for control group (n = 9) blood sampling at 5 time points, compared with MPA treated animals (n = 9) and normalised to the mean expression at start (100% = 2^0 = 1).

IMPDH isoforms I and II mRNA expression in WBC

In the untreated control group IMPDH I expression seems to be minor regulated, mainly down-regulated (data not shown). None of the trends in control group was significant. In the MPA treated group IMPDH I mRNA was expressed as a trend of up- and down-regulation as follows: in week one an up-regulation was detected (factor 6.2, $p = 0.14$) and then sharp drop of down-regulation by the factor 0.19 in the second week ($p = 0.13$). This down-regulation trend remained until the last experimental week.

IMPDH II mRNA expression in WBC was much more regulated (Figure 2). On the y-axes the mRNA IMPDH II expression level was plotted over nine weeks, compared to week 0 (before treatment). Data were shown as logarithmic expression levels (2-log) or mRNA up- or down-regulation gene regulation (x-fold levels compared to start). Week 0 of the MPA treatment was taken as 100% (1-fold expression level = 2^0). Within the untreated control group, IMPDH II mRNA was expressed as a trend of up-and down regulation. No differences in IMPDH II expression was obtained until week 1, whereas from that point on mRNA expression was lower in the MPA treated group ($p<0.05$). After the first week, IMPDH II expression was down regulated by the factor 1.8, followed by an up-regulation and reaching a peak after two weeks. Subsequently, the IMPDH II expression fell again until the end of the experiment to reach almost the starting value. Despite variations in the mRNA expression, none of the regulations in the control group was significant. Within the treated group, a down-regulation after the first week could be recognized. Until week 5, the mRNA expression of IMPDH II seemed to be increased, starting to decrease to the level of week 1 up to week 9. Comparing both groups over the entire feeding experiment, significant 1.4-fold down regulation ($p<0.05$) of IMPDH II gene expression was observed in the MPA treated group.

IMPDH I and II mRNA expression in tissues

To get an impression on the mean IMPDH I, IMPDH II and β-actin mRNA expression level in different tissues, all 18 investigated animals were grouped (Table 3). The IMPDH type I and II levels were normalized via the internal β-actin expression. For IMPDH I high expression levels were examined in kidney > mesenterial lymph node > jejunum > spleen > pharyngeal lymph node. Medium and low abundance expression for IMPDH I was found in ileum > WBC > liver, and even lowest expression in thymus. In liver > thymus > jejunum high mRNA expression levels were found for IMPDH II, whereas in pharyngeal lymph node > spleen > ileum > mesenterial lymph node medium and low abundance expression was observed. Lowest gene expression for IMPDH II was detected in kidney.

The graph (figure 3) shows the effects of MPA treatment on the IMPDH I expression levels compared to the non treated control. The expression of the MPA effect on absolute IMPDH I mRNA gene in tissues stated a significant up-regulation in jejunum (5.7-fold, $p < 0.05$). Tendencies of up-regulation were observed in thymus (4.3-fold, $p = 0.28$), ileum (3.1-fold, $p = 0.25$), liver (3.0-fold, $p = 0.28$), kidney (2.8-fold, $p = 0.15$), spleen (2.7-fold, $p = 0.58$) and mesenterial lymph node (1.7-fold, $p = 0.65$), but none of the expression level changed significantly. In pharyngeal lymphnode a trend of 3.1-fold down-regulation was examined ($p=0.33$)

Figure 4 shows the effects of MPA treatment on the IMPDH II expression levels in different tissues compared to the non treated group. Tendencies in down-regulation of IMPDH II expression could be seen in jejunum (5.1-fold, $p = 0.14$). In pharyngeal and mesenterial lymphnode, thymus, spleen, liver and kidney expression of IMPDH II did not change significantly ($p = 0.85$). Only in ileum a significant down-regulation ($p<0.01$) by the factor 3.4 could be observed.

Table 2. Linear regression of housekeeping gene (β-actin) expression data versus time (0 to 9 weeks). Data are shown on crossing point (CP) basis, in control group (0 mg/day/animal; n = 67) and MPA treated group (300 mg MPA/day/animal; n = 66).

control group:	CP = 18.451 + [0.003 * week of treatment]	p = 0.96
MPA group:	CP = 19.421 + [0.107 * week of treatment]	p = 0.27

	β-actin control group	**β-actin MPA treatment group**
Mean CP ± std. dev. CP	18.5±1.8	19.1±2.6
n	67	66
p values	0.96	0.27
Coefficient of correlation	0.006	-0.27
Coefficient of regression	0.0031	-0.107

Table 3. Mean expression level of IMPDH I, IMPDH II and β-actin mRNA in investigated tissues. Calculation is based on mean CP data of 18 animals normalized via the internal housekeeping expression of β-actin (Δ CP = mean CP_{IMPDH} – mean $CP_{\beta actin}$, and ΔΔ CP) and conversed in x-fold expression compared to the lowest expression level in thymus (= 1.0).

Tissue	IMPDH I [CP ± std. dev.]	β-actin [CP ± std. dev.]	Δ CP	ΔΔ CP	$2^{\Delta\Delta CP}$
White blood cells (WBC)	32.31 ± 2.89	18.44 ± 0.92	13.87	+2.41	5.3
Mesenterial lymph node	30.05 ±11.08	18.17 ± 7.16	11.87	+4.41	21.3
Pharyngeal lymph node	31.55 ± 8.49	18.93 ± 5.13	12.36	+3.92	15.1
Spleen	31.55 ± 8.22	19.58 ± 5.11	11.97	+4.31	19.8
Thymus	37.73 ± 4.66	21.45 ± 2.64	**16.28**	**0.00**	**1.0**
Liver	37.93 ± 3.33	23.79 ± 2.30	14.15	+2.13	4.4
Kidney	29.23 ± 2.17	17.60 ± 1.67	11.63	+4.65	25.1
Jejunum	32.68 ± 2.74	20.73 ± 2.40	11.96	+4.32	20.0
Ileum	33.38 ± 2.64	20.34 ± 2.10	13.04	+3.24	9.4
	IMPDH II [CP ± std. dev.]	**β-actin [CP ± std. dev.]**	**Δ CP**	**ΔΔ CP**	**$2^{\Delta\Delta CP}$**
White blood cells (WBC)	28.69 ± 1.79	18.44 ± 0.92	**10.25**	**0.00**	1
Mesenterial lymph node	27.02 ± 3.8	18.17 ± 7.16	8.85	+1.40	2.6
Pharyngeal lymph node	27 ± 2.22	18.93 ± 5.13	8.07	+2.18	4.5
Spleen	27.96 ± 3.16	19.58 ± 5.11	8.38	+1.87	3.7
Thymus	28.72 ± 2.5	21.45 ± 2.64	7.27	+2.98	7.9
Liver	29.96 ± 1.91	23.79 ± 2.30	6.17	+4.08	16.9
Kidney	27.09 ± 0.82	17.60 ± 1.67	9.49	+0.76	1.7
Jejunum	28.45 ± 2.35	20.73 ± 2.40	7.72	+2.53	5.8
Ileum	28.86 ± 1.67	20.34 ± 2.10	8.52	+1.73	3.3

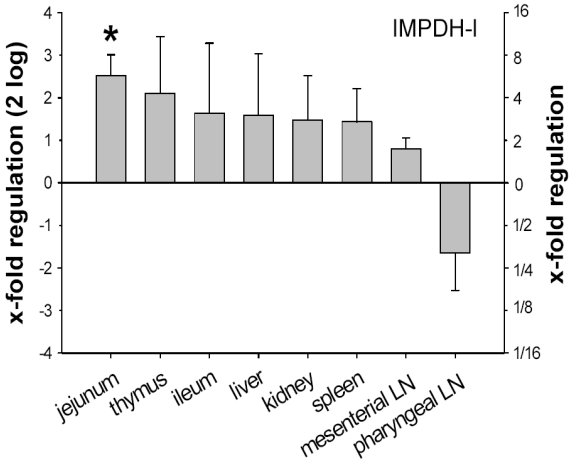

Fig. 3. Effect of long term MPA treatment on IMPDH I mRNA gene expression changes (mean ± std. dev.) in immunological active tissues [jejunum ($p<0.05$), thymus, ileum, liver, kidney, spleen, mesenterial and pharyngeal lymph node] in sheep (n = 9) compared to untreated control group (n = 9).

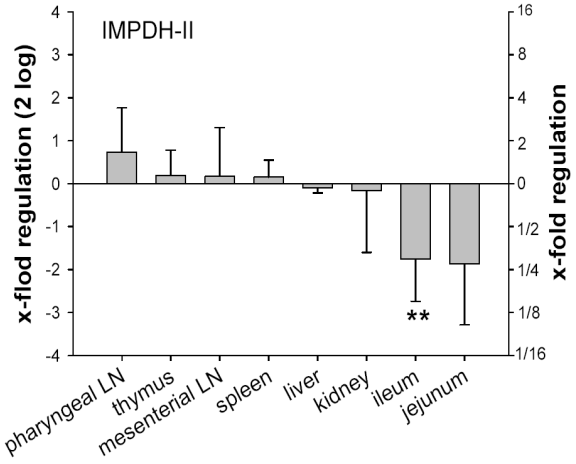

Fig. 4. Effect of long term MPA treatment on IMPDH II mRNA gene expression changes (mean ± std. dev.) in immunological active tissues [pharyngeal lymph node, thymus, mesenterial lymph node, spleen, liver, kidney, ileum ($p<0.01$) and jejunum] in sheep (n = 9) compared to untreated control group (n = 9).

Discussion

Mycotoxin contaminated silage showed clinical symptoms in cows: e.g. ketosis, mastitis, paralysis, and abortion (Häggblom, 1990). To detect the immune suppressive potency of MPA, total RNA contents, marker gene IMPDH I and IMPDH II mRNA expression levels were quantified. Quantitative real-time RT-PCR is a very sensitive method to quantify the low amounts of mRNA molecules in various tissues and offers important insights into the local expression of regulation processes. The reliability of such a relative quantification assay depends on the sensitivity, low assay variations and on a sufficiently wide quantification range. Herein we described the design and validation of a relative-quantification using an efficiency-corrected calculation model in real-time RT-PCR (Pfaffl, 2002) according to the demands of a fast cycle PCR (Rasmussen, 2001).

The central questions of our investigations in sheep were, whether or not MPA contamination in silage has an influence on the gene expression in WBC and numerous tissues, and which tissues are more sensitive to MPA. High MPA concentrations were measured in liver (2.6 µg/g) or kidney (3.6 µg/g) where MPA is metabolised and excreted (Mohr, 2003). High MPA concentrations exert potential immunosuppressive properties.

The chosen housekeeping gene β-actin showed no regulation in all investigated tissue samples. In jejunum a significant up-regulation of IMPDH I expression could be shown. For ileum, liver and kidney only trends of regulation could be observed, which correlates with the high MPA concentrations found. A significant change in IMPDH II gene expression could only be detected in ileum with a down-regulation by the factor 3.4. The numbers of IgG or IgM positive plasma cells decreased in the ileum with increasing MPA-doses. These results suggest, that MPA in a high concentration may affect the morphology of immune organs of sheep (Baum et al., 2005).

Additional in jejunum a down-regulation with tendency to significance was visible ($p<0.14$). The results in ileum an jejunum meets the expectations as these tissues are maximally exposed to MPA because of its position in the body. Surprisingly in the immunological active tissues like spleen, thymus, pharyngeal- and mesenterial lymph node no significant regulation of IMPDH type I and II isoforms could be observed. A trend of IMPDH I down-regulation was observed in pharyngeal lymph node. This might be due to a frequent ruminant activity and possible absorption through the oral cavity mucus, even at low effective MPA concentrations. With increasing dose of MPA, germinal centres in the retropharyngeal lymph nodes displayed an activated morphology with numerous centroblasts. In the highest dose group, the sheep showed shrinkage of thymic lobules (Baum et al., 2005). In the digestive tract, next to the immunological active ileum, we expected a much more significant suppression of the marker gene expression.

Total RNA concentrations were quantified in all described tissues, but no significant effects were evident after drug application. Further the expression levels for IMPDH I and II for both groups were monitored in WBC over the nine weeks treatment period. We observed no significant regulation of the proposed marker gene IMPDH I mRNA as well as of the housekeeping gene β-actin. A possible explanation for the missing significance, was the high inter-individual variability in the IMPDH I mRNA expression. In contrast to these results a significant down-regulation of IMPDH II gene expression in WBC could be seen in the MPA treated sheeps compared to the control group. MPA is known to be a fivefold more potent inhibitor of this isoform than of IMPDH I and has stronger cytostatic effect on lymphocytes than on other cell types (Allison & Eugui, 2000). The results of this investigation support this findings. These effects could also be noticed in the small bowel mucosa (jejunum, ileum), where large numbers of intraepithelial lymphocytes are found in the villus epithelium (Guy-Grand *et al.*, 1998). But in both cases, the

Table 4. MPA dose comparison between our study in sheep in relation to a mice model (Bentley, 2000)

mice model (BW 18-21 g)	MPA ppm [µg/g tissue]	effect	sheep model (BW 60 kg)	MPA ppm [µg/g tissue]
1 mg MPA/mouse/day	47	"no effect"	10 mg MPA/sheep/day	0.17
5 mg MPA/mouse/day	238	"prolonged sickness"	70 mg MPA/sheep/day	1.16
10 mg MPA/mouse/day	476	"lethal dose"	300 mg MPA/sheep/day	5.00

greatest effects caused by MPA in WBC seemed to be achieved within the first weeks of treatment.

It was reported that MPA dose-dependently blocks the proliferation of human lymphocytes after stimulation with T- or B-cell mitogens *in vitro* and inhibits IMPDH type I and type II enzymes almost equipotently (Jain *et al*., 2001; Eugui *et al.* 1991). Our results are in contrast to Jain *et al.* (2004), who showed that treatment of human peripheral blood mononuclear cells with up to 1 µM MPA does not result in any significant changes in IMPDH I or type II mRNA and protein levels induced in response to mitogenic stimulation and in unstimulated cells. This may have been due to the different technologies used for quantifying mRNA expression resulting in no significant changes of IMPDH gene expression levels. On the other hand it might be assumed that MPA has no influence on the IMPDH mRNA steady-state level in lymphocytes. The observed missing immunosuppressive effects of MPA via IMPDH expression inhibition in our study may have resulted from the absence of substantial stimulation of the immune system by pathogens. No clinical signs and significant alterations in the monitored haematological and clinical parameters were noted from our observations in sheep treated with natural occurring MPA concentrations even in the applied *"worst case scenario"* group.

The experimental MPA concentrations in sheep were designed according to a physiological silage uptake, an average MPA concentration of 1.8 mg of MPA/kg body weight in ruminants (Schneweis *et al.,* 2000). Taking all available concentration of MPA uptake data together and create a *"worst case scenario"*, an oral MPA application of 300 mg/sheep/day is possible (Auerbach *et al.,* 1998). We applied a maximal dose of 5.0 µg MPA/g sheep BW (= 5 ppm) in relation to 250 µg MPA/g mouse body weight (= 238 ppm), where an effective immune system suppression and prolonged sickness was achieved (Table 4). The highest applied doses in sheep was about 50-fold less than in mice model. However, in mice such high *per os* doses are required for an effective immune suppression (Bentley, 2000).

There might be degradation of the drug in the rumen by low pH values or degradation by micro organisms (Sweeney & Dobson, 1998). It is clear that microbial metabolism of mycotoxins take place and there is evidence of total mineralization of certain mycotoxins, the enzymatic pathway and degradation products produced in that manner is not yet fully known. A biotechnology approach to the detoxification of mycotoxins requires certain input from a research point of view and could be a vital tool (Sweeney & Dobson, 1998).

In future studies valuable data could be obtained by direct administration of MPA in tissue cell culture in order to see physiological pathways mechanisms on gene expression level of MPA immunosuppressive action. Possible candidates are cultured WBC and tissue cell lines with a large dose range of MPA. There, we could have better insight using real-time RT-PCR assays analyzing the IMPDH I and II mRNA and other immune system activation marker gene responses.

Conclusion

To conclude, in this study we have demonstrated:
(1) Significant down-regulation of IMPDH II gene expression in WBC over nine week MPA treatment;
(2) MPA treatment showed significant up-regulation of IMPDH I in jejunum and a significant down-regulation of IMPDH II gene expression in ileum;
(3) MPA treatment showed tendencies of up-regulation of IMPDH I mRNA in thymus, ileum, liver, kidney, spleen and mesenterial lymph node;
(4) MPA treatment showed tendencies of down-regulation of IMPDH I in pharyngeal lymph node and IMPDH II in jejunum;
(5) MPA may have immuno-suppressive effects via lower IMPDH I expression in pharyngeal lymph node, and via IMPDH II expression in the gastro intestinal immune system in ileum and jejunum;
(6) After feeding with natural occurring MPA concentrations in contaminated silage, immune suppressive effect are only given via IMPDH II expression inhibition;

Acknowledgments

This research was supported by a DFG grant. It is a pleasure to acknowledge Prof. D. Schams for reading this manuscript and fruitful discussion. We thank D. Tetzlaff for excellent technical assistance.

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