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Effects of conjugated linoleic acids on the function of bovine immune cells *ex vivo* and *in vitro*

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Abbreviations

AB	Alamar blue
ANOVA	analysis of variance
ap	ante partum
BHB	β-hydroxybutyrat
BP	base pair
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
CLA	conjugated linoleic acid
CON	control group
ConA	concanavalin A
COX-2	cyclooxygenase-2
C_T	cycle threshold
DM	dry matter
FA	fatty acid
FAME	fatty acid methyl ester
FBS	fetal bovine serum
FLI	Friedrich-Loeffler-Institute
for	forward
IBDV	infectious bursal disease virus
IC_{50}	half maximal inhibitory concentration
IFN-γ	interferon γ
Ig	immunoglobulin
IG	initial group
IL	interleukin
Io	ionomycin
LPS	lipopolysaccharide
LTB_4	leukotriene B4
MHC	major histocompatibility complex
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MUFA	monounsaturated fatty acids
NADH	nicotinamide adenine dinucleotide hydroxide
NEFA	nonesterified fatty acids in plasma
ns	not significant
OD	optical density
p	probability
PA	phytanic acid
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PG	prostaglandin
PHA	phytohemagglutinin
PMA	phorbol 12-myrisat 13 acetate
PMN	polymorphonuclear leucocytes
PMR	partial mixed ration
pp	post partum
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid

PWM	pokeweed mitogen
qRT-PCR	quantitative real-time polymerase chain reaction
RAW	mouse macrophage cell line
rev	reverse
Rn	derivative reporter
RPMI	Rosewell Park Memorial Institute
RT	room temperature
RXR	retinoid X receptor
SFA	saturated fatty acids
SI	stimulation index
TCR	T cell receptor
TNF-α	tumor necrosis factor-α
WC	workshop cluster

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General Introduction

Conjugated linoleic acids (CLA) are a group of C18:2 fatty acids (FA) which are characterized by the conjugated position of their double bonds. CLA mainly occur in dairy products and meat from ruminants (FRITSCHE AND STEINHART 1998). The discovery that CLA is an anticancerogenic compound in ground beef was the starting point of a special interest in this group of FA (HA ET AL. 1987). Besides its anticancerogenic effects (reviewed by LEE ET AL. (2005)) CLA shows several other health beneficial properties (BHATTACHARYA ET AL. 2006). CLA act antidiabetic (HOUSEKNECHT ET AL. 1998) and antiatherogenic which was first described by LEE ET AL. (1994) for rabbits. The effects of CLA on atherosclerosis might be due to decreasing the development of early atherosclerotic lesions and altering the production of atherogenic lipoproteins by the liver (MCLEOD ET AL. 2004). Furthermore, CLA alter the body composition towards lean body mass and reduced body fat content, which is mainly shown in animal models (DELANY ET AL. 1999; STANGL 2000). The effects on body composition in humans, however, are not as dramatic as those shown in animal studies (BHATTACHARYA ET AL. 2006).

Although CLA are produced by ruminal microorganisms (KEPLER ET AL. 1966) and therefore naturally occurring in ruminants, CLA are supplemented to dairy cows rations. This is done since CLA decrease milk fat synthesis, which is ascribed to the *trans*-10,*cis*-12 isomer (BAUMGARD ET AL. 2000). Dairy cows experience a period of huge energy demands and reduced dry matter (DM) intake around parturition, which is associated with a negative energy balance (GOFF AND HORST 1997; GRUMMER ET AL. 2004). Furthermore, this time period is characterized by a suppressed immune state of the dairy cow (MALLARD ET AL. 1998). Reducing the energy output of the milk through a decreased fat content might improve the energy balance of the cow. This aspect is of great interest in dairy cattle and was therefore frequently investigated (e. g. by BERNAL-SANTOS ET AL. (2003) and CASTAÑEDA-GUTIÉRREZ ET AL. (2005)). The effects of CLA on immune functions in dairy cows were not part of these studies. Effects of CLA on immune functions are reported e. g. for chicken (ZHANG ET AL. 2005A; ZHANG ET AL. 2005B), dogs (NUNES ET AL. 2008), pigs (CHANGHUA ET AL. 2005; LAI ET AL. 2005A), rats (RAMIREZ-SANTANA ET AL. 2009A; RAMÍREZ-SANTANA ET AL. 2011) and humans (TRICON ET AL. 2004; SONG ET AL. 2005). Studies investigating the effect of CLA on immune functions in ruminants are rare and especially feeding trials with dairy cows in order to investigate these aspects were not performed yet. Hence, research in this area is needed.

Background

1 Structure, Isomers and Synthesis of CLA

CLA are a group of positional and geometric isomers of linoleic acid. Linoleic acid is a fatty acid with 18 C atoms and 2 double bonds. CLA are characterized by the conjugated position of the double bonds. Various isomers, depending on the position of the conjugated double bonds and their *cis-trans* configuration, exist. Altogether, the term CLA includes 28 different isomers (COLLOMB ET AL. 2006). The most important isomers are the *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 isomer (Figure 1). These two isomers are the main components in CLA supplements.

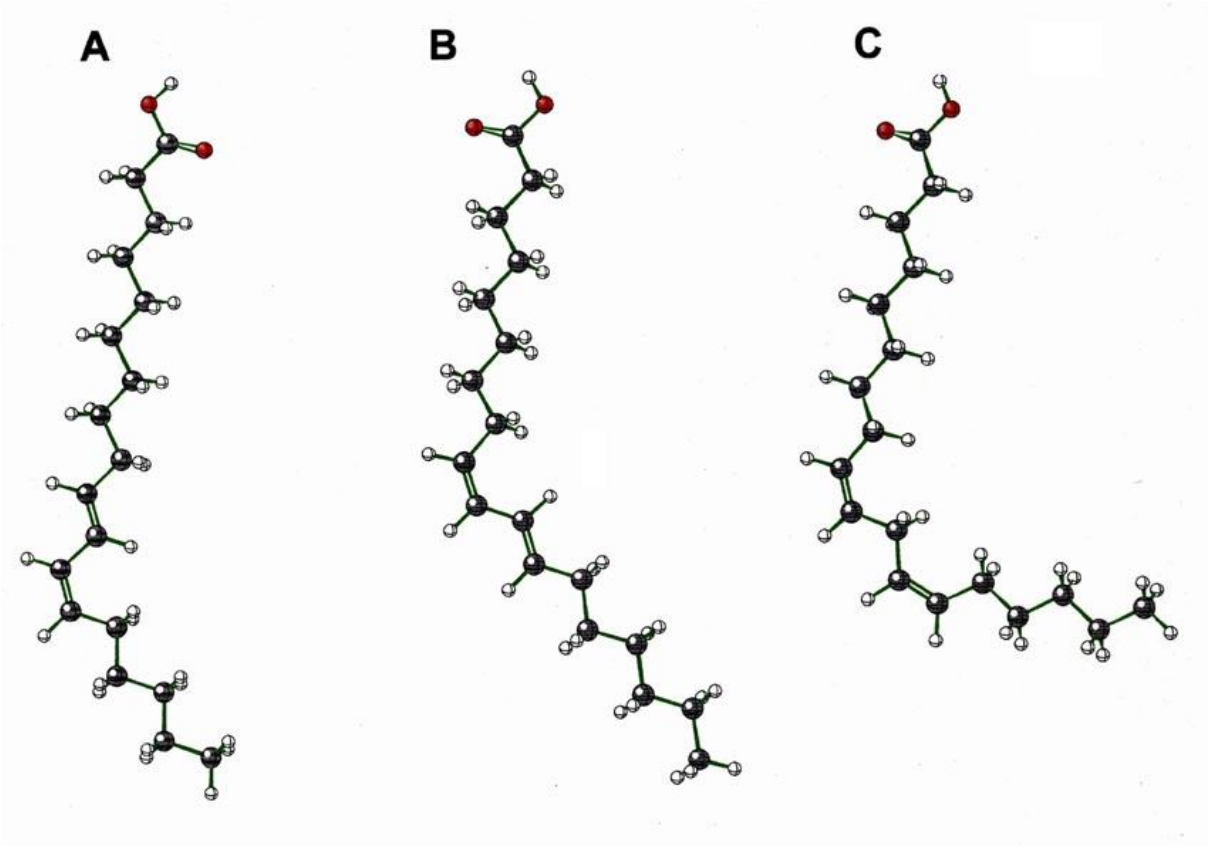

Figure 1 Chemical structure of conjugated linoleic acid isomers and linoleic acid. Fatty acids are *trans*-10,*cis*-12 octadecadienoic cid (A), *cis*-9,*trans*-11 octadecadienoic acid (B) and *cis*-9,*cis*-12 octadecadienoic acid (linoleic acid) (C), according to BAUMAN ET AL. (1999).

CLA, especially the *cis*-9,*trans*-11 isomer are predominantly found in dairy products and in meat from ruminants (FRITSCHE AND STEINHART 1998). There are two synthesis pathways of *cis*-9,*trans*- CLA (Figure 2): first of all *cis*-9,*trans*-11 CLA is an intermediate product of the biohydrogenation of linoleic acid by ruminal microorganisms (KEPLER ET AL. 1966). The

reaction starts with the isomerization of the *cis*-12 double bond by linoleate isomerase. The product of the reaction is *cis*-9,*trans*-11 CLA. This initial step is followed by a reduction of the *cis* double bond and *trans*-11 C18:1 arises. The hydration of the remaining double bond is slower, thus the *trans*-11 C18:1 accumulates in the rumen and is available for absorption (BAUMAN ET AL. 1999).

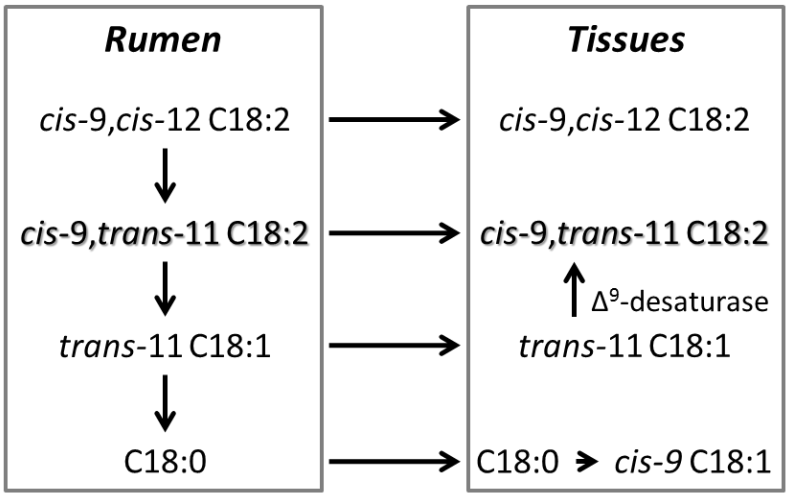

Figure 2 Synthesis pathways of *cis*-9,*trans*-11 CLA via hydrogenation by microorganisms in the rumen (left) and Δ^9-desaturase in tissues (right), modified according to BAUMAN ET AL. (1999)

The other source of CLA is the endogenous synthesis by Δ^9-desaturase, whereby *trans*-11 C18:1 serves as a precursor (GRIINARI ET AL. 2000). This synthesis pathway is also observed in non-ruminants, e. g. in humans (TURPEINEN ET AL. 2002). The *trans*-10,*cis*-12 isomer is a major component in CLA supplements, but its amount in dairy products and meat is rather low (STEINHART ET AL. 2003). However, it is also formed by ruminal microorganisms (LEE AND JENKINS 2011).

Most commercially available CLA supplements contain a mixture of CLA isomers, whereby *trans*-10,*cis*-12 and *cis*-9,*trans*-11 make the largest amounts. They are produced industrially by hydrogenation or alkali-isomerization of linoleic acid (BANNI 2002).

2 CLA and the immune system

FA in general play an important role within immune cells (e. g. for energy generation, as components of the cell membrane and precursor of lipid mediators). Thus, it is likely that an alteration of the FA supply influences the immune function (CALDER 2008). Possible effects, which result from an altered FA supply and therefore cause changes in the profile of phospholipids in immune cells, are presented in Figure 3. For CLA two main mechanisms are discussed. One is the suppression of generating inducible eicosanoids. The other one is based

on targeting peroxisome proliferator-activated receptors (PPAR) (O'SHEA ET AL. 2004; BASSAGANYA-RIERA AND HONTECILLAS 2006).

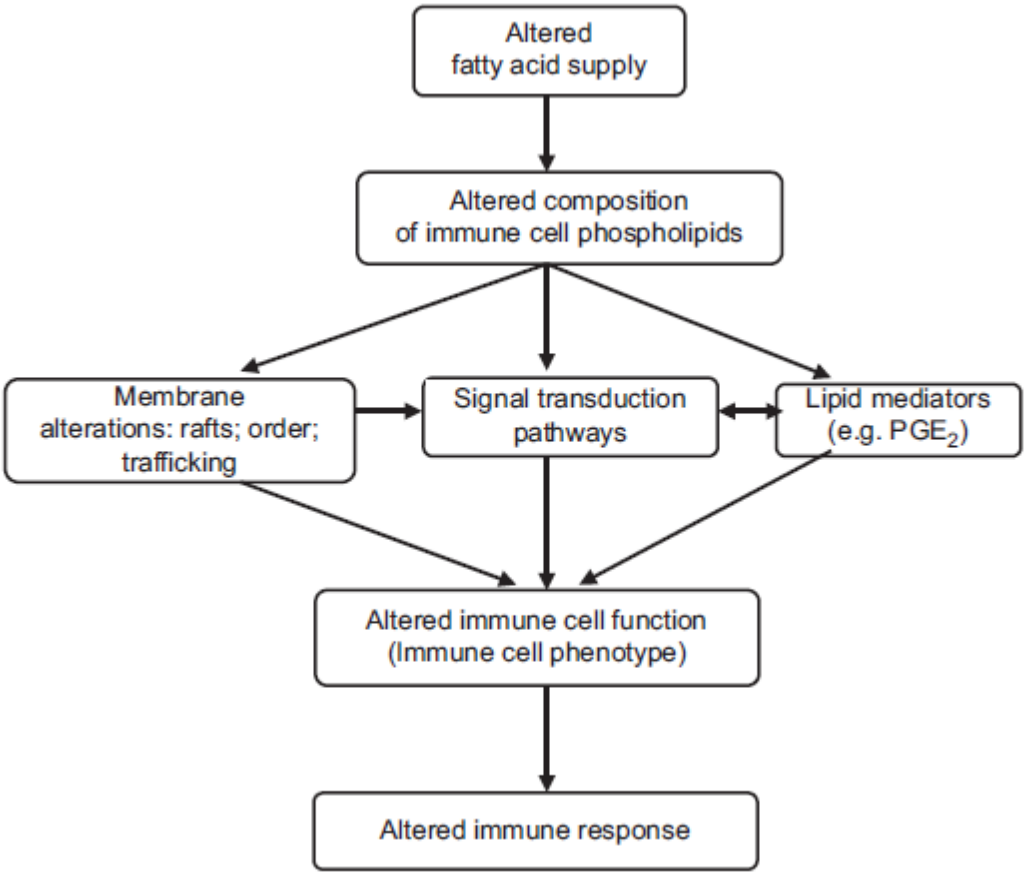

Figure 3 Mechanisms by which an altered FA supply can affect immune response (CALDER 2008)

MOYA-CAMARENA ET AL. (1999) showed that CLA, especially the *cis*-9,*trans*-11 isomer, are potent PPAR-α ligands and activators. CLA feeding differently modulated the expression of PPAR-α and PPAR-γ in virally infected pigs (BASSAGANYA-RIERA ET AL. 2003). An up regulation of PPAR-γ in response to CLA in peripheral blood mononuclear cells (PBMC) was found in several studies (KANG ET AL. 2007; KIM ET AL. 2011A). KIM ET AL. (2011A) indicated that the effects of the *trans*-10,*cis*-12 isomer on TNF-α expression in porcine PBMC are PPAR-γ dependent.

Various CLA isomers are capable of reducing the mRNA expression of cyclooxygenase-2 (COX-2), its promoter activity, and the end product prostaglandin (PG) E_2 in mouse macrophages in response to interferon γ (IFN-γ) *in vitro* (YU ET AL. 2002). In human vascular smooth muscle cells CLA isomers inhibited the tumor necrosis factor α (TNF-α) induced release of prostaglandins (PGE_2 and PGI_2). In this process PPAR-γ is at least partly involved (RINGSEIS ET AL. 2006). *In vivo* the secretion of PGE_2 in plasma was deceased in CLA fed

pigs (LAI ET AL. 2005A). However, CLA supplementation did not alter the secretion of PGE_2 (ALBERS ET AL. 2003) and leukotriene B4 (LTB_4) (KELLEY ET AL. 2001) from human mitogen stimulated PBMC *ex vivo*.

2.1 Effects of CLA on immune function in monogastric species

Proliferation assays and the expression or production of cytokines is often investigated to characterize immune functions. A cell fraction that is frequently used for these studies are PBMC. The advantage of this cell fraction is its easy isolation from the blood of donors by density gradient centrifugation (BOYUM 1968). PBMC mainly contain lymphocytes and monocytes (BOYUM 1968). Besides, immune cells from secondary organs like the spleen (so called splenocytes) are often investigated.

2.1.1 Effects of CLA on the proliferation of immune cells

The effects of CLA on the proliferation of immune cells differ among the investigated species, cell types, and the applied supplement (isomer composition of the supplement).

In rats, the proliferative response of phorbol 12-myrisat 13 acetate (PMA) and ionomycin (Io)-stimulated splenocytes was 10% lower in CLA fed rats (RAMIREZ-SANTANA ET AL. 2009A). The supplement contained 80% *cis*-9,*trans*-11 and 20% *trans*-10,*cis*-12 CLA. This down regulation was also observed when rats were supplemented with CLA during suckling and in early infancy (RAMÍREZ-SANTANA ET AL. 2011). No effect on proliferation of splenocytes was observed when pups were supplemented during gestation and suckling (via supplementation of the dam) or oral supplementation during suckling (RAMIREZ-SANTANA ET AL. 2009B). Supplementation of mice with the *cis*-9,*trans*-11 or the *trans*-10,*cis*-12 isomer did not alter the proliferation of splenocytes in response to the T cell mitogen Concanavalin A (ConA) or the B cell mitogen lipopolysaccharide (LPS) (KELLEY ET AL. 2002).

CLA increased the stimulation index of PBMC and the percentage of $CD8^+$ cells from CLA fed pigs and therefore had a positive effect on cellular immune response (LAI ET AL. 2005A). In contrast, MORAES ET AL. (2012) did not observe effects of CLA feeding on LPS or ConA stimulated proliferation of lymphocytes or $CD4^+$/$CD8^+$ subsets. This is in line with another study carried out with pigs where no effect on immune parameters was found (no effect on T-cell response to ConA, the percentages of white blood cells, monocytes, lymphocyte number, and T cell subpopulations) (WIEGAND ET AL. 2011).

In chicken, CLA feeding increased the mitogen stimulated proliferation of PBMC (ZHANG ET AL. 2005A; ZHANG ET AL. 2005B) or bursa lymphocytes (LONG ET AL. 2011), whereas the

proliferation of mononuclear cells from the spleen was not altered compared to cells from chicken fed a basal diet without fat supplementation (TAKAHASHI ET AL. 2007).

No influence of dietary CLA on the proliferation of PBMC was also found in several human studies. There was no influence of CLA on the proliferation of B and T lymphocytes obtained from healthy women who received 3.9 g CLA isomers per day for a period of 63 days (KELLEY ET AL. 2000). Similarly, proliferation of mitogen-stimulated PBMC from healthy men was not altered by supplementation of CLA for 12 weeks (1.7 g per day 50% *cis*-9,*trans*-11 and 50% *trans*-10, *cis*-12 or 1.6 g per day 80% *cis*-9,*trans*-11 and 20% *trans*-10, *cis*-12) compared to control (sunflower oil) (ALBERS ET AL. 2003). In another human study, the authors did as well not observe differences in proliferation of PBMC following an 8-week CLA supplementation and stimulation with mitogenic or antigenic compounds, except the group who received a supplement which contained mainly the *cis*-9,*trans*-11 isomers, when stimulated with phytohemagglutinin (PHA). In that case, proliferation was stronger after CLA supplementation (NUGENT ET AL. 2005). A dose-dependent decrease in lymphocyte activation was observed in a study with healthy men after supplementation with either the *cis*-9,*trans*-11 or the *trans*-10,*cis*-12 isomer (contains 80-85% of the requested isomer) (TRICON ET AL. 2004).

However, the effects of CLA supplementation are diverse and do not allow a clear conclusion. Even within the same species, the effects are different.

2.1.2 Expression and production of cytokines

Cytokines are proteins which are secreted by a great variety of cells, particularly by immune cells. They regulate the differentiation and activation of these cells and play a major role in inflammatory reactions and immune response (FORTH ET AL. 2001). Studies investigating the effects of CLA on cytokine mRNA expression are summarized in Table 1, those on cytokine production in Table 2.

Table 1 Overview of studies investigating the mRNA expression of cytokines in response to CLA

Species	Isomer/diet	Cell/tissue	Stimulation	Findings	Reference
In vitro studies					
pig	*cis*-9,*trans*-11	PBMC	LPS	IL-1β, IL-6, TNF-α ↔	CHANGHUA ET AL. (2005)
	trans-10,*cis*-12			IL-1β, IL-6, TNF-α ↓	
	1:1 mix of *cis*-9,*trans*-11 and *trans*-10,*cis*-12			IL-1β, IL-6, TNF-α ↔	
pig	*trans*-10,*cis*-12	PBMC	-	TNF-α ↔	KANG ET AL. (2007)
pig	*trans*-10,*cis*-12	PBMC	-	TNF-α ↑	(KIM ET AL. 2011A)
			LPS	TNF-α ↓	
mice	*cis*-9,*trans*-11	dendritic cells	-	IL-12p70, IL-12p40 ↓	LOSCHER ET AL. (2005)
			LPS	IL-10 ↑	
mice	*trans*-9,*trans*-11	RAW cells	LPS	IL-1α, IL-1β, IL-6 ↓	LEE ET AL. (2009)
	cis-9,*cis*-11			TNF-α ↔	
	trans-10,*cis*-12				
	cis-11,*trans*-13				
	cis-9,*trans*-11			TNF-α ↔	
Ex vivo studies					
pig	2% CLA	spleen	LPS	IL-1β ↔ IL-6, TNF-α ↓ IL-10 ↑	CHANGHUA ET AL. (2005)
		thymus	LPS	IL-1β ↔ IL-6, TNF-α ↓ IL-10 ↑	
mice	CLA high beef diet vs. CLA low beef diet	bone marrow-derived dendritic cells	LPS	IL-10 ↑ IL-12p70 ↓	REYNOLDS ET AL. (2009)
humans	2.2 g/d CLA	PBMC	ConA	IL-2 ↔	MULLEN ET AL. (2007)
chickens	1% CLA	bursa tissue	IBDV challenge	IFN-γ, IL-6 ↓	LONG ET AL. (2011)

↑ parameter increased due to CLA supplementation, ↓ parameter decreased due to CLA supplementation, ↔ no effect of CLA; abbreviations: PBMC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor α; RAW, mouse macrophage cell line; IBDV, infectious bursal disease virus; IFN-γ, interferon γ

It is stated that CLA have anti-inflammatory properties (BASSAGANYA-RIERA AND HONTECILLAS 2006), which is indicated e. g. by altered cytokine profiles. TNF-α is an important proinflammatory cytokine (TIZARD 2004). Reduced TNF-α levels following CLA supplementation were found in the plasma of mice (YANG AND COOK 2003) and in serum of pigs (ZHAO ET AL. 2005). In addition to the serum TNF-α level in the latter study the gene expression and the production of TNF-α from PBMC were decreased as well. However, it is also reported that CLA enhanced the secretion of the inflammatory cytokines TNF-α and interleukin (IL)-6 in LPS-stimulated splenocytes from CLA fed mice (KELLEY ET AL. 2002). YAMASAKI ET AL. (2003) did find an increased production of TNF-α only in *cis*-9,*trans*-11 fed mice compared to control and *trans*-10,*cis*-12 fed mice. PHA-stimulation increased TNF-α production after 8 weeks of supplementation in a study carried out with healthy volunteers (NUGENT ET AL. 2005). However, the effect was seen after supplementation of CLA and linoleic acid, which served as control, and is thus considered as an effect of polyunsaturated fatty acids (PUFA) and not specifically of CLA.

An effect of the CLA dose on production of cytokines is described by TRICON ET AL. (2004). Both isomers (*cis*-9,*trans*-11 and *trans*-10,*cis*-12) affected the production of TNF-α and IL-1β from LPS-stimulated PBMC, but not IL-10, IL-6 and IL-8. Stimulation with ConA influenced the production of IFN-γ, TNF-α, IL-10 and IL-5, but not IL-2 and IL-4. IL-10 is an important immunoregulating cytokine which acts anti-inflammatory and protects against a pathological over-induction of inflammation (COUPER ET AL. 2008). Based on the cytokine profile *ex vivo* and *in vitro*, CHANGHUA ET AL. (2005) found clear anti-inflammatory effects of CLA on both mRNA and protein level. *In vitro*, TNF-α production of PBMC was reduced due to *trans*-10,*cis*-12 and *cis*-9,*trans*-11 as well as a mixture of both isomers, whereas the expression of TNF-α was only reduced by *trans*-10,*cis*-12 CLA. Therefore, the authors concluded that the anti-inflammatory effect is attributed to the *trans*-10,*cis*-12 isomer (CHANGHUA ET AL. 2005). No differences between CLA and control group in IL-10 secretion were observed in porcine PBMC in response to LPS or Io and PMA (MALOVRH ET AL. 2009).

Studies carried out with mice obtained rather conflicting results in cytokine secretion. Whereas KELLEY ET AL. (2002) and YAMASAKI ET AL. (2003) did not find an effect of CLA supplementation on IL-4 secretion in ConA-stimulated splenocytes a reduction of IL-4 was observed in a study of YANG AND COOK (2003).

In human studies, CLA supplementation did not influence the secretion of IL-10 (MULLEN ET AL. 2007), IL-4 (ALBERS ET AL. 2003; NUGENT ET AL. 2005), TNF-α (MULLEN ET AL. 2007), and INF-γ (ALBERS ET AL. 2003) from stimulated PBMC.

Studies investigating the effects of CLA supplementation on cytokines on both, the mRNA and protein level, demonstrate mostly similar effects on both parameters (LOSCHER ET AL. 2005; KIM ET AL. 2011B). In contrast (KANG ET AL. 2007) observed increased TNF-α production on protein level, but not on the expression from porcine PBMC.

Table 2 Overview of studies investigating the production of cytokines in response to CLA

Species	**Isomer/diet**	**Tissue**	**Stimulation**	**Findings**	**Reference**
In vitro studies					
pig	*cis*-9,*trans*-11	PBMC	LPS	IL-1β, IL-6, TNF-α ↓	CHANGHUA ET AL. (2005)
	trans-10,*cis*-12			IL-1β, IL-6, TNF-α ↓	
	1:1 mix of *cis*-9,*trans*-11 and *trans*-10,*cis*-12			IL-1β, IL-6, TNF-α ↓	
pig	*trans*-10,*cis*-12	PBMC	-	TNF-α ↑	KANG ET AL. (2007)
pig	*trans*-10,*cis*-12	PBMC	- LPS	TNF-α ↑ TNF-α ↓	(KIM ET AL. 2011A)
mice	*cis*-9,*trans*-11	RAW macrophages	LPS	TNF-α ↓	YANG AND COOK (2003)
	trans-10,*cis*-12			TNF-α ↔	
	CLA mixture			TNF-α ↓	
mice	*cis*-9,*trans*-11	dendritic cells	LPS	IL-10 ↑ IL-12p70, IL-12p40 ↓ TNF-α ↔	LOSCHER ET AL. (2005)
In vivo/*ex vivo* studies					
pig	2% CLA	plasma	LPS	IL-1β, IL-6, TNF-α ↓ IL-10 ↑	CHANGHUA ET AL. (2005)
pigs	2% CLA	plasma	-	IL-1β ↓	LAI ET AL. (2005A)
pigs	1.2% CLA	PBMC	PMA/Io	IFN-γ ↓ TNF-α, IL-10 ↔	MALOVRH ET AL. (2009)
mice	0.5% CLA	plasma	LPS	TNF-α ↓	YANG AND COOK (2003)
		resident peritoneal macrophages	LPS, IFN-γ	TNF-α ↔	
		spenocytes	ConA (*in vitro*)	IL-2 ↑ IL-4 ↓ IL-2/IL-4 ↑	

Table 2 continued

Species	Isomer/diet	Tissue	Stimulation	Findings	Reference
mice	5 g/kg *cis*-9,*trans*-11 and 5 g/kg *trans*-10,*cis*-12	spleen lymphocytes	ConA	IL-2, IL-4, IL-5, IFN-γ, TNF-α ↔	YAMASAKI ET AL. (2003)
	10 g/kg *cis*-9,*trans*-11			IL-2, IL-4, IL-5, IFN-γ ↔ TNF-α ↑	
	10 g/kg *trans*-10,*cis*-12			IL-2, IL-4, IL-5, IFN-γ, TNF-α ↔	
mice	5 g/kg *cis*-9,*trans*-11	splenocytes	LPS	TNF-α, IL-6 ↑	KELLEY ET AL. (2002)
			ConA	IL-2 ↔ IL-4 ↓	
	5 g/kg *trans*-10,*cis*-12		LPS	TNF-α, IL-6 ↑	
			ConA	IL-2 ↔ IL-4 ↓	
mice	CLA high beef diet vs. CLA low beef diet	bone marrow-derived dendritic cells	LPS	IL-12p70, IL-12p40, IL-1β, IFN-γ ↓	REYNOLDS ET AL. (2009)
rats (lean vs. obese rats)	0.4% *cis*-9,*trans*-11	splenocytes	PMA/Io	IFN-γ ↓ (lean), IL-4 ↓ (obese)	RUTH ET AL. (2008)
			ConA	IL-2 ↓ (lean), IL-4 ↓ (obese)	
			PWM	IL-2 ↓ (lean), IL-10 ↑ (obese)	
	0.4% *trans*-10,*cis*-12		LPS	TNF-α, IL-1β ↓ (obese)	
			PMA/Io	IL-4 ↓ (obese)	
			ConA	IL-2 ↓ (lean), IL-4 ↓ (obese)	
	0.4% mixture		PMA/Io	IL-2, IL-4 ↓ (obese)	
			ConA	IL-4 ↓ (obese), IL-2 ↓ (lean)	
			PWM	IL-2 ↓ (lean)	

Table 2 continued

Species	Isomer/diet	Tissue	Stimulation	Findings	Reference
rats	1% CLA (80% *cis*-9,*trans*-11, 20% *trans*-10,*cis*-12)	spleen lymphocytes lymphocytes from mesenteric lymph nodes	polyclonal stimulation PMA/Io	IL-2 ↓ IL-2 ↔	RAMIREZ-SANTANA ET AL. (2009A)
rats	20 mg/kg/d *cis*-9,*trans*-11	blood lymphocytes	LPS ConA	IL-6 ↔ TNF-α, IL-1β ↓ IL-2 ↑ IL-4, IFN-γ ↔	TURPEINEN ET AL. (2006)
	40 mg/kg/d *cis*-9,*trans*-11		LPS ConA	IL-6, TNF-α, IL-1β ↔ IL-2, IL-4, IFN-γ ↔	
rats	1% (80% *cis*-9,*trans*-11, 20% *trans*-10,*cis*-12)	splenocytes	PMA/Io	IL-2, IFN-γ, IL-4, IL-10 ↔	RAMIREZ-SANTANA ET AL. (2009B)
rats	1% (80% *cis*-9,*trans*-11, 20% *trans*-10,*cis*-12)	spleen lymphocytes	PMA/Io	IL-2, IFN-γ, IL-4, IL-10 ↔ IL-6 ↑	RAMÍREZ-SANTANA ET AL. (2011)
humans	3 g/d *cis*-9,*trans*-11 3 g/d *trans*-10,*cis*-12	PBMC, whole blood	LPS	IL-6, IL-8, TNF-α ↔	RAMAKERS ET AL. (2005)
humans	1.7 g CLA/d (50% *cis*-9,*trans*-11, 50% *trans*-10,*cis*-12)	PBMC	PHA LPS	IFN-γ, IL-2, IL-4, ↔ TNF-α, IL-1β, IL-6 ↔	ALBERS ET AL. (2003)
	1.6 g/d (80% *cis*-9,*trans*-11, 20% *trans*-10,*cis*-12)		PHA LPS	IFN-γ, IL-2, IL-4, ↔ TNF-α, IL-1β, IL-6 ↔	

Table 2 continued

Species	Isomer/diet	Tissue	Stimulation	Findings	Reference
humans	0.59, 1.19 or 2.38 g/d *cis*-9,*trans*-11	PBMC	LPS	TNF-α*, IL-10, IL-6, IL-1β*, IL-8 ↔	TRICON ET AL. (2004)
			ConA	IFN-γ*, TNF-α*, IL-10*, IL-5*, IL-4, IL-2 ↔	
	0.63, 1.26 or 2.52 g/d *trans*-10,*cis*-12		LPS	TNF-α*, IL-10, IL-6, IL-1β*, IL-8 ↔	
			ConA	IFN-γ*, TNF-α*, IL-10*, IL-5*, IL-4, IL-2 ↔	
humans	3 g/d CLA	PBMC	LPS	TNF-α, IL-1β ↓ IL-10 ↑ (compared to baseline)	SONG ET AL. (2005)
humans	2.2 g/d CLA	PBMC	-	TNF-α ↔	MULLEN ET AL. (2007)
			ConA	IL-2 ↓ TNF-α, IL-10 ↔	
		serum	-	IL-6 ↔	

↑ parameter increased due to CLA supplementation, ↓ parameter decreased due to CLA supplementation, ↔ no effect of CLA; *effect of CLA dose; abbreviations: PBMC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor α; RAW, mouse macrophage cell line; PMA, phorbol 12-myrisat 13 acetate; Io, ionomycin; IFN-γ, interferon γ, ConA, concanavalin A; PWM, pokeweed mitogen, PHA, phytohemagglutinin

2.1.3 Effects of CLA on B cells and humoral immune response

B cells are part of the adaptive immune system. They are activated by exogenous antigens. The activation leads a differentiation into plasma cells and B memory cells. Plasma cells are able to produce specific antibodies (immunoglobulins [Ig]). These specific antibodies bind to antigens making them better available for phagocytosis. CLA seems to impair humoral immune response by influencing Ig production. Serum anti-bovine serum albumin (BSA) IgG levels were increased in pigs fed a diet containing 1% CLA in response to intramuscularly administered BSA, whereas 2% CLA in diet had no effect on anti-BSA IgG production (MORAES ET AL. 2012). CLA feeding had no effect on IgG level in serum without further stimulation (MALOVRH ET AL. 2009).

CLA, especially the *cis*-9,*trans*-11 isomer, enhanced antibody production in rats during suckling and in early infancy (RAMIREZ-SANTANA ET AL. 2009B; RAMÍREZ-SANTANA ET AL. 2011). Long-term supplementation did not enhance serum levels of ovalbumin specific antibodies and the number of anti-ovalbumin-antibody secreting cells in the spleen and mesenteric lymph nodes when rats were immunized with ovalbumin. However, there were restricted effects of CLA supplementation on anti-ovalbumin IgA levels in the intestinal mucosa without altering total gut IgA (RAMIREZ-SANTANA ET AL. 2009A). In mice, the *trans*-10,*cis*-12 isomer enhanced the production of IgA and IgM, but not that of IgG and IgE. Furthermore, the percentage of B cells was increased in *trans*-10,*cis*-12 CLA-fed mice and decreased in *cis*-9,*trans*-11 CLA-fed mice (YAMASAKI ET AL. 2003).

The CLA supplementation did not alter the proliferation of PBMC stimulated with the B cell antigen influenza vaccine in young and healthy women (KELLEY ET AL. 2000), but the IgA and IgM concentration in plasma increased during CLA supplementation whereas IgE decreased in another human study (SONG ET AL. 2005). The seroprotection rate increased following hepatitis B vaccination, when subjects consumed a CLA supplement containing the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers in equal amounts in comparison to those consuming the reference substance (sunflower oil). However, the effect did not reach significance (ALBERS ET AL. 2003).

2.2 Effects of CLA on immune function in Ruminants

Only little information about the effects of CLA on immune parameters in ruminants is available yet. IgG levels in serum and colostrum were investigated in pregnant goats who received either 12 g/kg CLA or no CLA in the diet beginning in the third month of gestation and lasting until parturition. The blood serum IgG concentration decreased from 3 months of gestation until parturition and increased during the first 96 h *post partum* (pp), whereby the increase was stronger in the CLA-fed group. The colostrum IgG concentrations declined similarly in both feeding groups from 0 h to 96 h pp (CASTRO ET AL. 2006). CLA (final dose: 0, 1.4 and 5.6 g/kg, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA in a ratio 50:50) in the starter concentrate feeding of lambs had no effect on the number of seroconverted animals following ovalbumin vaccination. Therefore, the authors concluded that there is no effect of CLA feeding on humoral immunity in lambs (TERRE ET AL. 2011). In bovine whole blood cultures, the LPS-induced production of TNF-α was decreased dose dependently (≤100 µM) when the cultures were incubated with the *trans*-10,*cis*-12 isomer, whereas the addition of linoleic acid or the *cis*-9,*trans*-11 isomer did not alter TNF-α concentrations (PERDOMO ET AL. 2011).

3 CLA and Phytanic acid

Phytanic acid (PA) is a C20 branched chain fatty acid (3,7,11,15-tetramethylhexadecanoic acid). It is a metabolite of chlorophyll, more precisely of its side chain phytol which is released from the porphyrin ring of chlorophyll by ruminal microorganisms. Phytol is converted to PA by microorganism in the ruminal gut as well (PATTON AND BENSON 1966). Humans are not able to cleave phytol from chlorophyll. Hence, dairy products and meat are an important source of PA (WANDERS ET AL. 2011). PA can not undergo direct β-oxidation. Therefore, it is metabolized by α-oxidation to pristanic acid which undergoes regular β-oxidation afterwards (VERHOEVEN AND JAKOBS 2001; WANDERS ET AL. 2011). If the first enzyme of the α-oxidation pathway (phytanoyl-CoA hydroxylase) is lacking PA accumulates in plasma and tissue. This rare inherited dysfunction is called Refsum's disease. Clinical signs are pigmentary retinal degeneration, peripheral neuropathy, cerebellar ataxia and high concentrations of protein in the cerebrospinal fluid (VERHOEVEN AND JAKOBS 2001; HELLGREN 2010).

PA acts as a ligand for the retinoid X receptor (RXR) (KITAREEWAN ET AL. 1996) and PPAR-α (ELLINGHAUS ET AL. 1999). CLA are also ligands for PPARs (MOYA-CAMARENA ET AL. 1999). Hence, it is hypothesized that there are complementary interactions in their anti-diabetic activity (MCCARTY 2001). Because the concentration of PA in bovine serum is rather high (5.9 mg/100 ml compared to 0.04 to 0.21 mg/100 ml in human serum, (AVIGAN 1966)) and both FA (PA and CLA) share similar activation mechanisms via PPARs there are potential interactions of these FA in cows.

4 The periparturient dairy cow with a special focus on the immune status

The so-called transition period or periparturient period refers to the time around parturition and includes 3 weeks *ante partum* (ap) and 3 weeks pp (MALLARD ET AL. 1998). This period is characterized by metabolic, hormonal, nutritional, and immunological changes due to transition from late pregnancy to the beginning lactation. The DM intake is reduced, but due to demands of the fetus in late pregnancy and the beginning lactation the energy requirement is increasing (GRUMMER ET AL. 2004). Therefore, cows often experience a negative energy balance and mobilize fat from adipose tissue as an additional source of energy (GOFF AND HORST 1997). This lipomobilization is associated with increasing concentrations of nonesterified fatty acids (NEFA) and ketone bodies like β-hydroxybutyrat (BHB) in plasma

(GOFF AND HORST 1997; CONTRERAS AND SORDILLO 2011). Ketone bodies are a result of FA metabolization in the liver in situations of oversupplied FA.

During the periparturient period the dairy cow experiences an immunosuppressive state, where cows are susceptible for infectious diseases like mastitis (KEHRLI AND HARP 2001). This is seen at a reduced ability of mitogens to stimulate proliferation of PBMC during the first week pp (NONNECKE ET AL. 2003). In this study the immunosuppressive effect is ascribed to the mammary gland since mitogen stimulated proliferation, the secretion of IFN-γ and the production of IgM from mitogen stimulated PBMC was decreased in intact cows after parturition, but not in mastectomized cows. Increased NEFA concentrations are discussed as a possible reason for the immunosuppression around calving as well. *In vitro* analyses using bovine PBMC showed decreased proliferation when cells were incubated with different NEFA concentrations mimicking physiological conditions: 62.5-500 µmol/L for healthy cows and 1000 µmol/L for ketotic cows. The highest tested concentration (2000 µmol/L) was beyond physiological levels (LACETERA ET AL. 2004). The proliferation was significantly decreased at 2000 and 1000 µmol/L NEFA concentrations in response to the mitogens PHA and ConA and additionally at 500 µmol/L in response to pokeweed mitogen (PWM). Also, the secretion of IgM and the production of IFN-γ was reduced at concentrations between 250 and 2000 µmol/L and 125 and 2000 µmol/L respectively. Furthermore, a study carried out with thin, medium and overconditioned cows confirmed these results. NEFA concentrations were significantly higher in overconditioned than in thin or medium cows 3 and 7 days pp. Although mitogen stimulated proliferation of PBMC was not different between the three categories of cows, secretion of IgM was reduced in overconditioned compared to thin cows 14 and 35 days pp. The production of IFN-γ was decreased 7 days ap in overconditioned cows compared to thin and medium cows (LACETERA ET AL. 2005). In addition to lymphocytes the function of polymorphonuclear leucocytes (PMN) is influenced by NEFA as well. Although there was no effect of different NEFA concentrations (0 to 2000 µmol/L) on phagocytosis of PMN *in vitro*, the phagocytosis-induced oxidative burst was decreased at low and moderate NEFA concentrations (62.5, 125, 250 and 500 µmol/L) and did not differ from control at 2000 µmol/L (SCALIA ET AL. 2006).

However, these results indicate that fat mobilization and, as a result of that NEFA play a crucial role in immunosuppression in the transition period of dairy cows.

Scope of the thesis

Research in the context of CLA in dairy cows is mainly addressed to the milk fat reducing effects of CLA. However, knowledge is lacking about the impact of CLA on immune functions in dairy cattle. Therefore, the aim of this thesis was to investigate the long-term impact of CLA feeding on immunological parameters in dairy cows. Because of the various challenges during the periparturient period of dairy cows, this time is of special interest. The goal of the present thesis is to answer the following questions:

1. Does long term CLA supplementation of dairy cows affect the mitogen-stimulated proliferation of PBMC and splenocytes *ex vivo*? Are there any effects on the expression of cytokines involved in immune response?

2. Does CLA supplementation of dairy cows alter the fatty acid profile of PBMC and is there a relationship between fatty acid profile and their function?

3. Do CLA isomers alone and in combination with other FA affect the proliferation and cytokine expression of bovine PBMC *in vitro*?

The effects of CLA supplementation were investigated in two separate feeding trials with dairy cows. The first study investigated long-term effects of CLA supplementation on the FA profile of PBMC and their proliferation *ex vivo.* Primiparous and pluriparous dairy cows diets were supplemented with CLA. The supplementation began after calving and lasted for 182 days (**Paper I**). The second study was a slaughter trial with primiparous dairy cows. The *ex vivo* proliferation and cytokine expression of PBMC and splenocytes in response to CLA feeding was studied (**Paper II**). Furthermore, the effects of different CLA isomers and other FA on proliferation and cytokine expression of PBMC were investigated *in vitro* (**Paper III**).

Paper I

Fatty acid profile and proliferation of bovine blood mononuclear cells after conjugated linoleic acid supplementation

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Abstract

Background: Conjugated linoleic acids (CLA) are in focus of dairy cattle research because of its milk fat reducing effects. Little is known about the impact of CLA on immune function in dairy cows. Therefore, in the present study we investigated the effects of a long term supplementation of dairy cows with CLA on the fatty acid profile of peripheral blood mononuclear cells (PBMC) and their proliferation *ex vivo*.

Results: The supplementation of dairy cows with either 100 g/d of a control fat preparation (CON, n = 15), 50 g/d of the control fat preparation and 50 g/d CLA supplement – containing 12.0% *cis*-9,*trans*-11 and 11.9% *trans*-10,*cis*-12 CLA of total fatty acid methyl esters – (CLA-50, n = 15) or 100 g/d of the CLA supplement (CLA-100, n = 16) did not influence the major fatty acids (C18:0, C16:0, *cis*-9 C18:1, *cis*-9,*cis*-12 C18:2, *cis*-5,*cis*-8,*cis*-11,*cis*-14 C20:4) in the lipid fraction of PBMC. The proportion of *trans*-10,*cis*-12 CLA of total fatty acids was increased in both CLA supplemented groups, but there was no effect on the *cis*-9,*trans*-11 isomer. Furthermore, the proportion of *trans*-9 C18:1 and *cis*-12 C24:1 was reduced in the CLA-100 group. The mitogen-stimulated cell proliferation was not influenced by CLA feeding.

Conclusion: CLA supplementation influenced the FA profile of some minor FA in PBMC, but these changes did not lead to differences in the mitogen induced activation of the cells.

Keywords: CLA, Dairy cow, Peripheral blood mononuclear cells, Cell proliferation, Fatty acid profile

Background

Conjugated linoleic acids (CLA) are a group of positional isomers of linoleic acid, which are characterized by conjugated double bonds. They are intermediate products in the biohydrogenation of unsaturated fatty acids (FA) by microorganisms in the rumen (KEPLER ET AL. 1966). Additionally, it is reported, that CLA originate from endogenous synthesis in tissues like the mammary gland of ruminants (GRIINARI ET AL. 2000). Several positive physiological effects are reported for CLA, like anticarcinogenic (e. g. reviewed by (LEE ET AL. 2005; BHATTACHARYA ET AL. 2006), antiatherogenic (LEE ET AL. 1994) and immunomodulatory (O'SHEA ET AL. 2004) properties. In general, dietary FA are able to influence the function of immune cells due to different mechanisms, which include alteration of the membrane, changes in signal transduction pathways and in lipid mediators like Prostaglandin E_2 (CALDER 2008). CLA supplementation e. g. led to decreased lymphocyte

activation of healthy men (TRICON ET AL. 2004) and declined proliferative response in rat splenocytes (RAMIREZ-SANTANA ET AL. 2009). Dietary CLA are capable to change the FA profile of human peripheral blood mononuclear cells (PBMC), but did not alter their function, like the mitogen stimulated production of PGE_2, leukotriene B_4 (LTB_4), interleukin (IL)-1β, IL-2 and tumor necrosis factor α (TNF-α) (KELLEY ET AL. 2001).

Although CLA originally occur in dairy cattle, the supplementation of the cows` diet with CLA gains in importance, because it reduces the milk fat content, which is ascribed to the *trans*-10,*cis*-12 isomer (BAUMGARD ET AL. 2000). The impact of a CLA supplementation on the immune system of dairy cows has been rarely investigated. There was no effect of CLA supplementation on the stimulation index (SI) of PBMC obtained from primiparous lactating cows *ex vivo* 42 and 105 days *post partum* (pp). But the SI of splenocytes from the same animals were decreased following CLA supplementation (RENNER ET AL. 2012). It is unknown, if the effects are similar in pluriparous cows and over a longer supplementation period and if the supplementation changes the FA profile of immune cells, which might have further downstream effects. Therefore, in the present investigation the effects of a long term CLA supplementation were evaluated. In the study primiparous and pluriparous cows were involved. Effects on immune cells were evaluated by cell proliferation assays using PBMC and furthermore, the FA profile of PBMC was analyzed.

Results

Data concerning performance of the cows in the present study are reported by PAPPRITZ ET AL. (2011).

Fatty acid profile of PBMC

The main FA occurring in PBMC were C18:0 (stearic acid), C16:0 (palmitic acid), *cis*-9 C18:1 (oleic acid), *cis*-9,*cis*-12 C18:2 (linoleic acid) and *cis*-5,*cis*-8,*cis*-11,*cis*-14 C20:4 (arachidonic acid) (Table 1). CLA supplementation did not change the proportions of these FA significantly, but there was a tendency of increasing C16:0 when CLA was supplemented. Furthermore the proportion of saturated, monounsaturated and polyunsaturated FA as well as the sum of n-3 and n-6 FA were not influenced by CLA supplementation. Regarding CLA, no *trans*-10,*cis*-12 was found in PBMC of control animals, but the isomer significantly increased in both supplemented groups (Table 1).

Table 1 Fatty acid profile of peripheral blood mononuclear cells

Fatty acid	**Group**			**probability**
	CON	**CLA-50**	**CLA-100**	
C16:0	15.30 ± 0.58	16.29 ± 0.41	17.12 ± 0.57	*0.061*
C18:0	26.81 ± 1.02	27.53 ± 0.82	28.18 ± 0.75	0.532
C18:1*c*9	13.52 ± 0.51	12.94 ± 0.54	12.46 ± 0.46	0.333
C18:1*c*11	2.41 ± 0.13	2.25 ± 0.11	2.19 ± 0.08	0.338
C18:1 *t*9	0.27 ± 0.01[a]	0.23 ± 0.01[ab]	0.22 ± 0.01[b]	**0.017**
C18:2*c*9,*c*12	10.13 ± 0.23	11.07 ± 0.35	10.36 ± 0.27	*0.072*
CLA-*c*9,*t*11	0.17 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	*0.071*
CLA-*t*10,*c*12	0.00 ± 0.00[a]	0.01 ± 0.00[b]	0.02 ± 0.00[b]	**<0.001**
other CLA	0.07 ± 0.01[a]	0.06 ± 0.01[a]	0.03 ± 0.01[b]	**0.006**
C20:3n-6	3.06 ± 0.14	3.07 ± 0.13	2.95 ± 0.16	0.805
C20:4n-6	11.12 ± 0.42	9.92 ± 0.49	9.96 ± 0.65	0.214
C20:5n-3	1.03 ± 0.08	0.96 ± 0.06	0.94 ± 0.05	0.553
C22:4n-6	1.35 ± 0.07	1.29 ± 0.08	1.27 ± 0.10	0.791
C22:5n-3	4.11 ± 0.29	4.03 ± 0.44	4.05 ± 0.22	0.295
C24:1 *c*15	0.03 ± 0.01[a]	0.02 ± 0.01[ab]	0.01 ± 0.00[b]	**0.03**
Minor FA[†]	11.94 ± 0.61	11.39 ± 0.42	11.27 ± 0.30	0.547
SFA	46.39 ± 1.48	48.09 ± 1.22	49.73 ± 1.28	0.214
MUFA	20.80 ± 0.691	19.78 ± 0.73	18.95 ± 0.48	0.131
PUFA[‡]	32.81 ± 1.00	32.12 ± 0.90	31.32 ± 1.09	0.576
all C18:1 *trans*-FA	2.53 ± 0.11	2.34 ± 0.13	2.28 ± 0.13	0.346
all CLA	0.24 ± 0.01	0.23 ± 0.02	0.20 ± 0.01	*0.071*
all n-3 FA	6.37 ± 0.41	6.01 ± 0.42	6.10 ± 0.24	0.772
all n-6 FA	26.30 ± 0.71	25.98 ± 0.81	25.12 ± 1.00	0.602
n-3/n-6	0.24 ± 0.01	0.24 ± 0.02	0.25 ± 0.02	0.872
MC-FA (C10 > C14)	0.53 ± 0.07	0.68 ± 0.10	0.87 ± 0.17	0.176

Fatty acid profile of peripheral blood mononuclear cells from cows that received a control fat preparation (CON, n=15) or 50 g/d (CLA-50, n=15) and 100 g/d (CLA-100, n=16) of a CLA supplement. Blood samples were taken after 70 and 140 days of supplementation and samples were pooled for each cow. Results are expressed as % of total fatty acid methyl esters, means ± standard error.

[†] Minors contain fatty acids which concentration is less than 1% of all fatty acids except CLA

[‡] includes CLA

c = *cis*, t = *trans*, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, MC-FA = medium chain fatty acid

The effect was not seen for the other main supplemented isomer *cis*-9,*trans*-11, where no differences were observed between the 3 groups. Other CLA were significantly more frequently found in CON and CLA-50 group than in CLA-100 group. When all CLA isomers are considered together there were no differences among the groups. Furthermore, CLA supplementation did influence the proportion of *trans*-9 C18:1 and *cis*-15 C24:1 (Table 1). Both FA were significantly reduced in CLA-100 group compared to CON.

Ex vivo cell proliferation assay

Cell viability and mitogen stimulated proliferation of PBMC was evaluated 7, 21, 35, 49, 105 and 182 days pp by Alamar blue (AB) and MTT assay.

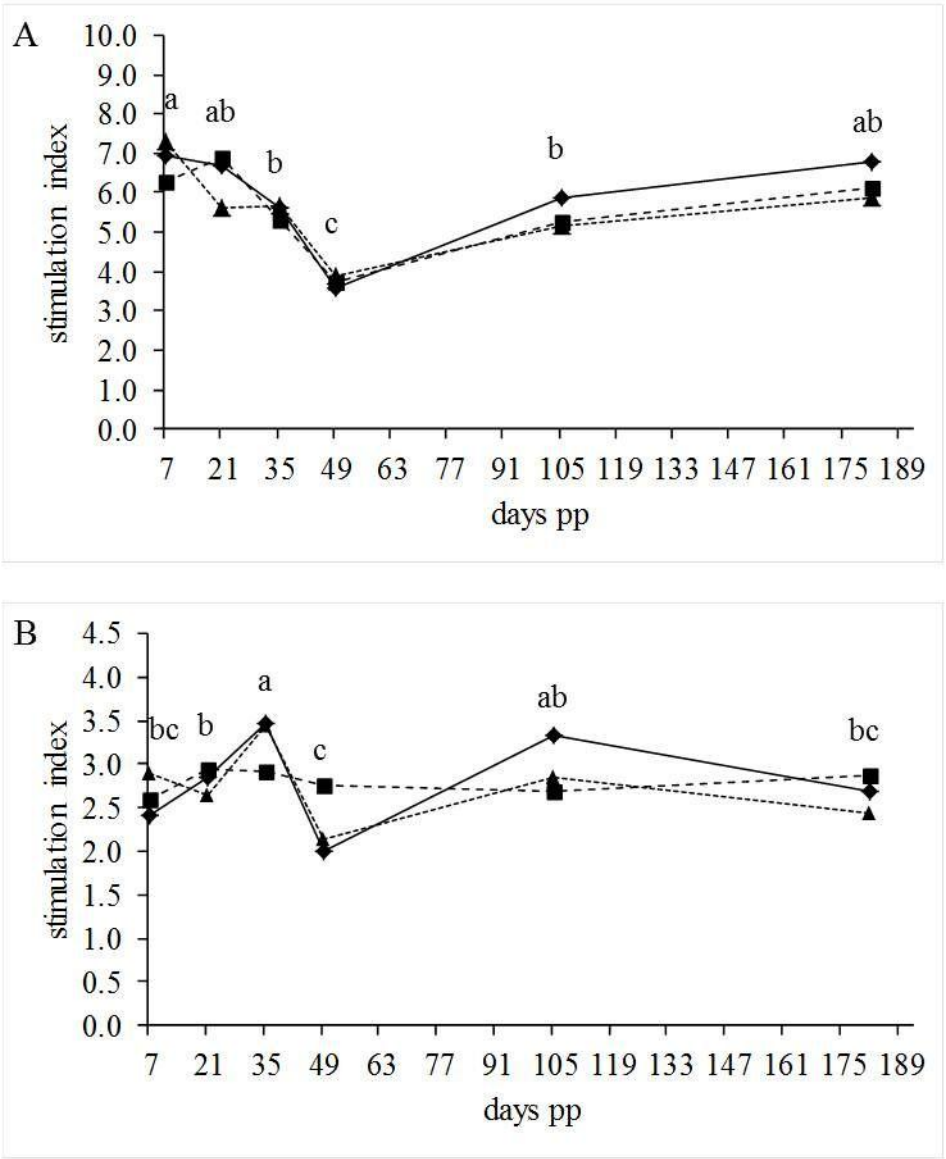

Figure 1 Stimulation index of peripheral blood mononuclear cells in MTT (A) and Alamar blue assay (B).
Cows were supplemented with a control fat preparation (CON, n = 15) or 50 g/d of a CLA supplement (CLA-50, n = 15) or 100 g/d of a CLA supplement (CLA-100, n = 16) abc: indicates significant differences between sampling days, $p < 0.05$

There was no effect of supplementation ($p = 0.742$ in MTT assay, $p = 0.955$ in AB assay) and lactation number ($p = 0.487$ in MTT assay and $p = 0.972$ in AB assay) on the stimulation index (SI) of PBMC. Furthermore, no interactions between day of lactation and lactation number ($p = 0.948$ in MTT assay and $p = 0.861$ in AB assay), supplementation and lactation number ($p = 0.702$ in MTT assay and $p = 0.792$ in AB assay) as well as supplementation and

day of lactation ($p = 0.821$ in MTT assay and $p = 0.128$ in AB assay) were found. Only the day of lactation had a significant effect ($p < 0.001$ in both assays, Figure 1). At day 49 pp the SI reached the minimum in both assays. In the AB assay the SI was rising from day 7 to day 35 pp and in contrast the SI decreased from day 7 pp until day 49 pp in the MTT assay.
The SI 105 days pp was not correlated with the proportion of *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, other CLA isomers or the sum of all CLA isomers in both assays.

Discussion

The FA profile of immune cells is influenced by FA in the diet, thus it is possible to modify the FA profile of these cells by altering the consumption of certain FA (CALDER 2008). This effect is described for n-3 polyunsaturated FA (PUFA) from fish oil or preparations of eicosapentaenoic acid or docosahexaenoic acid (THIES ET AL. 2001; KEW ET AL. 2004; REES ET AL. 2006), but also for CLA (KELLEY ET AL. 2001) in humans. The altered FA profile of the total lipids and phospholipids, respectively, might cause changes in the function of the cells, indicated by effecting signaling pathways or the pattern of lipid mediator production (CALDER 2008). Most studies were performed with humans, but also effects of certain FA on immune cell functions in dairy cows have been reported (LESSARD ET AL. 2003; LESSARD ET AL. 2004). In these studies the effects on FA profile of bovine immune cells were not investigated (CONTRERAS ET AL. 2010).
In the present study the proportion of CLA in the lipid fraction of PBMC was low (less than 1% of all fatty acid methyl esters [FAME]). Due to CLA supplementation the proportion of *trans*-10,*cis*-12 CLA was increased, but the *cis*-9,*trans*-11 isomer remained unchanged. The *trans*-10,*cis*-12 isomer was not found in PBMC of the control group and it accounted for only 0.004% of total FAME in the milk fat of the same animals (PAPPRITZ ET AL. 2011). The *cis*-9,*trans*-11 isomer is the major CLA isomer occurring in dairy products. It is formed in the rumen by microbial fermentation (KEPLER ET AL. 1966) and by endogenous synthesis via Δ^9-desaturase in the mammary gland (GRIINARI ET AL. 2000). These sources might have a greater impact on the proportion of *cis*-9,*trans*-11 CLA in bovine PBMC than the supplementation. There was also no effect of the diet on the percentage of *cis*-9,*trans*-11 in the milk fat of the same animals (PAPPRITZ ET AL. 2011). In humans, the proportion of CLA in PBMC was increased after 63 days CLA supplementation (3.9 g/d of CLA isomers). The *cis*-9,*trans*-11 isomer increased from 0.05 to 0.16% of all analyzed FA, which is in the same range as the proportion in bovine PBMC in the present study. The *trans*-10,*cis*-12 isomer increased from 0.04% to 0.19% (KELLEY ET AL. 2001), which is much higher than in the

present study. In humans, the main source of CLA is the consumption of dairy products and ruminant meat (FRITSCHE AND STEINHART 1998), but the endogenous synthesis via Δ^9-desaturase is also observed in humans, whereby *trans*-11 C18:1 serves as a precursor (TURPEINEN ET AL. 2002; KUHNT ET AL. 2007).

The two predominant FA in the lipid fraction of PBMC were C18:0 and C16:0 in the present study. That is in line with results from CONTRERAS ET AL. (2010) who investigated the FA composition of the phospholipid fraction of PBMC in dairy cows around parturition. In the present study, the major FA of the lipid fraction of PBMC were not significantly affected by CLA supplementation, but there was a trend of increased C16:0 following CLA supplementation. The increased percentage of C16:0 was also found in the FA profile of erythrocytes from new born calves whose mothers received the CLA supplement during a certain time of pregnancy (DÄNICKE ET AL. 2012). Reasons for this effect are not clarified yet. CLA, particularly the *trans*-10,*cis*-12 isomer, down regulate the expression of Δ^9-desaturase (CHOI ET AL. 2000; BAUMGARD ET AL. 2002) and inhibit its activity (CHOI ET AL. 2001) in different tissues. Therefore, a slight inhibition of Δ^9-desaturase might be accountable for the increased proportion of C16:0. Two minor FA (<1 % of total FAME), *trans*-9 C18:1 (elaidic acid) and *cis*-15 C24:1 (nervonic acid), were affected by CLA supplementation. Their percentage was decreased in CLA-100 group. Nervonic acid is an important FA in myelin sphingolipids (MARTINEZ AND MOUGAN 1998) and therefore in the nervous system. Elaidic acid is, like other *trans* C:18:1 FA, mainly found in partially hydrogenated vegetable oils, but also in fat of dairy products (PFEUFFER AND SCHREZENMEIR 2006). In splenocytes obtained from rats, elaidic acid was only found in phosphatidylethanolamine and phosphatidylcholine, when elaidic acid was supplemented. In this study the elaidic acid supplementation caused increased mitogen stimulated production of interleukin-6 (RUTH ET AL. 2010).

The mitogen-stimulated proliferation was investigated to obtain information about the functionality of PBMC. The function of the cells was not influenced by the CLA supplementation, although the fatty acid composition was slightly altered in the present study. Also the milk fat depression was observed in the present study. The milk fat content was reduced dose dependently by 7% and 12% in CLA-50 and CLA-100 group, respectively, in the time period from 49 to 182 days pp (PAPPRITZ ET AL. 2011). Changes in the SI were observed over the lactation period. It is known that the immune system of dairy cattle is suppressed after calving (NONNECKE ET AL. 2003), which was e. g. demonstrated by a decreased SI of ConA-stimulated PBMC *ex vivo* (RENNER ET AL. 2012). As a reason for the

immunosuppression in the pp period increased non esterified fatty acids (NEFA) concentrations, which result from increasing fat mobilization, are discussed. NEFA inhibit proliferation of bovine PBMC *in vitro* (LACETERA ET AL. 2004). The immunosuppressive effect in the pp period was not observed in the MTT assay of the present study, although NEFA concentrations in plasma were much higher from 7 to 49 days pp (0.70, 0.66 and 0.69 mmol/L in CON, CLA-50 and CLA-100 group, respectively) than between day 49 and 182 pp (0.29, 0.25, 0.22 mmol/L in CON, CLA-50 and CLA-100 group, respectively) (PAPPRITZ ET AL. 2011). In the AB assay the SI increased from day 7 to 35 pp, but reached the minimum at day 49 pp like in the MTT assay. At that time point the cows of the CLA fed groups turned from negative to positive calculated energy balance. Until 49 days pp the cows of the CON group were not in negative calculated energy balance (14.9±5.4 MJ/d), whereas the calculated energy balance of the CLA fed cows was negative during that period (CLA-50 -12.3±5.4 MJ/d and CLA-100 -8.3±5.2 MJ/d). These differences are based on a lower dry matter (DM) intake in CLA supplemented groups during the first weeks of lactation (CON 21.1±0.7 kg/d, CLA-50 18.5±0.7 kg/d and CLA-100 17.8±0.7 kg/d). In the following period (until the end of the supplementation 182 days pp), no differences between the feeding groups occurred in DM intake (CON 21.6±0.6 kg/d, CLA-50 22.4±0.6 kg/d and CLA-100 21.2±0.6 kg/) and energy balance (CON 15.3±2.7 MJ/d, CLA-50 10.4±2.6 MJ/d and CLA-100 10.8±2.5 MJ/d) (PAPPRITZ ET AL. 2011). The differences between AB and MTT assay might be due to different enzyme systems involved in reduction of the respective dye. MTT is mainly reduced by mitochondrial and microsomal enzymes and AB by mitochondrial and cytosolic enzymes (GONZALEZ AND TARLOFF 2001).

HUSSEN ET AL. (2011) examined the leukocyte profile of PBMC of the present investigation due to CLA supplementation. Although the SI of PBMC was not influenced by CLA supplementation, there were effects on their composition. The percentage of $CD4^+$ cells was decreased from 21 days pp onwards in the CLA-100 group compared to control and CLA-50 group and $CD8^+$ cells were slightly increased, starting 21 days pp. The percentage of monocytes, B cells and γδ-T cells was not altered by the diet. Furthermore, IgG1 and IgG2 levels in serum were significantly lower in the CLA-100 group throughout the supplementation period.

It is interesting to note that PBMC of calves (5 calves per group) of the CLA fed cows investigated in the present experiment showed an effect of the diet in the MTT assay (SI in CON 3.6±1.0, CLA-50 1.6±0.7, CLA-100 4.3±0.8), but not in the AB assay on mitogenic response immediately after partus and 1 day *post natum*. However, at this time, when CLA

supplementation was no longer fed, the cows still did not exhibit differences in the mitogen stimulated response of PBMC, but the SI was increasing from day 0 to day 21 pp in all groups (DÄNICKE ET AL. 2012).

In most studies investigating the effect of CLA on immune function, the FA profiles of the investigated cells were not analyzed. Altogether, the effects of CLA supplementation to dairy cows are low and effects seen in other species, e.g. ZHANG ET AL. (2005); NUNES ET AL. (2008), could not be observed.

Conclusions

Long term CLA supplementation to dairy cows did not alter the mitogen-induced proliferation of PBMC *ex vivo*, although the *trans*-10,*cis*-12 CLA isomer was increased in the lipid fraction of PBMC. Further investigations are necessary to evaluate if the increased proportion of *trans*-10,*cis*-12 CLA in the lipid fraction of PBMC has an impact on other immunological parameters.

Materials and methods

Experimental design

The experiment was carried out at the experimental station of the Friedrich-Loeffler-Institute (FLI) in Braunschweig, Germany. The study was conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany, File number 33.14.42502-04-071/07). In the study 46 cows, 32 pluriparous and 14 primiparous, were assigned to 3 feeding groups. The control group (CON, n = 15, out of them 5 primiparous cows) received 100 g/d of a control fat preparation, the CLA-50 group (n = 15, out of them 4 primiparous cows) received 50 g/d of the control fat preparation and the CLA supplement, respectively, and the CLA-100 group (n = 16, out of them 5 primiparous cows) received 100 g/d of the CLA supplement. The supplementation period began one day pp and lasted for 182 days. During that time the cows were fed a partial mixed ration (PMR) containing 37% concentrate and 63% silage (60% maize silage, 40% grass silage based on DM content) for ad libitum consumption by a computerized feeding station (Type RIC, Insentec, B.V., Marknesse, The Netherlands). The control fat preparation (Silafat®, BASF SE, Ludwigshafen, Germany) and the CLA supplement (Lutrell® pure, BASF SE, Ludwigshafen, Germany) were given with 4 kg additional concentrate, also via a

computerized concentrate feeding station. The CLA supplement contained mainly the *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 isomer (12.0% and 11.9% of FAME, respectively). The daily consumption of each isomer was 4 g/d in the CLA-50 group and 8 g/d in the CLA-100 group. In the control fat preparation CLA was substituted by stearic acid, which is also the main FA in the CLA supplement. Water was offered for ad libitum consumption. More detailed information about the animal experiment, including the FA profile of the supplements, is reported elsewhere (PAPPRITZ ET AL. 2011).

Sample preparation

At day 7, 21, 35, 49, 70, 105, 140 and 182 pp blood (30 mL) was taken by jugular venipuncture into heparinized vacutainer tubes. PBMC were isolated from whole-blood by density gradient centrifugation using Biocoll (Biochrom AG, Berlin, Germany, L 6115). The samples were processed as described by RENNER ET AL. (2011). Samples of day 7, 21, 35, 49, 105 and 182 pp were used to perform cell proliferation assays. The other 2 samples (70 and 140 days pp) were pooled for each cow and the FA profile of PBMC was analyzed. All samples were frozen and stored at −80°C in freezing medium containing fetal bovine serum (FBS, Biochrom AG, Berlin, Germany, S 0615) and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany, D 2438).

Analysis of fatty acid profile

The PBMC were washed 3 times with saline to remove freezing medium. Cellular lipids were extracted according to the procedure described by BLIGH AND DYER (1959) using a methanol/chloroform mixture. The extracted lipids were then transesterificated with boron trifluoride (BF_3) to produce FAME, followed by a purification of the extracts using thin-layer chromatography (SIL G-25 UV_{254}, Macherey-Nagel, Dueren, Germany). FAME were analyzed by gas chromatography ([GC], GC-17A Version 3, Schimadzu, Kyoto, Japan), fitted with an auto sampler and flame ionization detector. Two different procedures were necessary to identify all FAME and were conducted according to DEGEN ET AL. (2011). The general FA profile (FA, whose carbon length is 4 to 25) was analyzed using a medium polarity column (DB-225 ms, 60 m x 0.25 mm inner diameter; 0.25 µm film thickness; Agilent Technologies, Santa Clara, USA). Furthermore, the *cis* and *trans* isomers of C18:1 were separated via a high polarity column (Select^{TM} FAME, 200 m x 0.25 mm inner diameter, 0.25 µm film thickness; Agilent Technologies, Santa Clara, USA). The following reference standards were used as FAME mix to identify FA peaks: No. 463, 674, (Nu-Chek Prep, Inc., Elysian, USA), BR2,

BR4, ME 93 (Larodan; Malmö, Sweden), Supelco® 37 Component FAME Mix, PUFA No. 3, conjugated linoleic acid, linoleic-, linolenic- and octadecenoic acid methyl ester mix (Supelco; Bellefonte, USA). Results are expressed as percentage of total FAME.

Cell proliferation assays

PBMC viability and concanavalin A (ConA, Sigma–Aldrich, Steinheim, Germany, C 5275) stimulated proliferation were analyzed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and Alamar blue (AB) assay. The procedures were carried out as described in detail elsewhere (RENNER ET AL. 2012).

Calculations and statistics

Statistical analyses of the FA profile were performed by a one factorial analysis of variance (ANOVA) using the Statistica 8 for the Windows operating system, followed by a Tukey test. Probabilities below 0.05 were considered as statistically significant and $p < 0.1$ as a tendency.

The stimulation index (SI) was calculated by the following equation for the MTT assay:

SI = optical density (OD) of ConA stimulated PBMC/OD of non-stimulated PBMC

In the AB assay fluorescence instead of OD was used.

The PROC MIXED procedure with a compound symmetry covariance structure and supplementation, day of lactation and lactation number (primiparous vs pluriparous) as fixed factors as well as interactions of these factors was performed for statistical analyses of the SI using SAS (Software package, Version 9.1, SAS Institute, Cary, NC, USA). Because of frequent measurements during the experiment and the resulting individual cow effects, they were considered by the repeated procedure.

Correlations between the proportion of CLA isomers and the SI at 105 days pp were calculated using Statistica 8.

Abbreviations

AB, Alamar blue; ANOVA, analysis of variance; CLA, conjugated linoleic acid; CON, control group; ConA, concanavalin A; DM, dry matter; FA, fatty acid; NEFA, non esterified fatty acids; OD, optical density; PBMC, peripheral blood mononuclear cells; PUFA, polyunsaturated fatty acids; pp, *post partum*; SI, stimulation index

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LR performed isolation of PBMC and cell proliferation assays, did statistical analysis, participated in study design and wrote the manuscript. JP carried out the animal study. RK performed fatty acid analysis of PBMC. SK helped with the statistical analysis and to draft the manuscript. GJ participated in study design and fatty acid analysis. SD participated in study design, helped with statistical analysis and to draft the manuscript. All authors read and approved the final manuscript.

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The authors have declared no conflict of interest.

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Paper II

Effect of conjugated linoleic acid on proliferation and cytokine expression of bovine peripheral blood mononuclear cells and splenocytes ex vivo

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Abstract

Twenty five primiparous Holstein cows were divided into 5 experimental groups (5 animals per group) by different feeding (control fat preparation [CON] or CLA supplement [CLA]) and slaughtering times. The daily consumption of CLA was 6.0 g of the *trans*-10,*cis*-12 CLA-isomer and 5.7 g *cis*-9,*trans*-11 CLA isomer. An initial group (IG) was slaughtered one day *post partum* (pp) and the remaining 20 animals after 42 and 105 days pp, respectively. Blood for peripheral blood mononuclear cells (PBMC) separation was taken 7 days *ante partum* and immediately before slaughter. The spleen was removed during dissection for isolation of splenocytes and samples for histopathological examination. Cell viability and concanavalin A-stimulated proliferation was analyzed by MTT and Alamar blue assay. Basal expression of cytokines (interleukin [IL]-4, IL-10, IL-12, tumor necrosis factor α [TNF-α] and interferon γ [IFN-γ]) was measured by quantitative real time polymerase chain reaction (qRT-PCR) in unstimulated PMBC and splenocytes. With PBMC, stimulation indices increased from one day pp to 105 days pp with no differences between CLA and CON groups. With splenocytes the stimulation index of the CLA group was lower compared to CON group 105 days pp. Baseline expression of cytokines was not effected by CLA feeding comparing similar time points. Also, no differences occurred in the expression of IL-4 in PBMC and IL-10 as well as TNF-α in both cell populations, when comparing the feeding groups separately with IG. IL-4 was more frequently expressed in CLA group 42 days pp in splenocytes. IFN-γ expression was increased 105 days pp in CLA group in splenocytes and PBMC. IL-12 was higher expressed 105 days (PBMC) or 42 days pp (splenocytes) when compared to IG. There was no effect of CLA feeding or slaughter time on histopathology of the spleen.

In conclusion, the present results demonstrate an inhibiting effect of CLA on the mitogen-induced activation of splenocytes.

Keywords: conjugated linoleic acid, PBMC, splenocytes

1 Introduction

Conjugated linoleic acids (CLA) are a group of positional and geometric isomers of linoleic acid, which are originally produced by microbial fermentation in the rumen (KEPLER ET AL. 1966) and via endogenous synthesis by Δ^9-desaturase, whereby *trans*-11 C18:1 serves as a precursor (GRIINARI ET AL. 2000). Because of its milk fat reducing effects (BAUMGARD ET AL. 2000), CLA are frequently added to dairy cow rations and are thus in the focus of dairy cattle

research. Their mode of action is not yet fully understood. Also, little information is available about the effects of CLA on the bovine immune system. In general, fatty acids have the ability to modulate immune responses by altering the composition of phospholipids in immune cells and affecting downstream events like signal transduction pathways (CALDER 2008). This mechanism is also discussed for the mode of action of CLA in immune cells (O'SHEA ET AL. 2004). Furthermore, interactions of CLA with peroxisome proliferator-activated receptors (PPARs) are reported (BASSAGANYA-RIERA ET AL. 2002; O'SHEA ET AL. 2004).

The impact of CLA on the immune system of dairy cattle has, to our knowledge, not yet been investigated, but *in vitro* studies (LACETERA ET AL. 2004; LACETERA ET AL. 2007) and also *in vivo* studies (LESSARD ET AL. 2003) have demonstrated that the bovine immune system is sensitive for fatty acids. Furthermore, the immune function of dairy cows is characterized by an immune suppressed period around parturition. This immunosuppression is indicated e.g. by a reduced response of PBMC to mitogen-stimulation (NONNECKE ET AL. 2003; LOISELLE ET AL. 2009). Reasons for these effects are not fully understood yet, but several mechanisms are discussed. The immunosuppression might be caused by enhanced nonesterified fatty acids (NEFA) concentrations in the serum pp, which originate through increasing fat mobilisation in early lactation. NEFA inhibit the proliferation of bovine PBMC *in vitro* (LACETERA ET AL. 2004). Another starting point might be the mammary gland. Mastectomized cows do not show declined *in vitro* production of IFN-γ and DNA synthesis of pokeweed mitogen (PWM)-stimulated PBMC 0 to 4 days pp as PBMC from intact cows do (NONNECKE ET AL. 2003).

Because of its positive effects, the use of CLA in nutrition of dairy cows is rising and necessitates research in this area.

Therefore, a feeding trial was conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health to investigate these aspects about CLA as supplement in nutrition of dairy cows. The focus of the present work was to examine the effect of CLA on proliferation and cytokine expression of PBMC and splenocytes.

2 Materials and Methods

2.1 Experimental design

A feeding trial was conducted with 25 primiparous lactating German Holstein cows. The whole experiment is described by von Soosten et al. (2011) in detail. The cows were divided into 5 experimental groups of 5 animals in each with different feeding and different slaughter

times. One group got a diet without CLA and was slaughtered one day pp (initial group, [IG]). The other 4 groups got either a CLA mixture (CLA, Lutrell Pure, BASF SE, Ludwigshafen, Germany), or a control fat preparation (CON, Silafat, BASF SE, Ludwigshafen, Germany). Forty-two days *post partum* (pp) 5 animals per feeding group were slaughtered (42/CON and 42/CLA) and the remaining animals after 105 days pp (group 105/CON and 105/CLA). These time points were chosen because the mobilization of body fat is strongest within the first weeks of lactation (42 days pp) and milk yield is highest in the first third of lactation (105 days pp).

Up to 4 weeks before expected parturition all animals got a partial mixed ration (PMR) for *ad libitum* consumption and 2 kg concentrate per day by a computerized concentrate feeding station (Type RIC, Insentec, B.V., Marknesse, The Netherlands). The pp diet contained a PMR (25% grass silage, 38% corn (*Zea mays*) silage and 37% concentrate on DM basis) for *ad libitum* consumption. The control fat supplement or the rumen-protected CLA supplement (lipid encapsulation technique) was given with additional 3.5 kg concentrate (on DM basis) in pelleted form. The CLA concentrate contained 1.7% of the *trans*-10,*cis*-12 CLA isomer and 1.6% of the *cis*-9,*trans*-11 CLA isomer. That implies animals of the CLA group consumed 6.0 g/d of the *trans*-10,*cis*-12 CLA-isomer and 5.7 g/d *cis*-9,*trans*-11 CLA isomer (calculated based on the analyzed proportion in concentrates). In the control fat preparation, CLA was replaced by equal amount of stearic acid. The complete fatty acid profile of both supplements is reported by VON SOOSTEN ET AL. (2011). Water was offered *ad libitum*.

2.2 Sample preparation

If not otherwise stated, all chemicals were purchased from Biochrom AG (Berlin, Germany).

Isolation of PBMC was performed as described by GOYARTS ET AL. (2006). Blood was taken by jugular venipuncture 7 days *ante partum* (ap) and at the day of slaughter. Because some cows calved before expected parturition, it was not possible to take the sample 7 days ap from all cows (19 out of 25 were taken). PBMC were isolated by gradient centrifugation of heparinized blood. Blood (30 mL) was diluted 1:2 with phosphate buffered saline (PBS, L 1820) and layered over the Ficoll separation solution (L 6115). Following centrifugation at 400 x g for 30 min (room temperature [RT]), the PBMC containing interphase was harvested and washed with PBS (250 x g, 8 min, RT). The pellet was resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (F 1275), supplemented with 5% foetal bovine serum (FBS, S 0615), 1 M HEPES buffer (L 1603), 2 mM l-glutamin (K 0282), 5 mM β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany, M 7522), 100 U/mL penicillin and

0.1 mg/mL streptomycin (A 2212), and centrifuged for another 8 min (250 x g, RT). Finally, the cells were resuspended in ice-cold FBS and 10% DMSO (Sigma-Aldrich, Steinheim, Germany, D 2438) as freezing medium. PBMC were frozen and stored at -80°C until cell proliferation assays and cytokine expression analyzes were performed.

Splenocytes were obtained according to TIEMANN ET AL. (2006) with some modifications. Three pieces, approximately 4 g each, were cut out of the pulp of the spleen and rinsed with 70% ethanol. The pieces were put in sterile Hanks solution (Sigma-Aldrich, Steinheim, Germany, H 1387) immediately. The spleen-tissue was shredded through a cell strainer into petri dishes filled with ACK buffer (containing 8.3 g NH_4Cl, 1 g$KHCO_3$ and 200 µl 0.5 M dissolved in 1 L double distilled water) to lyse erythrocytes. The cell suspension was centrifuged at 181 x g for 5 min. The cells were washed with 30 mL Hanks solution and centrifuged for 5 min at 181 x g. The pellet was resuspended in RPMI medium supplemented with 10% FBS (S 0615), 2 mM l-glutamin (K 0282), 5 mM β-mercaptoethanol (Sigma®, M 7522), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (A 2212). Freshly isolated splenocytes were tested in the MTT assay. Remaining splenocytes were centrifuged at 181 x g for 5 min and the pellet was resuspended in freezing medium containing FBS and 10% DMSO. The cells were frozen and stored at -80°C until used for gene expression analysis.

2.3 Cell proliferation assays

For evaluating concanavalin A stimulated cell proliferation and cell viability of PBMC, MTT and Alamar blue (AB) assay were used. In the MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), a yellow tetrazolium salt, is reduced by NAD-dependent dehydrogenase to form a dark blue formazan product (MOSMANN 1983). The optical density (OD) of this product is measured. The AB assay is based on the reduction of the nonfluorescent resazurin to resorufin, which fluoresces. Possible enzymes which are involved in reducing resorufin are NADH-dehydrogenase (EC 1.6.99.3) and diaphorese (EC 1.8.1.4.) (O'BRIEN ET AL. 2000). Therefore, both tests require metabolically active cells.

Thawed PBMC or freshly isolated splenocytes were suspended in RPMI 1640 medium with supplements as described above. Cell viability was evaluated by trypan blue exclusion technique and cell number was adjusted to 1 x 10^6 viable cells/mL. Cell proliferation assays were performed in 96 well plates with 100 µL cell suspension (10^5 cells). Concanavalin A (2.5 µg/ml final, Sigma-Aldrich, Steinheim, Germany, C 5275) or medium (control) were added to each well up to a total volume of 200 µL. The plates were incubated for 72 h (splenocytes) or 48 h (PBMC) at 37°C and 5% CO_2. All set ups were done in 5 replicates.

After incubation, plates were centrifuged (200 x g, 6 min, RT) and 100 µL supernatant of each well was removed. MTT at 2.5 µg/mL was added to each well and incubated for another 4 h (PBMC and splenocytes). To PBMC, AB (AbDSerotec, Oxford, UK, BUF012A) was added at 1:10 for 2.5 h. MTT set ups were incubated over night with a solution containing 0.01 n HCL and sodium dodecyl sulfate to lyse the cells and to dissolve the resulting dye. The OD of the solutions were measured at a wavelength of 570 nm. The fluorescence of the AB reduction product resorufin was measured after 2.5 h at 540 nm (excitation) and 590 nm (emission).

2.4 RNA Isolation

RNA was isolated to investigate mRNA expression of IL-4, IL-10, IL-12, TNF-α and IFN-γ in unstimulated splenocytes and PBMC. Therefore, RNA was isolated using SV Total RNA Isolation kit (Promega, Madison, WI, USA, Z3100). The procedure followed the manufactures' protocol and RNA was quantified spectrophotometrically at 260 nm (Nanodrop). Purity was evaluated by the ratio of OD at 260 nm and 280 nm and was always greater than 1.9. The RNA was stored at -80°C.

2.5 cDNA synthesis

The mRNA concentration was adjusted to 100 ng/µL total RNA before transcribing it into cDNA (complementary DNA) using the enzyme Superscript™ II reverse transcriptase (Invitrogen, Karlsruhe, Gemany). RNA (10 µL), 1 µL Oligo-$(dt)_{12-18}$ primer and 1 µL trinucleotids were mixed and incubated at 65°C for 5 min. Afterwards, the samples were placed on ice and 7 µL master mix (containing first-stand-buffer (250 mmol/L Tris-HCL, 375 mmol/L KCL, 15 mmol/L $MgCl_2$), RNase inhibitor (RNaseOUT™, Invitrogen, Karlsruhe, Germany) and dithiothriol) were added. The samples were incubated at 42°C for 2 min. For reverse transcriptase reaction 1 µL Superscript™ II reverse transcriptase was added and incubated for 50 min at 42°C. The enzyme reaction was stopped by heat inactivation (70°C, 15 min) and the samples were stored in a freezer until quantitative real-time polymerase chain reaction (qrtPCR) was performed.

2.6 Quantitative Real time PCR

SYBR Green® PCR master mix (AppiledBiosystems, Darmstadt, Germany) was used for qrtPCR. Each measurement includes at least 5 points of a standard series (100 -10^6 copies) and a negative control (master mix and water) to determine copy numbers of individual

transcripts. Analyses were carried out in Micro Amp ™ fast 96 well plates in duplicates, each well filled with 1 µL cDNA and 24 µL reaction mix. The reaction mix contained SYBR Green® PCR master mix, RNAse-DNAse-free water and forward and reverse primer (Table 1) in proportions specific for the gene of interest. At the beginning of the reaction samples were heated up to 95°C for 10 min to denaturate DNA. This is followed by 40 cycles of denaturation at 95°C for 15 s and annealing of primer and elongation of the product at 60°C for 1 min. Afterwards, a melting curve was obtained by increasing temperature in 0.3°C steps beginning with 60°C up to 95°C.

Expression rates below 100 copies were beyond the detectable range of the standard curve and therefore indicated as zero.

Table 1 Primer sequences and concentrations used for real-time PCR analysis.

Gene	primer sequences (5´→3´)	concentration (nM)	Bp[1]	Reference
IL-4	for GCC ACA CGT GCT TGA ACA AA	900	63	ALMEIDA ET AL. (2007)
	rev TGC TTG CCA AGC TGT TGA GA	50		
IL-10	for CCT TGT CGG AAA TGA TCC AGT TTT	300	67	ALMEIDA ET AL. (2007)
	rev TCA GGC CCG TGG TTC TCA	300		
IL-12	for TGG TCG TTT CCT GGT TTT CC	300	205	novel design (Accession No NM 174356.1)
	rev GTT TTG CCA GAG CCC AAG AC	300		
INF-γ	for TTC AGA GCC AAA TTG TCT CCT TC	300	205	NEUVIANS ET AL. (2004) mod.
	rev AGT TCA TTT ATG GCT TTG CGC TG	50		
TNF-α	for CTT CTG CCT GCT GCA CTT CG	300	156	YANG ET AL. (2008)
	rev GAG TTG ATG TCG GCT ACA ACG	300		

1 Bp length of amplicons in base pair; for = forward, rev = reverse

2.7 Histopathology of the spleen

Tissue samples of spleens were taken and fixed in 10% formaldehyde solution buffered with $CaCO_3$ for at least 24 h. The samples were embedded in paraffin and sectioned (5 µm). For histological examination the sample was stained with haematoxylin and eosin (H&E).

2.8 Calculations and statistics

The stimulation index was calculated by dividing the OD or fluorescence of mitogen stimulated cells by OD or fluorescence of non-stimulated cells.

The stimulation index and cytokine mRNA expression data were not normally distributed; therefore Mann-Whitney-U-test was used for statistical analyses. The statistical test was

performed between the feeding groups at the corresponding time point and separately over the time within the feeding groups and the IG, respectively.

Spleens were histologically screened for the degree of lymphatic hyperplasia (low, medium and high) and the presence of neutrophil granulocytes in the marginal zone. The corresponding data were evaluated by the analysis of the resulting contingence table.

All statistical analyzes were carried out using Statistica for the Windows operating system.

3 Results

Data evaluating the performance of the cows in the present study are reported elsewhere (VON SOOSTEN ET AL. 2011).

3.1 Proliferation capacity of PBMC and splenocytes

The effect of CLA on proliferation of PBMC and splenocytes was tested at different times after calving and therefore different duration of CLA supplementation. Furthermore, proliferation of PBMC was tested 7 days ap. In the MTT assay of PBMC the stimulation index of the IG was significantly lower than the stimulation index of the 105/CON ($p = 0.016$) and the 105/CLA ($p = 0.047$) group (Figure 1 A).

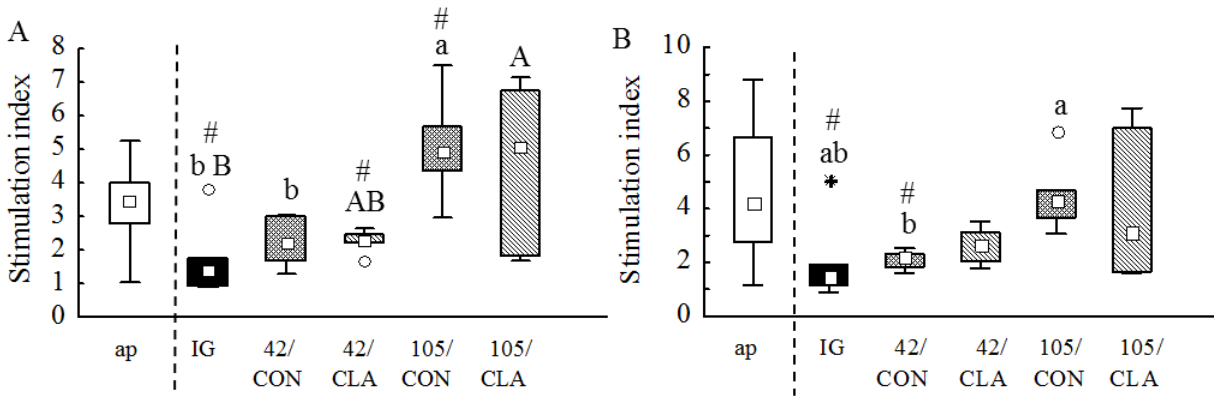

Figure 1 Stimulation index of peripheral blood mononuclear cells of cows 7 days *ante partum* (ap, □, pooled for 19 of the 25 animals), one day *post partum* (pp) (initial group IG, ■) or of cows fed a control (CON, ▩) or a CLA supplemented concentrate (▨), 42 days pp or 105 days pp *ex vivo* in MTT assay (panal A) or in Alamar blue assay (panal B).

Results are shown as median, box (= interquartile range), whisker (= minimum/maximum values), ○ outliers and ✳ extreme values.

Note: Significances: ab, different letters indicate significant differences between groups IG and CON, AB: Different letters indicate significant differences between groups IG and CLA, # indicates significant difference to stimulation index ap (all groups); no significant differences were observed between CON and CLA groups at the same time point, Mann-Whitney U test ($p < 0.05$).

Between 42 and 105 days pp the stimulation index differed only within the CON groups (p = 0.028). There were no differences between CLA-fed and control fat fed groups at similar time points. The stimulation index 7 days ap (pooled for 19 of the 25 animals, for the 6 remaining animals no sample was available) was higher than the stimulation index of IG (p = 0.030) and 42/CLA (p = 0.036) and lower than the stimulation index 105/CON group (p = 0.036). In AB assay (Figure 1 B) the stimulation index between 42 and 105 days pp was different in the CON groups (p = 0.009). As seen in the MTT assay there were no differences between CLA and CON groups comparing the same time in AB assay. The stimulation index ap was significantly higher than one day pp and 42 days pp in the CON group.

In contrast, the reduced ability to stimulate cells after calving was not found in splenocytes (Figure 2), but the stimulation index of the CLA feed group was reduced compared to the CON group 105 days pp (p = 0.028).

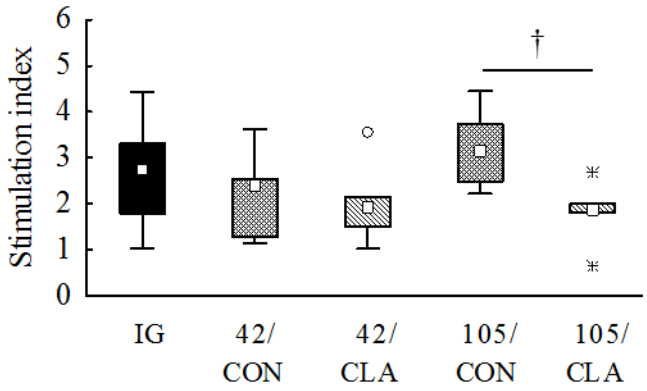

Figure 2 Stimulation index of splenocytes of cows one day *post partum* (pp) (initial group IG, ■) or of cows fed a control (CON, ▩) or a CLA supplemented concentrate (▧), 42 days pp or 105 days pp *ex vivo* in MTT assay.

Results are shown as median, box (= interquartile range), whisker (= minimum/maximum values), ○ outliers, and ✳ extreme values.

Note: Significances: † indicates significant difference between group CLA and CON at the same time point, no differences were observed concerning time within the feeding groups and IG, Mann-Whitney U test ($p<0.05$).

3.2 Expression of cytokines in PBMC and splenocytes

The basal mRNA expression of IL-4, IL-10, IL-12, TNF-α and IFN-γ was analyzed in PBMC and splenocytes (Table 2). Altogether, there was a strong variation in expression of cytokines among animals. IL-4 and IL-10 were expressed more abundantly in splenocytes than in PBMC. The expression of IL-4 in splenocytes was higher in the 42/CLA group than in IG (p = 0.047) and 105/CLA (p = 0.028). There was no effect of time within the feeding groups

and IG in the expression of IL-10 in splenocytes. Unstimulated PBMC occasionally expressed IL-4 and IL-10 with no differences among the groups.

Table 2 mRNA expression [number of mRNA copies] of cytokines in splenocytes and peripheral blood mononuclear cells (PBMC) of cows one day post partum (pp) (initial group, IG) or of cows fed a control (CON) or a CLA supplemented concentrate, 42 days pp or 105 days pp (median; range in brackets).

	Experimental groups					**Effect of CLA supplementation**	
	IG	**42/CON**	**42/CLA**	**105/CON**	**105/CLA**	**Day 42 pp**	**Day 105 pp**
IL-4							
Splenocytes	0^{b} (0-755)	0 (0-916)	804^{a} (108-1091)	0 (0-229)	0^{b} (0-188)	ns	ns
PBMC	0 (0-129)	0	0 (0-300)	0 (0-166)	0	ns	ns
IL-10							
Splenocytes	0 (0-571)	0 (0-534)	576 (0-654)	0 (0-199)	175 (0-305)	ns	ns
PBMC	0 (0-111)	0	0 (0-246)	0 (0-187)	0 (0-104)	ns	ns
TNF-α							
Splenocytes	0	0	0	0	0	ns	ns
PBMC	0 (0-182)	0 (0-894)	0 (0-968)	228 (0-1274)	124 (0-301)	ns	ns
IFN-γ							
Splenocytes	0^{b}	0	0^{b}	0 (0-16460)	513^{a} (0-2761)	ns	ns
PBMC	0^{b}	0	0^{b}	0 (0-2002)	264^{a} (0-9848)	ns	ns
IL-12							
Splenocytes	0^{b} (0-256)	198 (0-288)	321^{a} (136-381)	123 (0-203)	327^{ab} (0-554)	ns	ns
PBMC	117^{b} (0-201)	148 (0-400)	287^{ab} (0-2669)	163 (0-445)	251^{a} (143-2723)	ns	ns

Notes: IL = interleukin, TNF-α = tumor necrosis factor α, INF-γ = interferon γ, ns = not significant different.

Medians within a row not sharing the same superscript indicate significant differences concerning time of sampling (no differences were observed within the CON groups), Mann-Whitney U test ($p < 0.05$)

In PBMC TNF-α was expressed in some samples, but there were no significant differences between the experimental groups. Expression of TNF-α could not be detected in splenocytes. IFN-γ was only expressed 105 days pp in both cell populations, but the increased expression only reached significance in the CLA group ($p = 0.036$). IL-12 was expressed in almost all samples in PBMC and splenocytes. In splenocytes the expression was increased in 42/CLA group compared to IG ($p = 0.028$) and in PBMC in 105/CLA group compared to IG ($p = 0.028$).

There were no differences in cytokine expression between the feeding groups comparing the corresponding time points.

3.3 Histopathology of the spleen

Most animals showed a lymphatic hyperplasia, ranging from low to high degree of severity. In some samples increasing counts of neutrophil granulocytes in the marginal zone were also observed. However, the analysis of the corresponding contingence table did not reveal significant treatment effects ($p = 0.98$; data not shown).

4 Discussion

In the present study we investigated the effect of a CLA supplementation on the function of bovine immune cells. Therefore, PBMC and splenocytes were studied *ex vivo* around parturition.

The mitogen stimulated activation of PBMC was reduced in the present investigation in early lactation. In the time period after calving the immune response is rising, indicated by an increasing stimulation index. These findings are an indication for immunosuppression which is reported for the periparturent period (MALLARD ET AL. 1998; NONNECKE ET AL. 2003; LOISELLE ET AL. 2009). The effect occurred equally in both feeding groups in the MTT assay and in the AB assay within the CON groups. Therefore, an effect of dietary CLA supplementation on the proliferative response of PBMC can be excluded. Ante partum the stimulation index was higher than the first days pp. VON SOOSTEN ET AL. (2011) analyzed the NEFA concentrations of the same animals at corresponding time points as the cell proliferation tests were performed. Starting at day 7 ap the NEFA concentration increased and peaked at one day pp, the time point where the ability to stimulate the PBMC was lowest. Throughout lactation, NEFA concentrations decreased while the stimulation indices increased, which indicated a relation between immunosuppression, seen as decreased stimulation indices, and enhanced NEFA levels around parturition. *In vitro* experiments

showed that NEFA are able to inhibit the mitogen stimulated proliferation of bovine PBMC and therefore might be involved in pp immunosuppression (LACETERA ET AL. 2004). Although the function of PBMC was not influenced by CLA feeding, HUSSEN ET AL (2011) found that feeding the same amount of CLA to dairy cows caused changes in the composition of PBMC. Percentages of $CD4^+$ T cells decreased whereas those of $CD8^+$ T cells among viable mononuclear cells slightly increased.

In human studies mostly no influence of dietary CLA on the proliferation of PBMC was found (KELLEY ET AL. 2000; ALBERS ET AL. 2003). Stronger proliferation of PBMC was found in humans, when a 80:20 blend of the isomers *cis*-9 *trans*-11 and *trans*-10,*cis*-12 were supplemented and cells were stimulated with phytohemagglutinin *ex vivo* (NUGENT ET AL. 2005). Other stimulating agents (concanavalin A, murine monoclonal anti-human CD3 antibody OKT3 and murine monoclonal anti-mouse MHC antibody) or tested fatty acid compositions (50:50 blend of the isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12 or linoleic acid) did not affect proliferation in that study. Increased stimulation indices were found in PBMC obtained from beagle dogs who received a CLA supplement for 9 months (NUNES ET AL. 2008). A dose dependent decrease in lymphocyte activation was observed in a study with healthy men after supplementation with either the *cis*-9,*trans*-11 or the *trans*-10,*cis*-12 isomer (contains 80-85% of the requested isomer) (TRICON ET AL. 2004).

Besides PBMC the proliferation of splenocytes was also investigated with different results. After 105 days of CLA supplementation the stimulation index of splenocytes was decreased compared to control fed group. This is in line with observations obtained with splenocytes of CLA fed rats which proliferative response declined by 10% after phorbolmyristate acetate/ionomycin stimulation (RAMIREZ-SANTANA ET AL. 2009). Also, in male broiler chicks the stimulation index of concanavalin A stimulated splenocytes was reduced in CLA fed birds compared to birds fed the basal diet (TAKAHASHI ET AL. 2007). In contrast, both CLA isomers (*cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA) did not affect the lipopolysaccharide (LPS) or concanavalin A stimulated proliferation of murine splenocytes (KELLEY ET AL. 2002). This argues for species-specific effects of CLA in food supplements.

For more detailed information on the function of splenocytes and PBMC after CLA supplementation, the basal expression of cytokines (IL-4, IL-10, IL-12, TNF-α and IFN-γ) was analyzed in the present study. The anti-inflammatory cytokine IL-10, which is an important regulator of immune response in infection (COUPER ET AL. 2008), was more frequently expressed in splenocytes compared to PBMC. The expression was numerically increased in both CLA groups, but this effect was not significant compared to the control

groups at the corresponding slaughter time. The anti-inflammatory IL-4 basically followed the same expression pattern as IL-10 in PBMC. However, the expression in splenocytes displayed a temporally enhanced expression in the CLA group 42 days pp. In a study carried out with LPS-challenged pigs plasma concentration of IL-10 and furthermore the expression of IL-10 in spleen and thymus increased in CLA groups compared to control. This anti-inflammatory effect was confirmed by a decreased secretion of TNF-α and IL-6, which are both proinflammatory cytokines, in plasma. Additionally, the expression of IL-6 and TNF-α in spleen and thymus did not increase as much as in control animals after LPS injection (CHANGHUA ET AL. 2005). In the present study the expression of two tested inflammatory cytokines (TNF-a, IFN-γ) were not influenced by the CLA supplementation, but IFN- γ increased within the CLA fed groups during lactation. *In vitro* the LPS-stimulated TNF-α production was decreased dose-dependently in bovine whole blood cultures in response to the *trans*-10,*cis*-12 (≤ 100µM) isomer, but not for the *cis*-9,*trans*-11 isomer or linoleic acid (PERDOMO ET AL. 2011). CLA down regulated IL-12 expression in external inguinal lymph nodes in viral infected pigs (BASSAGANYA-RIERA ET AL. 2003). Conversely, the expression of inflammatory IL-12 was significantly enhanced in 42/CLA compared to IG in splenocytes and 105/CLA compared to IG in PBMC.

The expression of cytokines of CLA fed cows was investigated for the first time. No CLA effects were found comparing corresponding time points, but there were time effects within the CLA fed groups. Further research on this topic is needed, for instance using mitogen stimulated cells and furthermore, the expression of cytokines should be investigated at several time points.

Besides differences between splenocytes and PBMC in cell proliferation assays, similar differences are also observed in cytokine expression.

Like in the present study, mild lymphoid hyperplasia in the spleen was observed after histopathological examination in a study with CLA fed mice, but there were also no differences in the degree of severity between CLA and control group (DELANY ET AL. 1999).

5 Conclusion

The present results do not show effects of CLA on the function of bovine PBMC and expression of cytokines in PBMC and splenocytes, but the ability to stimulate splenocytes was reduced after CLA feeding. To evaluate consequences for the animal *in vivo* further studies are needed.

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Paper III

Effects of cis-9,trans-11 and trans-10,cis-12 Conjugated Linoleic Acid, Linoleic Acid, Phytanic Acid and the Combination of Various Fatty Acids on Proliferation and Cytokine Expression of Bovine Peripheral Blood Mononuclear Cells

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Abstract

Fatty acids may have an impact on immune functions, which is important in times of increased mobilization of body fat, e.g., around parturition. The aim of the present study was to investigate the effects of the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12, phytanic acid (PA), linoleic acid (LA) and a fatty acid (FA) mixture (containing 29.8% palmitic acid, 6.7% palmitoleic acid, 17.4% stearic acid and 46.1% oleic acid) on the proliferation of bovine blood mononuclear cells (PBMC) *in vitro* using Alamar blue (AB) and 5-bromo-2′-deoxyuridine (BrdU) assay. Quantitative real-time polymerase chain reaction analyses were performed to evaluate the expression of interleukin (IL)-4, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and peroxisome proliferator-activated receptor (PPAR)-γ in response to *cis*-9,*trans*-11 and LA. The IC_{50} values did not differ between the investigated FA, but there were differences within the proliferation in the response of these FA in a concentration range between 20 and 148 µM (e.g., increased proliferation after treatment with lower concentrations of LA). No differences occurred when different FA combinations were tested. ConA stimulation increased the expression of TNF-α and IFN-γ, whereas IL-10 decreased. In general, neither the baseline expression nor the ConA-stimulated mRNA expression of cytokines and PPAR-γ were affected by the FA. In conclusion, all FA inhibit the proliferation of PBMC dose dependently without significantly altering the induced cytokine spectrum of activated bovine PBMC.

Keywords: conjugated linoleic acid, phytanic acid, bovine peripheral blood mononuclear cells, proliferation, cytokine expression

1. Introduction

Conjugated linoleic acids (CLA) are positional isomers of the C18:2 fatty acid (FA) linoleic acid. They are characterized by the conjugated position of their double bonds. They occur predominantly in ruminants, because CLA, and especially the *cis*-9,*trans*-11 isomer, are intermediate products of the biohydrogenation of unsaturated fatty acids by ruminal microorganisms (KEPLER ET AL. 1966; FRITSCHE AND STEINHART 1998). The proportion of *cis*-9,*trans*-11 CLA in the serum is rather low (0.02 g/100 g total FA), but increased 10 fold due to feeding fresh pasture (KAY ET AL. 2005). An alternative pathway to form CLA is via endogenous synthesis by Δ^9-desaturase and *trans*-11 C18:1 as a precursor (GRIINARI ET AL. 2000), which is also observed in non-ruminants like humans (TURPEINEN ET AL. 2002). Although CLA is formed in ruminants (especially the *cis*-9,*trans*-11 isomer), the

supplementation of dairy cows with CLA gains in importance because of its milk fat reducing effect which is mainly ascribed to the *trans*-10,*cis*-12 isomer (BAUMGARD ET AL. 2000). However, information on immune modulating effects of CLA in dairy cows and bovine cells are scarce. Feeding a CLA mixture to dairy cows had no effect on the mitogen-stimulated proliferation of peripheral blood mononuclear cells (PBMC) *ex vivo* (RENNER ET AL. 2012A), but the proliferation of splenocytes was decreased in the CLA-fed group 105 days *post partum* (RENNER ET AL. 2012B). In the latter study, the effects on cytokine expression were rather conflicting. In these studies a mixture of different CLA isomers was used (mainly *cis*-9,*trans*-11 and *trans*-10,*cis*-12). The impact of different CLA isomers on the proliferation of bovine PBMC has not yet been investigated.

Another potential bioactive fatty acid is the C20 branched chain FA phytanic acid (PA, 3,7,11,15-tetramethylhexadecanoic acid) (HELLGREN 2010). PA originates from phytol, a side chain of chlorophyll, which is released from chlorophyll by ruminal microorganisms and converted into PA (PATTON AND BENSON 1966; VERHOEVEN AND JAKOBS 2001). The concentration of PA in serum of cows is indicated as 5.9 mg/mL (=188.8 µM) by AVIGAN (1966). THOMPSON AND CHRISTIE (1991) investigated the concentration of PA in triazylglyceroles of arterial and venous plasma of cows and reported 6.2 µM in arterial and 6.0 µM in venous plasma. The average PA plasma concentration in male subjects from Germany was 2.91 µM (results from the EPIC study (PRICE ET AL. 2010)). PA does not undergo direct β-oxidation. It is degraded to pristanic acid by α-oxidation (VERHOEVEN AND JAKOBS 2001; WANDERS ET AL. 2011). A lack of the first enzyme of α-oxidation is associated with increased levels of PA in plasma and tissues. This rare inherited dysfunction is called Refsum's disease and shows the following clinical signs: pigmentary retinal degeneration, peripheral neuropathy, cerebellar ataxia and high concentrations of protein in the cerebrospinal fluid (VERHOEVEN AND JAKOBS 2001; HELLGREN 2010). It is reported that PA serves as a ligand of retinoid X receptor (RXR) (KITAREEWAN ET AL. 1996) and peroxisome proliferator-activated receptor (PPAR)-α (ELLINGHAUS ET AL. 1999). Therefore, it is considered as beneficial in prevention of type-2 diabetes and metabolic syndrome (HELLGREN 2010). Because both FA share similar activation mechanisms, both are ligands of PPARs (MOYA-CAMARENA ET AL. 1999), there are probably complementary interactions in their anti-diabetic activity (MCCARTY 2001). Hence, it is hypothesized that CLA and PA share effects on bovine immune cells. Therefore, *in vitro* studies were performed investigating different FA - including CLA and PA - in various concentrations and combinations. The focus

of the research was on the effect of these FA on the proliferation of bovine PBMC. Furthermore, effects on the expression of cytokines were tested for selected FA.

2 Experimental Section

If not stated otherwise chemicals were purchased from Sigma-Aldrich, Steinheim, Germany.

2.1. Sample Preparation

PBMC were obtained from the blood of three different cows. The animals were chosen according to their age, lactation number and lactation stage. All three cows were in their second lactation. The blood samples were taken at two points in time (between 59 and 116 days in milk and 116 and 173 days in milk, respectively) by venipuncture of the *vena jugularis externa* using heparinized vacutainer tubes to obtain PBMC. PBMC were prepared following the procedure described by RENNER ET AL. (2011). The samples were frozen and stored at −80 °C until cell proliferation assays were performed. The concanavalin A (ConA) stimulated (2.5 µg/mL) cell proliferation was analyzed using Alamar blue (AB) and BrdU (5-bromo-2′-deoxyuridine) assay. In the AB assay, a nonfluorescent dye is reduced by metabolically active cells. The resulting dye resorufin fluoresces (O'BRIEN ET AL. 2000). The BrdU assay is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells.

FA were diluted in dimethyl sulfoxide (DMSO, D 2438) to obtain a stock solution (250 mM) and dose response studies were performed with various FA: linoleic acid *cis*-9,*cis*-12 C18-2 (L 1012), *cis*-9,*trans*-11 CLA (Matreya, Pleasant Gap, PA, 1245), *trans*-10,*cis*-12 CLA (Matreya, Pleasant Gap, PA, 1249), and a mixture of FA to mimic the FA composition of the subcutaneous adipose tissue according to RUKKWAMSUK ET AL. (2000). The FA mixture contained 29.8% palmitic acid C16:0 (P 0500), 6.7% palmitoleic acid *cis*-9 C16:1 (P 9417), 17.4% stearic acid C18:0 (85679), and 46.1% oleic acid *cis*-9 C18:1 (O 1383). Furthermore, a dose response study was performed with the branched chain FA phytanic acid (P 4060). The experiments were conducted with FA concentrations between 0 and 500 µM and 1:1.5 dilution steps. Additionally, the effect of the vehicle (0.2% DMSO) was tested at each setting (stated as 0 µM).

The goal of the dose-response studies was to determine reasonable concentrations for further experiments, in which the effect of a combination of different FA on the proliferation of bovine PBMC was investigated. The following FA combinations were tested at 33, 66, 99 and 500 µM: 60% FA mixture and 40% of LA, *cis*-9,*trans*-11, *trans*-10,*cis*-12 CLA or 13.3% of

each FA. The combination of 60% FA mixture and 40% PA or 20% PA and 20% *cis*-9,*trans*-11 and *trans*-10,*cis*-12, respectively was analyzed at 33, 66, 99, 150 and 500 µM. Each setting contained a vehicle control (0.2% DMSO) and a medium control (only medium without DMSO and FA).

2.2 Cell Culture Conditions and Cell Proliferation Assays

Frozen PBMC were thawed and washed with Roswell Park Memorial Institute (RPMI)-1640 medium (Biochrom AG, Berlin, Germany, F 1295) supplemented with 5% fetal bovine serum (FBS, Biochrom AG, S 0615), 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (Biochrom AG, L 1603), 2 mM L-glutamine (Biochrom AG, K 0282), 5 mM β-mercaptoethanol (M 7522), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Biochrom AG, A 2212) and a second time with phosphate buffered saline (PBS, Biochrom AG, L 1820). The samples were centrifuged at 250× *g* for 8 min at room temperature. Supernatants were discarded. After the second washing step, the pellet was suspended in a supplemented RPMI-1640 medium, and cells were adjusted to 1×10^6 cells/mL using the trypan blue exclusion technique and a Neubauer counting chamber. PBMC were seeded into 96-well plates (1×10^5 cells/well), the FA solution and ConA (2.5 µg/mL final, C 5275) or RPMI-1640 medium were added up to a final volume of 200 µL/well. Each set up was performed in 4 replicates. The plates were incubated for 72 h at 37 °C and 5% CO_2. After incubation, the plates were centrifuged at 200× *g* for 5 min and 100 µL of the supernatant per well were removed. The ConA concentration and the used incubation time is based on the study of GOYARTS ET AL. (2006).

Subsequently, AB, evaluating metabolic activity, and BrdU assays, evaluating DNA synthesis, were performed as indicators for ConA stimulated PBMC proliferation. AB (AbDSerotec, Oxford, UK, BUF012A) was added (1:10 final) and incubated for another 2.5 h. The fluorescence of the AB reduction product resorufin was measured at 540 nm (excitation) and 590 nm (emission).

The BrdU proliferation kit (Roche Diagnostic GmbH, Mannheim, Germany, 11 647 229 001) was used according to manufacturer's instructions.

2.3. RNA Isolation and cDNA Synthesis

The FA and their concentrations used for cytokine expression studies were selected based on the results of the cell proliferation assays. PBMC were incubated as described above. Set ups were done in 8 replicates (later pooled for RNA isolation). AB assays were performed as a

control in parallel. The RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany; 74104) was used for RNA isolation following the manufacturer's protocol. The RNA quantity was analyzed spectrophometrically at 260 nm (Nanodrop).

For cDNA synthesis the SuperScript™ II Reverse Transcriptase kit and oligo(dT)$_{12–18}$ primer (both from Invitrogen by Life Technologies, Darmstadt, Germany 18418012) were used according to manufacturer's instructions.

2.4. Quantitative Real Time PCR

The PCR was performed using SYBER green master mix (Invitrogen 4364344). Analyses were carried out in duplicates. Each reaction contained 24 µL reaction mix and 1 µL cDNA or water as negative control. The reaction mix contained SYBR Green® PCR master mix, RNAse-DNAse-free water and forward and reverse primer (sequences and concentrations presented in Table 1) in proportions specific for the gene of interest. The reaction started with heating up to 95 °C to denaturate the DNA. Subsequently 40 cycles of denaturation at 95°C for 15 s and annealing of primers and elongation of the product for 1 min at 60 °C were performed. Afterwards the products were heated from 60 to 95 °C in 0.3 °C steps to obtain a melting curve. The PCR products were quantified by a standard series of cDNA subclones with at least 5 points (10^2–10^6 copies) analyzed simultaneously to the samples.

Table 1 Primer sequences and concentrations used for real-time PCR analysis.

Gene	Forward (for) and reverse (rev) primer sequences (5′→3′)	concentration (nM)	Bp [a]	Reference
IL-4	for GCC ACA CGT GCT TGA ACA AA rev TGC TTG CCA AGC TGT TGA GA	900 50	63	ALMEIDA ET AL. (2007)
IL-10	for CCT TGT CGG AAA TGA TCC AGT TTT rev TCA GGC CCG TGG TTC TCA	300 300	67	ALMEIDA ET AL. (2007)
IL-12	for TGG TCG TTT CCT GGT TTT CC rev GTT TTG CCA GAG CCC AAG AC	300 300	205	novel design (Accession No. NM 174356.1)
INF-γ	for TTC AGA GCC AAA TTG TCT CCT TC rev AGT TCA TTT ATG GCT TTG CGC TG	300 50	205	NEUVIANS ET AL. (2004)
TNF-α	for CTT CTG CCT GCT GCA CTT CG rev GAG TTG ATG TCG GCT ACA ACG	300 300	156	modified form YANG ET AL. (2008)
PPAR-γ	for AAG AAT ATC CCC GGC TTT GT rev TTG GGC TCC ATA AAG TCA CC	300 300	200	novel design (Accession No. NM 181024)

[a] bp length of amplicons in base pair.

2.5. Calculations and Statistics

The stimulation index (*SI*) in the AB assay was calculated by the following equation:

$$SI = \frac{\text{Fluorescence or OD of ConA stimulated PBMC}}{\text{Fluorescence or OD of nonstimulated PBMC}} \quad (1)$$

The dose response curves (*SI*) were fitted to the following nonlinear regression equation (MERCER ET AL. 1987):

$$SI = \frac{R_0 \cdot K_{05}{}^{b} + R_{max} \cdot Con^{b}}{K_{05}{}^{b} + Con^{b}} \quad (2)$$

where: R_0 = intercept on ordinate (SI at 0 µM), R_{max} = asymptotic *SI* when Con converges to infinity, *Con* = FA concentration (µM), K_{05} = *SI* at 0.5 × (R_{max} + R_0), *b* = apparent kinetic order. According to mean values of all separate curves (fatty acids and animals) R_0 and R_{max} were defined for all variants. The *SI* was only calculated for the AB assay, because non-stimulated PBMC did not proliferate and therefore showed weak signals.

The proliferation of the ConA stimulated PBMC was calculated in relation to the control (no FA, but with DMSO), which was set at 100% in the AB and BrdU assay. For the BrdU assay, the proliferation of each FA was fitted to the following nonlinear regression equation:

$$\text{Resp} = \frac{100\text{-}R_{max}}{(1 + a^{b \cdot Con} + c^{d \cdot Con} - a - c) + R_{max}} \quad (3)$$

where: Resp = proliferation (%), R_{max} = asymptotic proliferation when *Con* converges to infinity, *Con* = FA concentration (µM) *a*, *b*, *c*, *d* = other estimation parameters. The resulting curves from Equations (1) and (2) were used to estimate the IC_{50} value of the investigated FA. A one factorial ANOVA was performed within the IC_{50} values of the AB assay and the results of mRNA expression analyses. A multifactorial ANOVA was used for analyses of the ConA stimulated proliferation (% of control) of BrdU and AB assay, where the FA and FA concentrations are fixed factors. In addition, interactions between these factors were calculated. The Tukey test was used as a post-hoc test.

All statistical analyses were calculated using the Statistica for the Windows operating system.

3. Results

3.1. Dose Response Studies

The dose response curves (based on the SI obtained in the AB assays) were fitted to equation 2 and used to calculate IC_{50} values. The IC_{50} values (means ± SD) were as follows:

LA 100.7 ± 18.4 µM, *cis*-9,*trans*-11 53.8 ± 11.9 µM, *trans*-10,*cis*-12 70.1 ± 12.5 µM, PA 94.7 ± 29.8 µM and the FA mixture 80.8 ± 21.4 µM. Differences between FA IC_{50} values were not significant ($p = 0.093$). For further cytokine expression analyses the FA with the lowest (*cis*-9,*trans*-11) and the highest (LA) IC_{50} value were used. Considering the ConA-stimulated proliferation (defined as % of control, without FA), there was an effect of FA, FA concentration and an interaction of these factors (always $p < 0.01$) on PBMC proliferation, in the AB assay (Figure 1). The proliferation decreased significantly compared to control starting at 99 µM for the FA mixture, 66 µM for *cis*-9,*trans*-11, 148 µM for *trans*-10,*cis*-12 and PA. For LA, the proliferation increased from 20 to 99 µM compared to control and decreased at concentrations ≥ 148 µM. An effect of the FA was seen between 20 and 148 µM, LA showed a higher proliferation than the remaining four FA.

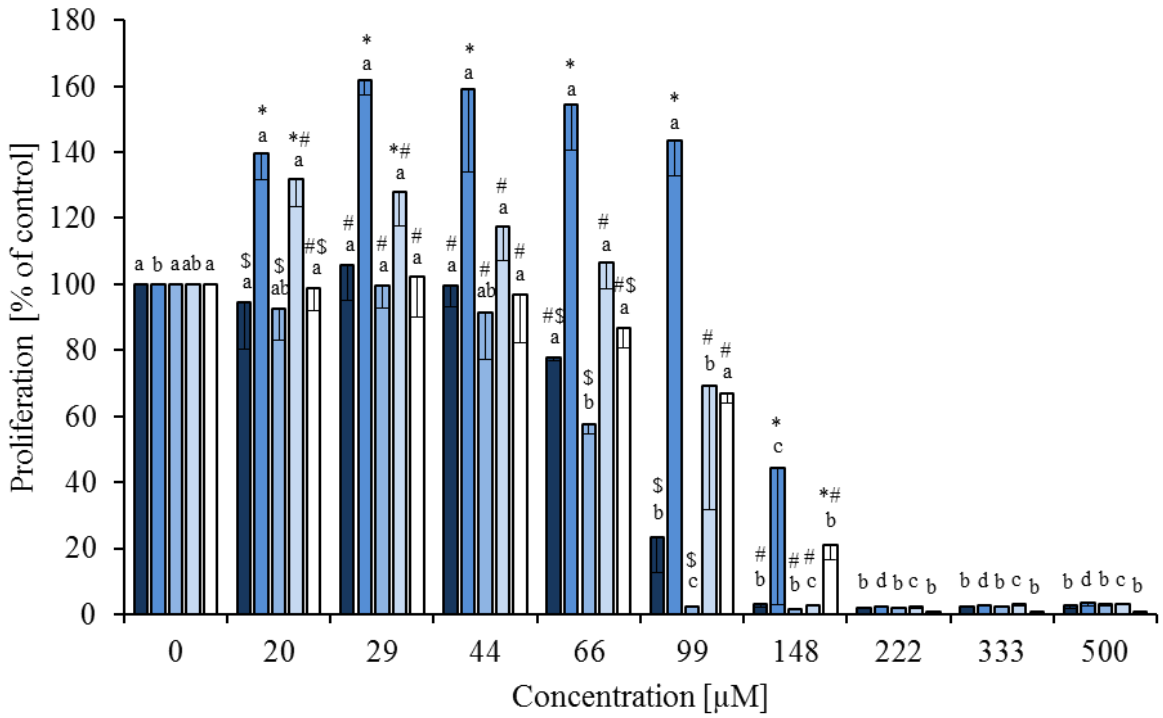

Figure 1 Effects of a fatty acid mixture* (■), linoleic acid (■), *cis*-9,*trans*-11 (■), *trans*-10,*cis*-12 (■) and phytanic acid (□) on concanavalin A stimulated proliferation of bovine peripheral blood mononuclear cells (n = 3) in the Alamar blue assay (means ± standard deviation). * containing 29.8% palmitic acid, 6.7% palmitoleic acid, 17.4% stearic acid and 46.1% oleic acid according to RUKKWAMSUK ET AL. (2000). a–d: different letters indicate significant differences within the same fatty acid, *, #, $ indicate significant differences between fatty acids at the same concentration, $p < 0.05$, Tukey test.

In the BrdU assay there was also an effect of the FA, the FA concentration and an interaction of these factors (always $p < 0.01$) on ConA-stimulated proliferation. The proliferation (shown as % of control) decreased starting at 66 µM for the FA mixture and *cis*-9,*trans*-11, at 99 µM for *trans*-10,*cis*-12 and 148 µM for LA and PA. Differences between the FA occurred

between 44 and 99 µM (Figure 2). The IC_{50} values obtained from dose-response curves (fitted to Equation (3)) were in the same range as those after AB assays: FA mixture (71.2 µM), LA (104.4 µM), *cis*-9,*trans*-11 (57.5 µM). The IC_{50} value of *trans*-10,*cis*-12 isomer obtained with the BrdU assay was lower (84.4 µM) and for PA higher (115.1 µM) compared to IC_{50} values obtained after AB assays.

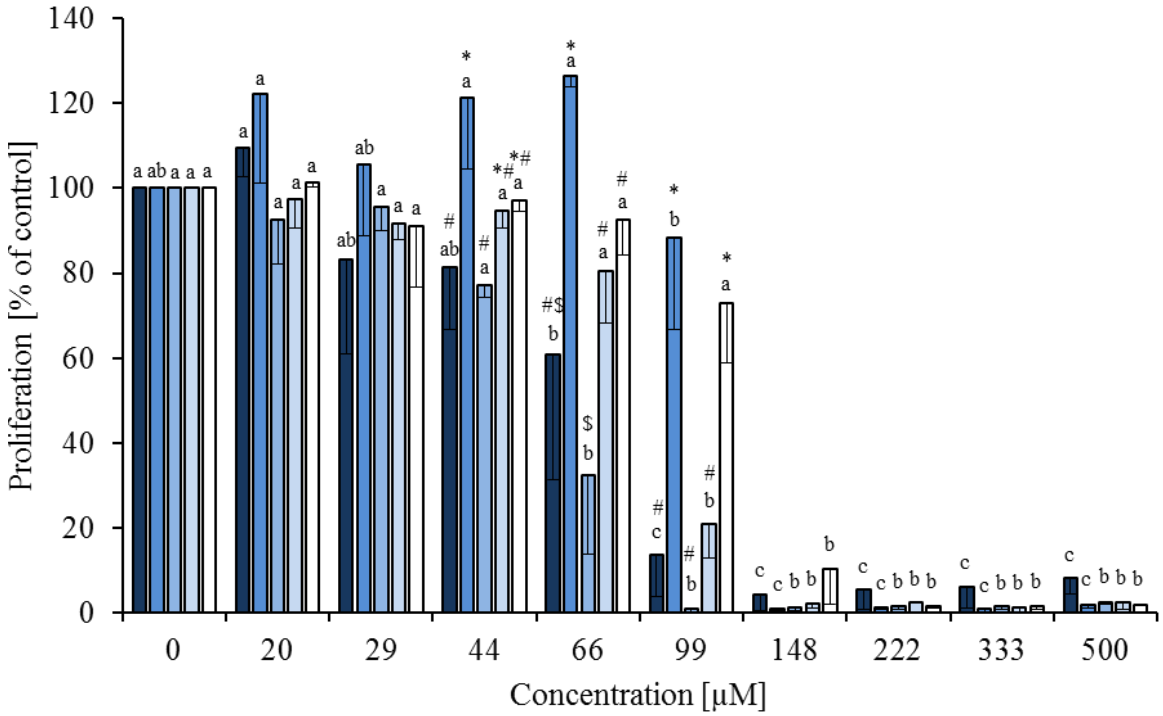

Figure 2 Effects of a fatty acid mixture* (■), linoleic acid (■), *cis*-9,*trans*-11 (■), *trans*-10,*cis*-12 (■) and phytanic acid (□) on concanavalin A stimulated proliferation of bovine peripheral blood mononuclear cells (n = 3) in the BrdU assay (means ± standard deviation). * containing 29.8% palmitic acid, 6.7% palmitoleic acid, 17.4% stearic acid and 46.1% oleic acid according to RUKKWAMSUK ET AL. (2000). a–c: different letters indicate significant differences within the same fatty acid, *, #, $ indicate significant differences between fatty acids at the same concentration, $p < 0.05$, Tukey test.

3.2. Fatty Acid Combinations

FA combinations were investigated at different concentrations (0, 33, 66, 99 and 500 µM): 60% of the FA mixture and 40% *cis*-9,*trans*-11 CLA or *trans*-10,*cis*-12 CLA or LA or a mixture of these 3 C18:2 FA (13.3% of each FA). There was no effect of FA combination ($p = 0.977$) and no interaction between FA combination and concentration ($p = 0.987$). Rising concentrations of the FA significantly inhibited PBMC proliferation ($p < 0.01$, Figure 3).

The FA mixture was also tested in combination with PA (60% FA mixture and 40% PA) and with PA and CLA (60% FA mixture, 20% PA and 20% *cis*-9,*trans*-11 and 60% FA mixture, 20% PA and 20% *trans*-10,*cis*-12) at 0, 33, 66, 99, 150 and 500 µM. There was an effect of FA combination ($p < 0.01$) and FA concentration ($p < 0.01$), but no interaction between FA combination and concentration ($p = 0.225$, Figure 4).

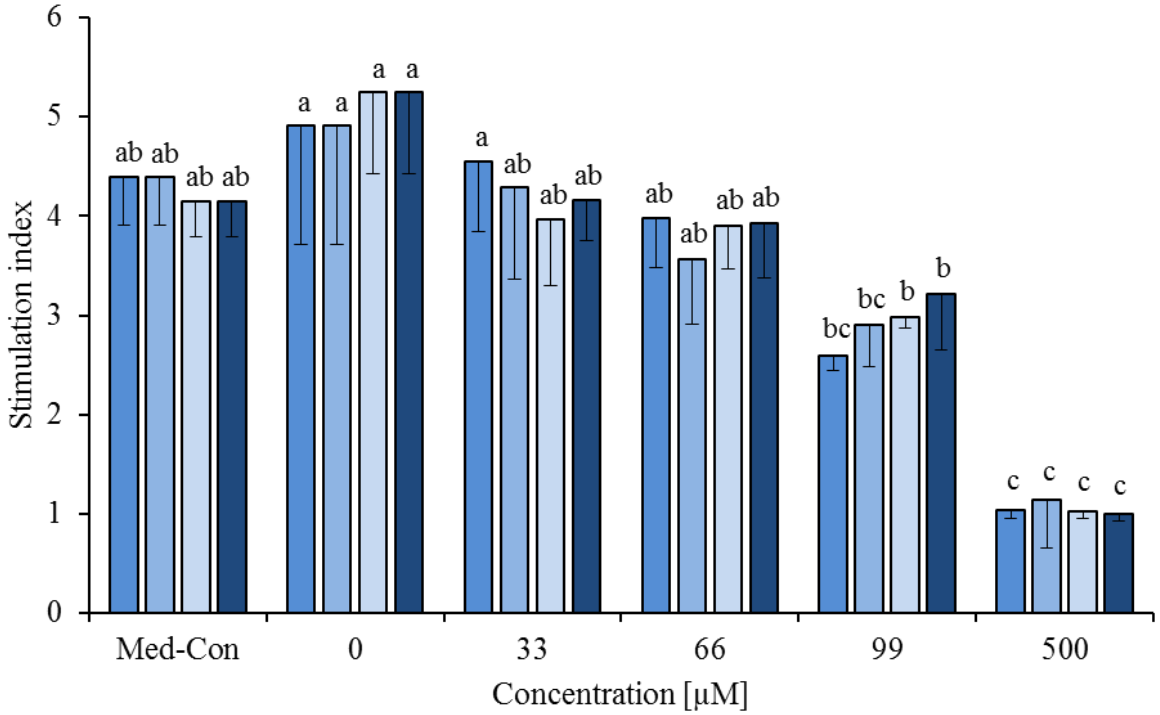

Figure 3 Effects of the combination of the fatty acid mixture* (60%) with either 40% linoleic acid (■), *cis*-9,*trans*-11 (■) or *trans*-10,*cis*-12 (■) or all 3 of them (each 13.3%, ■) on concanavalin A stimulated proliferation of bovine peripheral blood mononuclear cells (PBMC) (n = 3) in the Alamar blue assay (means ± standard deviation). * containing 29.8% palmitic acid, 6.7% palmitoleic acid, 17.4% stearic acid and 46.1% oleic acid according to RUKKWAMSUK ET AL. (2000). Med-Con = PBMC incubated without fatty acids and DMSO. a–c: different letters indicate significant differences within the same fatty acid, $p < 0.05$, Tukey test.

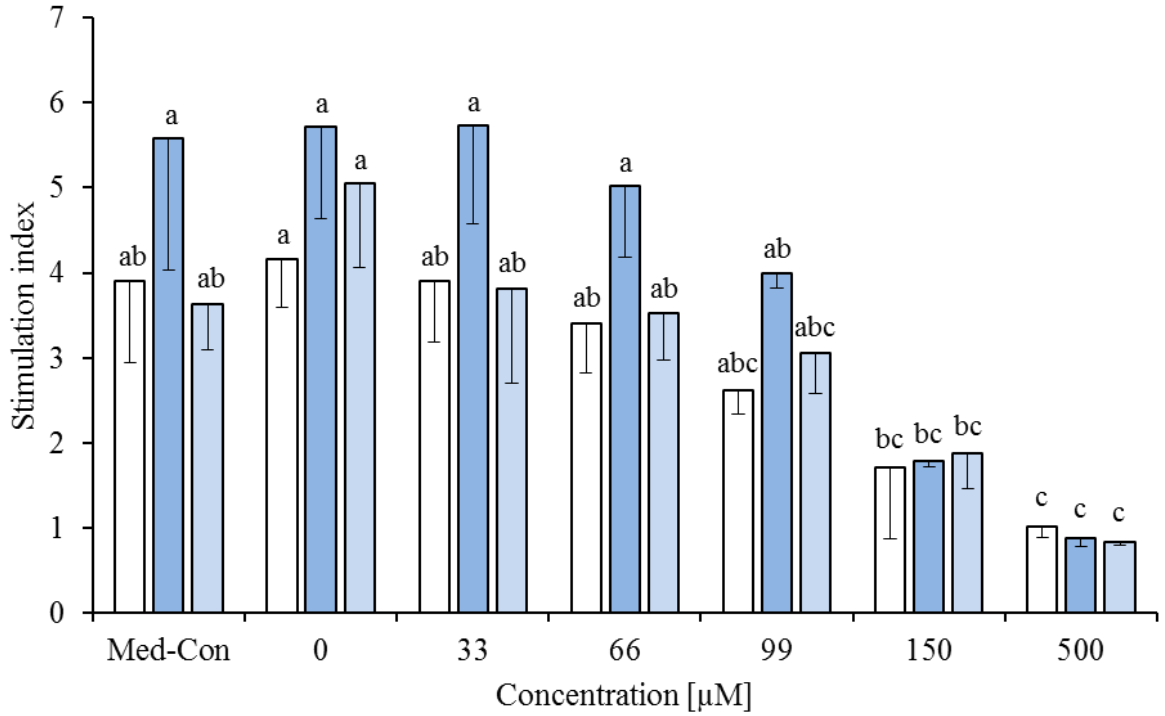

Figure 4 Effects of the combination of the fatty acid mixture* (60%) with either phytanic acid (40%, □) or phytanic acid (20%) and *cis*-9,*trans*-11 (20%, ■) or phytanic acid (20%) and *trans*-10,*cis*-12 (20%, ■) on concanavalin A stimulated proliferation of bovine peripheral blood mononuclear cells (PBMC) (n = 3) in the Alamar blue assay (means ± standard deviation). * containing 29.8% palmitic acid, 6.7% palmitoleic acid, 17.4% stearic acid and 46.1% oleic acid according to RUKKWAMSUK ET AL. (2000). Med-Con = PBMC incubated without fatty acids and DMSO. a–c: different letters indicate significant differences within the same fatty acid, $p < 0.05$, Tukey test.

3.3. Cytokine and PPAR-γ mRNA Expression

The *cis*-9,*trans*-11 CLA isomer and LA were chosen for cytokine and PPAR-γ expression analyses, because their IC_{50} values showed the greatest difference in proliferation (based on results obtained with the AB assay). Therefore, PBMC were incubated with the concentration of the IC_{50} value of the investigated FA and 77 µM, which is the mean concentration of the IC_{50} values of *cis*-9,*trans*-11 and LA. Furthermore, the cells were incubated with medium (Med-Con) or DMSO (DMSO-Con) in the concentration used to dissolve the FA. The expression was analyzed in non-stimulated and ConA-stimulated cells and the results are presented in Table 2.

There were no treatment effects (FA and ConA stimulation) for IL-4. The expression of IL-10 was higher in non-stimulated PBMC than in ConA-stimulated PBMC, which was significant for the Med-Con. TNF-α was more expressed in ConA-stimulated PBMC. Significant differences occurred for the Med-Con and the IC_{50} of LA. In addition, the mRNA expression of IFN-γ was induced by ConA stimulation, which also resulted in significant differences for the Med-Con and the IC_{50} of LA. Within the ConA-stimulated PBMC the expression of IFN-γ

was decreased in DMSO-Control and 77 µM *cis*-9,*trans*-11 compared to Med-Con. There were no differences of PPAR-γ expression between non-stimulated and ConA-stimulated PBMC, but the expression was significantly higher in the ConA-stimulated PBMC treated with 77 µM *cis*-9,*trans*-11 than that treated with 77 µM LA. IL-12 was not expressed in samples from non-stimulated cells and only weakly in some samples from ConA-stimulated cells (results not shown).

Table 2 Expression of cytokine and PPAR-γ mRNA.
Unstimulated (–) and concanavalin A (ConA) stimulated (+) bovine peripheral blood mononuclear cells were incubated with DMSO (0.2%), medium (without DMSO or fatty acids), 100.7 µM linoleic acid (IC_{50} LA), 77 µM linoleic acid (77 µM LA), 53.8 µM *cis*-9,*trans*-11 (IC_{50} *cis*-9,*trans*-11), 77 µM *cis*-9,*trans*-11 (77 µM *cis*-9,*trans*-11). Means ± standard deviation, n = 3.

Treatment	ConA	IL-4	IL-10	TNF-α	IFN-γ	PPAR-γ
DMSO	–	26,852 ± 3032	6201±1083	1039 ± 419	5181 ± 5759	4881 ± 790
	+	22,984 ± 4567	3808 ± 1765	4969 ± 1640	109,082 ± 56,582 [a]	3514 ± 1022 [a,b]
Medium	–	28,825 ± 5887	6700 ± 1848	900 ± 237	3617 ± 3273	4978 ± 565
	+	26,714 ± 3416	3719 ± 827 *	8509 ± 4635 *	295,989 ±167,635 [b,]*	3907 ± 694 [a,b]
IC_{50} LA	–	23,941 ± 4335	5312 ± 994	370 ± 145	613 ± 570	4019 ± 739
	+	34,703 ± 7345	4146 ± 1362	10,194 ± 5630 *	242,302 ± 126,184 [a,b,]*	3454 ± 365 [a,b]
77 µM LA	–	25,845 ± 1695	5635 ± 401	506 ± 179	1809 ± 1570	4547 ± 467
	+	28,348 ± 15,180	3520 ± 1019	9683 ± 7214	204,490 ± 159,761 [a,b]	2820 ± 635 [a]
IC_{50}	–	32,230 ± 4300	7245 ± 866	588 ± 87	743 ± 648	5507 ± 1203
cis-9,*trans*-11	+	32,640 ± 1566	5504 ± 1085	8957 ± 4128	97,534 ± 16,261 [a,b]	4641 ± 859 [a,b]
77 µM	–	29,684 ± 3774	6917 ± 617	511 ± 58	430 ± 319	5254 ± 712
cis-9,*trans*-11	+	31,867 ± 7269	5252 ± 349	8046 ± 4588	68,327 ± 28,935 [a]	5288 ± 439 [b]
p-value		0.182	<0.001	<0.001	<0.001	<0.001

* indicates significant differences between unstimulated and ConA stimulated cells; [a, b] different letters in a row indicate significant differences within ConA stimulated cells, $p < 0.05$.

4. Discussion

Around parturition the dairy cow is susceptible to infectious diseases like mastitis and experiences a state of immunosuppression (KEHRLI AND HARP 2001). Increased NEFA concentrations are discussed as a reason for this immunosuppression (LACETERA ET AL. 2004; STER ET AL. 2012). Due to the demands of the fetus in late pregnancy and the beginning of lactation, the energy requirements increase in this transition period while the dry matter intake is reduced (GRUMMER ET AL. 2004). Therefore, the cow mobilizes body fat from adipose tissue as an additional source of energy (GOFF AND HORST 1997). This lipomobilization has the effect that the concentration of NEFA and also of β-hydroxybutyrate increases in plasma

(GOFF AND HORST 1997; CONTRERAS AND SORDILLO 2011). Hence, the composition of the FA mixture used in the present study is based on the FA composition of subcutaneous adipose tissue (RUKKWAMSUK ET AL. 2000) in order to mimic the lipomobilization and the situation *in vivo*.

For the dose response studies on cell proliferation, two different assays were used for evaluation: AB and BrdU assay. The BrdU assay actually measures newly synthesized DNA, whereas the AB assay evaluates the metabolic activity of living cells (O'BRIEN ET AL. 2000; GONZALEZ AND TARLOFF 2001). Both assays showed similar results and the calculated IC_{50} values are in the same range for the FA mixture, LA and *cis*-9,*trans*-11. For *trans*-10,*cis*-12 and PA the differences in the IC_{50} values were slightly greater. In the BrdU assay the signal of non-stimulated cells was weak, as obviously the non-stimulated cells did not proliferate, so no SI was calculated. In the AB assay, more differences were observed between the FA (in the concentration range from 20 to 148 µM), than in the BrdU assay, where significant differences between FA occurred in concentrations from 44 to 99 µM.

In the present study, the FA mixture inhibited the mitogen-stimulated proliferation of PBMC with an IC_{50} value of 80.8 µM. These concentrations are much lower than results from another study that investigated different NEFA concentrations on the proliferation of bovine PBMC (LACETERA ET AL. 2004). In that study, the proliferation was only decreased at 1000 and 2000 µM in response to phythemagglutinin (PHA) and ConA, and in addition at 500 µM in response to pokeweed mitogen (PWM). The composition of the NEFA was slightly different. It contained 5% LA, which was not in the FA mixture of the present study, because LA was tested separately. LA increased the ConA-stimulated proliferation of PBMC in the present study. This was observed in comparison to the control and to the other investigated FA up to 99 µM. Stimulating effects of LA on bovine PBMC were also found by LACETERA ET AL. (2007) where the proliferation (BrdU assay) was increased at 9 µM LA in response to PWM, and THANASAK ET AL. (2005). In that study, LA increased the proliferation at 5 and 25 µM LA (without using a mitogen). At higher concentrations LA had inhibiting effects on the proliferation of PBMC which was seen in the present study (IC_{50} value 100.7 µM), by LACETERA ET AL. (2007) (reduced proliferation at 103 µM in response to ConA and PWM) and THANASAK ET AL. (2005) (reduced proliferation at 125 and 250 µM in response to ConA). In ewes the proliferation of PBMC was not affected by LA up to 100 µM (LACETERA ET AL. 2002).

The effects of CLA on cell proliferation *in vitro* have been predominantly investigated on various tumor/cancer cell lines, with inhibiting effects, e.g., on the mammary tumor cell line

MDA-MB-231 (MA ET AL. 2002), Jurkat cells (LUONGO ET AL. 2003) and human hepatoma HepG2 cells (IGARASHI AND MIYAZAWA 2001). In a study using non tumor cells, only the *trans*-9,*trans*-11 CLA isomer (5–60 µM) inhibited the proliferation of bovine aortic endothelial cells. Other CLA isomers (*cis*-9,*trans*-11; *cis*-9,*cis*-11; *trans*-10,*cis*-12 and *cis*-11,*trans*-13) had no effect on proliferation of these cells (LAI ET AL. 2005). In the present investigation the inhibiting effect of *cis*-9,*trans*-11 was observed at lower concentrations than for the *trans*-10,*cis*-12 isomer in both assays. However, these differences did not induce significantly different IC_{50} values of the 2 isomers. Although differences occurred between LA and the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12, these differences were not found when these FA were combined with the FA mixture. The combinations of different FA reflect the situation in the organism better than the single FA and the concentrations used in the study mimic the NEFA concentrations found in healthy cows (LACETERA ET AL. 2004). Furthermore, CLA supplements used for *in vivo* studies mostly contain a mixture of CLA isomers. The supplementation of dairy cows with a CLA supplement containing mainly *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA did not alter the mitogen-stimulated proliferation of PBMC *ex vivo* (RENNER ET AL. 2012A; RENNER ET AL. 2012B), but the SI of splenocytes was decreased in the CLA group after 105 days of supplementation (RENNER ET AL. 2012B).

PA has potential bioactive effects and is discussed in the context of type-2 diabetes (HELLGREN 2010). It is reported that PA decreases the proliferation of human prostate cancer cells PC-3 (50 µM) (TANG ET AL. 2007), although a positive correlation between prostate cancer and serum PA levels was found (XU ET AL. 2005). The effects of PA on immune cells have, to the authors' knowledge, not yet been investigated. The results of the present study show that PA reduces the proliferation of bovine PBMC beginning at a concentration of 148 µM and the calculated IC_{50} value was at 94.7 µM. AVIGAN (1966) indicated the concentration of PA in bovine serum with 5.9 mg/100 mL (=188.8 µM) without further information about the investigated animals. THOMPSON AND CHRISTIE (1991) analyzed the concentration of PA in triglycerides of arterial and mammary venous plasma, obtaining much lower concentrations (arterial plasma 6.2 µM, venous plasma 6.0 µM). As seen for the other investigated FA PA inhibited the ConA-stimulated proliferation of bovine PBMC dose-dependently. Only in the concentration range of 66 to 99 µM differences to the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomer occurred. It was hypothesized that there might be complementary effects of PA and CLA in their anti-diabetic activity (MCCARTY 2001). Therefore, a combination of the FA mixture with PA and the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12 was tested to investigate complementary effects on PBMC. No differences in

the SI were found between the tested FA combinations at the corresponding concentrations. Hence, no complementary effects of PA and CLA were observed on bovine PBMC.

Based on the results from the dose response studies, and the combinations of C18:2 FA with the FA mixture, cytokine expression analyses were performed in selected treatments. Because no differences occurred within the FA combinations, the pure FA were chosen for the analyses. The greatest difference in IC_{50} values was between *cis*-9,*trans*-11 CLA and LA, so these two FA were used. The expression of IL-4, IL-10, IL-12, TNF-α and IFN-γ was analyzed in non-stimulated PBMC and in response to ConA. ConA did not increase the expression of IL-4 and IL-10. Furthermore, the expression of IL-10 was significantly increased in PBMC cultured in medium without the addition of the FA or the vehicle control DMSO in non-stimulated cells compared to ConA-stimulated cells. In human PBMC the expression of IL-4 was only slightly increased by 2 mitogens (PHA and phorbol myristate acetate [PMA]) after 4 and 24 h, respectively (SULLIVAN ET AL. 2000). WATTEGEDERA ET AL. (2010) examined the expression of IL-10 and IFN-γ from ovine PBMC in response to ConA over 96 h. The expression of IFN-γ was higher than that of IL-10, which is in line with the present results. In contrast, ConA increased the expression of IL-10 in ovine PBMC, whereas ConA-stimulation did not increase its expression in bovine PBMC in the present study. The expression of TNF-α in non-stimulated PBMC might be due to compounds of the FBS in the culture medium. One of these compounds is bovine serum albumin which increased TNF-α production in murine macrophages (ZHENG ET AL. 1995).

TNF-α expression was not altered by *cis*-9,*trans*-11 or LA in PBMC incubated with or without ConA in the present study. *Ex vivo*, the basal expression of TNF-α from bovine PBMC and splenocytes was not affected by CLA supplementation (RENNER ET AL. 2012B), which is in line with the present results. The *cis*-9,*trans*-11 isomer and LA had no effect on the production of TNF-α in whole blood cultures from Holstein heifers stimulated with LPS, but the TNF-α production was decreased when cells were incubated in the presence of 50 or 100 µM *trans*-10,*cis*-12 CLA (PERDOMO ET AL. 2011). LPS is a major component of the outer membrane of gram negative bacteria that interacts with specific receptors and induces the release of inflammastory mediators (ADEREM AND ULEVITCH 2000). In PBMC from pigs the LPS-stimulated expression of TNF-α was decreased *in vitro* by the *trans*-10,*cis*-12 isomer (CHANGHUA ET AL. 2005; KIM ET AL. 2011). In contrast, TNF-α increased in the study of KIM ET AL. (2011) when non-stimulated cells were incubated with *trans*-10,*cis*-12 (10 µM). The *cis*-9,*trans*-11 isomer or a mix of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 (final concentration 100 µM) had no effect on TNF-α mRNA expression of LPS-stimulated porcine PBMC

(CHANGHUA ET AL. 2005). The anti-inflammatory effects were confirmed *in vivo* in LPS-challenged pigs who received a diet containing 2% CLA. The expression of TNF-α and IL-6, which is also an inflammatory cytokine, was decreased in thymus and spleen and furthermore, the anti-inflammatory cytokine IL-10 was increased (CHANGHUA ET AL. 2005). IL-10 expression and production was also increased in LPS-stimulated dendritic cells from mice in response to *cis*-9,*trans*-11 (50 µM) *in vitro*, whereas IL-12 was decreased (LOSCHER ET AL. 2005). In the present study there were no differences in IL-10 and IL-4 expression due to FA treatment. The expression of Il-4 in bovine splenocytes was increased after 42 days of supplementation compared to an initial group not supplemented with CLA, but there was no effect on expression in PBMC and the expression of IL-10 in both cell types (RENNER ET AL. 2012B).

Significant effects were observed in the expression of IFN-γ. The expression was decreased in ConA-stimulated cells treated with 77 µM *cis*-9,*trans*-11 and the vehicle control DMSO compared to the medium control. Treatment with the IC_{50} value of *cis*-9,*trans*-11 decreased the expression of IFN-γ only numerically compared to the medium control. It is questionable, if the decrease of IFN-γ mRNA expression is due to the treatment with *cis*-9,*trans*-11 or an effect of DMSO which served as an solvent for the FA. The expression of IFN-γ increased after 105 days of supplementation in bovine PBMC and splenocytes compared to a non-supplemented initial group and 42 days of CLA supplementation (RENNER ET AL. 2012B). The expression of IFN-γ and IL-6 in bursa tissue of chicken infected with the infectious bursal disease virus (IBDV) did not increase in CLA-fed birds as much as in control animals 3 days post infection (LONG ET AL. 2011).

The expression of PPAR-γ was significantly lower in ConA-stimulated PBMC when cells were treated with 77 µM LA than with 77 µM *cis*-9,*trans*-11. However, there were no differences between the other tested FA concentrations or controls, so there is no clear effect of enhanced PPAR-γ expression due to *cis*-9,*trans*-11 in bovine PBMC. The PPAR-γ antagonist GW9662 did not reverse the inhibiting effects of *cis*-9,*trans*-11 (25 and 50 µM) on proliferation of bovine PBMC (HUSSEN AND SCHUBERTH 2011). Hence, the authors concluded, that the inhibition of proliferation of bovine PBMC by *cis*-9,*trans*-11 CLA is independent from PPAR-γ. Enhanced expression of PPAR-γ in response to the *trans*-10,*cis*-12 isomer was found in porcine PBMC *in vitro* (KANG ET AL. 2007; KIM ET AL. 2011). In the latter study KIM ET AL. (2011) concluded that the immunostimulating effects (increased NFκB activity and expression of TNF-α in non-stimulated PBMC) and anti-inflammatory effects (decreased NFκB activity and TNF-α expression in LPS-stimulated PBMC) are PPAR-γ

dependent. In bovine immune cells the LPS induced production of TNF-α is attenuated by a PPAR-γ agonist (PERDOMO ET AL. 2011). The observations argue for isomer specific effects on PPAR-γ expression in PBMC. Isomer specific effects were also found in human preadipocytes, where the *cis*-9,*trans*-11 isomer increased the expression of PPAR-γ target genes. In contrast, the *trans*-10,*cis*-12 isomer decreased the expression of PPAR-γ (BROWN ET AL. 2003).

5. Conclusions

FA inhibit the ConA-stimulated proliferation of bovine PBMC *in vitro* in a dose dependent manner. Differences in the inhibition were only seen when single FA were tested but not in combination. Especially the effects in response to LA were different from the other investigated FA. The IC_{50} value of PA did not differ from that of the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12.

The effect of the *cis*-9,*trans*-11 CLA isomer on cytokine expression was marginal and does not indicate inflammatory or anti-inflammatory effects of CLA on bovine PBMC *in vitro*.

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Conflict of Interest

The authors declare no conflict of interest.

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General Discussion

1 Effects of CLA on cell proliferation and viability of bovine PBMC ex vivo and in vitro

The effect of CLA on the proliferation of PBMC was investigated *ex vivo* (**Paper I and II**) and *in vitro* (**Paper III**). The Alamar blue (AB) assay was used for *in vitro* and *ex vivo* tests. Furthermore, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was performed for *ex vivo* analyses and the BrdU (5-bromo-2'-deoxyuridine) assay for *in vitro* analyses. The PBMC investigated *ex vivo* were obtained from two different feeding trials. The first study was carried out with 46 primiparous and pluriparous dairy cows (**Paper I** and PAPPRITZ ET AL. (2011B)) who received different amounts of CLA (Table 3).

Table 3 Daily intake of the CLA supplement and the control fat preparation in both studies.

	Study 1			**Study 2 (Slaughter trial)**				
Groups	CON	CLA-50	CLA-100	IG	42/ CON	42/ CLA	105/ CON	105/ CLA
CLA supplement [g/d]	-	50	100	-	-	100	-	100
control fat preparation [g/d]	100	50	-	-	100	-	100	-
Intake of *cis*-9,*trans*-11 [g/d]*	-	4.0	8.0	-	-	5.7	-	5.7
Intake of *trans*-10,*cis*-12 [g/d]*	-	4.0	8.0	-	-	6.0	-	6.0

*daily intake of the CLA isomers via the CLA supplement

The other feeding trial was performed with primiparous dairy cows who received either the CLA supplement or the control fat preparation (Table 3, **Paper II** and VON SOOSTEN ET AL. (2011)). The study was designed as a slaughter trial in order to gain tissue-fixed immune cells such as splenocytes. Thus, the effect of the CLA supplementation on the proliferation of splenocytes was investigated using the MTT assay (**Paper II**). The sampling days in relation to calving were different for the 2 studies. In the first study, blood samples for isolation of PBMC were taken 7, 21, 35, 49, 70, 105, 140 and 182 pp (**Paper I**). In the slaughter trial blood was taken 7 days ap and before slaughter (1, 49 or 105 days pp, **Paper II**). In both studies no CLA effect but a time effect could be observed. It is reported, that the immune

system of dairy cows is suppressed after calving (KEHRLI AND HARP 2001; NONNECKE ET AL. 2003; LOISELLE ET AL. 2009). This effect became obvious in the slaughter trial (**Paper II**). In the other one, however, only in the AB assay and not in the MTT assay there was an increase of the SI from 7 to 35 days pp (**Paper I**). The first sample was taken at different times in relation to calving: 7 days pp in the first trial and 1 day pp in the slaughter trial. This might explain why the immunosuppressing effects seemed less pronounced in the first trial compared to the slaughter trial. NONNECKE ET AL. (2003) and LOISELLE ET AL. (2009) reported as well that the greatest suppressive effects on the function of PBMC were within the first week of lactation. In these studies pluriparous cows were investigated. This was also the case for $^{2}/_{3}$ of the cows in the first trial, but there was no effect of lactation number (primiparous and pluriparous cows were compared) on ConA-stimulated proliferation of PBMC (**Paper I**).

Although no CLA effect for PBMC was observed in both studies, CLA decreased the stimulation index (SI) of splenocytes 105 days pp (**Paper II**). Splenocytes are tissue-fixed immune cells and these cells act differently from peripheral immune cells in certain situations (SCHNEIDER ET AL. 2006). The spleen is a secondary lymphoid organ containing a large number of T and B cells. These cells are involved in immune response when antigens are entering the spleen via the bloodstream. The reduced response of splenocytes to mitogens of CLA fed animals is also reported for rats (RAMIREZ-SANTANA ET AL. 2009A), but not for mice (KELLEY ET AL. 2002). In chicken CLA feeding did not affect the SI of splenocytes compared to a basal diet or supplementation with safflower oil. *In vitro* the SI was significantly reduced in mitogen stimulated splenocytes incubated with 10 and 30 mg/L CLA (corresponds to 36 and 107 µM, respectively) compared to the same concentration of linoleic acid (TAKAHASHI ET AL. 2007).

Most studies investigating CLA effects on cell proliferation *in vitro* used tumor or cancer cell lines (e. g. LUONGO ET AL. (2003)). There are only a few studies using non tumor cells (e.g. LAI ET AL. (2005B)). The *cis*-9,*trans*-11 CLA isomer had no effect on viability of human PBMC up to 100 µM (JAUDSZUS ET AL. 2012). Because of the limited knowledge about the effect of different CLA isomers on immune cells *in vitro*, especially on bovine immune cells, these investigations became part of the present work. The strength of the *in vitro* experiments is the possibility to test single CLA isomers alone, but also in combination with other isomers and FA, respectively. Both *ex vivo* studies (**Paper I and II**) investigated a mixture of CLA isomers.

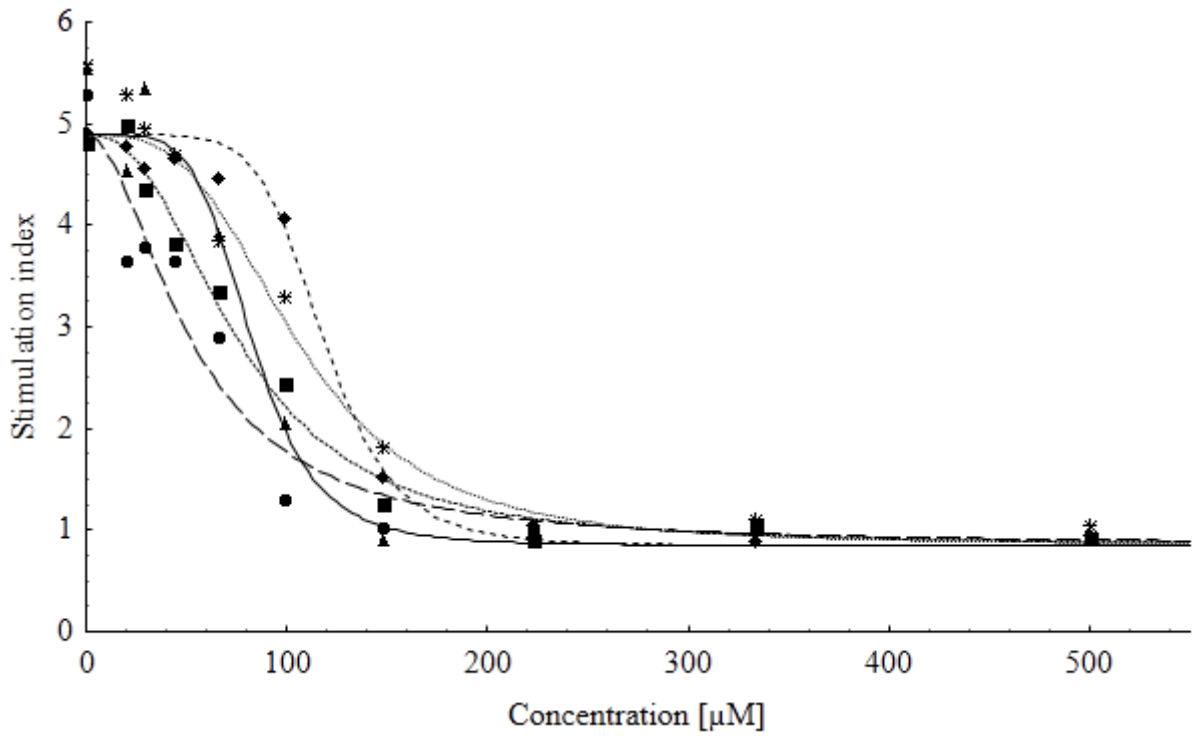

Figure 4 Effects of different fatty acids on the concanavalin A stimulated proliferation of bovine peripheral blood mononuclear cells (fitted dose response curves of stimulation index determined by Alamar blue assay).
Fatty acid mixture (—▲— containing 29.8% palmitic acid, 6.7% palmitoleic acid, 17.4% stearic acid and 46.1% oleic acid according to RUKKWAMSUK ET AL. (2000)), linoleic acid (- - ♦ - -), *cis*-9,*trans*-11 (— ● —), *trans*-10,*cis*-12 (---■---) and phytanic acid (·····✳·····) (symbols represent the mean of 3 assays using cells of 3 different animals tested in quadruplicates).

In vitro, there was no difference in the IC_{50} value between linoleic acid and the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12. The dose-response curves (Figure 4) were fitted to an nonlinear regression equation (based on MERCER ET AL. (1987), **Paper III**) which was used to estimate the IC_{50} values. However, there were differences between FA, when particular concentrations were compared (**Paper III**). The investigated FA concentrations in the dose response studies are based on the concentration of NEFA (LACETERA ET AL. 2004), but they occur as a mixture not as single FA *in vivo*. The FA mixture ought to mimic the condition *in vivo* (RUKKWAMSUK ET AL. 2000). The combination of the FA mixture (60%) with 40% linoleic acid, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA, or a mixture of these 3 FA did not affect the proliferation of the PBMC differently (**Paper III**). These results show that single CLA isomers affect the proliferation of bovine PBMC, but not in combination with other FA. That is in line with the missing CLA effects in the *ex vivo* investigations of PBMC (**Paper I and II**). Furthermore, there were no complementary effects of the CLA isomers *trans*-10,*cis*-12 and *cis*-9,*trans*-11 in combination with PA (**Paper III**).

2 Effects of CLA on cytokine expression ex vivo and in vitro

The expression of IL-4, IL-10, IL-12, TNF-α and IFN-γ was investigated *ex vivo* (**Paper II**) and *in vitro* (**Paper III**) using quantitative real-time polymerase chain reaction (PCR). The expression of the target genes was quantified by a standard curve using cDNA subclones (Figure 5).

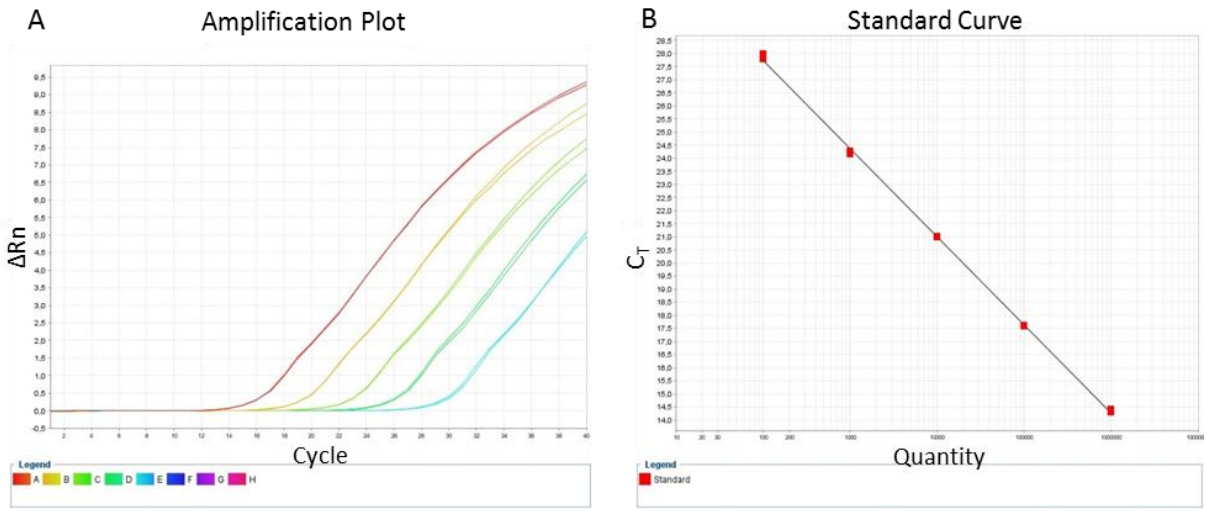

Figure 5 Example of amplification plot (A) and standard curve (B) used for quantification of gene expression in polymerase chain reaction.
Rn: derivative reporter (SYBR green fluorescence), ΔRn: slope of SYBR green fluorescence, C_T: cycle threshold

In general, the expression of the investigated cytokines was stronger in the *in vitro* experiments than in the *ex vivo* analyses. There were samples in the *ex vivo* analyses, where some cytokines were below 100 copies (lowest standard) and therefore indicated as not expressed (**Paper II**). One reason for the differences between *in vitro* and *ex vivo* might be the different treatment of the cells. The samples for the *ex vivo* analyses (**Paper II**) were directly used for mRNA isolation without culturing the cells. In contrast, the PBMC for *in vitro* analyses were cultured with different FA concentrations for 72 h before mRNA was isolated (**Paper III**). These different conditions have to be considered in the interpretation and comparison of the 2 experiments. IL-4 and IL-10 were only sporadically expressed in PBMC *ex vivo* (maximal 300 copies, **Paper II**). In contrast, the *in vitro* expression in unstimulated cells was up to 35,000 copies for IL-4 and about 6000 copies for IL-10. In ConA stimulated PBMC *in vitro*, the expression of IL-10 showed about 4000-5000 copies (**Paper III**). If TNF-α was expressed in the *ex vivo* experiments (no TNF-α expression in some samples), the expression was in the same range as in unstimulated PBMC *in vitro*. The TNF-α expression was much higher, when PBMC were stimulated with ConA although these differences reached statistical significance only for 2 tested combinations (**Paper III**). Differences were

also found between ConA stimulated and unstimulated cells for IFN-γ *in vitro* (**Paper III**). *Ex vivo*, there was a time effect in CLA supplemented cows for the expression of this cytokine. Copy numbers for the 105/CLA group were in the same range as those for unstimulated cells *in vitro*. IL-12 was excluded from evaluation of the *in vitro* experiments, since it was only expressed in a few samples. The melt curve analysis showed peaks that were not specific for the gene of interest (Figure 6). In the *ex vivo* analyses, IL-12 was expressed relatively high compared to the other investigated cytokines.

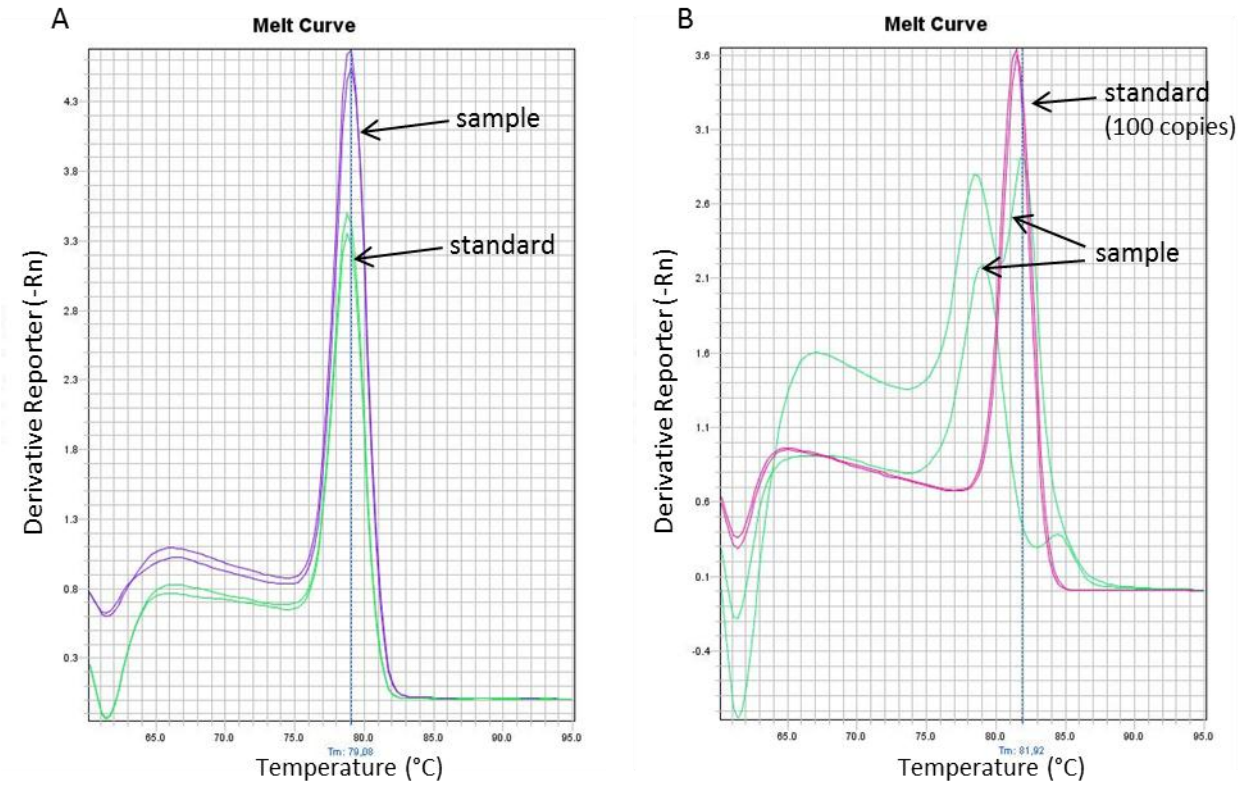

Figure 6 Examples of melt curves (obtained in in vitro experiment), example 1 (IFN-γ, A): gene of interest is amplified and example 2 (IL-12, B): random products are amplified

Another reason for the differences between *in vitro* and *ex vivo* expression of cytokines might be found in the different amount of complementary DNA (cDNA). cDNA was lower in the *ex vivo* analyses (**Paper II**), it was adjusted to 200 ng/µl in the *ex vivo* experiments (**Paper II**) and was used undiluted in the *in vitro* experiments (1169±179 ng/µl cDNA, **Paper III**). Furthermore, there are probably certain ingredients in the culture medium that promote the expression of cytokines. Such effects were shown for TNF-α by compounds of FBS (fetal bovine serum) like BSA in murine macrophages (ZHENG ET AL. 1995).

There were no clear CLA effects on the expression of the investigated cytokines in the *ex vivo* and in the *in vitro* experiments. *Ex vivo,* some time effects occurred within the CLA fed

groups (for IL-4 in splenocytes and IFN-γ and IL-12 in splenocytes and PBMC, **Paper II**). *In vitro*, differences between the treatment were found for the expression of IFN-γ in ConA-stimulated PBMC (**Paper III**). In pigs, CLA supplementation caused changes in cytokine expression from the spleen in LPS challenged animals only (increase of the anti-inflammatory cytokine IL-10 and lower expression of the inflammatory cytokines Il-6 and TNF-α), but not in untreated pigs (CHANGHUA ET AL. 2005).

3 Effects of CLA in relation to performance of cows and metabolites

The present *ex vivo* results are based on the feeding trials of PAPPRITZ ET AL. (2011B) and VON SOOSTEN ET AL. (2011). For interpretation of CLA effects on immune functions it is necessary to consider the performance of the investigated cows. The first trial was divided into 3 periods: day 7 to 49 pp (period 1), day 49 to 192 pp (period 2) and the depletion period beginning 182 days pp and lasting for 12 weeks (period 3) (PAPPRITZ ET AL. 2011B). The depletion period was not considered in **Paper I**. The slaughter trial was divided into 2 periods: day 1 to 42 pp and day 42 pp until 105 days pp (VON SOOSTEN ET AL. 2011).

A major effect of CLA, in particular the *trans*-10,*cis*-12 isomer, in dairy cows is the reduction of milk fat synthesis and therefore the reduction of milk fat yield (BAUMGARD ET AL. 2000). This result was observed after abomasal infusion of the *trans*-10,*cis*-12 isomer (BAUMGARD ET AL. 2000) and after oral supplementation with a mixture of CLA isomers (BERNAL-SANTOS ET AL. 2003; CASTAÑEDA-GUTIÉRREZ ET AL. 2005). In the feeding trials, the milk fat content was reduced by 7% and 12% dose dependently in period 2 (PAPPRITZ ET AL. 2011B). In the slaughter trial it was reduced by 14.1% in period 1 and 25.4% for the rest of the experiment (VON SOOSTEN ET AL. 2011) due to CLA supplementation. Only in the study of VON SOOSTEN ET AL. (2011) in the second period while the protein content was reduced also, did the milk yield decrease after CLA supplementation. Milk yield and milk lactose content and yield, respectively, were not affected by CLA feeding in both studies.

As a result of body fat mobilization, the concentrations of NEFA and BHB in plasma increase. NEFA are discussed to be involved in immunosuppression after calving. *In vitro*, NEFA inhibit the proliferation of bovine PBMC (LACETERA ET AL. 2004). In ewes NEFA and a combination of NEFA and BHB inhibit proliferation of PBMC *in vitro*, whereas BHA alone does not (LACETERA ET AL. 2002). In PBMC off midlactation dairy cows the proliferation capacity and the production of IFN-γ was reduced when incubated with serum from early lactation dairy cows (5 days pp). The inhibitory effects were also observed when serum from midlactation dairy cows (61 days pp) was supplemented with NEFA (equal amounts of NEFA

as in 5 days pp serum) (STER ET AL. 2012). These results support an involvement of NEFA in the immunosuppression in periparturient dairy cows. In the slaughter trial, the plasma NEFA concentrations peaked at day 1 pp and decreased until 42 and 105 days pp (VON SOOSTEN ET AL. 2011). The SI of PBMC was lowest 1 day pp, which is in line with the highest NEFA concentration at the same time point, and increased up to day 105 pp (**Paper II**). Neither the NEFA concentrations in plasma nor the SI values of the PBMC were different between CLA and control fat supplemented cows. BHB plasma concentrations were not affected by CLA treatment as well (VON SOOSTEN ET AL. 2011). In the other study, the plasma NEFA concentrations reached their maximum 7 days pp also and the plasma BHB concentrations reached theirs within the first 35 days of lactation (PAPPRITZ 2012). Decreased SI after calving were only found in the AB assay, but not in the MTT assay (**Paper I**). In contrast, the lowest SI was observed 49 days pp in MTT and AB assay for all experimental groups (except CLA-50 group in the AB assay) where NEFA plasma concentrations were already decreasing (PAPPRITZ 2012). Day 49 pp is the time point where the CLA fed cows reach a steady calculated energy balance, whereas the control animals were in a positive calculated energy balance for the whole time of the experiment. However, the DM intake was significantly lower in the CLA supplemented groups in the same time period, which explains the negative energy balance. In the subsequent course of the study (ongoing supplementation up to day 182 pp and depletion period), there were no differences in DM intake and calculated energy balance (PAPPRITZ ET AL. 2011B). SAREMI ET AL. (2012) observed a moderate correlation between haptoglobin serum concentrations and NEFA and BHB, especially between day 21 ap and 49 pp. This study is as well based on the trials of PAPPRITZ ET AL. (2011B) and VON SOOSTEN ET AL. (2011). The haptoglobin serum concentrations and the haptoglobin mRNA expression in the liver as well as in subcutaneous (sternum, withers and tail head) and visceral (retroperitoneal, mesenterial and omental) fat depots were investigated. Haptoglobin is an acute phase protein which is mainly expressed in the liver, but also expressed in adipose tissue (FRIEDRICHS ET AL. 1995). Therefore, it is classified as an adipokine, which are defined as proteins secreted from adipocytes (TRAYHURN AND WOOD 2004). CLA supplementation did not influence serum haptoglobin concentrations and haptoglobin mRNA expression in the liver. The haptoglobin mRNA abundance was reduced in omental and subcutaneous withers fat depots following CLA supplementation, but not in the other fat depots. This indicates for a local CLA effect only. These local effects of CLA are supported by the findings in **Paper II** where a local effect on the ability to stimulate splenocytes was observed, but not on PBMC.

As also seen in the SI of PBMC (**Paper II**) there was a time effect of haptoglobin serum concentration and mRNA abundance in relation to calving.

The transfer of the supplemented *trans*-10,*cis*-12 isomer into different tissues was rather low, altogether 0.10% until 42 days pp and 0.18% until 105 days pp. The fractions with the highest *trans*-10,*cis*-12 accumulation were retroperitoneal, mesenteric and subcutaneous fat and the mammary gland, but the transfer efficiency was always less than 0.1% (VON SOOSTEN ET AL.). In PBMC the *trans*-10,*cis*-12 isomer was not found in samples from the control group, but in both CLA supplemented groups (0.01 and 0.02% of total fatty acid methyl esters [FAME], **Paper I**). This indicates that at least the *trans*-10,*cis*-12 isomer reached the cells of interest. In the milk fat, the proportion of *trans*-10,*cis*-12 also increased significantly due to CLA supplementation (PAPPRITZ ET AL. 2011B). These results show that the supplemented CLA isomers were absorbed and had effects, especially on the milk fat content and the fatty acid profile of the milk. On the other hand, the duodenal availability of *trans*-10,*cis*-12 CLA was very low, 16% and 5%, respectively, when 50 g/d or 100 g/d of the CLA supplement was fed (PAPPRITZ ET AL. 2011A). In this study PAPPRITZ ET AL. (2011A) tested the duodenal availability of lipid-encapsulated CLA preparations (also used in the present studies) using double-fistulated cows.

4 Differences of CLA effects on the human and bovine immune system

Although the principles of immune response are similar in mammals, there are some differences between species. Three areas are reported where differences occur: the expression of MHC (major histocompatibility complex) class II molecules, leucocyte differentiation molecules, and the composition of the immune systems (DAVIS AND HAMILTON 1998). In contrast to most animals, the γδ T cells are a major subset of T cells in cattle (DAVIS ET AL. 1996) and might therefore be important in immune defense of cattle (GUZMAN ET AL. 2012). The composition of the major circulation T cell subsets differs in the peripheral blood from ruminants and humans or mice. Whereas the major T cells of mice and humans are α/β^+, $WC1^-$, $CD4^+$ and $CD8^-$, these from ruminants are $\gamma\delta^+$, $WC1^+$, $CD4^-$ and $CD8^-$ (TIZARD 2004). Considering these aspects, the effect of CLA on immune function might differ between monogastrics and ruminants, but there are no studies available. The effects of CLA on the human immune system are much more investigated than those on the bovine immune system. A difference between human studies and the present investigations are the applied CLA doses. Most human studies use a CLA supplement that provides 1.6 g/d (ALBERS ET AL. 2003) up to 3.9 g/d CLA (KELLEY ET AL. 2000). Considering the average body weight of humans

(70 kg) and cows (average live weight of cows in PAPPRITZ ET AL. (2011B) and **Paper I**: 627 kg), the lowest dose in human studies is in the same range as the highest tested concentration in the present study (**Paper 1 and II**). As also seen in the results of **Paper I** and **Paper II**, the effects of CLA on the human immune system are weak. The only effect on the mitogen-stimulated proliferation of immune cells was observed in splenocytes (**Paper II**), which are of course not available in human *in vivo* studies. Although using relatively high CLA doses (3.9 g/d), there were no CLA effects on immune function of healthy women (KELLEY ET AL. 2000; KELLEY ET AL. 2001). In contrast, TRICON ET AL. (2004) supplemented lower CLA doses (up to 2.38 g/d *cis*-9,*trans*-11 and 2.52 g/d *trans*-10,*cis*-12 CLA) and observed decreasing lymphocyte activation (measured by the expression of the early T cell activation marker CD69) within increasing CLA concentrations (both isomers).

Another variation between the different human studies is the duration of the CLA supplementation. In the studies of KELLEY ET AL. (2000) and KELLEY ET AL. (2001) the intervention period lasted for 9 weeks, in the study of TRICON ET AL. (2004) for 6 months. The trials described in **Paper I** and **Paper II** lasted for 182 days (corresponds to 6 months) and 105 days (corresponds to 15 weeks), respectively, and therefore were in the same range as most of the human studies.

However, the effects of CLA on human immune functions are inconsistent. Reasons for these discrepancies might be the different CLA doses used in the studies and the variation of the supplementation periods.

For the action of CLA in humans a PPAR-γ dependent pathway, especially for their antiatherogenic properties, is discussed (RINGSEIS ET AL. 2006; STACHOWSKA ET AL. 2011). In human PBMC, the *cis*-9,*trans*-11 isomer (100 µM) decreased IL-2 and TNF-α positive T-helper cells. The effect is at least partly attributable to a PPAR-γ dependent pathway (JAUDSZUS ET AL. 2012).

The role of PPAR-γ in the modulation of the bovine immune response due to CLA has not been clarified yet. HUSSEN AND SCHUBERTH (2011) indicate that a decrease of PBMC proliferation due to *cis*-9,*trans*-11 is PPAR-γ independent. In the present study (**Paper III**) the *cis*-9,*trans*-11 isomer increased the expression in ConA stimulated PBMC only when incubated with 77 µM compared to 77 µM LA. In both studies, HUSSEN AND SCHUBERTH (2011) and **Paper III**, only the *cis*-9,*trans*-11 isomer was investigated. In another *in vitro* study using bovine immune cells the *trans*-10,*cis*-12 isomer and the PPAR-γ agonist resiglitazone, but not the *cis*-9,*trans*-11 isomer and LA, attenuated the LPS induced TNF-α production (PERDOMO ET AL. 2011). Therefore, the anti-inflammatory effect of the

trans-10,*cis*-12 isomer in this study is PPAR-γ dependent. Altogether, the results argue for an isomer and species specific action of CLA where PPAR-γ is partly involved.

Conclusions

CLA supplementation of dairy cows did not affect the mitogen-stimulated proliferation of PBMC, but decreased the mitogen stimulated proliferation of splenocytes. The expression of cytokines from PBMC and splenocytes was not influenced by CLA feeding. However, in the CLA fed groups there was an effect of time in relation to calving for the expression of IL-4 in splenocytes and IFN-γ and IL-12 in PBMC and splenocytes.

Long-term CLA supplementation influenced the FA profile of PBMC, but only in minor FA. The proportion of the *trans*-10,*cis*-12 isomer was increased due to CLA supplementation, but other CLA (except the supplemented isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12) were decreased as well as the minor FA C24:1 *cis*-15 and C18:1 *trans*-9. These alterations in the FA profile of PBMC did not impair the mitogen-stimulated proliferation of PBMC.

FA influenced the mitogen-stimulated proliferation of PBMC *in vitro* in a dose dependent manner. Although the calculated IC_{50} values do not differ between LA, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA, PA and the FA mixture mimicking the FA composition of subcutaneous adipose tissue, differences were observed at certain concentrations. Furthermore, LA additionally stimulated the mitogen-induced proliferation in lower concentrations and therefore may have a greater impact on the function of PBMC than CLA. The c*is*-9,*trans*-11 CLA isomer slightly altered the expression of PPAR-γ and the cytokine IFN-γ.

Altogether, there were only minor effects of CLA on the function of bovine immune cells, seen as the decreased SI of splenocytes after CLA supplementation and minor effects on the expression of IFN-γ and PPAR-γ *in vitro*. It is unknown yet, if CLA impair the immune response of dairy cows in immune challenging situations. The focus of the present investigations was on the function of T cells and therefore on the adaptive immune system. Further research is needed in order to evaluate the impact of CLA in dairy cows feeding on cells of the innate immune system like the function of granulocytes.

Summary

Effects of conjugated linoleic acids on the function of bovine immune cells *ex vivo* and *in vitro*

Conjugated linoleic acids (CLA) are a group of C18:2 fatty acids (FA) that are characterized by the conjugated position of its 2 double bonds. Several health beneficial effects are proclaimed for CLA in animals and humans, such as anticancerogenic, antiatherosclerotic, antidiabetic and immunomodulating properties. Although CLA, especially the *cis*-9,*trans*-11 isomer, are intermediate products of biohydrogenation in the rumen and therefore naturally occur in dairy products and meat from ruminants, dairy cows feed is supplemented with CLA. This is done because CLA supplementation reduces the milk fat content. There is no information on the effects of CLA supplementation on the bovine immune system. Thus the following investigations were performed in order to clarify this open question.

Two feeding trials were conducted to evaluate the effects of CLA on immune functions in dairy cows. First, a long term feeding study with 46 cows was performed. After parturition, the cows received a diet either supplemented with 100 g/d of a control fat preparation (Con), 50 g/d of the control fat preparation and 50 g/d of the CLA supplement, respectively (CLA-50) or 100 g/d of the CLA supplement (CLA-100). The supplement contained 12% of the *cis*-9,*trans*-11 and 12% of the *trans*-10,*cis*-12 isomer. Thus the daily CLA consumption was 4 and 8 g of each isomer in the CLA supplemented groups, respectively. The supplements were fed from calving onward until day 182 *post partum* (pp). Blood was taken at day 7, 21, 35, 49, 70, 105, 140 and 182 pp. From the whole blood peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. Samples from day 70 and 140 were pooled for each animal and the FA profile of the lipid fraction of PBMC was analyzed. The samples from the remaining 6 sampling days were used to evaluate the concanavalin A (ConA) stimulated proliferation *ex vivo* by Alamar blue (AB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The most abundant FA in the lipid fraction of PBMC were not altered by CLA supplementation (stearic acid, palmitic acid, oleic acid, linoleic acid and arachidonic acid), but effects on the proportions of some minor FA were detected. The percentage of the *trans*-10,*cis*-12 isomer was significantly increased in both CLA supplemented groups, but there was no effect on the *cis*-9,*trans*-11 isomer. However, the proportion of CLA isomers other than *cis*-9,*trans*-11 and *trans*-10,*cis*-12 was decreased in the CLA-100 group compared

to Con, as well as the percentage of nervonic acid and elaidic acid. Although the FA profile was slightly altered, the mitogen stimulated proliferation of PBMC was not affected by CLA supplementation. The time in relation to calving had an effect on the stimulation index (SI, defined as optical density (OD) or fluorescence of ConA stimulated cells divided by OD or fluorescence of non-stimulated cells) of PBMC with a minimum at day 49 pp.

The second feeding study was conducted as a slaughter trial. In that study 25 primiparous cows were investigated. The animals received either the 100 g/d of the control fat preparation or 100 g/d of the CLA supplement beginning at day 1 pp. An initial group (IG), which was not supplemented with CLA or the control fat, was slaughtered 1 day pp. Five animals of each feeding group were slaughtered 42 days pp (42/CON and 42/CLA) and 105 days pp (105/CON and 105/CLA), respectively. Fourteen days *ante partum* and immediately before slaughter, blood samples were taken to isolate PBMC. The spleen was removed during dissection to obtain splenocytes. Mitogen stimulated cell proliferation of splenocytes was analyzed by MTT assay and that of PBMC by MTT and AB assay. As an additional characterization of immune functions the basal expression of interleukin (IL) 4, IL-10, IL-12, interferon γ (IFN-γ), and the tumor necrosis factor α (TNF-α) from splenocytes and PBMC was analyzed by quantitative real time polymerase chain reaction (PCR).

As in the previous study, there was no effect of CLA feeding on the SI of PBMC. The SI increased from day 1 pp to day 105 pp in the MTT assay, but only from day 42 pp until day 105 pp in the control fat fed groups in the AB assay. In contrast, there was no time effect on the SI of splenocytes, whereas CLA feeding significantly decreased the SI 105 days pp. Comparing the expression of the investigated cytokines at the same time point pp, there were no CLA effects. Increased expressions from day 1 pp (IG) up to day 105 pp were found within the CLA fed groups for IL-4 in splenocytes and for IFN-γ and IL-12 in both cell types. The expression of IL-4 in PBMC and of IL-10 and TNF-α was not effected by CLA or the time in relation to calving. TNF-α was not expressed in splenocytes.

In addition to the *ex vivo* analyses, the effects of CLA on the function of bovine PBMC was investigated *in vitro*. PBMC were obtained from 3 cows. The effect of linoleic acid (LA), *cis*-9,*trans*-11, and *trans*-10,*cis*-12 CLA, phytanic acid (PA), and a fatty acid mixture (mimicking the FA composition of the subcutaneous adipose tissue and containing 29.8% palmitic acid, 6.7% palmitoleic acid, 17.4% stearic acid and 46.1% oleic acid) on the ConA-stimulated proliferation of PBMC was tested by AB and 5-bromo-2'-deoxyuridine (BrdU) assay in a concentration range from 0 to 500 µM. Based on these results, a combination of the FA mixture (60%) with LA, *cis*-9,*trans*-11, *trans*-10,*cis*-12 (40%), and a combination of these

3 FA (each FA 13.3%) was tested in the AB assay (33, 66, 99 and 500 µM). The FA mixture (60%) was also combined with PA (40%), PA (20%), and *cis*-9,*trans*-11 (20%) as well as with PA (20%) and *trans*-10,*cis*-12 CLA (20%). Furthermore, the expression of PPAR-γ and the cytokines IL-4, IL-10, IL-12, IFN-γ and TNF-α from unstimulated and ConA-stimulated PBMC was analyzed by quantitative real-time PCR in response to *cis*-9,*trans*-11 and LA (concentrations: calculated IC_{50} values of the respective FA and 77 µM).

The IC_{50} values, calculated from the SI obtained from the dose response studies using AB assay, were 100.67 µM for LA, 53.83 µM for *cis*-9,*trans*-11, 70.12 µM for *trans*-10,*cis*-12, 94.67 µM PA and 80.83 µM for the FA mixture. The IC_{50} values did not differ significantly between the investigated FA, but the ConA stimulated proliferation (% of control) was different between the various FA at certain concentrations in the AB (20 to 148 µM) and BrdU assay (44 to 99 µM). These differences were not observed in the SI when LA, *cis*-9,*trans*-11, *trans*-10,*cis*-12, and PA were combined with the FA mixture. As in the investigations using single FA the SI decreased with increasing FA concentrations. No effects of FA treatment were observed in the expression of IL-4, IL-10 and TNF-α. The expression of IFN-γ was significantly reduced when PBMC were stimulated with ConA and incubated with DMSO or 77 µM *cis*-9,*trans*-11 compared to the ConA stimulated PBMC incubated without FA or DMSO (medium control). The expression significantly increased in response to ConA for TNF-α and IFN-γ (medium control and IC_{50} value of LA). ConA decreased the expression of IL-10 in the medium control. The expression of PPAR-γ was significantly lower in ConA treated cells incubated with 77 µM LA than in those treated with same concentration of *cis*-9,*trans*-11.

In conclusion, the effects of CLA on the investigated immune functions in dairy cows are marginal and only inhibiting effects on the SI of splenocytes were observed. Although the FA composition of PBMC was slightly altered by CLA supplementation, these changes did not impair the mitogen stimulated proliferation of the cells. *In vitro* FA dose-dependently inhibit the proliferation of bovine PBMC. Differences in ConA-stimulated proliferation occur at certain concentrations when single FA are investigated, but not within the tested FA combinations. The effects of CLA on the expression of cytokines were marginal in *ex vivo* and *in vitro* investigations.

Zusammenfassung

Einfluss konjugierter Linolsäuren auf die Funktion boviner Immunzellen *ex vivo* und *in vitro*

Konjugierte Linolsäuren (CLA) sind eine Gruppe von C18:2 Fettsäuren (FS), deren Doppelbindungen in konjugierter Form vorliegen. CLA werden verschiedene gesundheitsfördernde Eigenschaften (anticancerogene, antiatheriosclerotische, antidiabetische und immunmodulierende Wirkung) bei Mensch und Tier zugeschrieben. Obwohl CLA, hier besonders das *cis*-9,*trans*-11 Isomer, als Zwischenprodukt der Biohydrogenierung im Pansen entstehen und somit in Milchprodukten und Fleisch von Wiederkäuern vorkommen, werden sie Milchkühen in Form eines Supplements zu gefüttert. Der Grund hierfür liegt darin, dass CLA den Milchfettgehalt senken. Die Wirkung von CLA auf das bovine Immunsystem wurde bisher nicht untersucht und ist Gegenstand der vorliegenden Arbeit.

Um den Einfluss von CLA auf das Immunsystem von Milchkühen zu untersuchen, wurden 2 Fütterungsversuche durchgeführt. Der erste Versuch war ein Langzeitversuch mit 46 Kühen. Nach der Abkalbung erhielten die Kühe entweder ein Supplement bestehend aus 100 g/d eines Kontrollfettpräparats (Kon), 50 g/d dieses Präparats und 50 g/d des CLA-Supplements (CLA-50) oder 100 g/d des CLA Supplements (CLA-100). Das CLA-Supplement enthielt jeweils 12% der Isomere *cis*-9,*trans*-11 und *trans*-10,*cis*-12, so dass täglich 4 bzw. 8 g jedes Isomers in den supplementierten Gruppen aufgenommen wurden. Mit der Abkalbung begann die Supplementierung und dauerte bis zum 182. Tag *post partum* (pp). Blutproben wurden 7, 21, 35, 49, 70, 105, 140 und 182 Tage pp gezogen. Aus dem Vollblut wurden periphere mononukleäre Zellen (PBMC) mittels Dichtegradientenzentrifugation isoliert. Die Proben vom 70. und 140. Tag pp wurden zusammengeführt und für jedes Tier einzeln das FS-Muster der Lipidfraktion der PBMC analysiert. Die Proben der verbleibenden 6 Termine wurden für die Bestimmung der Concanavalin A (ConA)-stimulierten Proliferation mittels Alamar blue (AB) und 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) Assay verwendet.

Die quantitativ am meisten vorkommenden FS der Lipidfraktion der PBMC wurden von der CLA-Supplementierung nicht beeinflusst (Stearin-, Palmitin-, Öl-, Linol- und Arachidonsäure), allerdings gab es Verschiebungen im FS-Muster bei den quantitativ geringeren FS. Der Anteil an *trans*-10,*cis*-12 CLA war nach CLA-Supplementierung erhöht, diese hatte aber keinen Einfluss auf das *cis*-9,*trans*-11-Isomer. Der Anteil an den übrigen CLA-Isomeren, also alle außer *cis*-9,*trans*-11 und *trans*-10,*cis*-12, und der Anteil an Nervon- und Elaidinsäure war

in der CLA-100-Gruppe im Vergleich zur Kon-Gruppe signifikant erniedrigt. Obwohl das FS-Muster leicht verändert war, gab es keinen Einfluss der CLA-Supplementierung auf die mitogenstimulierte Proliferation der PBMC. Allerdings wurde ein Zeiteffekt in Relation zur Abkalbung auf den Stimulationsindex (SI, definiert als Quotient aus der optischen Dichte (OD) bzw. der Fluoreszenz der ConA stimulierten Zellen und OD bzw. Fluoreszenz der unstimulierten Zellen) der PBMC mit einem Minimum nach 49 Tagen pp gefunden.

Der zweite Fütterungsversuch war als Schlachtversuch mit 25 erstlaktierenden Kühen konzipiert. Beginnend am ersten Tag pp erhielten die Tiere entweder 100 g/d des Kontrollfettpräparats oder 100 g/d des CLA-Supplements. Die Basisgruppe (BG), die weder das Kontrollfettpräparat noch das CLA-Supplement erhielt, wurde einen Tag nach der Abkalbung geschlachtet. Aus jeder Fütterungsgruppe wurden jeweils 5 Tiere nach 42 Tagen pp (42/KON und 42/CLA) und 105 Tagen pp (105/KON und 105/CLA) geschlachtet. Blutproben wurden 14 Tage *ante partum* und direkt vor der Schlachtung genommen, um daraus die PBMC zu isolieren. Während der Schlachtung wurde die Milz entnommen, um daraus Splenozyten zu gewinnen. Die mitogenstimulierte Proliferation der Splenozyten wurde mittels MTT-Assay und die der PBMC mittels MTT und AB-Assay analysiert. Zusätzlich wurde die Immunfunktion durch Analyse der basalen Expression von Interleukin (IL) 4, IL-10, IL-12, Interferon (IFN)-γ und Tumornekrosefaktor α (TNF-α) der Splenozyten und PBMC mittels quantitativer Real-Time Polymerasekettenreaktion (PCR) charakterisiert. Wie auch in der vorangegangenen Untersuchung hatte die CLA-Fütterung keinen Einfluss auf den SI der PBMC. Der SI stieg vom ersten Tag pp bis 105 Tage pp im MTT-Assay an, aber nur von Tag 42 pp bis Tag 105 pp in der Kontrollgruppe im AB-Assay. Im Gegensatz dazu gab es keinen Zeiteffekt auf den SI der Splenozyten, allerdings war dieser nach 105 Tagen CLA-Fütterung signifikant reduziert. Kein CLA-Effekt wurde deutlich, wenn man die Zytokinexpression zum gleichen Zeitpunkt vergleicht. Innerhalb der CLA-Gruppe erhöhte sich die Expression von IL-4 in den Splenozyten und von IFN-γ und IL-12 in beiden Zelltypen von Tag 1 pp (BG) bis Tag 105 pp. Die Expression von IL-4 in den PBMC und von IL-10 und TNF-α wurde weder von CLA noch dem Zeitpunkt nach der Abkalbung beeinflusst. TNF-α wurde in den Splenozyten nicht exprimiert.

Zusätzlich zu den *ex vivo* Untersuchungen wurde der Einfluss von CLA auf PBMC *in vitro* analysiert. Die PBMC wurden von 3 Kühen gewonnen. Der Einfluss von Linolsäure (LS), *cis*-9,*trans*-11 und *trans*-10,*cis*-12 CLA, Phytansäure (PS) und einer FS-Mischung (entspricht der FS-Zusammensetzung der subkutanen Fettgewebes und besteht aus 29,8% Palmitin-, 6,7% Palmitolein-, 17,4% Stearin- und 46,1% Ölsäure) auf die ConA-stimulierte Proliferation der PBMC wurde mittels AB- und 5-Brom-2'-deoxyuridin (BrdU)-Assay in einem Konzent-

rationsbereich von 0 bis 500 µM untersucht. Auf diesen Ergebnissen basierend wurde eine Kombination der FS-Mischung (60%) mit 40% LS, *cis*-9,*trans*-11 oder *trans*-10,*cis*-12 und einer Kombination dieser 3 FS (jede FS 13,3%) im AB-Assay getestet (33, 66, 99 und 500 µM). Die FS-Mischung (60%) wurde außerdem mit PS (40%), PS und *cis*-9,*trans*-11 (jeweils 20%), sowie PS und *trans*-10,*cis*-12 (jeweils 20%) kombiniert. Die Expression von PPAR-γ und der Zytokine IL-4, IL-10, IL-12, IFN-γ und TNF-α von unstimulierten und ConA-stimulierten PBMC wurde mittels quantitativer Real-Time PCR nach Inkubation mit *cis*-9,*trans*-11 und LS analysiert (Konzentrationen: kalkulierte IC_{50}-Werte der jeweiligen FS und 77 µM). Folgende IC_{50}-Werte wurden anhand der SI aus den Dosis-Wirkungs-Versuchen mittels AB-Assay errechnet: 100,67 µM für LS, 53,83 µM für *cis*-9,*trans*-11, 70,12 µM für *trans*-10,*cis*-12, 94,67 µM für PS und 80,83 für die FS-Mischung. Die IC_{50}-Werte der untersuchten FS unterschieden sich nicht signifikant voneinander, allerdings unterschied sich die ConA-stimulierte Proliferation (% der Kontrolle) der FS bei bestimmten Konzentrationen im AB (20 bis 148 µM) und BrdU-Assay (44-99 µM). Keine Unterschiede der SI wurden beobachtet, wenn LS, *cis*-9,*trans*-11, *trans*-10,*cis*-12 und PS mit der FS-Mischung kombiniert wurden. Wie auch bei den Untersuchungen mit einzelnen FS verringerten sich die SI mit steigenden FS-Konzentrationen. Kein FS-Effekt wurde bei der Expression von IL-4, IL-10 und TNF-α beobachtet. Die Expression von IFN-γ war signifikant reduziert, wenn die PBMC mit ConA stimuliert und mit DMSO oder 77 µM *cis*-9,*trans*-11 inkubiert wurden (im Vergleich zu ConA-stimulierten PBMC ohne Zusatz von FS oder DMSO [Medium-Kontrolle]). Eine ConA-Stimulation bewirkte einen signifikanten Anstieg der Expression von TNF-α und IFN-γ (Medium-Kontrolle und IC_{50}-Wert von LS). Die Expression von IL-10 wurde bei ConA-Stimulation in der Medium-Kontrolle reduziert. PPAR-γ wurde signifikant weniger in ConA-stimulierten Zellen, die mit 77 µM LS inkubiert wurden im Vergleich zur gleichen Konzentration an *cis*-9,*trans*-11, exprimiert.

Es lässt sich schlussfolgern, dass die CLA-Effekte auf die untersuchten Immunparameter in Milchkühen eher schwach ausgeprägt sind und lediglich ein inhibierender Effekt auf die Splenozyten beobachtet wurde. Obwohl das FS-Muster der PBMC durch die CLA-Supplementierung leicht verändert wurde, hatte dies keinen Einfluss auf die mitogenstimulierte Proliferation der Zellen. *In vitro* wurde die Proliferation der bovinen PBMC dosisabhängig gehemmt. Die ConA-stimulierte Proliferation unterschied sich lediglich, wenn einzelne FS untersucht wurden, nicht aber wenn diese in Kombination getestet wurden. CLA-Effekte auf die Expression von Zytokinen waren sowohl in den *in vitro* als auch in den *ex vivo* Untersuchungen nur schwach ausgeprägt.

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Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Halle/Saale, den 20.09.2013 ……

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