Immunomodulation in foals

opportunities and challenges

Dax Vendrig

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Immunomodulation in foals

OPPORTUNITIES AND CHALLENGES

Immunomodulatie in veulens

KANSEN EN UITDAGINGEN

(met een samenvatting in het Nederlands)

Proefschrift

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Johannes Cornelis Vendrig

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Promotor: Prof.dr. J. Fink-Gremmels

Co- promotor: Dr. A.D. Kraneveld

The oldest, shortest words - yes and no –

are those which require the most thought.

Pythagoras

(ca. 570-495 BC)

In herinnering aan mijn vader

Contents

Chapter 1

Scope and aim of the thesis

Mammals are in close contact with both environmental and endogenous bacteria during their entire life. A well-balanced colonisation of epithelial surfaces, including the gastrointestinal tract, is required for homeostasis and a balanced immune system. If the host's defence throughout epithelial surfaces is impaired, both opportunistic and primary pathogenic bacteria are potentially harmful. The immune system has the critical task to tolerate the commensal flora and at the same time to help prevent bacteria, either commensal or primarily pathogenic, from crossing the epithelial barrier. In particular during the first period of life, when bacterial colonisation takes place and the immune system is primed, disturbance of these processes can easily result in either reduced bacterial defence or excessive inflammatory responses, both leading to increased susceptibility to bacterial infections and sepsis.

Foals are particularly prone to infections during the first months of life. Similar to other mammalian newborns, deficiencies in both innate and adaptive immune responses have been reported in neonatal foals in comparison with adult horses. In addition, immunity in young foals has been shown to strongly depend on the foal's uptake of colostrum. Conventionally, equine colostrum is mainly regarded as a source of maternal immunoglobulins. Foals lack circulating maternal immunoglobulins at birth and fully depend on the uptake of colostrum during the first day of life, as well as the quality of the mare's colostrum, to obtain sufficient protecting by antibodies during the first phase of life, until its own immune system is able to produce enough immunoglobulins. Beside this dependency on the transfer of passive immunity through colostrum, there is increasing evidence that other colostral constituents, in particular non-digestible carbohydrates, are essential in early life, as they influence the process of bacterial colonisation and the priming of the immune system for later stages in life.

In spite of the optimisation of management and therapy protocols for young foals, bacterial infections and sepsis in foals are still major concerns shared by horse owners and equine practitioners. Hence, it would be desirable to modulate the foal's immune responses during the first months of life, in a way that the susceptibility to bacterial infections is reduced.

In this thesis, methods to modulate immune responses in the horse are investigated. In Figure 1, a simplified illustration is given of the background and context of the chosen immunomodulatory approaches. Toll-like receptors (TLRs) play a central role, as these receptors recognise bacterial patterns and initiate subsequent innate and adaptive immune responses. TLRs stand on the crossroads between tolerance and immunity, or even inflammation in case of excessive TLR activity. This thesis will focus particularly on TLR-2 and TLR-4. The TLR-4 complex is triggered by lipopolysaccharide (LPS), derived from the cell wall of Gram-negative bacteria. TLR-4 activity is strongly associated with pro-inflammatory responses and inflammatory disorders. Hence, we applied LPS in cell

culture models to evaluate inflammatory responses and possible immunomodulatory properties of the compounds of interest *in vitro*. TLR-2 is activated mainly by constituents of Gram-positive bacteria. TLR-2/TLR-4 dysbalance has been related with inflammatory disorders, and the synthetic TLR-2 agonist Pam3-Cys-Ser-Lys4 (PCSK) has been documented to mitigate gastrointestinal inflammation in experimental animals. As we hypothesised that this TLR-2 ligand is a promising candidate for immunomodulation in foals, we investigated immunomodulatory effects of PCSK *in vitro*, in both murine and equine models.

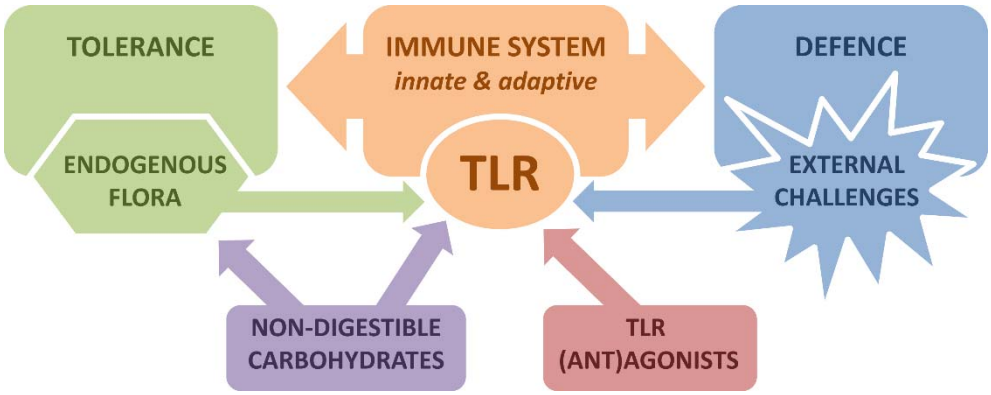

Figure 1: Schematic overview of the role of Toll-like receptors (TLRs) in tolerance and immunity and the proposed methods of immunomodulation in this thesis.

Next to direct modulation of TLR signalling by established TLR ligands, we investigated immunomodulatory properties of natural compounds, such as equine colostral carbohydrates and several defined fractions of non-digestible carbohydrates derived from cow's milk (galacto-oligosaccharides) and plants (fructo-oligosaccharides and acidic oligosaccharides). The chosen defined fractions of non-digestible carbohydrates contained specific oligosaccharides with proven prebiotic properties in humans and experimental animals *in vivo*. As of yet, the documented immunomodulatory effects of these dietary oligosaccharides have been mostly ascribed to the modulation of the gut flora, hence affecting TLR signalling in the epithelial barrier indirectly. However, there is increasing evidence for direct immunomodulation by oligosaccharides. Though the exact underlying mechanism of action is largely unknown, both indirect and direct influences of these substances on TLR signalling are debated throughout this thesis. In our *in vitro* experiments into immunomodulation in the horse, we included several oligosaccharide fractions, which previously have been shown to exhibit beneficial effects *in vivo* in other species by dietary supplementation. Eventually, we carried out an *in vivo* experiment in

newborn foals, looking into immunomodulatory effects of an orally applied galacto-oligosaccharide preparation. Furthermore, preliminary results of our current research, which focuses on specific equine colostral oligosaccharides and their immunomodulatory competence, were included.

The outline of the thesis

Chapter 2 gives an overview of the functional elements of the intestinal epithelial barrier and potential targets of therapeutic intervention, with particular focus on pattern recognition receptors. In **Chapter 3**, immune responses to a wide concentration range of acknowledged ligands for both TLR-2 and TLR-4 are characterised in a worldwide-established model of murine macrophages. **Chapter 4** describes two investigations into immunomodulation in murine intestinal epithelial cells, one concerning a TLR-2 ligand and one concerning the carbohydrate fraction of equine colostrum. In **Chapter 5**, the isolation and subculturing procedure of equine peritoneal macrophages is described, as well as the effects of a concentration range of a ligand for TLR-2 and TLR-4. Based on the data generated in the experiments of **Chapters 3, 4, and 5**, standard concentrations of the TLR ligands were chosen to do further research into immunomodulation in equine models. In the **Chapters 6, 7, and 8**, a model with equine peripheral blood mononuclear cells is used. Effects of separate and concomitant TLR-2 and TLR-4 activation in newborn foals and adult horses are explored in **Chapter 6**. Immunomodulatory properties of equine colostral carbohydrates and defined oligosaccharide fractions (galacto-, fructo-, and acidic oligosaccharides) are evaluated in **Chapters 7 and 8**, respectively. In **Chapter 9**, a pilot study is described with regard to the effects of orally supplemented galacto-oligosaccharides in foals. In **Chapter 10**, we present preliminary results of our current research into specific equine colostral oligosaccharides. **Chapter 11** comprises the general discussion of the thesis, in which an overview and critical evaluation of the findings is given, as well as the consequences of these findings for forthcoming research.

Chapter 2

Intestinal barrier function in neonatal foals: options for improvement

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Abstract

Gastrointestinal defence in the newborn is limited in comparison to adults, due to an immature epithelial barrier function and deficits in both innate and adaptive immune responses. Consequently, neonates (including foals) are at increased risk of disturbance to mucosal homeostasis during initial intestinal colonisation that may lead to excessive inflammation and bacterial translocation into the bloodstream, resulting in septicaemia. Bacterial recognition by Pattern Recognition Receptors (PRRs) and their downstream regulation of cytokine release have been shown to be pivotal for gastrointestinal mucosal homeostasis and the development of a functional intestinal barrier. Evidence suggests that selective PRR agonists limit the inflammatory responses and improve epithelial barrier function. Milk, and in particular colostrum, contain a broad array of oligosaccharides which seem to act as PRR agonists. This class of compounds forms a source for new dietary formulas that may orchestrate gut colonisation by the commensal flora in the early phase of life and so reduce the risks of inflammation and pathogen invasion.

Introduction

Septicaemia is the foremost cause of death in newborn foals (Cohen, 1994). Several retrospective studies have reported mortality rates from 21 to 68 % (Raisis et al., 1996; Barton et al., 1998; Gayle et al., 1998; Stewart et al., 2002; Corley et al., 2007; Russell et al., 2008; Sanchez et al., 2008). At birth, the foal is transited from a sterile womb into an environment that contains a myriad of microorganisms, including pathogens, challenging the innate immune system. In contrast to other mammalian species, the equine placenta is almost impermeable to immunoglobulins so the newborn foal has very few circulating maternal antibodies and strongly depends on the passive transfer of immunoglobulins via the colostrum of the mare and alternative defence mechanisms (LeBlanc et al., 1992).

The intake of colostrum during the first 24 hours of life is therefore extremely important and well recognised by horse breeders and equine practitioners. A pivotal early study which temporarily deprived neonates of colostrum found that almost all of them developed neonatal septicaemia (Robinson et al., 1993). Similarly, failure of passive transfer of maternal immunoglobulins (foal plasma IgG concentration < 8 g/L) increases the risk of developing septic illnesses in foals (Raidal, 1996). The transition of bacteria to the bloodstream indicates that colostrum deprivation results in more effects than just a deficiency in maternal antibodies and it may be assumed that other colostral constituents contribute to the foal's passive immunity.

Innate and adaptive immune responses are modulated by the colonisation of microbes on the mucosal surfaces of the neonate (Levy, 2007). Colonisation of the gut and other mucosal surfaces commences during birth and proceeds during the first phases of life parallel to the dietary pattern (Mshvildadze and Neu, 2010). The immune system responds to bacterial compounds through pattern recognition receptors (PRRs), comprising several Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domains (NODs). Both play a pivotal role in the recognition of bacteria and subsequent immune responses (Takeuchi and Akira, 2010). PRR-dependent signalling in response to bacteria triggers the release of pro- and anti-inflammatory cytokines, which in turn improve or damage the integrity of the intestinal barrier (Al-Sadi et al., 2009). Current research focuses on substances that directly or via PRRs enhance epithelial barrier function and consequently may have potential to prevent and treat neonatal sepsis. Many of these compounds are common natural constituents of milk and colostrum, but their quantity in either milk or colostrum and their chemical structure are largely unknown.

Functional elements of the intestinal barrier

A schematic illustration of the intestinal epithelial barrier is given in Figure 1. The primary barrier is formed by the epithelial monolayer and its tight junctions (TJs) (Marchiando et al., 2010). Intra-epithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), dendritic cells (DCs), macrophages (MPs) and polymorphonuclear neutrophils (PMNs) are the main cell types responsible for cellular immune responses (Acheson and Luccioli, 2004; Johansson-Lindbom and Agace, 2007). Antigen sampling is facilitated through M cells at the site of Peyer's Patches (PPs) or other lymphoid follicles. After antigen sampling, antigen presenting cells (APCs) migrate within the intestinal lamina propria to mesenteric lymph nodes (MLNs) in order to mediate subsequent immune responses (Kelsall, 2008). Luminal defence mechanisms include IgA secretion by plasma cells, production of mucin glycoproteins by Goblet cells and release of antimicrobial peptides and enzymes by Paneth cells resided in epithelial crypts (Mantis and Forbes, 2010; Schenk and Mueller, 2008).

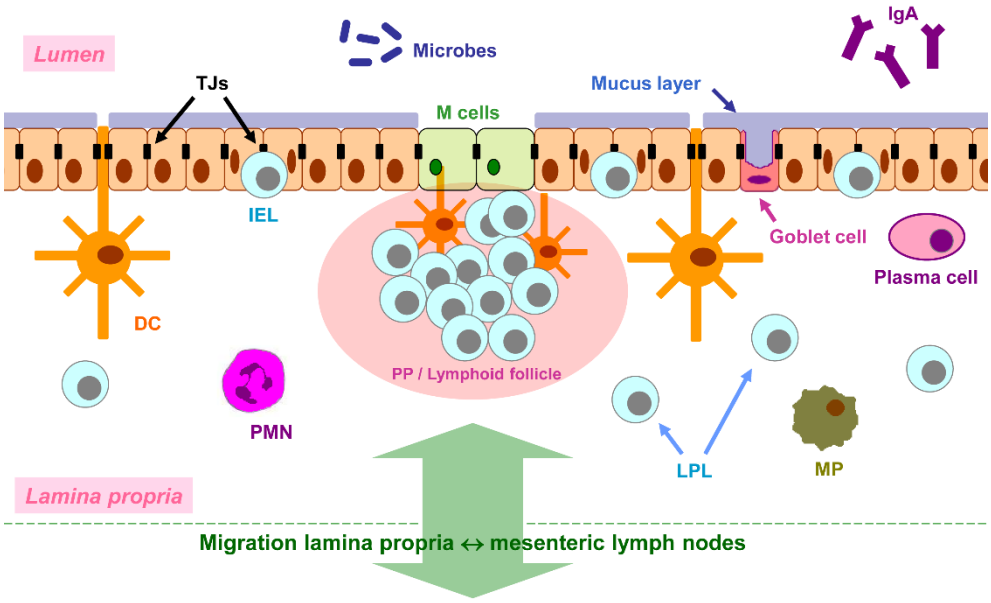

Figure 1: The intestinal barrier. Several mechanisms and cell types present in the epithelial monolayer and the lamina propria are accountable for effective barrier function and gut homeostasis.

The cellular immune response in the intestines is generated by antigens directly or via antigen presentation (mainly by dendritic cells) and T lymphocyte activation. Moreover, intestinal epithelial cells (IECs) and the immune cells present in the intestinal mucosa are

capable of altering cell-mediated immunity by producing pro- and anti-inflammatory cytokines (Le Bourhis et al., 2007; Shaw et al., 2010).

APCs respond directly to luminal bacteria or indirectly through the production of pro- or anti-inflammatory cytokines by the surrounding cells. In the presence of pathogenic microbes, APCs generate pro-inflammatory cytokines including interferon-γ (IFN-γ) and interleukin-12 (IL-12), which in adults enhance overall T helper (Th) cell responses including Th1, Th2 and Th17 cell populations (Acheson and Luccioli, 2004). Th1 cells provoke a cell mediated immune response by producing cytokines such as IL-2 and IFN-γ, while Th2 cells induce humoral immunity via several interleukins (IL-4, 5, 6, 13) (Acheson and Luccioli, 2004). Effector cytokines secreted by Th17 cells include IL-17 and IL-22 (Blaschitz and Raffatellu, 2010). In contrast, following contact with commensal micro- organisms, APCs secrete IL-10 and transforming growth factor beta (TGF-β), leading to an activation of T regulatory cells (Tregs) which will also produce IL-10 and TGF-β. As a result of these cytokines, Th1, Th2 and Th17 responses are suppressed (Borchers et al., 2009; Nyirenda et al., 2009).

In several mammalian species, neonates predominantly display Th2 responses and are deficient in eliciting Th1 responses, restricting their ability to mount a cellular defence (Adkins et al., 2004; Levy, 2007). Certain stimuli, however, have been shown to induce profound pro-inflammatory responses in neonates, comparable to or even more pronounced than responses in adults. An example is the marked tumour necrosis factor alpha (TNF-α) production in human cord blood monocytes compared to adult monocytes after a challenge with Group B Streptococci (Levy, 2007). This phenomenon has also been confirmed in neonatal foals in which decreased basal levels of IFN-γ and IL-6 were found in peripheral blood mononuclear cells, whereas the production of IL-8, IL-12(p35/p40) and IL-23(p19/p40) was similar or even increased in comparison to older individuals (Liu et al., 2009).

This finding fits with the hypothesis that Th1 responses are deficient in newborn foals, although they have been shown to have increased expression of IFN-γ and IL-6 in response to the equine specific pathogen *Rhodococcus equi* (Jacks et al., 2007; Liu et al., 2009; Nerren et al., 2009). In addition, infected foals displayed significantly lower IL-4 expression (compared to infected adults) eliciting a reduced Th2 response to *R. equi* (Jacks et al., 2007). More recent work on *in vitro* cytokine responses to *R. equi* in foals during the first weeks of life confirmed this absence of age-related impairment in Th1 response to this specific pathogen. Moreover, in those foals both IL-4 and IL-17 expression in response to *R. equi* were relatively reduced at birth, suggesting a decreased Th2 and Th17 response (Liu et al., 2011) and that the basal and stimulus-induced cytokine expression is selectively impaired in neonatal foals.

INNATE IMMUNITY AND BACTERIAL RECOGNITION

The innate immune response to bacteria depends on recognition of bacterial molecular patterns (pathogen associated molecular patterns, PAMPs) by PRRs and subsequent intracellular downstream signalling, ultimately leading to the production of pro-inflammatory cytokines. Specific PAMPs of both Gram-negative and Gram-positive bacteria are sensed by several PRRs, including TLR-1, -2, -4, -5, -6 and -9, as well as nucleotide-binding oligomerisation domain protein 1 (NOD-1) and NOD-2.

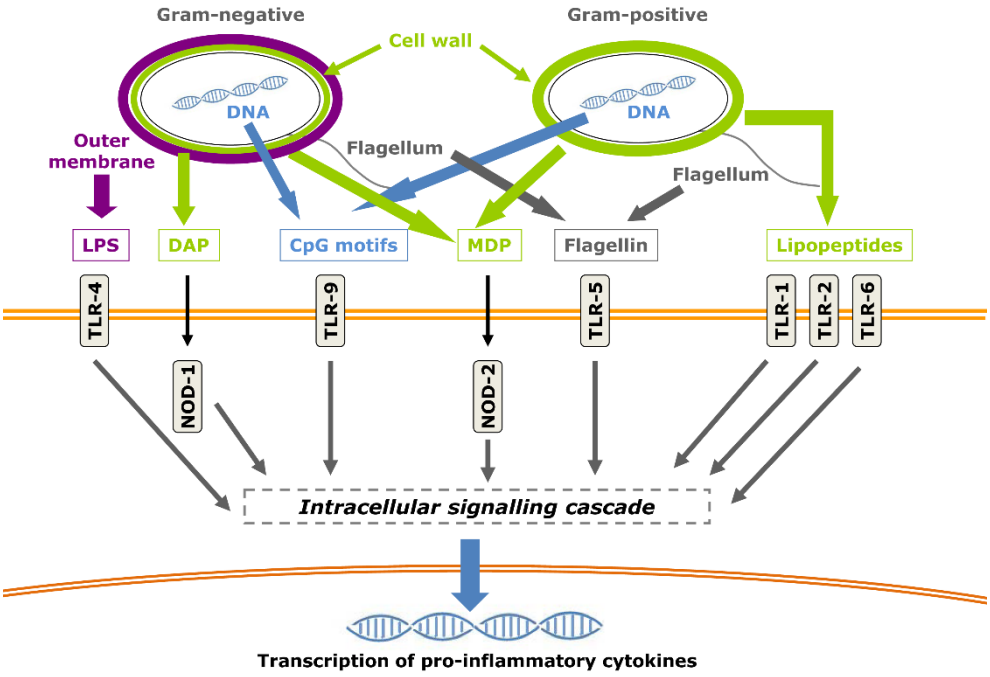

Figure 2: Bacterial recognition by PRRs.

Figure 2 illustrates the initiation of the PRR-signalling cascade by bacterial PAMPs. Whereas TLR-2 mainly recognises constituents of Gram-positive bacteria (including bacterial lipopeptides) in concert with TLR-1 or TLR-6 (Buwitt-Beckmann et al., 2006; Kang et al., 2009), TLR-4 senses Gram-negative microbes by responding to bacterial lipopolysaccharide (LPS) (Lu et al., 2008). TLR-5 and TLR-9 are triggered by bacterial flagellin and CpG[1] DNA motifs respectively, derived from both Gram-positive and Gram-negative bacteria (Hayashi et al., 2001; Hemmi et al., 2000; Rolli et al., 2010). NOD-1 and

[1] Cytosine-phosphate-guanine

NOD-2 sense specific motifs of bacterial peptidoglycan. NOD-1 detects diaminopimelic acid (DAP) derivatives, mainly present in peptidoglycan of Gram-negative bacteria. NOD-2 is triggered by muramyl dipeptide (MDP), a large peptidoglycan motif common to both Gram-negative and Gram-positive bacteria (Le Bourhis et al., 2007; Shaw et al., 2010). All above mentioned PRRs are capable of activating both NFκB, JNK and MAPK pathways[2] (Kumar et al., 2009). For a more detailed and complete overview of PRR-signalling see reviews by Kawai and Akira (2010), Shaw et al. (2010) and Takeuchi and Akira (2010).

Various cell types throughout the human gastrointestinal tract express TLRs constitutively or upon induction, including intestinal epithelial cells (IECs) (Cario and Podolsky, 2000), fibroblasts (Otte et al., 2003), entero-endocrine cells (Bogunovic et al., 2007) and professional immune cells in the lamina propria, such as T cells (Kabelitz, 2007), macrophages and dendritic cells (Visintin et al., 2001; Hart et al., 2005). Comparable investigations on expression and regulation of PRRs in intestinal tissues of horses are lacking. Limited data are available with regard to TLR signalling in equine immune cells and lung tissue. However, the genes of TLR-2, TLR-4 and TLR-9 have been characterised in the horse. In equine monocytes, induction of TLR-2 and TLR-4 expression was demonstrated after incubation with TLR-2 and TLR-4 ligands (Kwon et al., 2010). Furthermore, enhanced expression of pro-inflammatory cytokines and IL-10 has been reported in equine monocytes following activation of TLR-2 and TLR-4 (Figueiredo et al., 2009). For example, we demonstrated in a previous study that TLR-4, MD-2 and CD-14[3] are expressed by leukocytes of healthy horses (Werners et al., 2006).

In another study, expression of TLR-2 and TLR-4 in the equine lung was investigated, using immunocytochemistry and real-time polymerase chain reaction (RT-PCR) (Singh Suri et al., 2006). Immunocytochemistry was applied to demonstrate the presence of TLR-4 in pulmonary intravascular macrophages (PIMs), alveolar macrophages and septal endothelial cells of normal and LPS-exposed horses. RT-PCR revealed TLR-4 mRNA expression in lung tissue of normal horses, whereas TLR-2 mRNA expression was not detectable. In LPS-exposed horses, both TLR-2 and TLR-4 expression levels were found to be elevated (Singh Suri et al., 2006). TLR-4 expression was also reported in equine bronchial epithelial cells of healthy and horses affected with recurrent airway obstruction (RAO). Up-regulation of TLR-4 expression was seen in the RAO-affected group (Berndt et al., 2007).

[2] NFκB: nuclear factor kappaB, JNK: c-jun N-terminal kinase, MAPK: mitogen-activated protein kinase
[3] MD-2: myeloid differentiation protein 2, CD-14: cluster of differentiation 14

More recently, a study demonstrated TLR-9 expression in different cell types in the horse lung, including PIMs, alveolar macrophages and bronchial epithelial cells. After LPS exposure, TLR-9 expression in lung tissue increased (Schneberger et al., 2009). Interestingly, the LPS-induced increase in TLR-9 expression in horse lung tissue was inhibited by intravenous gadolinium chloride, leading to depletion of PIMs (Schneberger et al., 2009). Apparently, PIMs are an important source of TLR-9 in the equine lung. In contrast, a similar study reported no difference in TLR-2 and TLR-4 up-regulation after LPS exposure between normal and PIM-depleted horses although PIM depletion did reduce the expression of TLR-4 in lung tissue of normal horses, without affecting the presence of TLR-2 (Singh Suri et al., 2006). A possible explanation for this difference between normal and LPS-exposed horses may be that in the absence of PIMs, TLR-2 and TLR-4 are up-regulated in other lung cells. These results indicate that different cell types exhibit distinct patterns of TLR expression upon bacterial challenge.

An increased expression of TLR-2 and TLR-9 (next to TLR-4 itself) as a result of LPS exposure was documented earlier in murine dendritic cells. The study demonstrated that up-regulation of TLR-9 coincides with increased TNF-α production induced by LPS. LPS-induced up-regulation of TLR-2, TLR-4, and TLR-9 was inhibited by suppression of the NFκB pathway or the MAPK pathway, confirming the influence of these pathways on TLR expression levels (An et al., 2002). This effect of LPS on expression of other TLRs than TLR-4 possibly enhances overall responses of dendritic cells to bacteria by promoting cytokine production.

In horses, expression of TLR-9 on peripheral blood monocytes of both foals and adults has also been observed. Peripheral blood monocytes of foals and adult horses have shown to express TLR-9 at comparable levels (Flaminio et al., 2007) and Liu et al. (2009) demonstrated responsiveness of equine neutrophils to both CpG motifs and *R. equi* in foals (newborn and 2 months old) and adult horses.

Recently, it was shown that equine mononuclear cells lack surface expression of TLR-5 and are unresponsive to flagellin, unlike similar cells in other mammalian species. In contrast, equine neutrophils were shown to respond to TLR-5 activation and produced both mRNA and protein encoding for TLR-5 (Kwon et al., 2011).

Expression levels of PRRs are known to be subjected to many influences and to vary in different circumstances. Both the relatively low PRR expression levels in highly challenged parts of the gastrointestinal tract and the suppression of pro-inflammatory signals by anti-inflammatory cytokines after the recognition of commensal microbes contribute to this finding (Testro and Visvanathan, 2009), which, in turn, may contribute to colonic homeostasis by conveying tolerance to the resident microflora and simultaneously eliciting efficient immune responses to pathogens when necessary.

Intestinal disease associated with TLR polymorphisms

By 1998, hypo-responsiveness to LPS had been reported in TLR-4 mutated mouse strains (C3H/HeJ and C57BL/10ScCr) (Poltorak et al., 1998). These mutant mice were highly susceptible to infection by a number of Gram-negative bacteria, including *Salmonella typhimurium* and *Escherichia coli* (Cross et al., 1995; Bernheiden et al., 2001). Accordingly, TLR-2 dysfunction in mice has been associated with decreased resistance to Gram-positive bacteria, such as *Staphylococcus aureus* (Takeuchi et al., 2000). In human hospitalised patients, polymorphisms of the TLR-4 gene have been demonstrated to be related to increased susceptibility to Gram-negative systemic infections and sepsis (Agnese et al., 2002; Lorenz et al., 2002; Smirnova et al., 2003; Faber et al., 2006) and it was suggested that TLR-2 polymorphism in humans may be associated with the prevalence of Gram-positive infections (Lorenz et al., 2000).

In a previous study, we have investigated the genetic sequences of TLR-4, MD-2 and CD-14 in a group of healthy horses. Though TLR-4 polymorphisms were found, these mutations did not influence TLR-4 function and were not related to an altered LPS response in individual horses (Werners et al., 2006).

Targets for therapeutic intervention

Direct effects of PRR-mediated cytokine expression on the tight junctions (TJ) of the intestinal barrier have been extensively documented (reviewed by Al-Sadi et al., 2009). Whereas pro-inflammatory cytokines such as TNF-α and IFN-γ increase TJ permeability by disrupting the TJ barrier, anti-inflammatory cytokines such as IL-10 and TGF-β seem to have protective properties and preserve TJ integrity (Al-Sadi et al., 2009).

Cario et al. (2007) have demonstrated that TLR-2 stimulation with specific agonists protects mice against dextran sodium sulfate (DSS)-induced colitis by preserving the tight junction related epithelial barrier function. Moreover, in the same model, probiotics exerted anti-inflammatory effects which appeared to be TLR-9 mediated (Rachmilewitz et al., 2004). In newborn foals, activation of TLR-9 leads to a selective stimulation of cytokines which are normally deficient in the foal (Liu et al., 2009). Improvement of the TJ-related epithelial barrier and inhibition of mucosal inflammation limits the risk of bacteria crossing the epithelial barrier.

For decades, compounds of colostrum and milk have been thought to have protective effects on neonates and to influence bacterial colonisation and tolerance. For example, the presence of anti-inflammatory cytokines in human milk has been recognised to enhance TJ-related epithelial barrier function (Al-Sadi et al., 2009; Garofalo, 2010). Moreover, human breast milk contains soluble TLR-2 and CD-14, which may inhibit signalling through TLR-2 and TLR-4 in the neonatal gastrointestinal tract by

occupying binding sites for these signal molecules (Jones et al., 2002; LeBouder et al., 2003; LeBouder et al., 2006), and bovine colostrum was shown to decrease NFκB activation and subsequent pro-inflammatory cytokine production in intestinal epithelial cells (An et al., 2009; Jorgensen et al., 2010).

More recently, research has been undertaken on specific non-digestible compounds of milk and colostrum, in particular the oligosaccharides representing the dominant carbohydrate fraction. One of the first chemical characterisations of the different oligosaccharides in milk and colostrum was made in camels. In colostrum from Bactrian camels, 10 distinct oligosaccharides were characterised using a sophisticated ^{1}H-nuclear magnetic resonance spectroscopy technique. In contrast, milk from these animals contained only three distinct oligosaccharides (Fukuda et al., 2010). These findings are of clinical relevance, as oligosaccharides (and other glycans) are thought to play an important role in bacterial colonisation of the neonatal gastrointestinal tract and to protect neonates against bacterial invasion (Newburg, 2009). In several models with bacterial challenges, the anti-adhesive effects of milk oligosaccharides have been identified (Kunz et al., 2009). Prebiotic effects of both fructo-oligosaccharides and human milk oligosaccharides have also been illustrated by determining increased faecal *Bifidobacteria* counts after oral administration (Lindsay et al., 2006; Kunz and Rudloff, 2008).

Human milk-derived oligosaccharides exert direct immunomodulatory properties in addition to their recognised prebiotic properties (Eiwegger et al., 2010). *In vitro* experiments have demonstrated that these oligosaccharides were transferred across a monolayer of intestinal epithelial cells. Moreover, human milk-derived oligosaccharides reduced Th2 type cytokine production (IL-13) in T lymphocytes, while the Th1 type cytokine response (IFN-γ) was enhanced. Thus, the Th1/Th2 ratio (which is skewed to Th2 responses in neonates) was altered in the direction of Th1. In addition, an anti-inflammatory response was illustrated by inducing the IL-10 response following its activation by human milk-derived acidic oligosaccharides (Eiwegger et al., 2010). In a rat model of DSS induced colitis, oligosaccharides isolated from goat's milk were shown to reduce intestinal inflammation and enhance the recovery of the damaged colonic mucosa (Lara-Villoslada et al., 2006). Similar to these milk-derived carbohydrates, a plant-derived oligogalactan was recently shown to limit the up-regulation of TLR-4 and TNF-α in mice, using the DSS induced colitis model (Liu et al., 2010).

Milk of different mammalian species appears to display distinct oligosaccharide patterns. Oligosaccharides derived from human milk mainly comprise glucose, galactose, N-acetylglucosamine, fucose and N-adetylneuraminic acid (Kunz et al., 2009). Very few studies have looked at the composition of equine colostrum and milk and both similarities and differences have been described in comparing the structure of human and equine milk-derived oligosaccharides (Urashima et al., 1989; Urashima et al., 1991; Nakamura et

al., 2001). According to this preliminary research, the immunomodulatory capacity of the carbohydrate fraction of equine colostrum and milk is promising, as both similarities in the oligosaccharide patterns of humans and horses are evident as well as differences, revealing the presence of unique equine oligosaccharides.

Conclusions

Certain TLRs, such as TLR-2 and TLR-9, have been shown to have a protective role in experimental models of inflammatory bowel disease, and colostrum- and milk-derived compounds that are agonists for these receptors are promising candidates for therapeutic approaches. Direct immunomodulatory effects of milk-derived oligosaccharides have been demonstrated and the structural similarity of colostrum- and milk-derived oligosaccharides and known TLR ligands suggests that these will activate TLRs directly, causing comparable protective effects. The exact underlying mechanisms remain to be elucidated, but these findings, together with increasing evidence that colostrum- and milk-derived compounds have protective properties, indicate that their effects on gut health will be a promising topic for future research, aiming to boost the foal's resistance against invading pathogens.

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Chapter 3

Characterisation of TLR-2 and TLR-4 activation in RAW 264.7 cells

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PRELIMINARY DATA

Abstract

Toll-like receptors (TLRs) have shown to be key players in bacterial recognition and subsequent immune responses. TLR expression levels and the functionality of TLR signalling pathways have great influence in both physiological processes and the development of inflammatory disorders. Modulation of TLR-mediated immune responses can be desirable to either improve immunity or prevent exaggerated inflammatory responses from occurring. In this study, we investigated TLR-2 and TLR-4 signalling pathways in RAW 264.7 cells, which are widely acknowledged as a murine macrophage model. Aside from confirming the phagocytic capacity of these macrophages, we demonstrated by means of immunofluorescent imaging that, next to being a cell surface receptor, the TLR-4 complex resides intracellularly. Furthermore, our data show that both TLR-2 and TLR-4 activation lead to dose-dependent induction of inflammatory pathways in RAW 264.7 cells. Activation of both TLRs affected expression levels of the TLRs involved in bacterial recognition (TLR-1, -2, -4, -5, -6, and -9), comparably. Interestingly, the activation of one specific TLR resulted in altered expression levels of several TLRs, probably due to shared intracellular signalling pathways amongst these TLRs.

Introduction

Bacterial recognition by Toll-like receptors (TLRs) and the influence of TLR expression and functionality on bacterial tolerance and immunity are subjects of great interest and relevance in both human and veterinary research. The main TLRs involved in bacterial recognition are TLR-1, -2, -4, -5, -6 and -9. Whereas TLR-4 is widely acknowledged as the lipopolysaccharide (LPS)-receptor and thus responds to Gram-negative bacteria, TLR-2 mainly recognises constituents of Gram-positive bacteria such as lipoproteins, lipoteichoic acid and lipopeptides (Kawai and Akira, 2010). TLR-2 interacts with triacylated or diacylated lipopeptides by formation of heterodimers with TLR-1 and TLR-6 respectively. However, recognition of (diacylated) bacterial lipopeptides by TLR-2 homodimers has been described as well (Buwitt-Beckmann et al., 2005; Buwitt-Beckmann et al., 2006). LPS signal transduction by TLR-4 requires other proteins, including LPS binding protein (LBP), CD-14 and MD-2[1] (Lu et al., 2008). TLR-5 senses bacterial flagellin (Hayashi et al., 2001) and TLR-9 recognizes cytosine-phosphate-guanine (CpG) motifs of bacterial or viral DNA (Hemmi et al., 2000).

Downstream TLR signalling is schematically illustrated in Figure 1. All TLRs (as well as the type I IL-1 receptor) are capable of activating the MyD88-dependent pathway[2]. After recruitment of the adaptor molecule MyD88, IL-1 receptor kinases (IRAKs) are bound and TRAF-6 is activated[3]. TRAF-6 enables TAK-1 to form a complex with TAK binding proteins[4]. In the end, TAK-1 activates both the NFκB (nuclear factor κB) pathway, the JNK (c-Jun N-terminal Kinase) pathway and the MAPK (Mitogen-Activated Protein Kinase) pathway, leading to induction of inflammatory cytokines and type 1 interferons (IFNs) (Kawai and Akira, 2006). Both TLR-2 and TLR-4 require the adaptor protein TIRAP (Mal)[5] next to MyD88 to activate the MyD88-dependent signalling pathway, as TIRAP-deficient mice display defects in inflammatory responses mediated by ligands of TLR-2 and TLR-4 (Horng et al., 2002; Yamamoto et al., 2002; Mansell et al., 2004). Besides, TLR-4 is capable of activating NFκB and MAP kinases in a MyD88-independent manner. MyD88-deficient murine macrophages and dendritic cells fail to produce inflammatory cytokines in response to TLR-4 activation, but do produce type I IFNs (Kawai and Akira, 2006). TRAM and Trif[6] are identified as functional adapter proteins in this MyD88-independent signalling pathway (Kawai and Akira, 2006). Crosstalk between different TLRs has been described in several studies (Becker and O'Neill, 2007; Geisel et al., 2007; van Aubel et al., 2007).

[1] CD-14: cluster of differentiation 14, MD-2: myeloid differentiation protein 2
[2] MyD88: myeloid differentiation primary response protein
[3] TRAF: tumour necrosis factor receptor-associated factor
[4] TAK: transforming growth factor-β-activated protein kinase
[5] TIRAP: Toll/interleukin-1 receptor domain containing adapter protein, Mal: MyD88-adapter like
[6] TRAM: Trif-related adapter protein, Trif: Toll/interleukin-1 receptor domain containing adapter inducing IFN-β

TLRs, in particular TLR-2 and TLR-4, have shown to play a key role in various inflammatory diseases. Malfunctioning of these receptors and signalling pathways leads to an increased susceptibility to for instance inflammatory bowel disease, bacterial infection and sepsis (Viemann et al., 2005; Cario et al., 2007; Gribar et al., 2008). In this study, we aimed to characterise the responses of RAW 264.7 cells to a broad concentration range of LPS and Pam3-Cys-Ser-Lys4 (PCSK), which is a synthetic bacterial lipopeptide that specifically activates TLR-2. We chose to use the RAW 264.7 cell line, which is a widely acknowledged murine macrophage model, to establish the optimal conditions for future studies to look more specifically into immunomodulation by these TLR ligands, in this model as well as in *ex vivo* cell cultures of other species. Next to the production and expression of pro- and anti-inflammatory cytokines, we investigated the expression of the TLRs involved in bacterial recognition following TLR-2 and TLR-4 activation. Moreover, we looked further into the functionality of the TLR-4 complex, by performing phagocytosis assays and immunofluorescent imaging of both TLR-4 and internalised LPS particles.

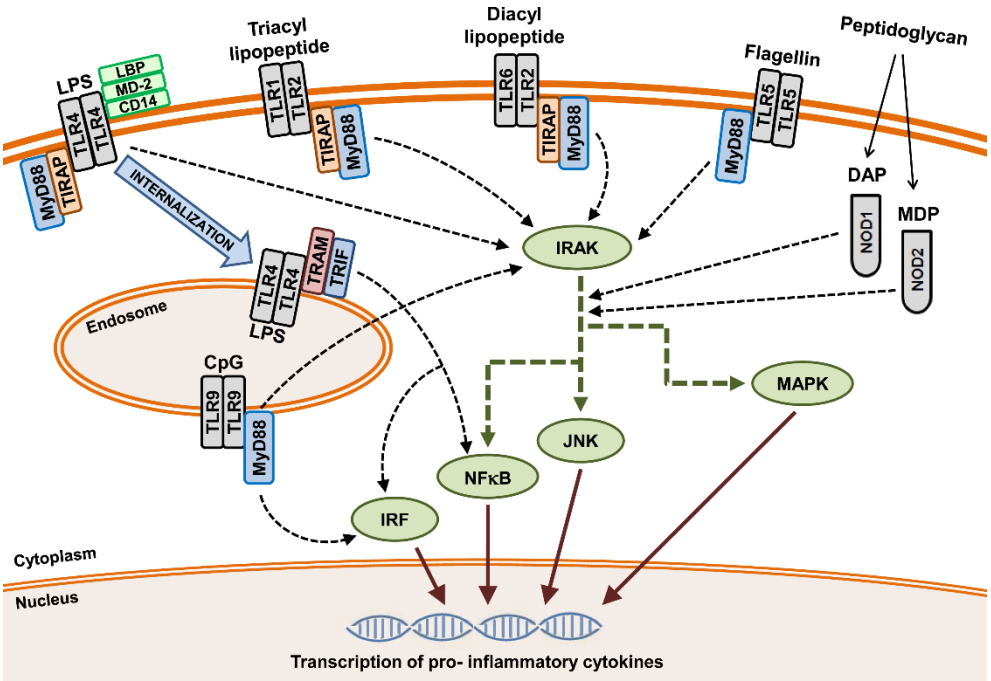

Figure 1: Schematic illustration of TLR activation by specific bacterial patterns. Ultimately, inflammatory responses are influenced through modulation of intracellular signalling pathways. The TLR family shares signalling pathways, hence enabling TLR crosstalk.

Materials and Methods

Cell culture conditions

RAW 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% foetal bovine serum (Life technologies, Carlsbad, CA, USA), 100 µg/ml penicillin and streptomycin (Lonza, Basel, Switzerland), 1 mM sodium pyruvate (Life technologies, Carlsbad, CA, USA) and 2 mM glutamine (Lonza, Basel, Switzerland).

Phagocytosis assay

For the phagocytosis assay, RAW 264.7 cells were seeded in 96-well plates at a density of $2*10^4$ cells/100 µl/well (in supplemented DMEM as described above) and incubated overnight (37 °C and 5% CO_2) to allow the cells to adhere. Thereafter, the medium was replaced with supplemented DMEM containing fluorescein conjugated *E.Coli* (K12-strain) Bioparticles® (Molecular Probes, Eugene, OR, USA) at a concentration of 5 mg/ml and 10 mg/ml, including blank controls. All incubations were performed in triplicate. The plates were covered to protect them from light, and subsequently incubated for 2 hours (37 °C and 5% CO_2). After removing the solution and washing the cells with sterile PBS (Lonza, Basel, Switzerland), 100 µl of a solution of 250 µg/ml of trypan blue (Sigma- Aldrich, St. Louis, MO, USA) in PBS was added immediately and kept on the cells for 1 minute. Ultimately, trypan blue was removed and fluorescence was measured at 485 nm excitation and 520 emission wavelength, using a Fluostar Optima fluorometer (BMG Labtechnologies, Offenburg, Germany).

Confocal fluorescence microscopy of phagocytosis

To obtain microscopic images of the above stated phagocytosis, we seeded RAW 264.7 cells in chamber slides (0.8 cm^2/well) at a density of $4*10^4$ cells/200 µl/well (in supplemented DMEM as described above) and incubated overnight (37 °C and 5% CO_2) to allow the cells to adhere. The next day, the medium was replaced with supplemented DMEM containing 5 mg/ml fluorescein conjugated *E.Coli* (K12-strain) Bioparticles® (Molecular Probes, Eugene, OR, USA). The plates were covered to protect them from light, and subsequently incubated for 2 hours (37 °C and 5% CO_2). Subsequently, the medium was removed, cells were washed with PBS, and a fluorescent label for actin was added to the cells (phalloidin diluted 1:100; Life technologies, Carlsbad, CA, USA). Ultimately, a drop of mounting medium was added (FluorSave™; Merck Chemicals Ltd., Darmstadt, Germany) with a cover slip on top. Slides were stored at 4°C whilst protected from light.

Immunofluorescent labelling of TLR-4

For immunofluorescent labelling of TLR-4, chamber slides (0.8 cm^2/well) were coated with 0.01% poly-L-lysine (Sigma- Aldrich, St. Louis, MO, USA) for 1 hour at room temperature. After rinsing the slides 3 times with sterile H_2O, slides were allowed to dry and subsequently sterilised under UV light for 4 hours. Then, RAW 264.7 cells were seeded in the chamber slides at a density of $4*10^4$ cells/200 µl/well (in supplemented DMEM as described above) and incubated overnight (37 ºC and 5% CO_2) to allow the cells to adhere. The next day, RAW 264.7 cells were washed with sterile PBS and fixated with 4% paraformaldehyde (Merck Chemicals Ltd., Darmstadt, Germany) in PBS, pH 7.4, for 15 minutes at room temperature. The fixative was washed off with ice cold PBS and cells were incubated with 1% bovine serum albumin (BSA; Sigma- Aldrich, St. Louis, MO, USA) in PBST (PBS containing 0.1% Tween® 20; Merck Chemicals Ltd., Darmstadt, Germany) for 30 minutes to block unspecific binding of the antibodies. Subsequently, cells were incubated with the primary antibody for murine TLR-4 (rabbit polyclonal to TLR-4; Abcam, Cambridge, UK), 1:100 diluted in 1% BSA in PBST for 1 hour at room temperature. Afterwards, the cells were washed 3 times with PBS for 5 minutes and incubated for 1 hour with the secondary antibody (goat polyclonal to rabbit immunoglobulin containing an Alexa Fluor® 488 dye; Abcam, Cambridge, UK) at room temperature in the dark. Ultimately, cells were washed again 3 times for 5 minutes and a drop of mounting medium was added (FluorSave™; Merck Chemicals Ltd., Darmstadt, Germany) with a cover slip on top. Slides were stored at 4ºC whilst protected from light.

Cell culture experiments

For the experiments, RAW 264.7 cells were seeded in 24-well plates at a density $2*10^5$ cells/ml/well and incubated overnight (37 ºC and 5% CO_2) to allow the cells to adhere. The next day, experiments were started by replacing the medium with medium containing different concentrations (0, 0.001, 0.01, 0.1 and 1 µg/ml) of either LPS (*Escherichia coli* O111:B4; Sigma- Aldrich, St. Louis, MO, USA) or Pam3-Cys-Ser-Lys4 (PCSK; Invivogen, San Diego, CA, USA). All experiments were performed in triplicate. For qPCR analyses, samples were collected after 4 hours of incubation: cells were lysed using RNA lysis buffer (Promega, Madison, WI, USA) and samples were stored at -80 ºC until RNA isolation was resumed. For the ELISAs and the nitric oxide (NO) measurements, supernatants were collected after 4 hours and 24 hours of incubation and stored at -80 ºC.

Nitric oxide (NO) measurements

NO concentrations in the cell culture supernatants were estimated trough determination of the nitrite concentrations by means of the Griess reaction, as described previously (Green et al., 1982).

Cytokine measurements

To measure protein levels of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-10, ELISA was performed on the supernatants using ELISA MAX™ Deluxe kits for murine TNF-α, IL-6, and IL-10 (Biolegend, San Diego, CA, USA). Standard operating procedures of the manufacturer were followed, applying all required buffers and solutions in the form provided by the manufacturer (Biolegend, San Diego, CA, USA). The lower limits of detection of the ELISAs were 7.8 pg/ml (TNF-α), 7.8 pg/ml (IL-6), and 31.3 pg/ml (IL-10), respectively.

RNA Isolation

RNA was isolated from the RAW 264.7 cells using SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Isolated fractions were dissolved in 100 µl ribonuclease free water and stored at -80 °C. Quality and quantity of RNA was determined spectrophotometrically (Nanodrop).

Real-time PCR analysis

cDNA was generated using iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA, USA) according to the manufacturer's protocol. For reverse transcriptase reaction, 1000 ng RNA was applied per sample. Expression of mRNA was assessed by real-time PCR using a Biorad iQ5 Multicolor Real-time PCR detection system and iQ™ SYBR® Green Supermix (Biorad, Hercules, CA, USA). Specific primer pairs were designed and tested for efficiency and accuracy, after having checked their specificity using the NCBI-BLASTN search program. Primer pairs were synthesised commercially (Eurogentec, Maastricht, The Netherlands). For this study, mRNA expression of TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, TLR-9, IL-6, IL-10, TNF-α, β-actin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was determined using the following primer pairs:

TLR-1:	F 5'-TTGCTGGTGTTAGGAGATGCT-3'
	R 5'-TCCAAACCGATCGTAGTGCT-3'
TLR-2:	F 5'-TCCCAGATGCTTCGTTGTTC-3'
	R 5'-AGTGGTTGTCGCCTGCTTC-3'
TLR-4:	F 5'-AGAATGAGGACTGGGTGAGA-3'
	R 5'-TGTAGTGAAGGCAGAGGTGA-3'
TLR-5:	F 5'-CACCGAAGACTGCGATGAAGAG-3'
	R 5'-CAAGGGTGATGACGAGGAATAGAG-3'
TLR-6:	F 5'-CTGAAGTCACTGATGATAGAGCAC-3'
	R 5'-GCACACCATGTGGATGAAAG-3'
TLR-9:	F 5'-CCTCATGGCCTGGTGGACT-3'
	R 5'-AGGTGGTGGATACGGTTGGA-3'

IL-6:	F 5′-CCGGAGAGGAGACTTCACAG-3′
	R 5′-TCCACGATTTCCCAGAGAAC-3′
IL-10:	F 5′-TCAATTGCTCTCATCCCTGA-3′
	R 5′-GGATCTCCCTGGTTTCTCTTC-3′
TNF-α:	F 5′-GGACTAGCCAGGAGGGAGAACA-3′
	R 5′-GCCAGTGAGTGAAAGGGACAGA-3′
GAPDH:	F 5′-GTGGAGCCAAAAGGGTCATC-3′
	R 5′-TGTCATATTTCTCGTGGTTCACAC-3′
β-actin:	F 5′-ACCCTAAGGCCAACCGTGAAAAG-3′
	R 5′-CGACCAGAGGCATACAGGGACA-3′

Cell viability assessment

To investigate possible influence of the applied TLR ligands on cell viability, 2 assays were performed according to manufacturer's instructions. The MTT assay was used as a colorimetric assay to determine mitochondrial activity, through measuring the degree of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA). The neutral red (NR) uptake assay was used to estimate the number of viable cells, capable of taking up NR (Sigma- Aldrich, St. Louis, MO, USA). Before viability assessment, RAW 264.7 cells were incubated for 24 hours with supplemented DMEM containing 0, 0.1, 1, or 10 μg/ml LPS or PCSK.

Data analysis

In the figures of this chapter, data are displayed as mean values and standard deviations. The qPCR data were assumed to be normally distributed. Mean values and standard deviations were calculated for each incubation. Subsequently, the differences between the mean value of each incubation and the mean value of the blank controls were calculated, as well as the standard deviations of these differences. Ultimately, all differences and their error bars were translated to the scale of relative expression by means of the following transformation: 2^{-x}. The data obtained from all other assays were assumed to be log-normally distributed. Mean values and standard deviations were calculated from the log-transformed data. Error bars were calculated and transformed back to the unit scale.

Results

Phagocytosis assay

Figure 2 illustrates the results of the phagocytosis assay. The capacity of RAW 264.7 cells to phagocytise bacterial particles was confirmed by these results (most evident at 10 mg/ml). Moreover, the confocal microscopy images clearly showed that the fluorescent particles were situated intracellularly (Figure 3).

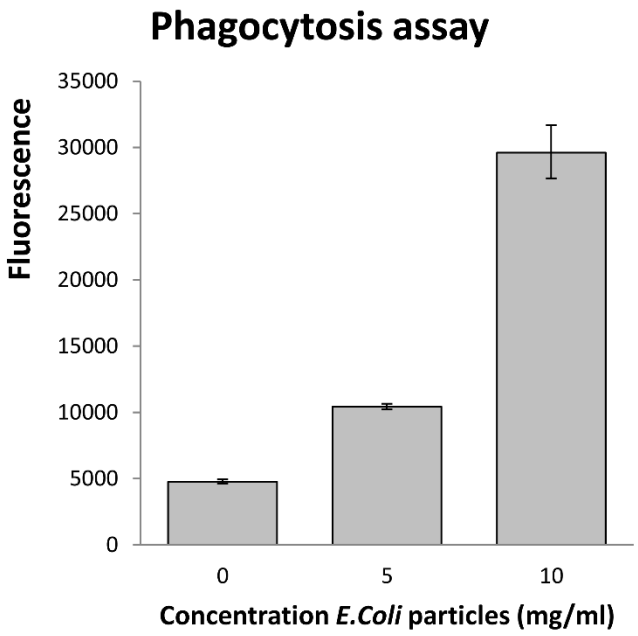

Figure 2: Mean fluorescence values for the phagocytosis assay, using fluorescein conjugated *E.Coli* particles, including standard deviations. Experiments were conducted in triplicate.

Immunofluorescent labelling of TLR-4

Figure 4 shows the microscopic images of one RAW 264.7 cell, both naively and with Alexa 488 labelled TLR-4.

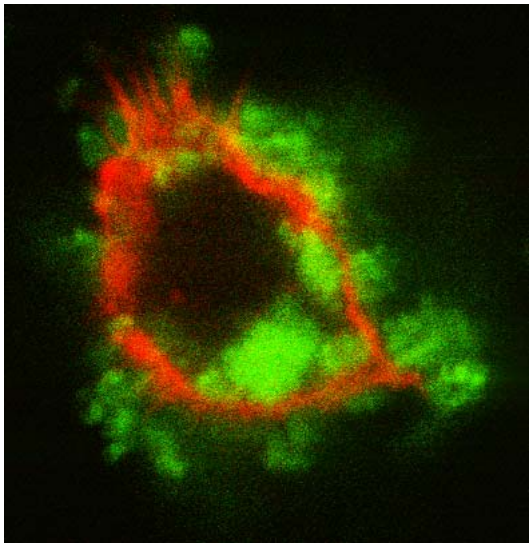

Figure 3: Confocal fluorescence microscopy image of a RAW 264.7 cell with internalised *E.Coli* particles. The intracellular localisation was confirmed by means of multiple cross section images (not shown).

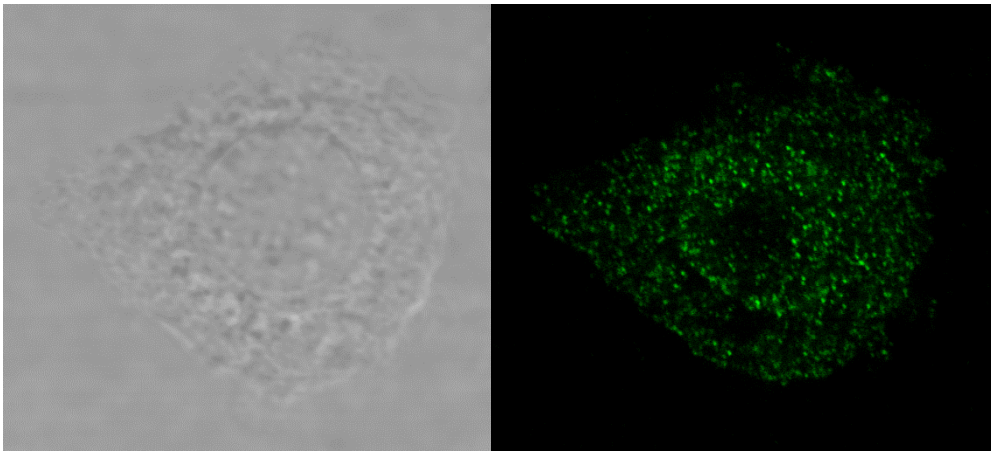

Figure 4: Microscopic images of one RAW 264.7 cell with Alexa 488 labelled TLR-4, normal light microscopy on the left and fluorescence microscopy on the right. Negative control not shown.

TLR expression levels

In Figure 5, relative mRNA expression levels are illustrated for TLRs involved in bacterial recognition. TLR-5 is not included in the figure, as the expression levels of TLR-5 in all investigated incubations were too low to quantify validly. Our data showed that LPS and PCSK have comparable effects on the expression levels of TLR-1, -2, -4, -6, and -9. TLR-2 expression was dose-dependently enhanced by both LPS and PCSK, whereas expression of TLR-4 was generally reduced after incubation with both ligands. TLR-1 expression was dose-dependently up-regulated by both ligands, but this up-regulation appeared to decrease again at the top of the applied concentration range (1 µg/ml). The expression of TLR-6 was not influenced evidently under the investigated circumstances. TLR-9 expression levels were dose-dependently enhanced by both ligands, though after incubation with 1 µg/ml LPS, TLR-9 expression levels were comparable to blank controls.

Cytokine expression levels

Figure 6 illustrates the relative mRNA expression levels of TNF-α, IL-6, and IL-10 after 4 hours of incubation with LPS or PCSK. Up-regulation of all measured cytokines was evident after incubation with LPS or PCSK, mostly dose-dependent as well. When comparing effects of LPS and PCSK, generally LPS had a more profound impact on cytokine expression levels than PCSK, in particular regarding the pro-inflammatory cytokines TNF-α and IL-6. For both ligands a dose-dependency could be seen throughout the applied concentration range. Though, cytokine expression levels appeared to reach their maximum level yet at lower concentrations of both TLR ligands compared with the actual cytokine response (produced protein levels, Figures 7 and 8).

Housekeeping gene expression

Variation of mRNA expression could be excluded as a confounding factor, as no significant differences in either β-actin or GAPDH expression were detected amongst control samples and the incubations with LPS and PCSK (data not shown).

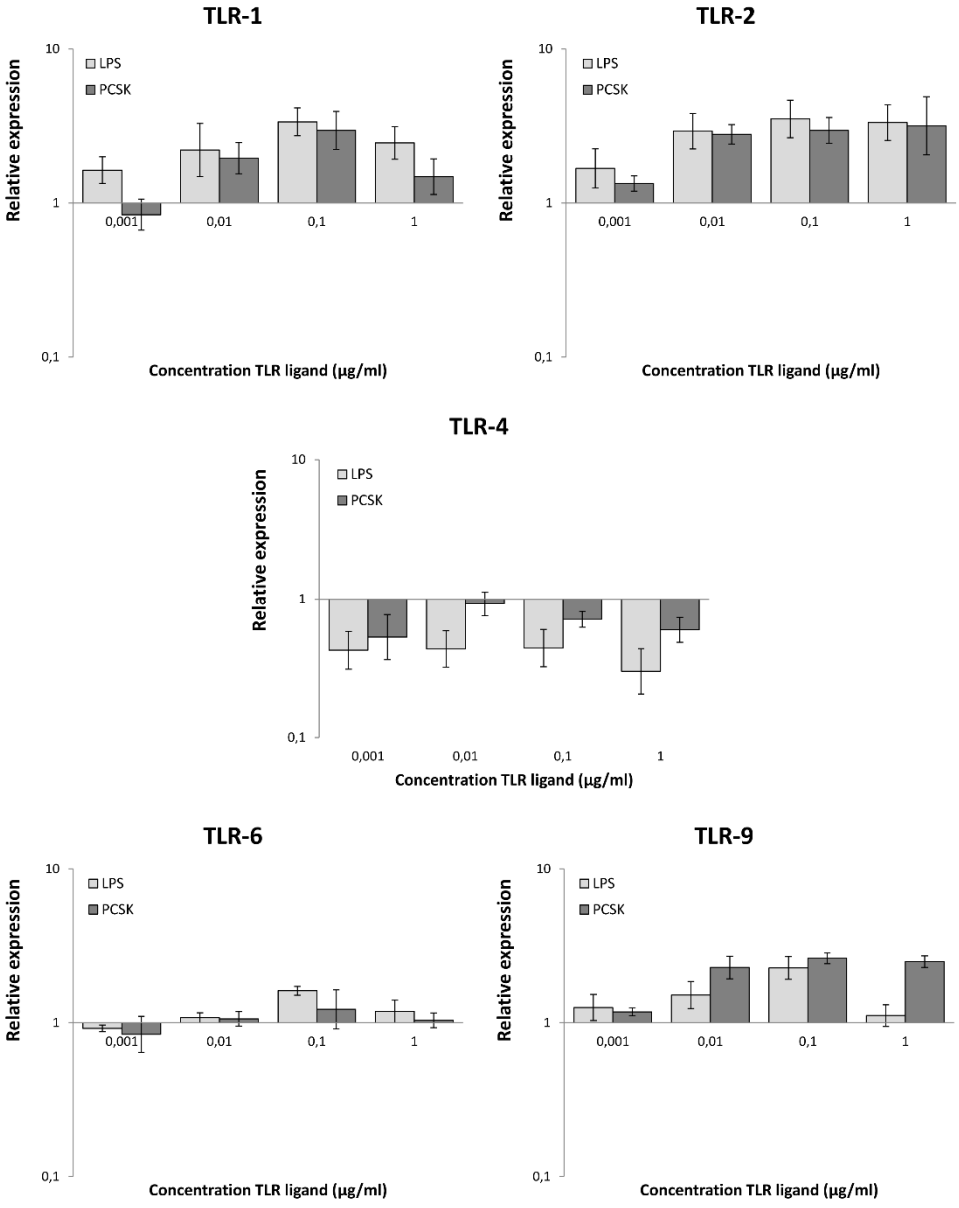

Figure 5: Mean values and standard deviations for the relative expression levels of TLRs after 4 hours of incubation with LPS or PCSK, compared with blank controls. Experiments were conducted in triplicate.

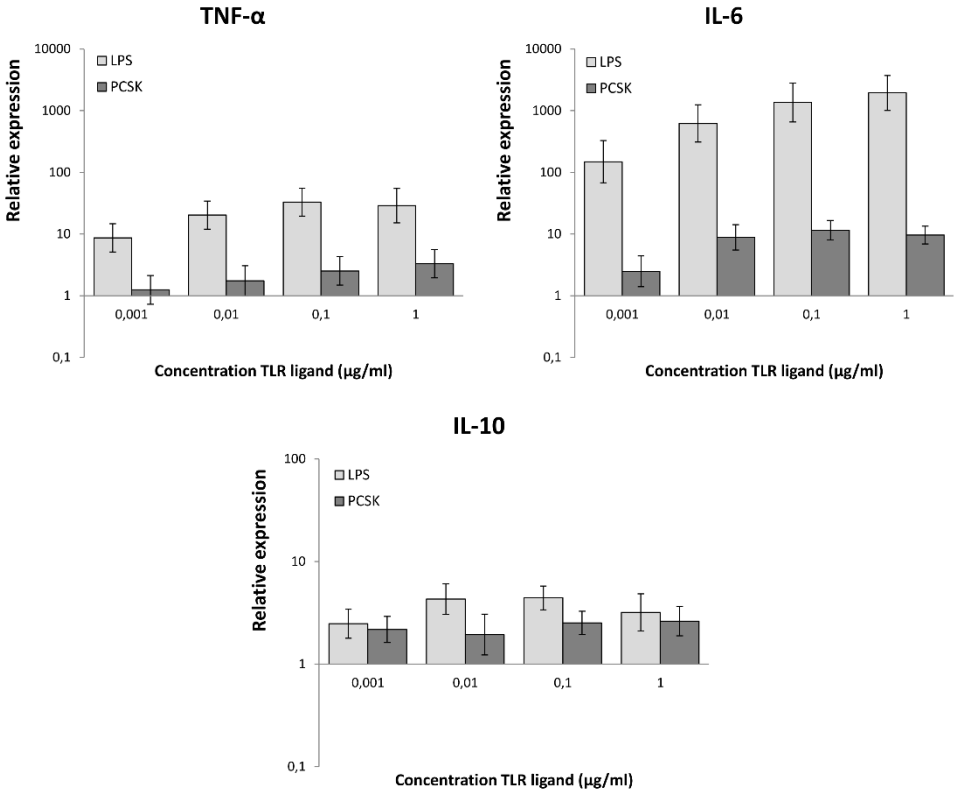

Figure 6: Mean values and standard deviations for the relative expression levels of TNF-α, IL-6, and IL-10 after 4 hours of incubation with LPS or PCSK, compared with blank controls. Experiments were conducted in triplicate.

Functional cytokine response

In Figure 7, the measured supernatant concentrations of TNF-α, IL-6, and IL-10 are illustrated after 4 hours of incubation with the chosen concentrations of LPS and PCSK, including blank controls. For LPS, a dose-dependent increase of both TNF-α, IL-6, and IL-10 production was evident in the applied concentration range after 4 hours of incubation. After a 4 hours incubation of RAW 264.7 cells with PCSK, there was a dose-dependent increase of TNF-α production, which appears to come at a maximum at PCSK concentrations of 0.1 µg/ml and higher. IL-10 production after 4 hours was limited in all incubations, but more evident in LPS-challenged cells. Again, no increase of IL-10 production was observed whilst comparing challenges of 0.1 µg/ml and 1 µg/ml PCSK. IL-6 production was hardly detectable in RAW 264.7 cells challenged with this concentration range of PCSK for 4 hours. Measurements below the detection limit of the ELISAs were stated equal to the lower detection limit of the concerning ELISA (evident in Figure 7 for TNF-α production in blank controls, for IL-6 at 0 and 0.001 µg/ml (for both LPS and PCSK)

as well as 0.1 and 1 µg/ml (for PCSK), and for IL-10 at 0, 0.001, and 0.01 µg/ml (both LPS and PCSK).

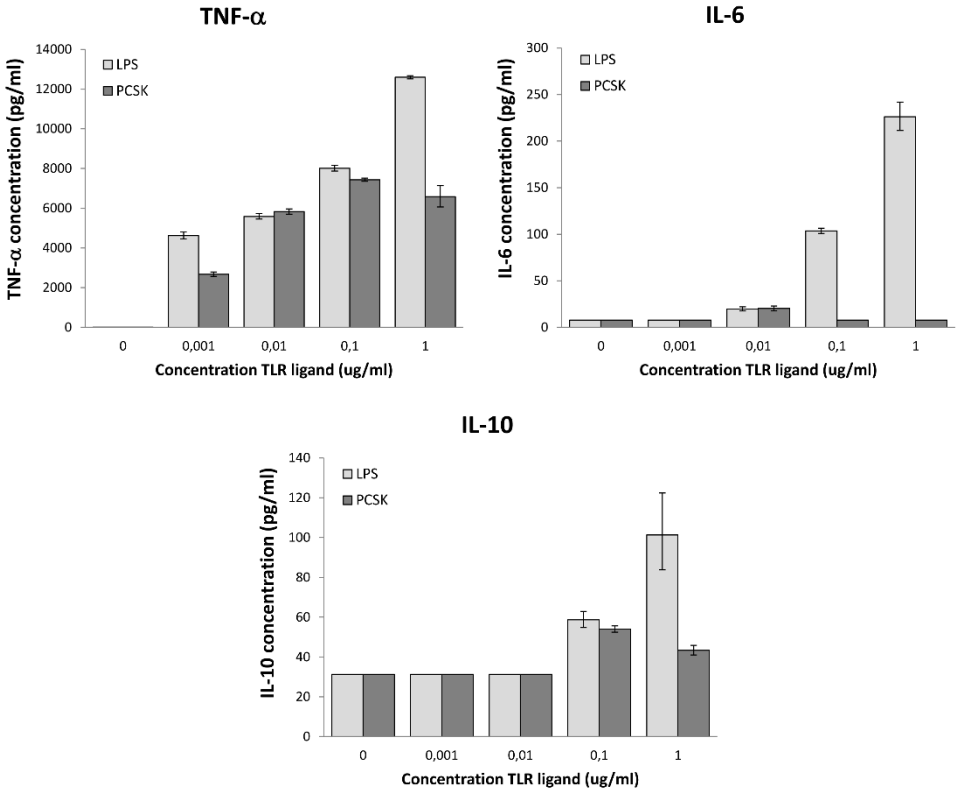

Figure 7: Mean supernatant concentrations of TNF-α, IL-6, and IL-10 (pg/ml) after 4 hours of incubation with LPS or PCSK, including standard deviations. Experiments were conducted in triplicate.

In Figure 8, the measured supernatant concentrations of TNF-α, IL-6, and IL-10 are illustrated after 24 hours of incubation. For TNF-α, throughout the entire concentration range a dose-dependent increase in production was evident for both TLR ligands. However, similar to the results after 4 hours of incubation, for PCSK the dose-dependent increase appeared to stop at concentrations higher than 0.1 µg/ml. The results for IL-6 and IL-10 production largely fit the data concerning TNF-α production. Though, IL-6 and IL-10 were generally produced to a lesser extent and after a low dose (0.001 µg/ml) challenge with LPS or PCSK, no IL-6 or IL-10 production was detectable. Furthermore, LPS-stimulated cells were shown to produce IL-6 and IL-10 to a higher extent than cells incubated with PCSK (most evident at 1 µg/ml), similar to the results after 4 hours of incubation.

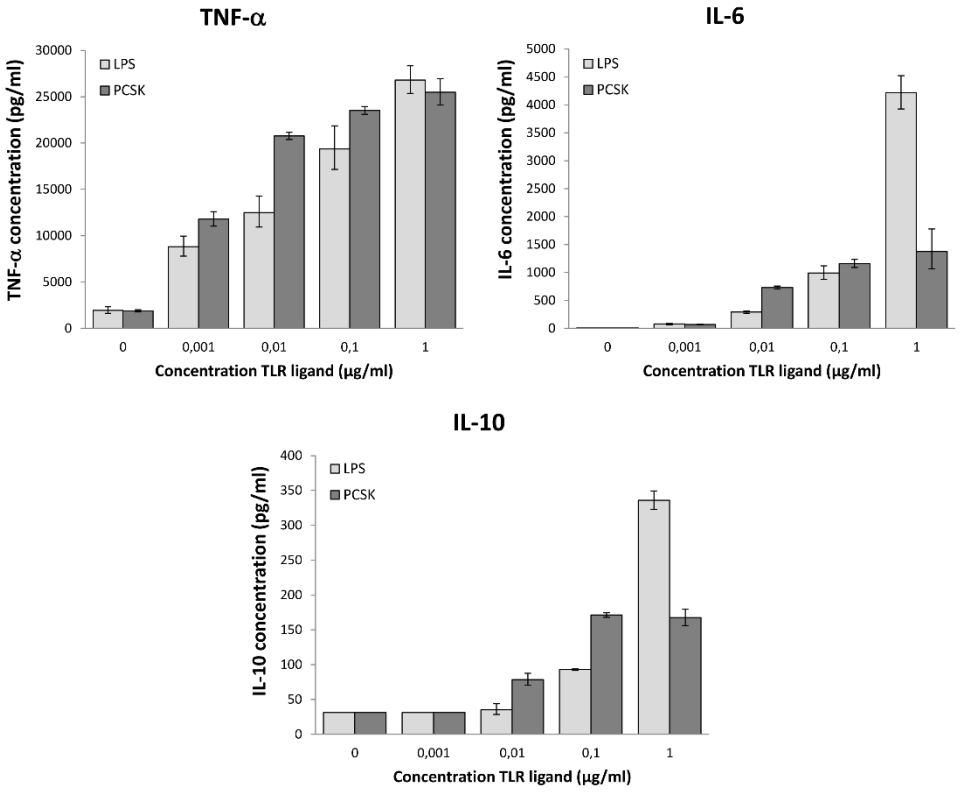

Figure 8: Mean supernatant concentrations of TNF-α, IL-6, and IL-10 after 24 hours of incubation with LPS or PCSK, including standard deviations. Experiments were conducted in triplicate.

NO production

The NO concentrations after 4 hours of incubation with LPS or PCSK were not detectable and are therefore not shown. After 24 hours of incubation, a dose-dependent increase in NO production was observed for both TLR ligands throughout the entire concentration range (Figure 9).

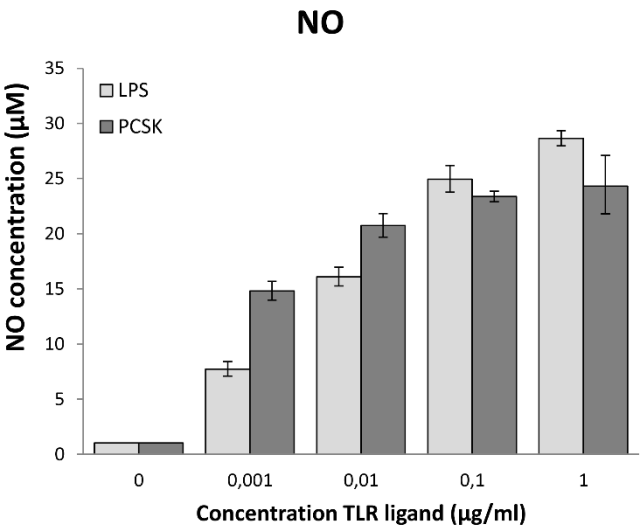

Figure 9: Mean supernatant concentrations of NO after 24 hours of incubation with LPS or PCSK, including standard deviations. Experiments were conducted in quadruplicate.

Cell viability assays

Cell viability, as estimated by means of the MTT and NR cell viability assay, was not influenced after 24 hours of incubation with LPS or PCSK, in concentrations up to 10 µg/ml (Figure 10).

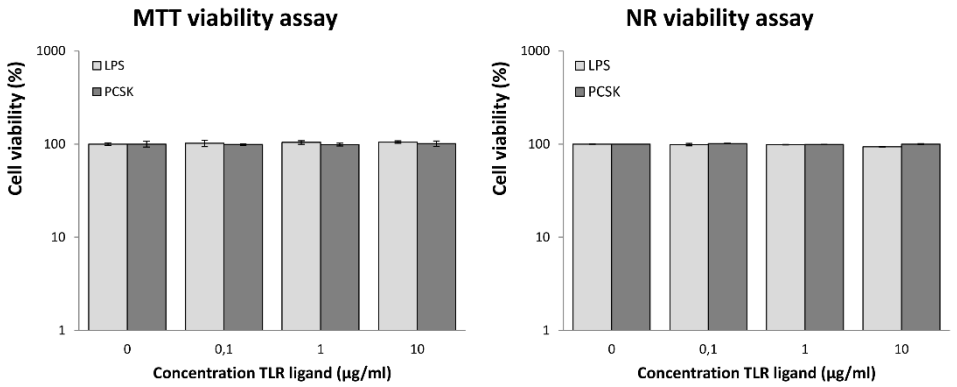

Figure 10: Mean cell viability of RAW 264.7 cells after 24 hours of incubation with LPS or PCSK, including standard deviations. Experiments were conducted in quadruplicate.

Discussion

Internal localisation of TLR-4

Our results in RAW 264.7 cells confirm the findings of published studies, which state that TLR-4 is expressed in internal cell compartments, such as endosomes and the Golgi apparatus, next to being present at the cell surface (Latz et al., 2002; Hornef et al., 2003; Latz et al., 2003). Though TLR-4 seems to be activated at the cell surface to generate inflammatory signals, the process of trafficking LPS between the Golgi apparatus and the cell membrane, while bound to the TLR-4/MD-2/CD-14 complex, appears to take place independent of the initiation of signal transduction (Latz et al., 2002; Latz et al., 2003). More recently, it was shown that CD-14 is required for the initiation of both endocytosis and further downstream signal transduction (Zanoni et al., 2011).

TLR crosstalk

The effects of LPS and PCSK on TLR expression levels in this model were largely comparable. Interestingly, both TLR-2 and TLR-4 activation resulted in down-regulation of TLR-4 and up-regulation of TLR-1, TLR-2, and TLR-9. Apparently, the regulation of expression of several TLRs can be influenced by the activation of one specific TLR. As all

TLRs involved in bacterial recognition share similar signalling pathways, we hypothesised that the activation of these shared pathways can initiate regulatory feedback mechanisms, which affect the expression of multiple TLRs. The increased expression of TLR-2 and TLR-9 as a result of LPS treatment was documented previously in murine dendritic cells (An et al., 2002). LPS-induced up-regulation of TLR-2 and TLR-9 was inhibited by suppression of the NFκB pathway or the MAPK pathway, confirming the influence of these pathways on TLR expression levels (An et al., 2002).

TLR-4 down-regulation

An interesting finding was the down-regulation of TLR-4 after incubation with LPS or PCSK, whilst the expression of the other investigated TLRs was not influenced of up-regulated by both ligands. It is known from literature that TLR activation can eventually result in suppression of the concerning signalling pathways. Down-regulation of TLR-4 pathways leads to endotoxin tolerance (LPS tolerance), which is thought to be a process to either prevent inflammation physiologically or suppress defensive immune responses, leading to pathology (Nahid et al., 2011; Quinn et al., 2012). Ongoing research focuses on the involvement of microRNA in the regulation of endotoxin tolerance. It appears that activation of the NFκB pathway can lead to induction of several subtypes of microRNA, which regulate the TLR-4 signalling pathway at the level of the receptor, intracellular pathways, gene transcription and translation (Nahid et al., 2011; Quinn et al., 2012). The down-regulation of TLR-4 expression after LPS stimulation was documented before, for instance in murine peritoneal macrophages (Nomura et al., 2000). Next to conforming this in our experiments, our data showed TLR-4 down-regulation after TLR-2 activation as well. These findings are in line with the above stated studies, which documented NFκB-dependent regulation of the TLR-4 signalling pathway by microRNA, as TLR-2 activation triggers the NFκB pathway as well.

RAW 264.7 cells express TLR-5 to a minor extent

Our data could not provide a valid estimation of TLR-5 expression, as the detected PCR signals were not within the efficient range of detection of our primer set (the found Ct's[7] were higher than 35). We did detect a qPCR signal with a fitting melting curve for TLR-5 and our primer set, thus RAW 264.7 cells do express TLR-5. However, as we previously tested other murine tissues as well and we detected high expression levels of TLR-5 in for instance murine lung tissue using the same primer set (mean Ct 23.0), we can conclude that, based on our findings, RAW 264.7 cells express TLR-5 to a minor extent and that LPS and PCSK do not enhance TLR-5 expression in these cells.

[7] Ct: cycle threshold

Cytokine profiles

Our data show that both LPS and PSCK induce both mRNA expression and protein production of the pro-inflammatory cytokines TNF-α and IL-6. LPS appeared to be a more potent inducer of inflammatory pathways compared with PCSK. In this study, the expression and production of the regulatory cytokine IL-10 was modulated to a lesser extent. This is possibly due to the fact that regulatory mechanisms are induced as a consequence of the primary inflammatory response, and that IL-10 production is not modulated directly through activation of TLR-2 or TLR-4.

Conclusion

Our data show that both TLR-2 and TLR-4 activation lead to dose-dependent induction of inflammatory pathways in this murine macrophage cell line. LPS appeared to be a more potent inducer of inflammation compared with PCSK. Moreover, both TLR ligands affected the investigated TLR expression levels comparably. Interestingly, the activation of one specific TLR resulted in altered expression levels of several TLRs, which are involved in bacterial recognition, probably due to shared intracellular signalling pathways amongst these TLRs.

Acknowledgements

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Chapter 4

Modulation of lipopolysaccharide-induced inflammatory responses in a murine intestinal epithelial cell line

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PRELIMINARY DATA

Abstract

Expression and function of Toll-like receptors (TLRs) is known to vary amongst different cell types and situations. Intestinal epithelial cells normally exhibit relatively low activity of TLR signalling pathways, preventing the initiation of exaggerated inflammatory responses in the intestinal tract to the present endogenous flora. On the other hand, dysfunction of TLRs has been related to decreased defence mechanisms in the epithelial barrier and subsequent pathology as well. Present research focuses on TLR-mediated improvement of epithelial barrier function, whilst preserving immunological balance and preventing excessive inflammatory responses. In this study, we investigated the direct influence of a synthetic TLR-2 ligand and the carbohydrate fraction of equine colostrum on the inflammatory response in a murine intestinal epithelial cell line (mICcl2) following TLR-4 activation. Our data give evidence for functional signalling of TLR-2 and TLR-4 in mICcl2 cells. Whereas TLR-2 activation did not alter the lipopolysaccharide-induced inflammatory response, equine colostral carbohydrates did suppress pro-inflammatory cytokine production in this model. Future investigations are required to investigate the effects of TLR-2 ligands and equine colostral carbohydrates in more extensive experimental setups, to elucidate both underlying immunomodulatory mechanisms as well as the effects on inflammatory processes in other animal species.

Introduction

Expression of the different Toll-like receptors (TLRs), which are activated by bacterial patterns, is known to vary amongst different cell types. The basal expression and functionality of TLRs on a specific cell type as well as the induced regulatory processes in case of a bacterial challenge determine the eventual immune response. Hence, the degree of TLR functionality has been shown to play a pivotal role in the functional defence against pathogens, as well as tolerance to commensal microorganisms (Testro and Visvanathan, 2009; Takeuchi and Akira, 2010; Abdelsadik and Trad, 2011). For instance, in healthy human individuals, intestinal epithelial cells (IECs) express TLR-5, whilst TLR-2 and TLR-4 expression levels are barely detectable (Cario and Podolsky, 2000; Melmed et al., 2003; Otte et al., 2004). Consequently, pro-inflammatory signalling pathways are very mildly activated in the epithelial monolayer, or not at all (Abreu et al., 2002).

In general, dysfunction of TLRs has been associated with the occurrence of various inflammatory disorders in mammals, amongst others an increased susceptibility to bacterial infections and sepsis (Takeuchi et al., 2000; Bernheiden et al., 2001). Elevated expression levels of TLR-2 and TLR-4 are documented in IECs and lamina propria macrophages of human individuals with inflammatory bowel disease (IBD) (Hausmann et al., 2002; Cario et al., 2004; Szebeni et al., 2008). There is still debate on what is more important in the pathogenesis of IBD: TLR deficiency or TLR over-activity. The increased expression levels of TLRs in the epithelial barrier, in particular TLR-2 and TLR-4, could be causing overproduction of pro-inflammatory mediators in the first place, leading to disruption of the epithelial barrier and progressive disease. On the other hand, TLR deficiency could primarily cause decreased epithelial barrier function, followed by invasion of the resident intestinal flora. Subsequently, the progressive damage could be caused by the presence of these bacteria and inflammatory mediators, which cause TLR up-regulation in the second place. The latter theory is supported by the fact that various research groups have found correlations between IBD and TLR polymorphisms in certain populations (Lorenz et al., 2000; Hugot et al., 2001; Cuthbert et al., 2002; Franchimont et al., 2004; Torok et al., 2004; Arnott et al., 2005; Lakatos et al., 2005; Pierik et al., 2006; De Jager et al., 2007; Torok et al., 2009). In mice, disabling of TLR-5 resulted in spontaneous development of colitis, as a result of excessive TLR-4 activation by the endogenous flora (Vijay-Kumar et al., 2007; Carvalho et al., 2012a; Carvalho et al., 2012b).

As TLR function has been shown to be pivotal in health and disease, various studies have been performed to investigate possibilities to modulate immune response by interfering with TLR signalling, aiming to prevent or treat inflammatory disorders. Previously published *in vivo* studies in experimental animals have indicated that activation of certain TLRs, in particular TLR-2, can result in suppression of inflammatory

gastrointestinal pathology, by improving epithelial barrier function (Cario et al., 2004; Cario et al., 2007). In the latter studies, Pam3-Cys-Ser-Lys4 (PCSK) was used as a TLR-2 ligand. PCSK is a synthetic triacylated lipopeptide, which is recognised by TLR-2 in concert with TLR-1 by formation of heterodimers. However, TLR-1 independent recognition of PCSK by TLR-2 homodimers has been described as well (Buwitt-Beckmann et al., 2005; Buwitt-Beckmann et al., 2006).

The aim of the current study was to explore direct immunomodulatory properties of PCSK. For this purpose, we investigated the influence of PCSK on the lipopolysaccharide (LPS)-induced inflammatory response in mICcl2 cells, a murine intestinal epithelial cell line. In previously published studies, TLR-4 function of mICcl2 cells has been investigated extensively, documenting marked inflammatory responses following LPS stimulation (Hornef et al., 2002; Hornef et al., 2003). The most evident pro-inflammatory cytokine, which these IECs produced, was documented to be macrophage inflammatory protein 2 (MIP-2), a small cytokine belonging to the CXC chemokine family (also known as CXCL2). Therefore, in this study we used MIP-2 as the most important functional parameter.

Next to effects of PCSK, we investigated possible immunomodulation by the carbohydrate fraction of equine colostrum, using the same experimental model. Limited research has been conducted with equine colostrum, despite known immunomodulatory properties of colostrum of other species, such as humans and cattle (Biswas et al., 2007; An et al., 2009; Newburg. 2009; Eiwegger et al., 2010; Jenny et al., 2010; Jorgensen et al., 2010). Moreover, chemical analysis of equine colostrum has revealed specific oligosaccharide patterns, which could be of interest for the development of new formulas with immunomodulatory capacity (Urashima et al., 1989; Urashima et al., 1991; Nakamura et al., 2001). In the present study, we investigated the effects of the total equine colostral carbohydrate fraction, containing both digestible and non-digestible carbohydrates (such as oligosaccharides).

Materials and Methods

Cell culture experiments: separate and concomitant TLR-2 and TLR-4 activation

mICcl2 cells were obtained from Inserm Research Institute (Paris, France) and cultured according to the provided general culturing conditions. Culture medium comprised DMEM F12 (Life Technologies, Carlsbad, CA, USA), supplemented with 2% decomplemented foetal bovine serum (Life Technologies, Carlsbad, CA, USA), $5*10^{-8}$ M Dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 5 µg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 60 nM selenium (Sigma-Aldrich, St. Louis, MO, USA), 5 µg/ml transferrin (Sigma-Aldrich, St. Louis, MO, USA), 10^{-9} M triiodothyronine (Sigma-Aldrich, St. Louis, MO, USA), 10 ng/ml murine epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA), 20 mM HEPES (Life Technologies, Carlsbad, CA, USA), 2.2 mg/ml D-Glucose (Sigma-Aldrich, St. Louis, MO, USA), 100 µg/ml penicillin and streptomycin (Lonza, Basel, Switzerland), and 2 mM glutamine (Lonza, Basel, Switzerland). For the experiments, mICcl2 cells were seeded in 12-well plates at a density $4*10^5$ cells/1.5 ml/well and incubated overnight (37 °C and 5% CO_2) to allow the cells to adhere. The next day, experiments were started by pre-incubating the cells with supplemented DMEM F12 containing 0 or 1 µg/ml Pam3-Cys-Ser-Lys4 (PCSK; Invivogen, San Diego, CA, USA). After pre-incubation, medium was replaced with supplemented DMEM F12 containing 0 or 1 µg/ml LPS (*Escherichia coli* O111:B4; Sigma- Aldrich, St. Louis, MO, USA) and 0 or 1 µg/ml PCSK. After placing the plates in the incubator for another 4 hours, samples were collected for qPCR and ELISA. Each combination of incubations was investigated in triplicate or quadruplicate. For the ELISAs, supernatants were stored at -80 °C. For qPCR analyses, the cells were lysed using RNA lysis buffer (Promega, Madison, WI, USA) and stored at -80 °C until RNA isolation was resumed.

Cell culture experiments: effects of equine colostral carbohydrates (eCC) on the LPS-induced inflammatory response

In addition to the experiments as described above, an identical experimental setup was used to investigate possible effects of equine colostral carbohydrates (eCC) on the LPS-induced inflammatory response in mICcl2 cells. The carbohydrate fraction of the colostrum samples was extracted as described by Fukuda et al (Fukuda et al., 2010). In short, a colostrum sample of 50 ml was thawed and mixed with 210 ml chloroform:methanol 2:1 v/v. This emulsion was centrifuged in glass tubes for 30 minutes at 4 °C and 4000*g and the lower chloroform layer and the denatured protein were discarded. Methanol was removed from the upper layer using a vacuum centrifuge. The residue was dissolved in 35 ml supplemented DMEM F12 medium (as described above) and filtered through a 0.2 µm filter. This solution was called 1.000 eCC (stock solution) and eCC fractions of 0.500, 0.250, 0.125, 0.063, 0.031, and 0.016 were obtained by diluting

1:1 each step using supplemented DMEM F12. The experiments were carried out identical to the experiments using PCSK. mICcl2 cells were pre-incubated for 2 hours with all different concentrations of eCC, including blank controls. Then, cells were incubated for another 4 hours with eCC and 0 or 1 µg/ml LPS and samples were collected for qPCR and ELISA.

MIP-2 determination

Supernatant protein levels of MIP-2 were determined by means of ELISA, using Duoset® ELISA Development System for murine MIP-2 (R&D Systems, Minneapolis, MN, USA). Standard operating procedures of the manufacturer were followed, applying all required buffers and solutions in the form provided by the manufacturer (R&D Systems, Minneapolis, MN, USA). The lower limit of detection of the ELISA was 15.6 pg/ml.

RNA Isolation

RNA was isolated from the cells using SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Isolated fractions were dissolved in 100 µl ribonuclease free water and stored at -80 °C. Quality and quantity of RNA was determined spectrophotometrically (Nanodrop).

Real-time PCR analysis

cDNA was generated using iScriptTM cDNA Synthesis Kit (Biorad, Hercules, CA, USA) according to the manufacturer's protocol. For reverse transcriptase reaction, 1000 ng RNA was applied per sample. Expression of mRNA was assessed by real-time PCR using a Biorad iQ5 Multicolor Real-time PCR detection system and iQTM SYBR® Green Supermix (Biorad, Hercules, CA, USA). Specific primer pairs were designed and tested for efficiency and accuracy, after having checked their specificity using the NCBI-BLASTN search program. Primer pairs were synthesised commercially (Eurogentec, Maastricht, The Netherlands). For this study, mRNA expression of TLR-2, TLR-4, interleukin-6 (IL-6), IL-10, MIP-2, transforming growth factor-β1 (TGF-β1), tumour necrosis factor-α (TNF-α), β-actin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was determined using the following primer pairs:

TLR-2:	F 5′-TCCCAGATGCTTCGTTGTTC-3′
	R 5′-AGTGGTTGTCGCCTGCTTC-3′
TLR-4:	F 5′-AGAATGAGGACTGGGTGAGA-3′
	R 5′-TGTAGTGAAGGCAGAGGTGA-3′
IL-6:	F 5′-CCGGAGAGGAGACTTCACAG-3′
	R 5′-TCCACGATTTCCCAGAGAAC-3′
IL-10:	F 5′-TCAATTGCTCTCATCCCTGA-3′
	R 5′-GGATCTCCCTGGTTTCTCTTC-3′

MIP-2:	F 5′-GCCAAGGGTTGACTTCA-3′
	R 5′-TGTCTGGGCGCAGTG-3′
TGF-β1:	F 5′-CGAAGCGGACTACTATGC-3′
	R 5′-ACTGTGTGAGATGTCTTTGG-3′
TNF-α:	F 5′-GGACTAGCCAGGAGGGAGAACA-3′
	R 5′-GCCAGTGAGTGAAAGGGACAGA-3′
GAPDH:	F 5′-GTGGAGCCAAAAGGGTCATC-3′
	R 5′-TGTCATATTTCTCGTGGTTCACAC-3′
β-actin:	F 5′-ACCCTAAGGCCAACCGTGAAAAG-3′
	R 5′-CGACCAGAGGCATACAGGGACA-3′

Cell viability assessment

To investigate possible influence of the applied incubations on cell viability, 2 assays were performed according to manufacturer's instructions. The MTT assay was used as a colorimetric assay to determine mitochondrial activity, through measuring the degree of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA). The neutral red (NR) uptake assay was used to estimate the number of viable cells, capable of taking up NR (Sigma- Aldrich, St. Louis, MO, USA). Before viability assessment, mICcl2 cells were incubated for 4 hours with supplemented DMEM containing LPS (0, 0.1, 1 or 10 µg/ml), PCSK (0, 0.1, 1 or 10 µg/ml), or eCC (entire applied concentration range) .

Data analysis

In the figures of this chapter, data are displayed as mean values and standard deviations. For the ELISA data, data were assumed to be log-normally distributed. Mean values and standard deviations were calculated from the log-transformed data. Error bars were calculated and transformed back to the unit scale. The qPCR data were assumed to be normally distributed. Mean values and standard deviations were calculated for each incubation. Subsequently, the differences between the mean value of each incubation and the mean value of the blank controls were calculated, as well as the standard deviations of these differences. Ultimately, all differences and their error bars were translated to the scale of relative expression by means of the following transformation: 2^{-x}. Significance of differences was investigated by means of an unpaired t-test with unequal variance. Differences with p-values<0.05 were considered significant.

Results: effects of TLR-2 activation on the LPS response in mICcl2 cells

Functional MIP-2 response

In Figure 1, MIP-2 production is illustrated in mICcl2 cells incubated with LPS and PCSK alone, and in LPS-challenged mICcl2 cells, which were pre- and co-incubated with PCSK. LPS and PCSK both significantly induced MIP-2 production compared with blank controls. LPS had a more pronounced effect on MIP-2 production in these experiments compared with PCSK. Pre- and co-incubation of LPS-challenged mICcl2 cells with PCSK did not affect MIP-2 production compared with mICcl2 cells incubated with LPS alone.

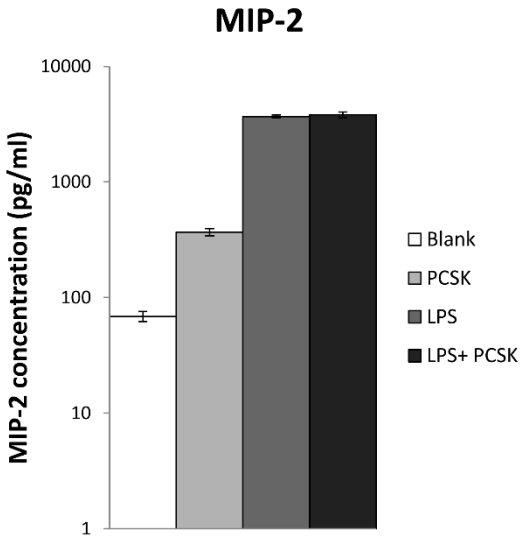

Figure 1: Mean supernatant MIP-2 concentrations (pg/ml) including standard deviations, after incubation with PCSK, LPS, and PCSK+LPS (all 1 µg/ml), including blank controls. Experiments were conducted in triplicate.

Cytokine mRNA expression levels

Figure 2 displays the relative mRNA expression levels of MIP-2, TNF-α, IL-6, IL-10, and TGF-β1 after 4 hours of incubation with LPS, PCSK, and LPS+PCSK. The relative expression was determined by comparing the qPCR signal (cycle threshold; Ct) with the qPCR signal in blank controls (2^ΔCt). Up-regulation of MIP-2 mRNA expression was the most pronounced and these qPCR data fit the above described ELISA data completely. Both TLR ligands significantly induced MIP-2 mRNA expression in mICcl2 cells, and this

effect was more prominent for LPS than for PCSK. Whereas LPS enhanced mRNA expression levels of the other pro-inflammatory cytokines TNF-α and IL-6 as well, incubation with PCSK for 4 hours did not alter the mRNA expression of these cytokines compared with blank controls. LPS stimulation of mICcl2 cells for 4 hours influenced the mRNA expression of the regulatory cytokines TGF-β1 and IL-10 to a minor extent, whilst incubation with PCSK did not affect the mRNA expression of these cytokines compared with blank controls. Pre- and co-incubation of LPS-challenged mICcl2 cells with PCSK did not affect the LPS-induced cytokine response on a transcriptional level in these experiments.

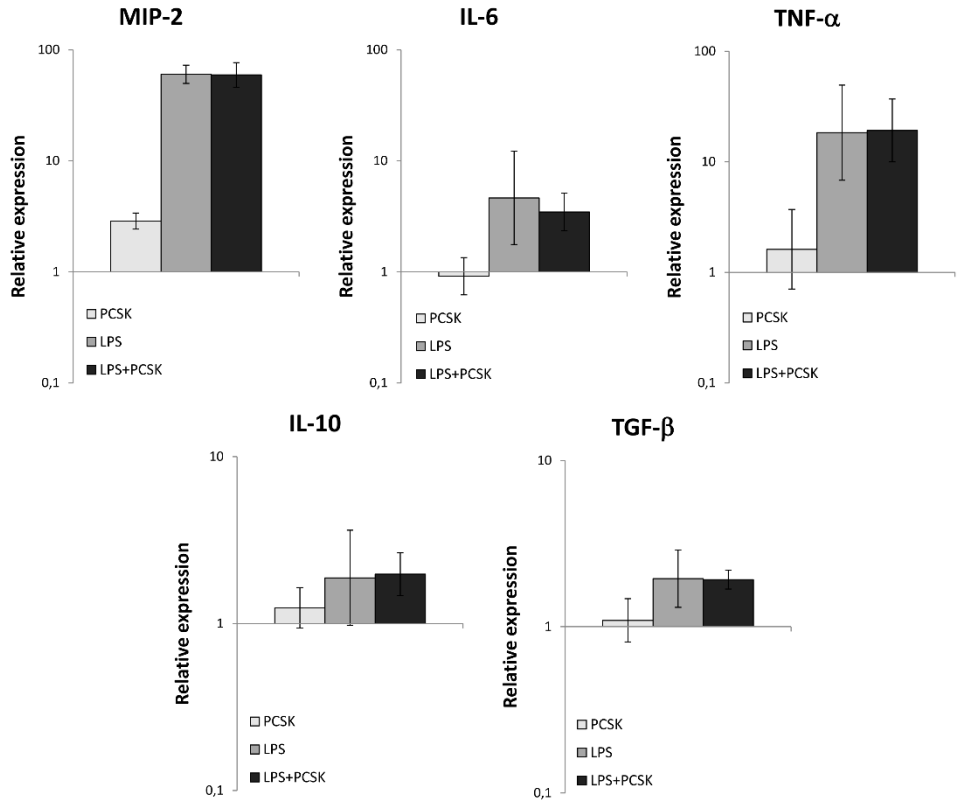

Figure 2: Mean values and standard deviations for relative cytokine mRNA expression levels, related to blank controls, after incubation with PCSK, LPS, and PCSK+LPS (all 1 µg/ml). Experiments were conducted in triplicate.

TLR-2 and TLR-4 mRNA expression levels

In Figure 3, relative mRNA expression levels are given for TLR-2 and TLR-4. None of the investigated incubations influenced the mRNA expression of TLR-4 compared with blank controls. Whereas LPS significantly induced relative mRNA expression of TLR-2, incubation with PCSK did not alter TLR-2 mRNA expression compared with blank controls. Again, no difference was observed between LPS stimulated cells and cells, which were pre-incubated with PCSK and subsequently incubated with LPS+PCSK.

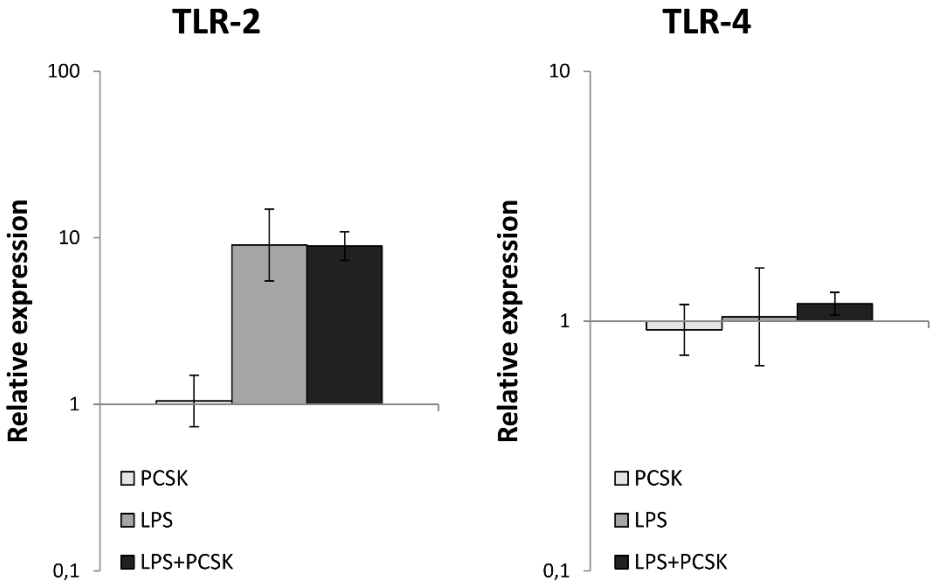

Figure 3: Mean values and standard deviations for relative TLR mRNA expression levels, related to blank controls, after incubation with PCSK, LPS, and PCSK+LPS (all 1 µg/ml). Experiments were conducted in triplicate.

Housekeeping gene expression

Variation of mRNA expression could be excluded as a confounding factor, as no significant differences in either β-actin or GAPDH mRNA expression were detected amongst blank controls and the incubations with LPS and/or PCSK.

Cell viability assays

Cell viability of mICcl2 cells, as estimated by means of the MTT and NR cell viability assay, was not influenced after 4 hours of incubation with LPS and/or PCSK (compared with blank controls).

Results: effects of equine colostral carbohydrates (eCC) on the LPS response in mICcl2 cells

Functional MIP-2 response

In Figure 4, supernatant concentrations of the MIP-2 protein are illustrated in unchallenged and LPS-challenged mICcl2 cells, pre- and co-incubated with eCC. In unchallenged cells, no induction of MIP-2 production was detected. In LPS-challenged cells, a marked MIP-2 induction was observed. A significant reduction of the LPS response was observed for eCC fractions of 0.500 (p-value=0.008) and 1.000 (p-value <0.001). For 0.250 eCC, this reductive effect was nearly significant (p-value=0.054).

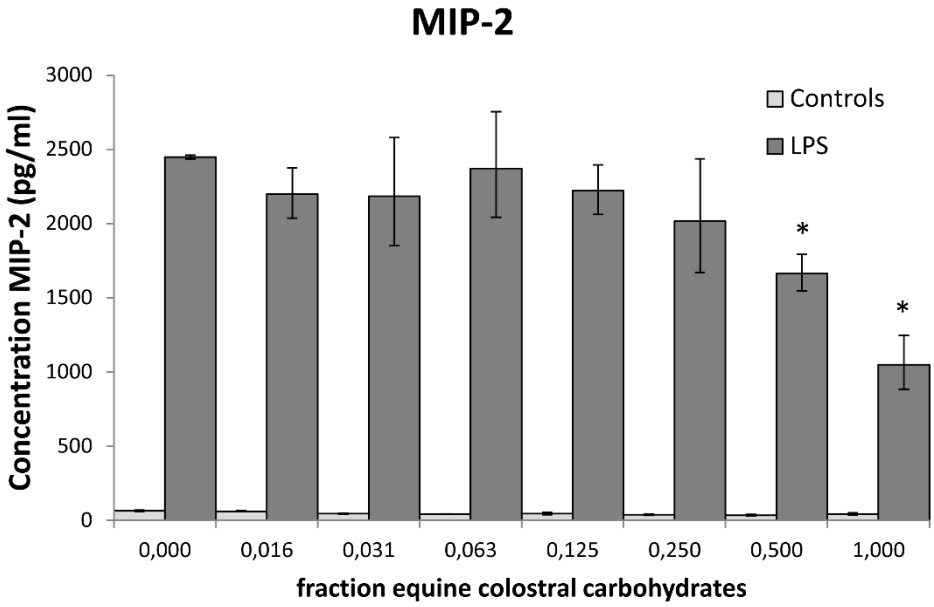

Figure 4: Mean supernatant MIP-2 concentrations (pg/ml) including standard deviations, in unchallenged and LPS-challenged mICcl2 cells incubated with equine colostral carbohydrates (eCC), including blank controls. The asterisk signs (*) indicate a significant difference (p-value <0.05) with the blank controls. Experiments were conducted in quadruplicate.

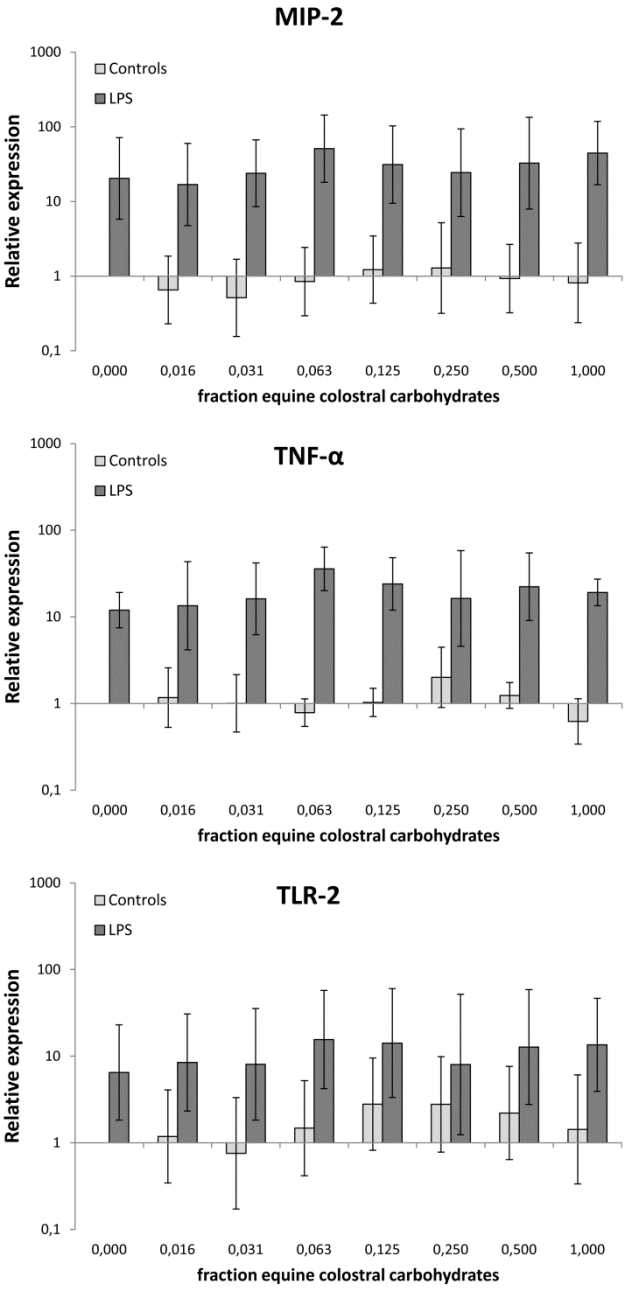

Figure 5: Mean values and standard deviations for relative mRNA expression levels of MIP-2, TNF-α, and TLR-2, in unchallenged and LPS-challenged mICcl2 cells incubated with equine colostral carbohydrates (eCC), including blank controls. Experiments were conducted in quadruplicate.

Effects of eCC on mRNA expression levels of MIP-2, TNF-α and TLR-2

In Figure 5, relative mRNA expression of MIP-2, TNF-α, and TLR-2 are illustrated. No effects were observed on a transcriptional level of the investigated genes when comparing mRNA expression levels in mICcl2 cells incubated with or without eCC, neither with nor without an LPS challenge. The LPS challenge did significantly induce mRNA expression of MIP-2, TNF-α, and TLR-2 compared with unchallenged cells, but pre- and co-incubation of the cells with eCC did not influence the LPS response on a transcriptional level at this time point.

Housekeeping gene expression

Variation of mRNA expression could be excluded as a confounding factor, as no significant differences in either β-actin or GAPDH expression were detected amongst blank controls and the incubations with eCC (with and without 1 μg/ml LPS).

Cell viability assays

Cell viability of mICcl2 cells, as estimated by means of the MTT and NR cell viability assay, was not influenced in comparison with blank controls after 4 hours of incubation with the entire investigated concentration range of eCC (with and without 1 μg/ml LPS).

Discussion

Effects of TLR activation in mICcl2 cells

Our data show that the TLR-4 ligand LPS is a potent inducer of inflammatory pathways in mICcl2 cells. In addition, TLR-2 (and TLR-1) activation by PCSK increased mRNA expression and protein production of the pro-inflammatory chemokine MIP-2, but this effect was more pronounced after an LPS challenge. Moreover, LPS enhanced mRNA expression of the pro-inflammatory cytokines TNF-α and IL-6 as well, whereas PCSK did not affect mRNA expression levels of these genes. The mRNA expression of the regulatory cytokines TGF-β1 and IL-10 were modulated to a minor extent in this model. This is possibly due to the fact that regulatory mechanisms are induced as a consequence of the primary inflammatory response, and that the induction of TGF-β1 and IL-10 is not modulated directly through activation of TLR-2 or TLR-4. Additional experiments with a more extensive setup, for instance co-cultures of IECs with challenged immune cells, are required to clarify such mechanisms.

Though TLR-4 was expressed by mICcl2 cells and was shown to be fully functional as well, the mRNA expression of TLR-4 was not influenced in this model, neither by its own ligand nor by the applied ligand for TLR-2. Interestingly, mRNA expression encoding for TLR-2 was markedly up-regulated by LPS, but not after incubation with its own ligand, confirming the phenomenon of TLR crosstalk in this

model. The increased expression of TLR-2 as a result of LPS treatment was documented earlier in murine dendritic cells (An et al., 2002). In these dendritic cells, LPS-induced up-regulation of mRNA encoding for TLR-2 was inhibited by suppression of the NFκB pathway or the MAPK pathway, confirming the influence of these pathways on TLR expression levels (An et al., 2002). This effect of a single LPS challenge on mRNA expression of other TLRs than TLR-4 is possibly a functional defence mechanism, through enhancement of overall responses to bacteria by promoting cytokine production. However, our data did not show an alteration of the LPS-induced measured immune response by concomitant incubation with LPS and PCSK at this time point. To explain this discrepancy, supplementary experiments would be required with longer incubation periods and sample collection at multiple time points. Furthermore, the influence of LPS on the functionality of the TLR-2 complex could be investigated by performing LPS challenge prior to TLR-2 activation and subsequently comparing the functional response with controls, which were not exposed to LPS.

Modulation of the LPS response in mICcl2 cells

Though previously published *in vivo* studies have reported beneficial effects of PCSK on gastrointestinal inflammatory processes in rodents (Cario et al., 2004; Cario et al., 2007), pre- and co-incubation with PCSK did not influence the LPS-induced response in this murine intestinal epithelial cell line. This could be due to indirect effects of PCSK on intestinal epithelial cells in the *in vivo* studies on targets, which were not present in our model, such as immune cells residing in the gastrointestinal epithelial barrier. Direct immunomodulatory effects of PCSK on IECs can be excluded in this model, as our data showed that, next to the previously reported functional signalling of TLR-4 (Hornef et al., 2002; Hornef et al., 2003), mICcl2 cells express mRNA encoding for TLR-2 and functionally respond to TLR-2 activation. Future experiments with co-cultures of IECs and immune cells, such as dendritic cells or peripheral blood mononuclear cells, could elucidate indirect immunomodulatory effects of PCSK on IECs *in vitro.*

The eCC fraction did exhibit immunomodulatory properties in our model. MIP-2 production in mICcl2 cells following LPS challenge was significantly reduced by eCC in a dose-dependent manner. Strangely, mRNA expression of MIP-2 and the other investigated genes at the same time point was not influenced by incubation with eCC. Unspecific binding of LPS by the eCC fraction could be largely excluded as a cause of the reduced LPS response, as the qPCR results did clearly show up-regulation of MIP-2, TNF-α, and TLR-2 mRNA expression levels as a result of LPS stimulation, with and without concomitant incubation with the eCC fraction. Possibly, additional experiments with collection of samples for qPCR and ELISA at more time points can explain this controversy, as the observed influence on protein production by the cells should be detectable on a transcriptional level at an earlier time point. Moreover, the extraction of

specific oligosaccharides from equine colostrum and subsequent research into immunomodulatory properties of specific equine oligosaccharides could lead to the discovery of new compounds with high potential for therapeutic or preventive purposes.

Conclusion

Our data give evidence for functional signalling of TLR-2 and TLR-4 in mICcl2 cells. Whereas LPS elicited extensive effects on both transcription and protein production in mICcl2 cells in a short period of time, PCSK exhibited comparable properties to a much lesser extent. Pre- and co-incubation with PCSK did not alter the LPS-induced inflammatory response in mICcl2 cells. However, the eCC fraction did suppress MIP-2 production following LPS challenge in the same model. Future investigations are required to investigate the effects of the eCC fraction and specific compounds within the eCC fraction (such as oligosaccharides) in more extensive experimental setups, to elucidate both underlying immunomodulatory mechanisms as well as the effects of eCC on inflammatory processes in other animal species.

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Chapter 5

Effects of TLR-2 and TLR-4 activation in equine peritoneal macrophages ex vivo

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PRELIMINARY DATA

Abstract

The importance of Toll-like receptor (TLR) signalling in health and disease is generally acknowledged in mammalian species. However, up to now, a limited amount of research has been published with regard to TLR expression and functionality in the horse. In search of an equine model to investigate TLR signalling in the horse and to obtain data concerning effects of TLR-2 and TLR-4 activation in the horse, we isolated equine peritoneal macrophages and incubated them with a concentration range of specific ligands for TLR-2 and TLR-4. A significant pro-inflammatory cytokine response was observed following incubation with ligands for both TLRs. The amplitude of the cytokine response in equine macrophages was higher after activation of TLR-4 compared with activation of TLR-2. We conclude that *ex vivo* equine peritoneal macrophages can be used as a model for specific research questions, particularly with regard to horse immunology. Future research could focus on methods or compounds, which affect the LPS-induced immune response in equine macrophages.

Introduction

Up to date, a limited amount of research has been published concerning Toll-like receptor (TLR) expression and signalling in the horse. Nevertheless, in other mammalian species, TLR functionality has been shown to play a crucial role in health and disease, accounting for both the functional defence against pathogens, as well as tolerance to commensal microorganisms (Testro and Visvanathan, 2009; Takeuchi and Akira, 2010). Hence, dysfunction of TLRs has been associated with the occurrence of various inflammatory disorders in mammals, including an increased incidence of bacterial infections and sepsis (Takeuchi et al., 2000; Bernheiden et al., 2001). In line with this, several *in vivo* studies in experimental animals have indicated that activation of certain TLRs, in particular TLR-2, can result in suppression of inflammatory pathology (Cario et al., 2004; Re and Strominger, 2004; Cario et al., 2007). In the horse, limited research has been performed in this area, mainly focusing on TLR-9 ligands. *Ex vivo* activation of TLR-9 in peripheral blood mononuclear cells (PBMCs) isolated from neonatal foals has been shown to enhance the production of cytokines, which are functional in defensive immune responses (Liu et al., 2009). However, antigen presenting cells derived from foals did not respond to TLR-9 activation with increased expression of cytokines (Flaminio et al., 2007). Likewise, *in vivo* administration of a TLR-9 ligand in foals did not result in a modulation of cytokine expression patterns (Liu et al., 2011). To our knowledge, effects of TLR-2 activation on basal or stimulus-induced immune responses in the horse have not yet been described. Though, modulation of TLR-mediated immune responses in the horse, particularly in foals, would be a possible option to lower susceptibility to infections.

The aim of this study was to develop a model to carry out further investigations into TLR functionality in the horse, and to study the effects of a specific ligand for both TLR-2 and TLR-4 on the expression of these TLRs and the following cytokine response in the horse. For this purpose, we isolated peritoneal macrophages from healthy Dutch warmblood horses. Subsequently, macrophages were cultured *ex vivo* and incubated with a concentration range of the TLR-4 ligand lipopolysaccharide (LPS), and the TLR-2 ligand Pam3-Cys-Ser-Lys4 (PCSK). The obtained data were aimed to be a prerequisite to perform future studies in the horse concerning TLR-mediated immunomodulation.

Materials and Methods

Animals and sample collection

The horses, which were sampled in this study, were involved in surgical training sessions for pre-graduate students and residents at the Faculty of Veterinary Medicine, Utrecht University. The horses were all adult, healthy, Dutch warmblood horses, which were selected for these surgical trainings due to consistent orthopaedic problems. All surgical procedures were conducted under general anesthesia, and the horses were euthanised at the end of the training session.

Before starting experimental procedures, the horses were generally anesthetised and situated in a lateral position. Then, after surgical preparation of the area, one incision was made in the dorsolateral flank, and one incision was made just lateral of the ventral midline. Subsequently, the peritoneum was perforated with a sterile, blunt needle and through each incision a sterile catheter was inserted into the peritoneal cavity. Six litres of sterile NaCl 0.9% were infused into the abdomen via the dorsolateral entrance, and fluids were collected into sterile glasswork through the ventral catheter. During collection and transport to the lab, fluids were kept on ice and softly rotated to prevent adherence of macrophages. All experimental procedures were approved by the committee of ethical considerations in animal experiments of Utrecht University (DEC Utrecht).

Isolation of macrophages

In the laboratory, lavage fluid was centrifuged for 30 minutes at 4°C and 150**g* in aliquots of 50 ml. Cell pellets were washed twice using sterile PBS (Lonza, Basel, Switzerland) containing 3% fetal bovine serum (Life technologies, Carlsbad, CA, USA) and centrifuged consecutively for 15 minutes at 4°C and 150**g*. Cells were then resuspended in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 2mM glutamine (Lonza, Basel, Switzerland), 100 IU/ml penicillin (Lonza, Basel, Switzerland), 100 µg/ml streptomycin (Lonza, Basel, Switzerland) and 10% horse serum (prepared in our own laboratory by aseptically collecting blood, allowing clotting, and centrifuging). Morphological features of the macrophages were investigated by light microscopy. Cells were counted using trypan blue, resuspended to a density of $2*10^6$ cells/ml medium, and seeded into 24 well plates at a density of $2*10^6$ cells/ml medium/well. After incubation overnight (37°C, 5% CO_2) to allow the cells to adhere, cells were washed twice with fresh, warm PBS.

Cell culture experiments

Experiments were started by adding supplemented RPMI containing 0, 0.1, 1, or 10 µg/ml of either LPS (*Escherichia coli* O111:B4; Sigma- Aldrich, St. Louis, MO, USA) or Pam3-Cys-Ser-Lys4 (PCSK; Invivogen, San Diego, CA, USA), a synthetic bacterial lipopeptide that specifically activates TLR-2. Plates were placed in the incubator again and samples for

qPCR and ELISA were taken after 4 hours. All incubations was investigated in duplicate or triplicate for each horse. For ELISA, supernatants were collected and stored at -80 °C. For qPCR analyses, the macrophages were lysed using RNA lysis buffer (Promega, Madison, WI, USA) and stored at -80 °C until RNA isolation was resumed.

TNF-α protein determination

To measure protein levels of tumour necrosis factor-α (TNF-α), ELISA was performed on the supernatants using the Duoset® ELISA Development System for equine TNF-α (R&D Systems, Minneapolis, MN, USA). Standard operating procedures of the manufacturer were followed, applying all required buffers and solutions in the form provided by the manufacturer (R&D Systems, Minneapolis, MN, USA). The lower limit of detection of the ELISA was 15.6 pg/ml.

RNA Isolation

RNA was isolated from the cells using SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Isolated fractions were dissolved in 50 µl ribonuclease free water and stored at -80 °C. Quality and quantity of RNA was determined spectrophotometrically (Nanodrop).

Real-time PCR analysis

cDNA was generated using iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA, USA) according to the manufacturer's protocol. For reverse transcriptase reaction, 500 ng RNA was applied per sample. Expression of mRNA was assessed by real-time PCR using a Biorad iQ5 Multicolor Real-time PCR detection system and iQ™ SYBR® Green Supermix (Biorad, Hercules, CA, USA). Specific primer pairs were designed and tested for efficiency and accuracy, after having checked their specificity using the NCBI-BLASTN search program. Primer pairs were synthesised commercially (Eurogentec, Maastricht, The Netherlands). For this study, mRNA expression of TLR-2, TLR-4, interleukin-6 (IL-6), IL-10, TNF-α, β-actin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was determined using the following primer pairs:

TLR-2:	F 5′-TGCTGCCATTCTCATTCTTC-3′
	R 5′-GGGCCACTCCAGGTAGGT-3′
TLR-4:	F 5′-CCCTTTCAACTCTGCCTTCACT-3′
	R 5′-GGGACACCACGACAATAACTTTC-3′
IL-6:	F 5′-TGGCTGAAGAACACAACAACT-3′
	R 5′-GAATGCCCATGAACTACAACA-3′
IL-10:	F 5′-GAGAACCACGGCCCAGACATCAAG-3′
	R 5′-GACAGCGCCGCAGCCTCACT-3′
TNF-α:	F 5′-TCCAGACGGTGCTTGTGC-3′
	R 5′-GGCCAGAGGGTTGATTGACT-3′
β-actin:	F 5′-CAAGGCCAACCGCGAGAAGATGAC-3′
	R 5′-GCCAGAGGCGTACAGGGACAGCA-3′
GAPDH:	F 5′-TGGCATGGCCTTCCGTGTCC-3′
	R 5′-GCCCTCCGATGCCTGCTTCAC-3′

Data analysis

In the figures of this chapter, data are displayed as mean values and standard deviations. The qPCR data were assumed to be normally distributed. Mean values and standard deviations were calculated for each incubation. Subsequently, the differences between the mean value of each incubation and the mean value of the blank controls were calculated, as well as the standard deviations of these differences. Ultimately, all differences and their error bars were translated to the scale of relative expression by means of the following transformation: 2^{-x}. The data obtained from all other assays were assumed to be log-normally distributed. Mean values and standard deviations were calculated from the log-transformed data. Error bars were calculated and transformed back to the unit scale.

Results

Functional TNF-α response

In Figure 1, the measured supernatant concentrations of TNF-α are illustrated after 4 hours of incubation with the chosen concentrations of LPS and PCSK, including blank controls. TNF-α concentrations in supernatants of the blank controls were not detectable, hence the concentrations in Figure 1 were set equal to the lower detection limit of the applied ELISA. Incubation with LPS resulted in a marked increase in TNF-α production, which appeared to be already maximal at the lowest investigated concentration (0.1 µg/ml). Incubation with PCSK caused an increase in TNF-α production to a lesser degree than LPS. For PCSK, a dose-dependent increase was observed in the investigated concentration range, and TNF-α production was maximal as from 1 µg/ml PCSK.

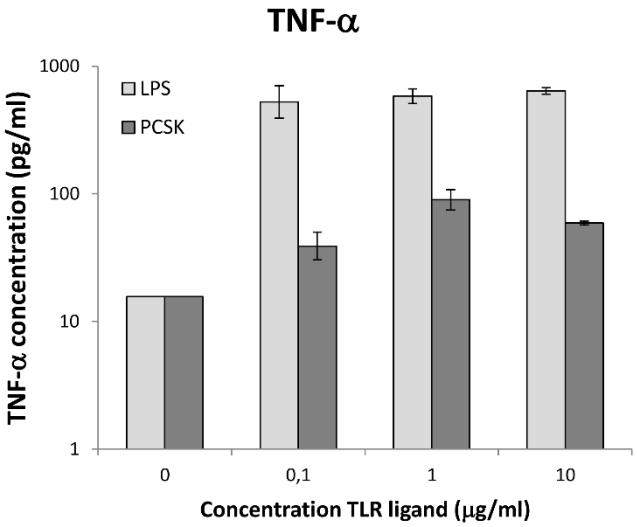

Figure 1: Mean supernatant concentrations of TNF-α (pg/ml) after 4 hours of incubation, including standard deviations. Experiments were conducted in triplicate.

Cytokine expression

Figure 2 illustrates the relative mRNA expression levels of TNF-α, IL-6, and IL-10 after 4 hours of incubation with LPS or PCSK. The relative expression was determined by comparing the qPCR signal (cycle threshold; Ct) with the qPCR signal in blank controls (2^ΔCt). Up-regulation of all measured cytokines was evident after incubation with LPS or PCSK, except for IL-10 production after incubation with 0.1 or 1 µg/ml PCSK. When comparing effects of LPS and PCSK, generally LPS had a more profound impact on

cytokine expression levels than PCSK, in particular regarding the pro-inflammatory cytokines TNF-α and IL-6. Whereas incubation with LPS already resulted in a maximal production of TNF-α protein at a concentration of 0.1 μg/ml, at a transcriptional level, a clear dose-dependent increase in expression was seen through the entire investigated concentration range, in particular for TNF-α and IL-6. For PCSK, cytokine expression levels were enhanced to a lesser degree and no increase was observed when comparing incubation with 1 μg/ml and 10 μg/ml PCSK.

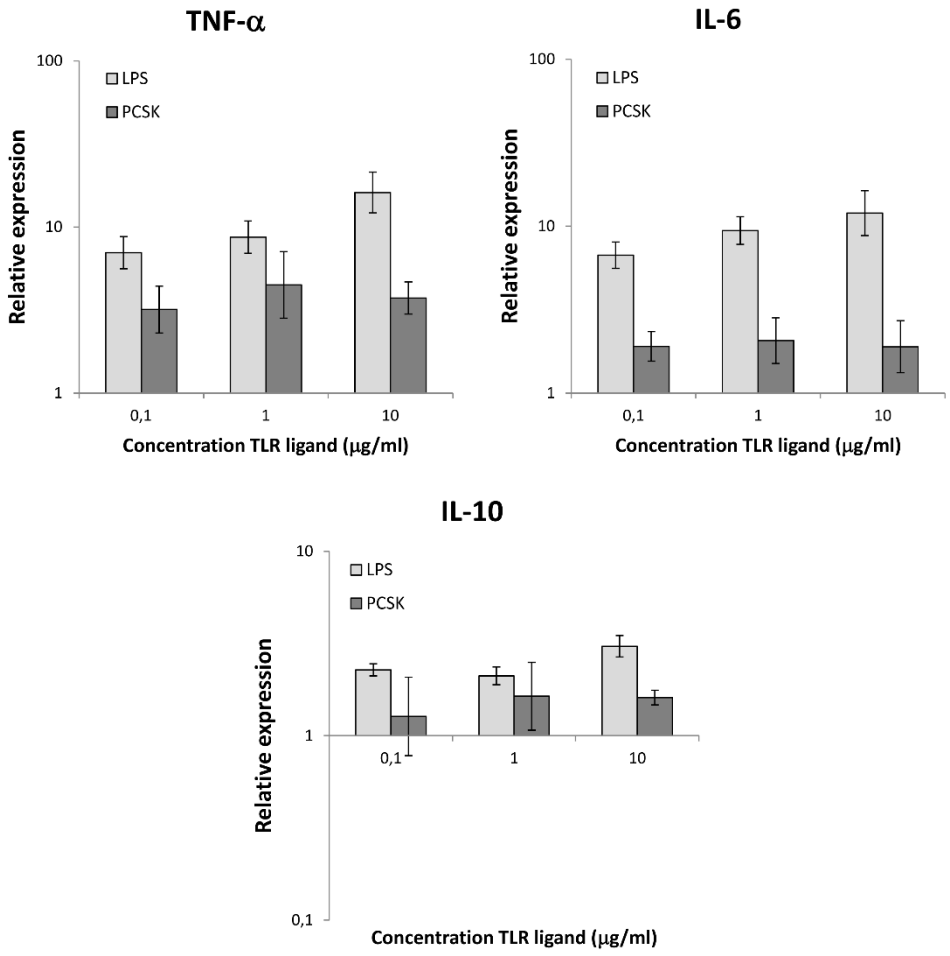

Figure 2: Mean relative expression levels of TNF-α, IL-6, and IL-10 after 4 hours of incubation, including standard deviations. Experiments were conducted in triplicate.

TLR expression

In Figure 3, the relative mRNA expression levels of TLR-2 and TLR-4 are illustrated after 4 hours of incubation with LPS or PCSK. Compared with blank controls, the expression levels of TLR-4 were not significantly influenced by incubation with LPS or PCSK. Generally, a mild up-regulation of TLR-2 was observed after incubation with both ligands (not significant for PCSK 1 µg/ml).

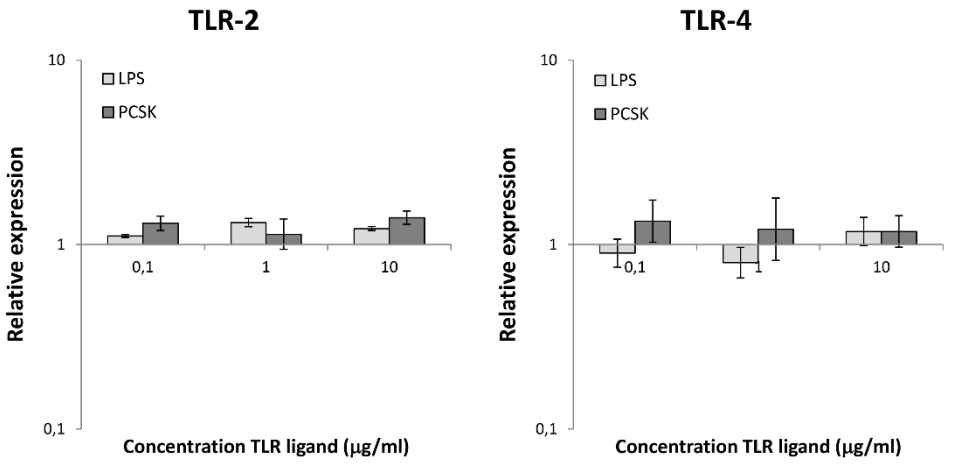

Figure 3: Mean relative expression levels of TLR-2 and TLR-4 after 4 hours of incubation, including standard deviations. Experiments were conducted in triplicate.

Discussion

Our results show that this model, in which *ex vivo* equine macrophages are cultured, is suitable as an equine model to assess both basal and stimulus-induced immune responses. The described results are in line with previously published studies, looking into the effects of TLR ligands in equine monocytes (Figueiredo et al., 2009; Kwon et al., 2010).

The limitations of this model are mainly the invasive character of the procedure, the relatively limited amount of isolated macrophages, and the restricted time frame, in which *ex vivo* cultured cells, in particular equine cells, remain sufficiently viable. Due to the invasive character of the procedure, we would not apply this method on horses, which are not anesthetised. Furthermore, despite sterile operating procedures, these horses would be at risk to develop a peritonitis. Thus, next to particular circumstances like the surgical training sessions, in which the horses we used in this study were involved, the

method is limitedly applicable. Though, probably the isolation of peritoneal macrophages according to this method will also be successful in horses, right after euthanasia. This could be interesting for further research with the use of equine peritoneal macrophages.

We isolated approximately 60-100 $*10^6$ macrophages from the abdominal lavage fluid of each horse. These amounts were sufficient to perform basic, small experiments like the executed experiments in this study. Though, for more extensive research, more macrophages would be required. Hence, this method would be especially appropriate for future experiments into for instance the effects of specific compounds in an equine model (proof of principle) or the specific functionality and physiology of equine (peritoneal) macrophages.

Cell viability of *ex vivo* cultured equine macrophages, according to the described method, was sufficient during the time span of our experiments. However, we observed that longer incubation periods resulted in a decrease in cell viability, in spite of several attempts optimise the subculturing conditions. For instance, after a 24 hours incubation, the morphology of the cells was visibly affected and the basal expression levels of all investigated genes (including housekeeping genes) were much lower compared to the 4 hours incubations. Therefore, we feel that incubation of these cells for longer than 4 hours does not result in reliable data. Due to the limited amounts of cells and the invasive method, cytotoxicity assays for LPS and PCSK were not performed in these cells. Though, in our laboratory, cytotoxicity assays for both ligands in concentrations up to 10 µg/ml have been previously executed in several other cell culture models, including murine macrophages, and no effects on cell viability were reported up to now.

Conclusion

Our described model, using *ex vivo* cultured equine peritoneal macrophages, is a suitable model for specific research questions, particularly with regard to horse immunology. A significant pro-inflammatory cytokine response was observed following incubation with either LPS or PCSK. As expected, the amplitude of the cytokine response in equine macrophages was higher after an LPS challenge than after a challenge with the TLR-2 ligand PCSK. Future research could focus on methods or compounds, which affect the LPS-induced immune response in equine macrophages.

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Chapter 6

Effects of separate and concomitant TLR-2 and TLR-4 activation in peripheral blood mononuclear cells of newborn and adult horses

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Abstract

Deficient innate and adaptive immune responses cause newborn mammals to be more susceptible to bacterial infections than adult individuals. Toll-like receptors (TLRs) are known to play a pivotal role in bacterial recognition and subsequent immune responses. Several studies have indicated that activation of certain TLRs, in particular TLR-2, can result in suppression of inflammatory pathology. In this study, we isolated peripheral blood mononuclear cells (PBMCs) from adult and newborn horses to investigate the influence of TLR-2 activation on the inflammatory response mediated by TLR-4. Data were analysed in a Bayesian hierarchical linear regression model, accounting for variation between horses. In general, cytokine responses were lower in PBMCs derived from foals compared with PBMCs from adult horses. Whereas in foal PBMCs expression of TLR-2, TLR-4, and TLR-9 was not influenced by separate and concomitant TLR-2 and TLR-4 activation, in adult horse PBMCs, both TLR ligands caused significant up-regulation of TLR-2 and down-regulation of TLR-9. Moreover, in adult horse PBMCs, interleukin-10 protein production and mRNA expression increased significantly following concomitant TLR-2 and TLR-4 activation (compared with sole TLR-4 activation). In foal PBMCs, this effect was not observed. In both adult and foal PBMCs, the lipopolysaccharide-induced pro-inflammatory response was not influenced by pre-incubation and co-stimulation with the specific TLR-2 ligand Pam3-Cys-Ser-Lys4. This indicates that the published data on other species cannot be translated directly to the horse, and stresses the necessity to confirm results obtained in other species in target animals. Future research should aim to identify other methods or substances that enhance TLR functionality and bacterial defence in foals, thereby lowering susceptibility to life-threatening infections during the first period of life.

Introduction

Due to deficits in both innate and adaptive immune responses, newborn mammals display increased susceptibility to bacterial infections compared with adult individuals (Levy. 2007). With regard to T helper cell (Th) responses, newborns predominantly display Th2 responses and are deficient in eliciting Th1 responses (Adkins et al., 2004; Levy. 2007). In *ex vivo* foal models, basal levels of Th1-related cytokines such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6) have been shown to be decreased compared with older individuals (Breathnach et al., 2006; Liu et al., 2009; Merant et al., 2009). In addition, monocyte-derived dendritic cells (MoDCs) of foals were classified as less mature, and were shown to express limited levels of IFN-γ following LPS stimulation compared with cells derived from adult horses (Merant et al., 2009). Expression levels of regulatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) were limited in foals as well (Merant et al., 2009). In contrast, basal and stimulus-induced levels of Th2-related cytokines such as IL-8, IL-12, and IL-23 were comparable in foals and older individuals (Liu et al., 2009; Merant et al., 2009). Furthermore, in response to the specific pathogen *Rhodococcus equi*, no Th1-deficits were found in neonatal foals (Jacks et al., 2007; Liu et al., 2009; Liu et al., 2011) Thus, the basal and stimulus-induced cytokine expression in neonatal foals appears to be selectively impaired. Similarly, in human infants, who are biased towards Th2 responses as well, mature Th1 responses have been documented in response to Group B Streptococci (Levy. 2007).

Bacterial recognition by Toll-like receptors (TLRs) and the subsequent induction of signalling cascades have been shown to play a pivotal role in the functional defence against pathogens, as well as tolerance to commensal microorganisms (Testro and Visvanathan, 2009; Takeuchi and Akira, 2010). The induction of cytokine production by TLRs is crucial for both the innate immune response itself, and the initiation of adaptive immune responses (Abdelsadik and Trad, 2011). Whereas TLR-4 responds to Gram-negative bacteria by sensing LPS, TLR-2 mainly recognises components of Gram-positive bacterial cell walls such as lipoproteins, lipoteichoic acid, and lipopeptides (Takeuchi and Akira, 2010). TLR-2 interacts with bacterial lipopeptides either independently by formation of TLR-2 homodimers, or in concert with TLR-1 or TLR-6 by formation of heterodimers (Buwitt-Beckmann et al., 2006). TLR-5 and TLR-9 are activated by bacterial flagellin and specific bacterial DNA motifs, respectively, derived from both Gram-positive and Gram-negative bacteria (Hemmi et al., 2000; Hayashi et al., 2001).

Dysfunction of TLRs has been associated with the occurrence of various inflammatory disorders in mammals, amongst others an increased susceptibility to bacterial infections and sepsis (Takeuchi et al., 2000; Bernheiden et al., 2001). Several *in vivo* studies in experimental animals have indicated that activation of certain TLRs, in particular TLR-2, can result in suppression of inflammatory pathology (Cario et al., 2004;

Re and Strominger, 2004; Cario et al., 2007). In the horse, limited research has been performed in this area, mainly focusing on TLR-9 ligands. *Ex vivo* activation of TLR-9 in peripheral blood mononuclear cells (PBMCs) isolated from neonatal foals has been shown to enhance the Th1 response (Liu et al., 2009). However, in another study, antigen presenting cells derived from foals did not respond to TLR-9 activation with increased expression of cytokines (Flaminio et al., 2007). Likewise, *in vivo* administration of a TLR-9 ligand in foals did not result in a modulation of cytokine expression patterns (Liu et al., 2011). Effects of TLR-2 activation on basal or stimulus-induced immune responses in the horse have not yet been described. Current research into immunomodulation focuses either on enhancement of TLR-dependent defensive immune responses, or suppression of excessive TLR activity, leading to inflammation and tissue damage. Both scenarios could apply to immunomodulation in horses, particularly in foals, and could contribute to lowering susceptibility to early, often life-threatening infections.

Hence, the aim of the current study was to explore the immunomodulatory properties of TLR-2 ligands in foals and adult horses. For this purpose, we used PBMCs from adult horses and newborn foals (<12 hours postpartum) to investigate the influence of TLR-2 activation on the LPS-induced inflammatory response. We investigated protein levels of TNF-α and IL-10, and mRNA expression levels of TNF-α and IL-6 (Th1-related cytokines), IL-10 (regulatory cytokine), and TLR-2, TLR-4, and TLR-9 (TLRs involved in bacterial recognition, for which the genetic sequence is known in the horse). Data were analysed with a Bayesian hierarchical regression model, accounting for variation between horses.

Materials and Methods

Animals and sample collection

Six healthy adult mares and foals (crossbreed Arabian*New Forest) were sampled during this study. Foals were sampled within 12 hours postpartum. 60 ml of blood was collected by jugular venipuncture directly into sterile heparinised blood collection tubes (BD Vacutainer Systems, Plymouth, United Kingdom). Blood samples were kept cooled during transport to the laboratory, where PBMC isolation started within 2 hours after collection. All experimental procedures were approved by the committee of ethical considerations in animal experiments of Utrecht University (DEC Utrecht, Permit Number: 2011.II.06.101).

PBMC isolation

Blood samples were diluted 1:1 in fresh PBS (Lonza, Basel, Switzerland) containing 2mM EDTA (Sigma- Aldrich, St. Louis, MO, USA) and subsequently layered over Ficoll-PaqueTM plus (GE Healthcare, Waukesha, WI, USA). After centrifugation (400*g*, 30 minutes at room temperature) PBMCs were pipetted from the Ficoll layer and washed twice in PBS/EDTA. PBMCs were resuspended in RPMI 1640 Medium (Lonza, Basel, Switzerland) containing 2mM glutamine (Lonza, Basel, Switzerland), 100 IU/ml penicillin (Lonza, Basel, Switzerland), 100 µg/ml streptomycin (Lonza, Basel, Switzerland) and 10% horse serum (prepared in our own laboratory by aseptically collecting blood, allowing clotting, and centrifuging). PBMCs were counted using trypan blue and resuspended to a density of $4*10^6$ cells/ml medium. Following storage overnight at 4 °C to attenuate possible stimulatory effects of the applied Ficoll, PBMCs were seeded in 24 well plates at a density of $4*10^6$ cells/ml medium/well.

Cell culture experiments

After seeding the PBMCs in 24 well plates, the plates were incubated for 2 hours at 37 °C and 5% CO_2. Thereafter, the plates were centrifuged for 10 minutes at 400*g* before refreshing the medium without removing PBMCs. Before starting the experiments, PBMCs were pre-incubated for 2 hours with subculturing medium containing 0 or 1 µg/ml Pam3-Cys-Ser-Lys4 (PCSK; Invivogen, San Diego, CA, USA), a synthetic bacterial lipopeptide that specifically activates TLR-2. After pre-incubation, the experiments were started by replacing the medium with medium containing 0 or 1 µg/ml LPS (*Escherichia coli* O111:B4; Sigma- Aldrich, St. Louis, MO, USA) and 0 or 1 µg/ml PCSK. Plates were placed in the incubator and samples for qPCR and ELISA were taken after 4 hours. Each combination of incubations was investigated in triplicate for each horse. For the ELISAs, supernatants were stored at -80 °C. For qPCR analyses, the PBMCs were lysed using RNA

lysis buffer (Promega, Madison, WI, USA) and stored at -80 ºC until RNA isolation was resumed.

Cytokine measurements
To measure protein levels of TNF-α and IL-10, ELISA was performed on the supernatants using Duoset® ELISA Development System for equine TNF-α and equine IL-10 (R&D Systems, Minneapolis, MN, USA). Standard operating procedures of the manufacturer were followed, applying all required buffers and solutions in the product sheet provided by the manufacturer (R&D Systems, Minneapolis, MN, USA). The detection limits of the ELISAs were 15.6 pg/ml (TNF-α) and 156.3 pg/ml (IL-10), respectively.

RNA Isolation
RNA was isolated from PBMCs using SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Isolated fractions were dissolved in 50 µl ribonuclease free water and stored at -80 °C. Quality and quantity of RNA was determined spectrophotometrically (Nanodrop).

Real-time PCR analysis
cDNA was generated using iScriptTM cDNA Synthesis Kit (Biorad, Hercules, CA, USA) according to the manufacturer's protocol. For reverse transcriptase reaction, 1000 ng RNA was applied per sample. Expression of mRNA was assessed by real-time PCR using a Biorad iQ5 Multicolor Real-time PCR detection system and iQTM SYBR® Green Supermix (Biorad, Hercules, CA, USA). Specific primer pairs were designed and tested for efficiency and accuracy, after having checked their specificity using the NCBI-BLASTN search program. Primer pairs were synthesised commercially (Eurogentec, Maastricht, The Netherlands). For this study, mRNA expression of TLR-2, TLR-4, TLR-9, IL-6, IL-10, TNF-α, β-actin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was determined using the following primer pairs:

TLR-2:	F 5'-TGCTGCCATTCTCATTCTTC-3'
	R 5'-GGGCCACTCCAGGTAGGT-3'
TLR-4:	F 5'-CCCTTTCAACTCTGCCTTCACT-3'
	R 5'-GGGACACCACGACAATAACTTTC-3'
TLR-9:	F 5'-GACTGGCTACCTGGCAAGAC-3'
	R 5'-GAAGCTGGCACGCAAGAG-3'
IL-6:	F 5'-TGGCTGAAGAACACAACAACT-3'
	R 5'-GAATGCCCATGAACTACAACA-3'
IL-10:	F 5'-GAGAACCACGGCCCAGACATCAAG-3'
	R 5'-GACAGCGCCGCAGCCTCACT-3'

TNF-α:	F 5′-TCCAGACGGTGCTTGTGC-3′
	R 5′-GGCCAGAGGGTTGATTGACT-3′
β-actin:	F 5′-CAAGGCCAACCGCGAGAAGATGAC-3′
	R 5′-GCCAGAGGCGTACAGGGACAGCA-3′
GAPDH:	F 5′-TGGCATGGCCTTCCGTGTCC-3′
	R 5′-GCCCTCCGATGCCTGCTTCAC-3′

Data analysis and statistical methods

Data from the second time point (4 hours incubation) were analysed in a Bayesian hierarchical linear regression model. Log-transformed ELISA results (log-concentrations) and qPCR results (cycle threshold value or Ct value) were assumed to be normally distributed. For all incubations (controls, LPS, PCSK, or LPS+PCSK) population means of the log-concentrations and Ct values were modelled in mares and foals, allowing responses to differ between mares and foals. Inter-individual variation was modelled by means of an error term at the level of horses (i.e. assuming that PBMCs from some horses are more 'reactive' in general than PBMCs from others) and incubation types within horses (i.e. assuming that horse PBMCs may react differently to specific incubations). Because TNF-α ELISA signals of all control samples were below the detection limit, we assumed signal values equal to the detection limit, leading to a conservative estimate of differences between controls and other samples. Because IL-10 ELISA signals were mostly above the detection limit, those few values that were below the detection limit (n=2) were left-censored; i.e. assuming that the 'true', unobserved IL-10 concentration is such that the 'observed' value is likely to be under the detection limit.

Analyses were performed in JAGS, a program for analysis of Bayesian models using Markov Chain Monte Carlo (MCMC) simulation (version 3.2.0; Plummer, 2012). Simulations in JAGS were set up and analysed in R (version 2.14.2; R Development Core Team, 2011), using packages rjags (version 3-5; Plummer, 2011) and R2jags (version 0.03-06; Su, 2011). Posterior distributions were estimated based on uninformative prior distributions (normal distributions with mean 0 and standard deviation 100 for parameter means; inverse gamma distributions with mean 1 and variance 10,000 for parameter variances). Bayesian credible intervals (BCI) for parameter estimates were based on the 2.5% and 97.5% percentiles of posterior distributions. Posterior distributions were simulated by means of four Markov chains, each consisting of 30,000 Monte Carlo samples. The first 10,000 samples were discarded for burn-in, allowing the model to converge. Model convergence was assessed by checking whether chains converged to the same posterior distribution, based on Gelman and Rubin's convergence diagnostic, the potential scale reduction factor (Gelman and Rubin, 1992).

Differences were stated significant, based on the calculated 95% BCI. In the results section, significant differences are quantified by factors or percentages, though

not all significant differences are quantified in the results section. An overview of all data and the corresponding 95% BCI's is given in an additional file, annexed to this chapter (Annex I).

qPCR data validation

Variation of mRNA expression was excluded as a confounding factor, as no significant differences in either β-actin or GAPDH expression were detected amongst control samples and the incubations with LPS, PCSK, and LPS+PCSK.

In addition, we analysed the data with a correction for these housekeeping genes, according to the method described by Livak and Schmittgen (Livak and Schmittgen, 2001). In our case, normalisation of data (based on housekeeping genes) did not change point estimates for differences between incubation types. Furthermore, normalisation did not reduce the variation in the data (as should be expected in case of confounding due to variation in total mRNA levels). This indicated that the error due to variation in mRNA expression, if any, was negligible compared to basic measurement error in the data. Based on this knowledge and the fact that housekeeping genes for equine PBMCs have not yet been validated, we decided to present the data in this manuscript as they are (i.e. not normalised).

Results

Cytokine production

ELISA results for equine TNF-α and IL-10 are illustrated in Figure 1. In comparison with controls, all tested conditions (LPS, PCSK, and LPS+PCSK) led to a significant increase of TNF-α and IL-10 production by PBMCs of both adult and newborn horses (not indicated in the figure). In both age groups, TNF-α production by PBMCs after LPS stimulation was not significantly influenced by pre-incubation and co-stimulation of the cells with PCSK. In contrast, IL-10 production by adult horse PBMCs challenged with LPS increased significantly (1.6-fold) when the cells were co-incubated with PCSK. In foals, this effect was not significant. Compared with adult horse PBMCs, foal PBMCs produced significantly lower amounts of TNF-α after LPS stimulation (87% lower) and after incubation with both LPS and PCSK (83% lower).

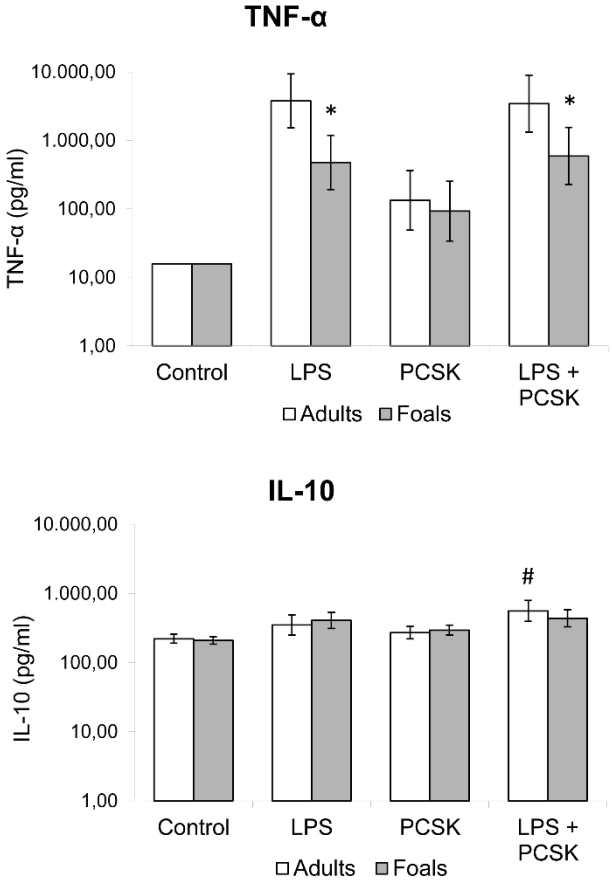
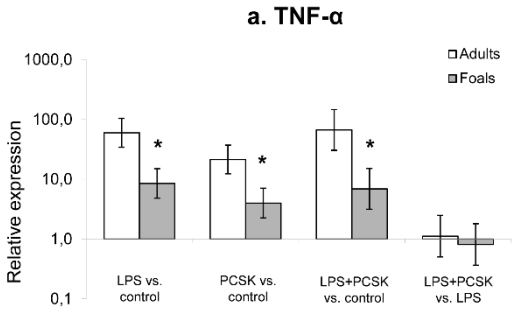
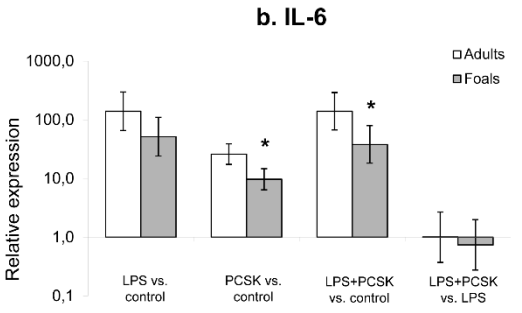
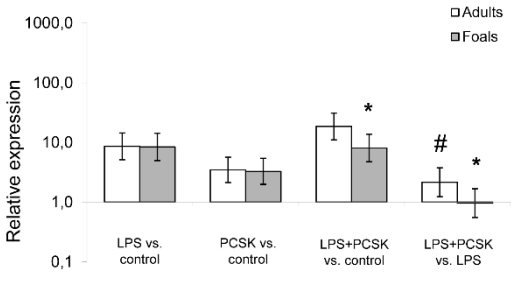


Figure 1: ELISA results for TNF-α and IL-10 in PBMCs of adult horses and neonatal foals. Mean concentrations of cytokines (pg/ml) and 95% Bayesian credible intervals are included for PBMCs incubated with blank medium (Control), with 1 µg/ml LPS, with 1 µg/ml Pam3-Cys-Ser-Lys4 (PCSK), or with a combination of both LPS and PCSK. Compared to control samples, there was a significant increase of both TNF-α and IL-10 in response to all tested compounds in both groups (not indicated in the figure). Significant differences between responses in adults and foals are indicated with an asterisk (*). A significant difference between the LPS response and the response to LPS+PCSK (within the same age group) is indicated with a hash (#).







d. Basal expression (controls)

	Adults			Foals		
	point estimate	*lower bound*	*upper bound*	***point estimate***	*lower bound*	*upper bound*
TNF-α	**26,21**	25,11	27,32	**25,03**	23,91	26,13
IL-6	**26,18**	24,99	27,37	**27,06**	25,87	28,25
IL-10	**26,72**	25,92	27,54	**26,46**	25,64	27,28

Figure 2: qPCR results for TNF-α, IL-6, and IL-10 in PBMCs of adult horses and neonatal foals. In Figure 2a, 2b, and 2c, mean values and 95% Bayesian credible intervals are given for relative expression levels (compared to controls and LPS stimulated cells) in PBMCs incubated with blank medium (Control), 1 µg/ml LPS, with 1 µg/ml Pam3-Cys-Ser-Lys4 (PCSK), or with a combination of both LPS and PCSK. Compared to control samples, there was a significant increase of both TNF-α, IL-6, and IL-10 in response to all tested conditions in both age groups (not indicated in the figure). In Figure 2d, estimated means for Ct values and 95% Bayesian credible intervals are stated for basal cytokine expression levels in both groups. Significant differences between responses in adults and foals are indicated with an asterisk (*). A significant difference between the LPS response and the response to LPS+PCSK (within the same age group) is indicated with a hash (#).

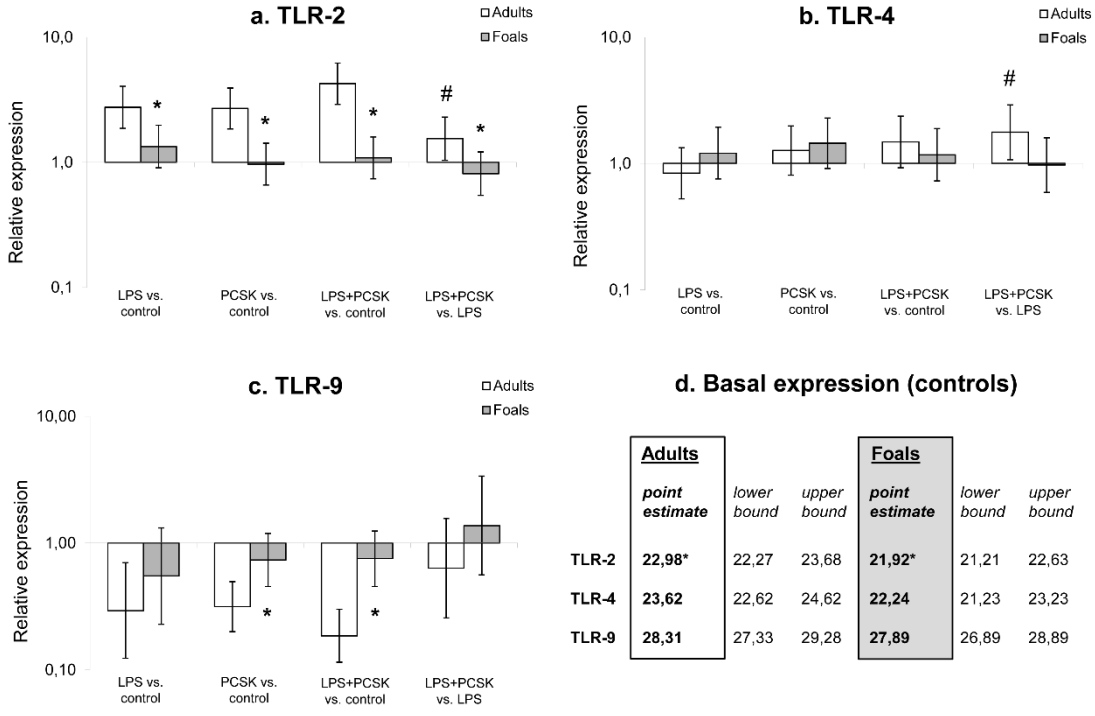
Cytokine expression

In Figure 2, the qPCR results are summarised for equine TNF-α, IL-6, and IL-10. Basal expression levels of these cytokines did not differ significantly between the two age groups (figure 2d). In both age groups, expression of all measured cytokines was relatively higher for incubations with LPS, PCSK, or both, compared with control samples, similar to cytokine protein levels (not indicated in the figure). Furthermore, in both age groups, TNF-α and IL-6 expression in response to LPS was not significantly influenced by co-stimulation with PCSK. However, in adult horse PBMCs, incubation with LPS and PCSK resulted in a significant increase in IL-10 expression (2.2-fold), whilst in PBMCs of neonates this effect was not significant. Relative expression of TNF-α in foal PBMCs was significantly lower compared to adult horse PBMCs in all incubations (86% lower in foal PBMCs incubated with LPS, 81% lower for PCSK, and 90% lower for LPS+PCSK). In addition, relative expression of IL-6 was significantly lower in foal PBMCs incubated with PCSK (63% lower) and LPS+PCSK (73% lower). For IL-10, relative expression was only significantly different between adults and foals in PBMCs incubated with LPS and PCSK (57% lower in foals).

TLR expression

Figure 3 summarises qPCR results for TLR-2, TLR-4, and TLR-9. In control samples, TLR-2 expression was significantly higher in foals (2.1-fold) in comparison with adults (average Ct values were 22.98 for adult horse PBMCs, and 21.92 for foal PBMCs). Basal expression of TLR-4 and TLR-9 did not differ significantly among the two age groups (Figure 3d). The difference in basal expression of TLR-4 among the two age groups was very close to being significant. Mean Ct values for TLR-4, calculated from this model for adult and foal PBMCs, were 23.62 and 22.24 respectively (average ΔCt=1.39, 95% BCI: -0.02–2.80), indicating a factor 2.6 difference in expression level.

In foal PBMCs, no significant change in the expression of TLR-2, TLR-4, or TLR-9 was found in response to LPS, PCSK, or LPS+PCSK in comparison with control samples. In contrast, in PBMCs of adults there was a significant up-regulation of TLR-2 expression and a significant down-regulation of TLR-9 in response to all tested TLR ligands, whereas TLR-4 expression did not change significantly in comparison to controls. Furthermore, TLR-2 expression was relatively lower in foal PBMCs compared to adult PBMCs under all tested conditions (51% lower in foal PBMCs incubated with LPS, 64% for PCSK, and 75% for LPS+PCSK). TLR-9 expression was relatively higher in foal PBMCs incubated with PCSK (2.3-fold) and LPS+PCSK (4.1-fold) compared to adult PBMCs. In adult PBMCs, a significant increase of TLR-2 and TLR-4 expression was found after incubation with LPS+PCSK compared to LPS alone (1.6-fold increase for TLR-2, 1.8-fold for TLR-4). In contrast, in LPS-challenged foal PBMCs, pre-incubation and co-stimulation with PCSK did



	Adults			Foals		
	point estimate	*lower bound*	*upper bound*	*point estimate*	*lower bound*	*upper bound*
TLR-2	**22,98***	22,27	23,68	**21,92***	21,21	22,63
TLR-4	**23,62**	22,62	24,62	**22,24**	21,23	23,23
TLR-9	**28,31**	27,33	29,28	**27,89**	26,89	28,89

Figure 3: qPCR results for TLR-2, TLR-4, and TLR-9 in adult and neonatal PBMCs. In Figure 3a, 3b, and 3c, mean values and 95% Bayesian credible intervals are given for relative expression levels (compared to controls and LPS stimulated cells) in PBMCs incubated with blank medium (Control), 1 µg/ml LPS, with 1 µg/ml Pam3-Cys-Ser-Lys4 (PCSK), or with a combination of both LPS and PCSK. In adult horse PBMCs, significant up-regulation of TLR-2 expression and significant down-regulation of TLR-9 was found in response to LPS, PCSK, and LPS+PCSK (not indicated in the figure). In Figure 3d, estimated means for Ct values and 95% Bayesian credible intervals are stated for basal TLR expression levels in both groups. Significant differences between responses in adults and foals are indicated with an asterisk (*). A significant difference between the LPS response and the response to LPS+PCSK (within the same age group) is indicated with a hash (#).

not lead to changes in TLR-2 or TLR-4 expression levels. TLR-9 expression in response to LPS was not significantly influenced by co-stimulation with PCSK in either group.

Discussion

Cytokine production and expression

Our data show that the TNF-α responses in foal PBMCs following LPS stimulation do not reach the level of adult horse PBMCs. This finding is in line with previously published results, demonstrating that in newborn foals, the cytokine response to external challenges like LPS, including Th-1 related and regulatory cytokines, is lower than that of adult individuals (Adkins et al., 2004; Levy. 2007; Liu et al., 2009). However, Mérant et al (2009) documented comparable TNF-α expression levels after LPS challenge in foal and adult MoDCs. Most likely, this apparent discrepancy is caused by the difference in age of the tested foals. Mérant et al (2009) sampled 2-3 weeks old animals, whereas the study population in the experiment described here comprised true neonates, sampled within 12 hours postpartum. The limited expression and production of IL-10 we documented in LPS-challenged foal PBMCs compared to adult horse PBMCs was consistent with the limited IL-10 mRNA expression levels in LPS-challenged MoDCs derived from foals (Merant et al., 2009). Apparently, in the foal, the TNF-α response matures earlier in life compared with the IL-10 response.

In our experimental model, TNF-α production after LPS challenge was not significantly influenced by pre-incubation and co-stimulation with PCSK in either age group. On the other hand, TLR-2 stimulation in addition to TLR-4 activation resulted in a significant increase of IL-10 production in adult horse PBMCs (1.6-fold). In foals, this effect was not observed. This increase of IL-10 production (and not TNF-α) as a result of concomitant TLR-2 stimulation fits the hypothesis that TLR-2 ligands exhibit anti-inflammatory properties, as the secretion of IL-10 activates regulatory T cells, which subsequently will produce IL-10 and TGF-β. As a result of these cytokines, Th1, Th2, and Th17 responses are suppressed (Borchers et al., 2009; Nyirenda et al., 2009).

Regarding the differences in cytokine production between PBMCs incubated with TLR-2 or TLR-4 ligands, our results are in accordance with the existing literature on mammalian species (Dowling et al., 2008; Figueiredo et al., 2009). We found that TLR-2 activation resulted in a relatively mild inflammatory response, whereas TLR-4 activation triggered a marked inflammatory response, characterised by a high peak of TNF-α production after 4 hours of incubation with LPS. This pattern was evident in both adult and foal PBMCs.

TLR expression

TLR expression levels in foals were stable under all different experimental conditions. In contrast, in adult PBMCs, significant up-regulation of TLR-2 and down-regulation of TLR-9 were evident after both separate and concomitant TLR-2 and TLR-4 activation. Expression of TLR-4 did not change significantly. Besides clear differences in the regulation of TLR expression levels between PBMCs derived from newborn and adult horses, these qPCR results also demonstrate TLR crosstalk in this experimental model, as the expression of TLR-2 and TLR-9 was significantly influenced by both TLR-2 and TLR-4 activation. Remarkable was that though TLR expression rates in foal PBMCs were not influenced by the used TLR ligands (in contrast to adult horse PBMCs), basal TLR expression levels in PBMCs tended to be higher in foals than in adults.

Our observation of coinciding lower cytokine responses and higher TLR expression levels in PBMCs derived from neonatal foals suggests that TLR signalling is impaired in newborn foals compared with adult horses. Moreover, our data illustrate that in foals, TLR expression levels were not influenced by a challenge with bacterial patterns, representing either Gram-negative or Gram-positive bacteria, in contrast to the situation in adults. Such a limited TLR function is essential in neonates, as the transition from the sterile womb into an environment full of micro-organisms would lead to exaggerated inflammatory responses and subsequent pathology if all TLR signalling pathways were fully operational. Tolerance to commensal flora is a prerequisite for bacterial colonisation of epithelial surfaces including the gut. The relatively high TLR expression levels, which were found in foals, might facilitate more profound defensive responses towards specific pathogens, if necessary. For instance, *Rhodococcus equi* is a specific pathogen that elicits mature defensive responses in young foals (Jacks et al., 2007; Liu et al., 2011). The challenge for future research in this area is to find methods or ligands that enhance TLR functionality and bacterial defence mechanisms in young foals without interfering with the physiological process of bacterial tolerance and colonisation.

Ligation of TLR-2 by different compounds

PCSK is a confirmed TLR-2 agonist, which is often applied in the published literature throughout the past decades. Nonetheless, other compounds have been described as TLR-2 ligands as well. PCSK binds to TLR-2/TLR-1 heterodimers and does not interact with TLR-6, unlike other TLR-2 agonists (Ozinsky et al., 2000). Long et al (2009) compared effects of four different TLR-2 agonists, including PCSK. Though all tested TLR-2 ligands were shown to activate NFκB and MAPK signalling pathways[1] in murine macrophages, the degree of activation differed among the tested compounds. Moreover, significant differences were documented in cytokine responses as well as the *in vivo* recruitment of leukocytes in a murine model of acute inflammation. Thus, although we

[1] NFκB: nuclear factor kappaB, MAPK: mitogen-activated protein kinase

can conclude that TLR-2 was ligated in our model, we cannot exclude that other TLR-2 agonists would possibly modulate the inflammatory responses in this equine model differently.

Conclusion

The obtained data demonstrate a distinct regulation of cytokine and TLR expression levels in PBMCs of foals and adult horses in response to TLR-2 and TLR-4 activation. While TLR expression levels were high in foals, the lower functional response following TLR activation in neonatal foals can be considered as a strategy to limit the immune responses to the bacterial colonisation of epithelial surfaces during the first period of life. In turn, the relatively high TLR expression levels in foals might be responsible for the previously described marked immune responses to specific pathogens, such as *Rhodococcus equi*. PCSK, which has been shown to alter inflammatory responses beneficially in previously studied experimental models by activating TLR-2, did not exhibit comparable properties in our model. Hence, PCSK does not seem to be a promising candidate for immunomodulation in foals. This indicates that translation of findings from one animal species to another is limitedly possible and requires confirmation. It remains a challenge for future research to identify substances or methods, which enhance TLR functionality and bacterial defence in young foals without causing an exaggerated inflammatory response or interfering with bacterial colonisation.

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Annex I: Model parameter estimates and 95% Bayesian Credible Intervals

Significant differences are marked grey

a. TNF-α; protein levels

	Adults (pg/ml)			**Foals (pg/ml)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**15.63**	15.61	15.64	**15.63**	15.61	15.64	**1.00**	1.00	1.00
LPS	**3763.11**	1517.77	9339.43	**473.90**	190.38	1178.50	**0.13**	0.04	0.46
PCSK	**132.95**	48.81	361.04	**92.39**	33.78	252.40	**0.69**	0.17	2.86
LPS+PCSK	**3439.22**	1320.81	8955.29	**589.34**	225.43	1540.71	**0.17**	0.04	0.67

b. TNF-α; relative protein levels

	Adults (fold change)			**Foals (fold change)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**240.81**	97.13	597.65	**30.33**	12.18	75.41	**0.13**	0.04	0.46
PCSK vs. Control	**8.51**	3.12	23.10	**5.91**	2.16	16.15	**0.69**	0.17	2.85
LPS+PCSK vs. Control	**220.08**	84.52	573.07	**37.71**	14.43	98.59	**0.17**	0.04	0.67
LPS+PCSK vs. LPS	**0.91**	0.64	1.32	**1.24**	0.86	1.79	**1.36**	0.81	2.28

c. IL-10; protein levels

	Adults (pg/ml)			**Foals (pg/ml)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**220.30**	190.00	255.95	**208.51**	184.38	235.57	**0.95**	0.78	1.15
LPS	**348.28**	250.39	485.90	**407.08**	310.13	533.79	**1.17**	0.76	1.79
PCSK	**270.16**	220.08	331.62	**293.83**	248.39	347.23	**1.09**	0.84	1.42
LPS+PCSK	**558.36**	395.05	789.97	**436.59**	329.64	578.82	**0.78**	0.50	1.23

D. IL-10; RELATIVE PROTEIN LEVELS

	Adults (fold change)			**Foals (fold change)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**1.58**	1.15	2.16	**1.95**	1.51	2.52	**1.23**	0.83	1.85
PCSK vs. Control	**1.23**	1.03	1.46	**1.41**	1.22	1.62	**1.15**	0.92	1.44
LPS+PCSK vs. Control	**2.53**	1.83	3.53	**2.09**	1.60	2.73	**0.83**	0.54	1.26
LPS+PCSK vs. LPS	**1.60**	1.03	2.50	**1.07**	0.75	1.54	**0.67**	0.38	1.18

E. TNF-α; MRNA EXPRESSION LEVELS

	Adults (number of cycles)			**Foals (number of cycles)**			**Foals vs. adults (ΔCt)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**26.21**	25.11	27.32	**25.03**	23.91	26.13	**-1.19**	-2.75	0.37
LPS	**20.31**	19.17	21.42	**21.94**	20.81	23.07	**1.64**	0.05	3.23
PCSK	**21.79**	20.66	22.92	**23.03**	21.89	24.17	**1.24**	-0.35	2.83
LPS+PCSK	**20.14**	18.78	21.53	**22.24**	20.86	23.60	**2.09**	0.15	4.01

F. TNF-α; RELATIVE MRNA EXPRESSION LEVELS

	Adults (fold change)			**Foals (fold change)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**60.05**	34.34	104.98	**8.50**	4.82	15.01	**0.14**	0.06	0.31
PCSK vs. Control	**21.42**	12.30	37.22	**3.99**	2.25	7.07	**0.19**	0.08	0.41
LPS+PCSK vs. Control	**67.14**	30.68	146.73	**6.92**	3.16	15.23	**0.10**	0.03	0.31
LPS+PCSK vs. LPS	**1.12**	0.50	2.49	**0.81**	0.37	1.81	**0.73**	0.23	2.26

G. IL-10; mRNA expression levels

	Adults (number of cycles)			Foals (number of cycles)			Foals vs. adults (ΔCt)		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**26.72**	25.92	27.54	**26.46**	25.64	27.28	**-0.27**	-1.42	0.89
LPS	**23.62**	22.76	24.48	**23.39**	22.52	24.25	**-0.23**	-1.45	0.98
PCSK	**24.94**	24.11	25.76	**24.75**	23.91	25.58	**-0.19**	-1.36	0.99
LPS+PCSK	**22.51**	21.64	23.38	**23.45**	22.58	24.32	**0.93**	-0.29	2.16

H. IL-10; relative mRNA expression levels

	Adults (fold change)			Foals (fold change)			Foals vs. adults (fold change)		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**8.60**	5.11	14.34	**8.38**	4.97	14.20	**0.97**	0.47	2.04
PCSK vs. Control	**3.46**	2.12	5.63	**3.26**	1.98	5.40	**0.94**	0.47	1.90
LPS+PCSK vs. Control	**18.51**	11.02	31.02	**8.06**	4.75	13.65	**0.43**	0.21	0.91
LPS+PCSK vs. LPS	**2.15**	1.23	3.74	**0.96**	0.55	1.67	**0.45**	0.20	0.97

I. IL-6; mRNA expression levels

	Adults (number of cycles)			Foals (number of cycles)			Foals vs. adults (ΔCt)		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**26.18**	24.99	27.37	**27.06**	25.87	28.25	**0.89**	-0.80	2.57
LPS	**19.06**	17.54	20.59	**21.38**	19.86	22.90	**2.33**	0.18	4.47
PCSK	**21.48**	20.25	22.70	**23.79**	22.57	25.00	**2.31**	0.58	4.04
LPS+PCSK	**19.05**	17.56	20.55	**21.82**	20.32	23.30	**2.77**	0.64	4.88

J. IL-6; RELATIVE MRNA EXPRESSION LEVELS

	Adults (fold change)			**Foals (fold change)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**139.10**	65.39	296.11	**51.30**	24.13	109.67	**0.37**	0.13	1.08
PCSK vs. Control	**26.01**	17.40	38.96	**9.68**	6.41	14.67	**0.37**	0.21	0.66
LPS+PCSK vs. Control	**139.78**	67.37	290.42	**37.98**	18.23	79.45	**0.27**	0.10	0.77
LPS+PCSK vs. LPS	**1.00**	0.37	2.69	**0.74**	0.28	1.99	**0.74**	0.18	2.97

K. TLR-2; MRNA EXPRESSION LEVELS

	Adults (number of cycles)			**Foals (number of cycles)**			**Foals vs. adults (ΔCt)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**22.98**	22.27	23.68	**21.92**	21.21	22.63	**-1.05**	-2.06	-0.06
LPS	**21.52**	20.79	22.23	**21.50**	20.78	22.23	**-0.01**	-1.04	1.01
PCSK	**21.55**	20.83	22.26	**21.97**	21.26	22.69	**0.43**	-0.59	1.43
LPS+PCSK	**20.89**	20.17	21.60	**21.80**	21.09	22.52	**0.92**	-0.09	1.93

L. TLR-2; RELATIVE MRNA EXPRESSION LEVELS

	Adults (fold change)			**Foals (fold change)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**2.75**	1.87	4.05	**1.34**	0.90	1.98	**0.49**	0.28	0.84
PCSK vs. Control	**2.69**	1.85	3.92	**0.96**	0.66	1.42	**0.36**	0.21	0.62
LPS+PCSK vs. Control	**4.26**	2.90	6.21	**1.08**	0.74	1.59	**0.25**	0.15	0.44
LPS+PCSK vs. LPS	**1.55**	1.04	2.30	**0.81**	0.54	1.21	**0.52**	0.30	0.92

m. TLR-4; mRNA expression levels

	Adults (number of cycles)			Foals (number of cycles)			Foals vs. adults (ΔCt)		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**23.62**	22.62	24.62	**22.24**	21.23	23.23	**-1.39**	-2.80	0.02
LPS	**23.88**	22.85	24.90	**21.96**	20.94	22.98	**-1.91**	-3.36	-0.46
PCSK	**23.28**	22.27	24.29	**21.70**	20.69	22.71	**-1.58**	-2.99	-0.15
LPS+PCSK	**23.05**	22.03	24.09	**22.01**	20.98	23.04	**-1.05**	-2.50	0.39

n. TLR-4; relative mRNA expression levels

	Adults (fold change)			Foals (fold change)			Foals vs. adults (fold change)		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**0.84**	0.52	1.33	**1.21**	0.75	1.94	**1.44**	0.74	2.79
PCSK vs. Control	**1.27**	0.81	1.99	**1.45**	0.91	2.29	**1.14**	0.60	2.16
LPS+PCSK vs. Control	**1.48**	0.92	2.37	**1.17**	0.73	1.89	**0.79**	0.41	1.55
LPS+PCSK vs. LPS	**1.77**	1.07	2.91	**0.97**	0.59	1.60	**0.55**	0.27	1.11

o. TLR-9; mRNA expression levels

	Adults (number of cycles)			Foals (number of cycles)			Foals vs. adults (ΔCt)		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**28.31**	27.33	29.28	**27.89**	26.89	28.89	**-0.42**	-1.81	0.99
LPS	**30.08**	28.62	31.54	**28.75**	27.29	30.21	**-1.34**	-3.40	0.73
PCSK	**29.98**	28.95	30.99	**28.33**	27.30	29.36	**-1.64**	-3.09	-0.19
LPS+PCSK	**30.74**	29.70	31.78	**28.29**	27.25	29.34	**-2.45**	-3.93	-0.96

P. TLR-9; RELATIVE MRNA EXPRESSION LEVELS

	Adults (fold change)			**Foals (fold change)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**0.29**	0.12	0.70	**0.55**	0.23	1.32	**1.88**	0.54	6.41
PCSK vs. Control	**0.31**	0.20	0.50	**0.73**	0.45	1.19	**2.33**	1.20	4.53
LPS+PCSK vs. Control	**0.19**	0.11	0.30	**0.75**	0.45	1.25	**4.08**	2.02	8.19
LPS+PCSK vs. LPS	**0.63**	0.26	1.56	**1.37**	0.56	3.37	**2.16**	0.60	7.76

Q. GAPDH (HOUSEKEEPING GENE); MRNA EXPRESSION LEVELS

	Adults (number of cycles)			**Foals (number of cycles)**			**Foals vs. adults (ΔCt)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**21.27**	20.47	22.08	**20.60**	19.79	21.42	**-0.67**	-1.81	0.48
LPS	**21.96**	21.06	22.84	**21.21**	20.31	22.11	**-0.75**	-2.01	0.52
PCSK	**21.51**	20.68	22.33	**20.90**	20.06	21.72	**-0.62**	-1.78	0.55
LPS+PCSK	**21.50**	20.68	22.32	**21.05**	20.23	21.87	**-0.45**	-1.61	0.70

R. GAPDH (HOUSEKEEPING GENE); RELATIVE MRNA EXPRESSION LEVELS

	Adults (fold change)			**Foals (fold change)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**0.62**	0.37	1.05	**0.66**	0.39	1.12	**1.06**	0.50	2.23
PCSK vs. Control	**0.85**	0.53	1.34	**0.82**	0.51	1.31	**0.96**	0.50	1.87
LPS+PCSK vs. Control	**0.86**	0.54	1.36	**0.73**	0.46	1.18	**0.86**	0.45	1.66
LPS+PCSK vs. LPS	**1.38**	0.80	2.36	**1.12**	0.65	1.92	**0.81**	0.38	1.74

s. β-actin (housekeeping gene); mRNA expression levels

	Adults (number of cycles)			**Foals (number of cycles)**			**Foals vs. adults (ΔCt)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
Control	**18.55**	17.51	19.58	**17.99**	16.93	19.05	**-0.57**	-2.05	0.91
LPS	**18.81**	17.70	19.92	**19.06**	17.95	20.16	**0.25**	-1.31	1.80
PCSK	**17.91**	16.85	18.96	**18.55**	17.48	19.62	**0.64**	-0.86	2.15
LPS+PCSK	**17.88**	16.82	18.95	**19.10**	18.03	20.16	**1.22**	-0.29	2.72

t. β-actin (housekeeping gene); relative mRNA expression levels

	Adults (fold change)			**Foals (fold change)**			**Foals vs. adults (fold change)**		
Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*	*Point Estimate*	*Lower Bound*	*Upper Bound*
LPS vs. Control	**0.84**	0.44	1.59	**0.47**	0.25	0.91	**0.57**	0.23	1.42
PCSK vs. Control	**1.56**	0.85	2.86	**0.68**	0.36	1.26	**0.43**	0.18	1.03
LPS+PCSK vs. Control	**1.59**	0.87	2.93	**0.67**	0.36	1.25	**0.42**	0.18	1.01
LPS+PCSK vs. LPS	**1.91**	0.98	3.70	**0.97**	0.50	1.90	**0.51**	0.20	1.30

Chapter 7

Equine colostral carbohydrates reduce lipopolysaccharide-induced inflammatory responses in equine peripheral blood mononuclear cells

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Abstract

Increasing evidence suggests that reactions to lipopolysaccharide (LPS), particularly in the gut, can be partly or completely mitigated by colostrum- and milk-derived oligosaccharides. Confirmation of this hypothesis would open the way to new therapeutic concepts. This study investigates the influence of equine colostral carbohydrates on inflammatory responses in an *in vitro* model with equine peripheral blood mononuclear cells (PBMCs). For this purpose, we extracted carbohydrates from mare colostrum. In PBMCs isolated from the same mares, the influence of equine colostral carbohydrates on the LPS-induced inflammatory response was determined. mRNA expression of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-10 was measured as well as the protein levels of TNF-α and IL-10. The results demonstrated that, following an LPS challenge of PBMCs, colostral carbohydrates modulated the IL-10 response and reduced the determined levels of the pro-inflammatory cytokines TNF-α and IL-6 dose-dependently. Moreover, cell viability significantly increased in the presence of high concentrations of colostral carbohydrates. We conclude that carbohydrates derived from equine colostrum reduced the LPS-induced inflammatory response in equine PBMCs. Hence, colostrum and milk-derived carbohydrates are promising candidates for new concepts in preventive and regenerative medicine.

Introduction

For decades already, colostral compounds are thought to have protective effects on neonates. In foals, the importance of colostral intake is well recognised by equine breeders and practitioners. Insufficient intake of colostrum by neonatal foals increases the risk of developing septic illnesses (Robinson et al., 1993). Recent investigations demonstrate that next to the essential passive transfer of maternal immunoglobulins via colostrum during the first day of the foal's life (LeBlanc et al., 1992), mammalian colostrum contributes significantly to the maturation of the developing gut-associated immune system and probably to the total immune competence in the first period of life. For example, it could be demonstrated that human breast milk contains soluble Toll-like receptor 2 (TLR-2) and CD-14 (essential for TLR-4 signalling) which could inhibit signalling through TLR-2 and TLR-4 in the gastrointestinal tract of neonates by occupying binding sites for these signal molecules (Jones et al., 2002; LeBouder et al., 2003; LeBouder et al., 2006). This may be important during the early bacterial colonisation of the intestines and the development of tolerance to the commensal bacterial flora. Furthermore, the presence of anti-inflammatory cytokines in human milk are thought to enhance the tight junction related epithelial barrier function (Al-Sadi et al., 2009; Garofalo. 2010). Recently, the colostral transfer of tumour necrosis factor-α (TNF-α) was documented in newborn foals, eliciting a possible immunomodulatory role of this pro-inflammatory cytokine in the early life phase (Secor et al., 2012). Comparable experiments in calves had already reported a stimulation of the immune system by pro-inflammatory cytokines present in bovine colostrum (Yamanaka et al., 2003). Another colostral constituent with well documented immunomodulatory and antimicrobial capacity is (bovine) lactoferrin (Haversen et al., 2002; Berlutti et al., 2006; Tomita et al., 2009).

In consideration of the immunomodulatory effects of colostrum in several models, recently more mechanistic studies were published, which illustrated that the application of bovine colostrum decreased NF□B activation and subsequent production of pro-inflammatory cytokines in intestinal epithelial cells (An et al., 2009; Jorgensen et al., 2010). In a model with human peripheral blood mononuclear cells (PBMCs), the immune response to several stimuli was modulated by bovine colostrum (Biswas et al., 2007; Jenny et al., 2010). Distinct human milk-derived oligosaccharides were shown to exhibit immunomodulatory as well as prebiotic properties (Eiwegger et al., 2010). Moreover, human milk glycans have been proven to protect neonates against bacterial invasion and are thought to play an important role in the optimal bacterial colonisation of the neonatal gastrointestinal tract (Newburg. 2009).

Limited research has been conducted with equine colostrum, despite the known differences in chemical composition and pattern of individual oligosaccharides between different animal species and humans. Chemical analysis of equine colostrum reveals both

similarities and differences in oligosaccharide patterns of humans and horses (Urashima et al., 1989; Urashima et al., 1991; Nakamura et al., 2001). In this study, we investigated the effects of carbohydrates derived from equine colostrum on the lipopolysaccharide (LPS)-induced inflammatory response in equine PBMCs.

Materials and Methods

Animals and sample collection

Six healthy adult mares (NRPS and New Forest breed) were sampled during this study. Within 1 hour postpartum, 50 ml of colostrum was collected and immediately stored at -20 ºC. Within 12 hours postpartum, 150 ml of blood was collected by jugular venipuncture directly into a sterile blood collection bag containing citrate phosphate dextrose adenine as an anticoagulant (Macopharma, Mouvaux, France). Blood samples were kept cooled during transport to the laboratory, where PBMC isolation started within 2 hours after collection. Colostrum was processed simultaneously with PBMC isolation, and colostral extracts of each horse were tested on PBMCs of the same horse (homologous testing). All experimental procedures were approved by the committee of ethical considerations in animal experiments of Utrecht University (DEC Utrecht).

PBMC isolation

Blood samples were diluted 1:1 in fresh phosphate buffered saline (PBS; Lonza, Basel, Switzerland) containing 2mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) and subsequently layered over Ficoll- PaqueTM plus (GE Healthcare, Waukesha, WI, USA). After centrifugation (400*g, 30 minutes at room temperature) PBMCs were pipetted from the Ficoll layer and washed twice in PBS/EDTA. PBMCs were resuspended in RPMI 1640 Medium (Lonza, Basel, Switzerland) containing 2mM glutamine (Lonza, Basel, Switzerland), 100 IU/ml penicillin (Lonza, Basel, Switzerland), 100 µg/ml streptomycin (Lonza, Basel, Switzerland) and 10% horse serum (prepared in our own laboratory according to standard procedures). PBMCs were counted using trypan blue and resuspended to a density of $4*10^6$ cells/ml medium. Following storage overnight at 4 ºC to attenuate possible stimulatory effects of the applied Ficoll, PBMCs were seeded in 24 well plates at a density of $4*10^6$ cells/ml medium/well.

Colostrum extraction

The carbohydrate fraction of the colostrum samples (equine colostral carbohydrates, eCC) was extracted as described by Fukuda et al (Fukuda et al., 2010). A colostrum sample of 50 ml was thawed and mixed with 210 ml chloroform:methanol 2:1 v/v. This emulsion was centrifuged in glass tubes for 30 minutes at 4 ºC and 4000*g and the lower chloroform layer and the denatured protein were discarded. Methanol was removed from

the upper layer using a vacuum centrifuge. The residue was dissolved in 35 ml fortified RPMI 1640 medium (as described above) and filtered through a 0.2 µm filter. This solution was called 1.00 eCC (stock solution) and dilutions of 0.50 eCC and 0.25 eCC prepared in RPMI 1640 medium were used in the following experiments.

Cell culture experiments

After seeding the PBMCs in 24 well plates, the plates were incubated for 2 hours at 37 °C and 5% CO_2. After this, the plates were centrifuged for 10 minutes at 400**g* to refresh the medium without removing PBMCs. Before starting the experiments, PBMCs were pre-incubated for 2 hours with 0, 0.25, 0.50 or 1.00 eCC medium solutions as described above. After pre-incubation, the experiments were started (t-0) by replacing the medium with medium containing 0 or 1 µg/ml LPS (*Escherichia coli* O111:B4; Sigma-Aldrich, St. Louis, MO, USA) and 0, 0.25, 0.50 or 1.00 eCC. The used concentration of LPS was chosen after preliminary experiments in comparable models. Plates were placed in the incubator and samples for qPCR and ELISA were taken at 2 and 4 hours (t-2 and t-4). All different combinations and time points were investigated in triplicate for each horse. For the ELISA's, supernatants were collected and stored at -80 °C. For qPCR, the PBMCs were lysed using RNA lysis buffer (Promega, Madison, WI, USA) and stored at -80 °C until RNA isolation was resumed.

Cell viability assessment

To investigate possible influence of the colostral extract on cell viability, 2 assays were performed using Alamar Blue (Sigma-Aldrich, St. Louis, MO, USA) and CCK-8 (Sigma-Aldrich, St. Louis, MO, USA). Cell viability assays were performed according to manufacturer's instructions. Before viability assessment, PBMCs were incubated with 0, 0.0625, 0.125, 0.25, 0.50, 0.75 and 1.00 eCC for 6 hours in total (similar to the maximal exposure to eCC in the experiments; 2 hours of pre-incubation and 4 hours of experiment).

ELISA

To measure protein levels of TNF-α and interleukin-10 (IL-10), ELISA was performed on the supernatants using Duoset® ELISA Development System for equine TNF-α and equine IL-10 (R&D Systems, Minneapolis, MN, USA). Standard operating procedures of the manufacturer were followed, applying all required buffers and solutions in the form provided by the manufacturer (R&D Systems, Minneapolis, MN, USA). The lower limit of detection of the ELISAs were 15.6 pg/ml (TNF-α) and 156.3 pg/ml (IL-10), respectively.

RNA Isolation

RNA was isolated from PBMCs using SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Isolated fractions were dissolved in 50 µl ribonuclease free water and stored at -80 °C. Quality and quantity of RNA was determined spectophotometrically (Nanodrop).

Real-time PCR analysis

cDNA was generated using iScriptTM cDNA Synthesis Kit (Biorad, Hercules, CA, USA) according to the manufacturer's protocol. For reverse transcriptase reaction, either 1000 or 500 ng RNA was applied per sample. Expression of mRNA was assessed by real-time PCR using a Biorad iQ5 Multicolor Real-time PCR detection system and iQTM SYBR® Green Supermix (Biorad, Hercules, CA, USA). Specific primer pairs were designed and tested for efficiency and accuracy (before testing specificity was investigated using the NCBI-BLASTN search program). Primer pairs were synthesised commercially (Eurogentec Nederland B.V., Maastricht, The Netherlands). For this study, expression of IL-6, IL-10, TNF-α and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA was determined using the following primer pairs:

IL-6;	F 5'-TGGCTGAAGAACACAACAACT-3'
	R 5'-GAATGCCCATGAACTACAACA-3'
IL-10;	F 5'-GAGAACCACGGCCCAGACATCAAG-3'
	R 5'- GACAGCGCCGCAGCCTCACT-3'
TNF-α;	F 5'-TCCAGACGGTGCTTGTGC-3'
	R 5'- GGCCAGAGGGTTGATTGACT-3'
GAPDH (HK gene);	F 5'-TGGCATGGCCTTCCGTGTCC-3'
	R 5'-GCCCTCCGATGCCTGCTTCAC-3'.

Data analysis and statistical methods

Data were analysed by means of linear regression. Independent variables were LPS (1 µg/ml), several concentrations of colostrum extract, and time (t-2 and t-4), allowing for interaction between all three. Control samples from t-0 were left out as they were not informative for the model (i.e. there were no other t-0 measurements). For the qPCR measurements, the dependent variable was time until qPCR gave a positive signal, expressed as the number of PCR cycles (decimals allowed). Parameter estimates (other than the intercept) represented the difference in number of cycles between the reference and another experimental setup (for example LPS vs. control). Consequently, two to the power of any parameter estimate represented the relative expression of a gene in a sample compared to the reference, being the blank measurements of the same time point (blanks at t-4 were compared to blanks at t-2). For the ELISA measurements, the

dependent variable was the log-transformed concentration. Both dependent variables were assumed to be normally distributed.

Parameters were estimated in a generalised linear mixed model. Clustering of data was modelled through random intercepts at the level of horses/plates (48 samples for each of the six horses, with samples from each horse on one ELISA or qPCR plate). ELISA measurements below the detection limit were assumed to be equal to the detection limit (TNF-α: 15.6 pg/ml; IL-10: 156.3 pg/ml). Models were fitted by means of residual maximum likelihood (REML) approach in R^+ (R Foundation for Statistical Computing, Vienna, Austria, version 2.14.0), using the function 'lmer' from the package 'lme4' (http://lme4.r-forge.r-project.org/). REML-ratio tests indicated that addition of aforementioned random intercept significantly improved the model (specifically, there was significant inter-horse variation). *P*-values and confidence intervals (highest posterior density intervals) were estimated by means of Markov chain Monte Carlo sampling from the posterior distribution of parameter values (10,000 iterations).

Results

Cytokine production by PBMCs is reduced by eCC

In Figure 1, the results of the ELISA's are illustrated (TNF-α and IL-10). LPS stimulation of PBMCs led to a remarkable and highly significant increase of TNF-α production at both t-2 and t-4, with a factor 40.09 and 216.41 respectively (p-value<0.01, compared to blank controls). In contrast, IL-10 production after LPS challenge was not influenced at t2 and only mildly (factor 1.53) -but significantly- increased at t-4 (p-value<0.01). The addition of eCC to the medium did not influence basal TNF-α production by PBMCs. However, at both t-2 and t-4 the 1.00 solution of eCC did significantly reduce the TNF-α response after LPS challenge with 88% at t-2 and 92% at t-4 (p-value<0.01). IL-10 concentrations were significantly decreased (p-value<0.01) at all tested concentrations at both time points in both unchallenged and LPS-treated cells (compared to blank controls in unchallenged cells and to the response to LPS alone in LPS treated cells).

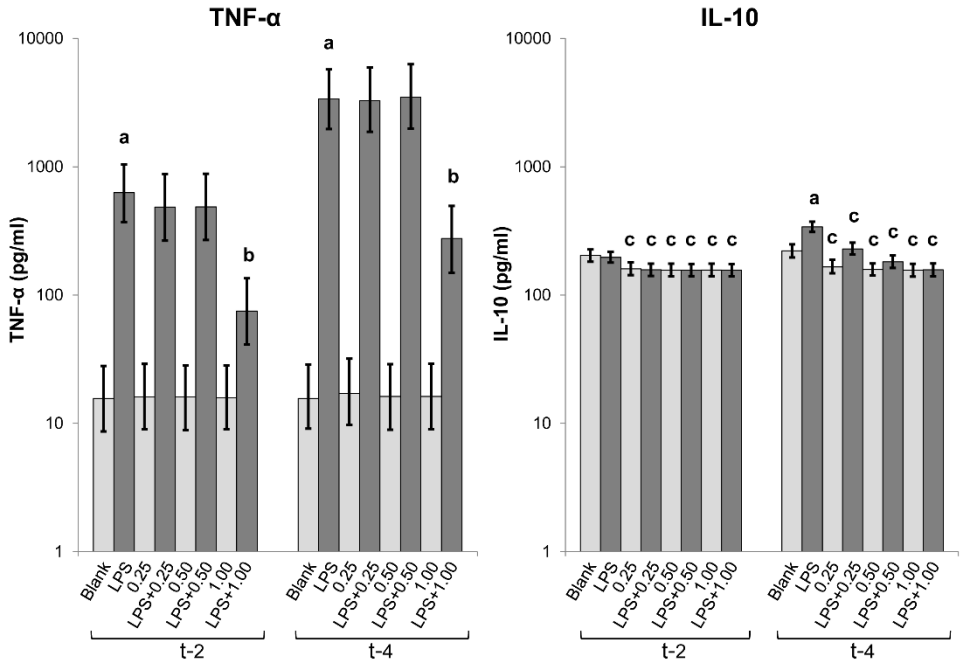

Figure 1: ELISA results for equine TNF-α and equine IL-10. Mean concentrations of cytokines (pg/ml) and 95% confidence intervals are given for all investigated incubations, being different fractions of equine colostral carbohydrates (eCC) with and without concomitant LPS stimulation. a. Significant LPS response compared to blank at same time point (*p*-value<0.01), b. Significantly decreased LPS response in cells incubated with 1.00 eCC (*p*-value<0.01). c. Significantly decreased IL-10 levels in presence of all tested eCC fractions (*p*-value<0.01).

Induction of cytokine mRNA expression is reduced by eCC

In Figure 2, the results of the qPCR measurements are illustrated (TNF-α, IL-6 and IL-10). For all tested cytokines, mRNA expression was increased significantly after LPS challenge at both time points (*p*-value<0.01). Moreover, in all cases this LPS response was significantly reduced by 1.00 eCC at both timepoints (*p*-value<0.01). In non-LPS-challenged cells, only IL-10 mRNA expression was decreased by eCC, dependent on the concentration of eCC (significant at t-4 for 0.25, 0.50 and 1.00 eCC, *p*-value<0.01).

mRNA expression of the housekeeping gene GAPDH was found not to be significantly influenced in these experiments under all investigated circumstances, confirming its suitability as a reference gene.

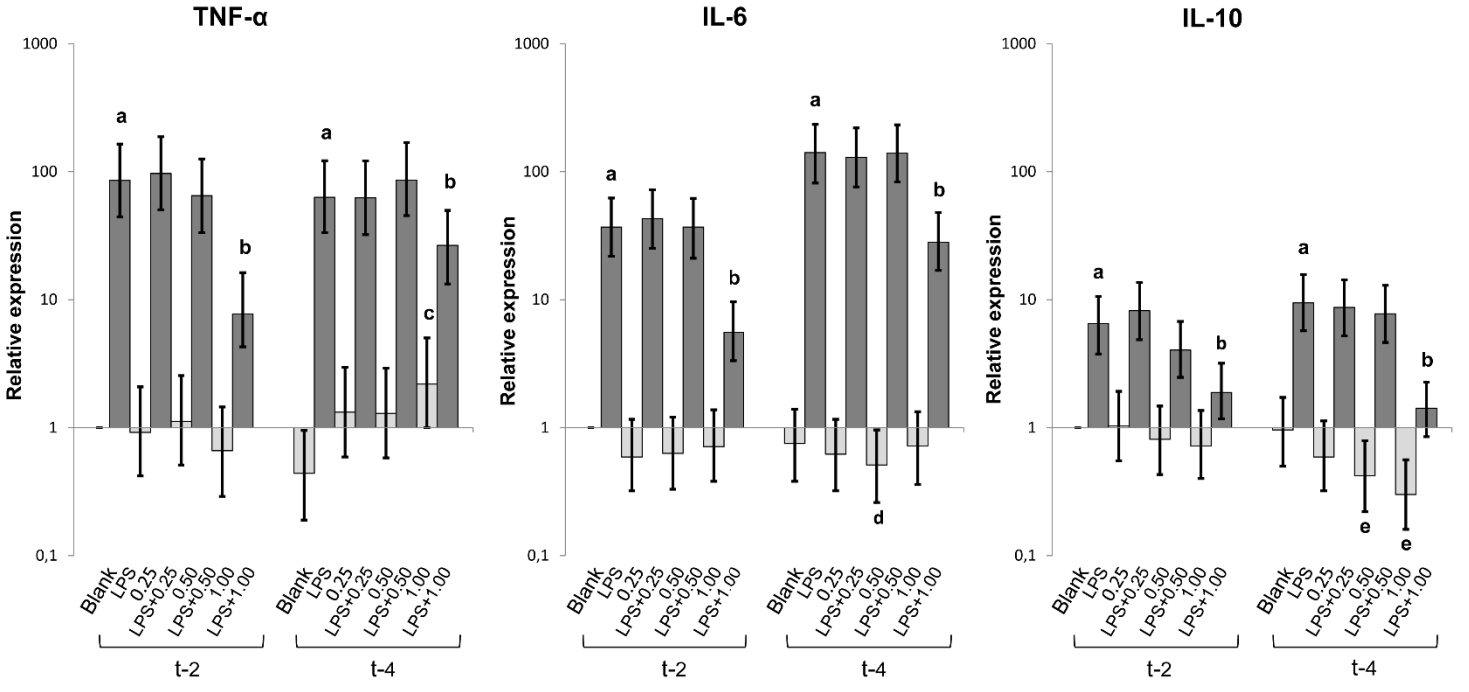

Figure 2: qPCR results for equine TNF-α, equine IL-6 and equine IL-10. Relative expression given and 95% confidence intervals are given for all investigated incubations, being different fractions of equine colostral carbohydrates (eCC) with and without concomitant LPS stimulation. a. Significant LPS response compared to blank at same time point (p-value<0.01), b. Significantly decreased LPS response in cells incubated with 1.00 eCC (p-value<0.01). c. Significantly increased TNF-alpha expression after incubation with 1.00 eCC compared to blank at same time point (p-value<0.05). d. Significantly decreased IL-6 expression after incubation with 0.50 eCC compared to blank at same time point (p-value<0.05). e. Significantly decreased IL-10 expression after incubation with both 0.50 eCC (p-value<0.05) and 1.00 eCC (p-value<0.01) compared to blank at same time point.

Cell viability is improved by eCC

In Figure 3, the results of both cell viability assays are illustrated (Alamar Blue reduction assay and CCK-8 assay). A concentration-dependent rise in cell viability was found, with significantly higher cell viability (*p*-value<0.05) for 0.50 eCC (CCK-8), 0.75 eCC (CCK-8) and 1.00 eCC (CCK-8 and Alamar Blue) than in control samples.

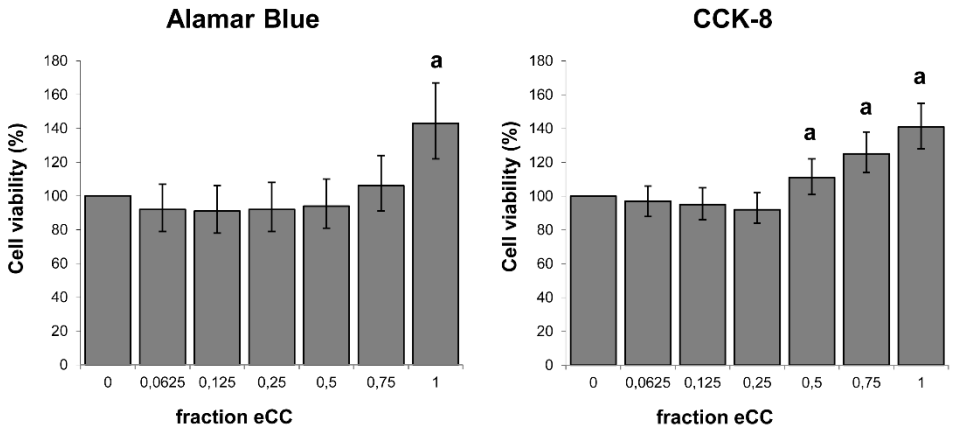

Figure 3: Results for the cell viability assays. In both assays viability is given in percentages, compared with the viability of PBMCs incubated without eCC. a. Significantly higher cell viability (*p*-value<0.05) in cells incubated with 0.50 eCC (CCK-8), 0.75 eCC (CCK-8) and 1.00 eCC (CCK-8 and Alamar Blue) in comparison with blank

Discussion

This study was based on the hypothesis that the inflammatory response induced in equine PBMCs by challenging these cells *in vitro* with LPS, could be suppressed by biologically active carbohydrates in colostrum. In the LPS-challenged cells, mRNA expression of pro-inflammatory cytokines (TNF-α and IL-6) as measured by qPCR and production of TNF-α as measured by ELISA were significantly increased as expected. In line with the hypothesis, eCC suppressed the pro-inflammatory response to LPS, as confirmed by the reduced TNF-α and IL-6 mRNA expression and TNF-α protein levels. The effect was dose-dependent. Non-specific binding of TNF-α protein by the colostral extract could be largely excluded due to the differences in the suppression of TNF-α concentrations at different time points (88% reduction at t-2 and 92% at t-4) and the fact that ELISA and qPCR results were comparable. Moreover, fractions of 0.25 and 0.50 eCC did not reduce TNF-α protein levels significantly. In case of non-specific binding, lower eCC fractions would probably have had a suppressive effect as well (given the great impact of eCC at a fraction of 1.00). In contrast to TNF-α, all tested eCC concentrations caused a reduction of IL-10 protein levels in comparison to cells incubated without eCC. This finding could indicate that IL-10 binds to eCC or that eCC components interfere with antibody binding in the ELISA. However, the significant and dose-dependent reduction of IL-10 mRNA expression by eCC matches the ELISA results and make non-specific binding of IL-10 by eCC components less plausible.

Alternatively, it needs to be considered that in this model the reduction of pro-inflammatory signals is not modulated through IL-10. If that was the case, IL-10 production should increase in the presence of eCC to regulate the inflammatory response to LPS. In contrast, we observed a suppression of IL-10 production by eCC, concomitant to the suppression of pro-inflammatory cytokines. A possible explanation for this observation is the short time-span of the experiments. The functional response by IL-10 protein as a reaction to pro-inflammatory signals is possibly limited in this short-term model. This assumption is supported by the fact that the LPS challenge induced a clear increase in IL-10 mRNA expression, whereas at these time points only limited protein levels were measured (also in PBMCs not incubated with eCC). This could be caused by posttranscriptional regulation of IL-10 mRNA, but it cannot be excluded that in comparable experiments with longer incubations IL-10 protein levels would indeed be up-regulated.

Because in the presence of eCC all investigated cytokine levels decreased simultaneously in LPS-challenged PBMCs, there probably is a direct influence of eCC in this model. This could be a direct influence of eCC on Toll-like receptors (TLRs), as similarities in both structure and immunomodulatory capacity of colostral oligosaccharides and known TLR ligands are evident (Cario et al., 2007; Newburg. 2009;

Eiwegger et al., 2010). TLRs are activated by (among others) bacterial constituents (such as LPS, which activates TLR-4) and subsequently induce an intracellular signalling cascade leading to the production of various cytokines (Kawai and Akira, 2010). In this study, next to suppression of cytokine protein and expression levels by eCC (certainly in LPS-stimulated cells), transcription of TNF-α was slightly up-regulated in presence of 1.00 eCC (t-4). This could indicate that similar to known immunomodulatory agents such as TLR agonists, eCC alone trigger intracellular signalling pathways and, in case of parallel activation of the immune system, modulate the cytokine response in favour of the host.

To exclude possible cytotoxic effects of the colostral extract (and possible traces of chloroform and/or methanol), we performed two different cell viability assays. Instead of solely excluding cytotoxic effects, we reported a significant increase of PBMC viability in the high concentrations of eCC in both assays. Interestingly, this increase in cell viability was demonstrated especially in the presence of those concentrations of colostral carbohydrates coinciding with the suppression of the inflammatory response to LPS.

In this model, we have primarily demonstrated that mixtures of colostrum-derived oligosaccharides exert a dose-dependent immunomodulatory effect. Fingerprint-analysis (data not shown) showed clear differences between equine and bovine colostral fractions, but at present, the exact composition and quantity of galacto-oligosaccharides in these samples await further characterisation. Our study warrants further research of milk- and colostrum-derived oligosaccharides, with the aims of identifying the most active compounds and providing more insight in the molecular processes involved in its anti-inflammatory properties.

Conclusion

The presented results indicate that equine colostral carbohydrates are capable of altering the inflammatory response to LPS in equine PBMCs challenged *in vitro*. Moreover, cell viability of PBMCs was increased under the same experimental conditions. Despite the fact that more research is needed to elucidate the immunomodulatory mechanisms of specific constituents of the tested carbohydrate fractions, these compounds are promising candidates for the development of new strategies for prevention and intervention of intestinal inflammatory pathology.

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Chapter 8

In vitro evaluation of defined oligosaccharide fractions in an equine model of inflammation

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Abstract

Dietary supplementation with oligosaccharides has been proven to be beneficial for health in several mammalian species. Next to prebiotic effects resulting in a modulation of gut microbiota, immunomodulatory effects of oligosaccharides have been documented *in vivo*. Supplementation with defined oligosaccharide fractions has been shown to attenuate allergic responses and enhance defensive immune responses. Despite the accumulating evidence for immunomodulatory effects, very limited information is available regarding the direct mechanism of action of oligosaccharides. This study aims to elucidate the effects of selected oligosaccharide fractions on the lipopolysaccharide (LPS)-induced inflammatory response in equine peripheral blood mononuclear cells (PBMCs). We investigated three different products containing either galacto-oligosaccharides (GOS) alone, a combination of GOS with fructo-oligosaccharides (FOS), and a triple combination of GOS and FOS with acidic oligosaccharides (AOS), at different concentrations. These products have been used in an identical composition in various previously published *in vivo* experiments. As the selected oligosaccharide fractions were derived from natural products, the fractions contained defined amounts of mono- and disaccharides and minor amounts of endotoxin, which was taken into account in the design of the study and the analysis of data. Acquired data were analysed in a Bayesian hierarchical linear regression model, accounting for variation between horses. Our results indicated that exposing cultured PBMCs to either GOS or GOS/FOS fractions resulted in a substantial dose-dependent increase of tumour necrosis factor-α (TNF-α) production in LPS-challenged PBMCs. In contrast, incubation with GOS/FOS/AOS resulted in a dose-dependent reduction of both TNF-α and interleukin-10 production following LPS challenge. In addition, incubation with GOS/FOS/AOS significantly increased the apparent PBMC viability, indicating a protective or mitogenic effect. Furthermore, mono- and disaccharide control fractions significantly stimulated the inflammatory response in LPS-challenged PBMCs as well, though to a lesser extent than GOS and GOS/FOS fractions. In conclusion, we found distinct immunomodulating effects of the investigated standardised oligosaccharide fractions, which either stimulated or suppressed the LPS-induced inflammatory response in PBMCs. Both scenarios require additional investigation, to elucidate underlying modulatory mechanisms, and to translate this knowledge into the clinical application of oligosaccharide supplements in foals and other neonates.

Introduction

During the past decade, numerous *in vivo* studies in humans (and experimental animals) have reported beneficial effects of dietary supplementation with oligosaccharides derived from natural products such as milk, fruits and vegetables. The original goal of supplementing infant formulas with oligosaccharide fractions was to mimic prebiotic effects of human milk oligosaccharides in non-breastfed infants. Several oligosaccharide fractions were synthesised as possible surrogates of human milk oligosaccharides. Short chain galacto-oligosaccharides (GOS) are oligomers of lactose (degree of polymerisation (dp) 2-6), produced by elongating lactose using β-galactosidase enzymes (Rijnierse et al., 2011). GOS is applied either alone or in combination with long-chain fructo-oligosaccharides (FOS), using a GOS:FOS ratio of 9:1. FOS fractions are acquired by removing the short-chain fructans from chicory inulin, resulting in fructan mixtures with terminal glucose monomers and a minimal dp of 22 (Rijnierse et al., 2011). During the last few years, additions of methylated pectin-derived acidic oligosaccharides (AOS) to infant formulas were investigated as well, mostly combined with both GOS and FOS (GOS:FOS:AOS ratio of 9:1:2)(Rijnierse et al., 2011). The prebiotic properties of these commercially produced GOS/FOS and GOS/FOS/AOS fractions have been proven in various studies (Boehm et al., 2002; Moro et al., 2002; Salvini et al., 2011; Westerbeek et al., 2013).

Moreover, immunomodulatory properties of GOS and combinations of both GOS/FOS and GOS/FOS/AOS have been documented *in vivo*. Dietary supplementation with these oligosaccharide fractions was shown to be beneficial for both allergic immune responses (Moro et al., 2006; Vos et al., 2007b; Arslanoglu et al., 2008; van Hoffen et al., 2009; Yasuda et al., 2012) and defensive immune responses following viral or bacterial challenge (Vos et al., 2007a; Gopalakrishnan et al., 2012; Schijf et al., 2012). Furthermore, there is evidence that early supplementation with GOS/FOS (with or without AOS) lowers the incidence of infections in human infants (Arslanoglu et al., 2007; Westerbeek et al., 2010). In most of these studies, the effects of oligosaccharides on the intestinal microbiotica were discussed as the primary mode of action of the administered oligosaccharides. In contrast, very limited information is available concerning any direct immunomodulatory effects of these oligosaccharide fractions and the underlying mechanisms. Eiwegger et al (2004) reported that human milk-derived oligosaccharides and plant-derived oligosaccharides (low-molecular-weight fucoidan) affect the cytokine production and activation of unchallenged cord blood derived T cells *in vitro*. In a more recent study by the same group, the *in vitro* incubation of unchallenged human cord blood mononuclear cells with low concentrations (10-100 µg/ml) of AOS or GOS combined with FOS did not result in an alteration of cytokine production, whereas incubation with similar concentrations of acidic human milk-derived oligosaccharides did

significantly induce the production of Interferon-γ and Interleukin-10 (Eiwegger et al., 2010). The latter study also provides in vitro evidence for epithelial transport of prebiotic oligosaccharides, enabling direct contact between oligosaccharides and cells of the immune system.

Neonatal foals possess limited defence mechanisms, in particular due to the impermeability of the equine placenta to maternal immunoglobulins. Consequently, newborn foals are strongly dependent on the transfer of immunoglobulins through the uptake of colostrum (LeBlanc et al., 1992). Moreover, similar to newborns of other mammalian species, both innate and adaptive immune responses are immature at the time of delivery (Levy, 2007). Dietary supplementation of oligosaccharides would be one of the possible options to improve the development of the immune system and consequently lower the incidence of infections in foals, which are often life-threatening. However, up to now no research has been published regarding immunomodulatory effects of oligosaccharides in the horse, neither in vivo nor in vitro.

In this study, we investigated the effects of defined oligosaccharide fractions on the inflammatory response in equine peripheral blood mononuclear cells (PBMCs) following a standardised lipopolysaccharide (LPS) challenge. We chose to use equine PBMCs as a readily available representative model of immune cells in this animal species.

Materials and Methods

Animals and sample collection

Twelve healthy, adult, Dutch warmblood horses (K.W.P.N. studbook) were sampled in total during this study. PBMCs from five horses were used for experiments with oligosaccharide fractions, four horses were sampled for control experiments with glucose/lactose solutions, and PBMCs of three horses were used for cell viability assays. Experiments could not all be executed in the same horses, due to the large amounts of PBMCs required to investigate all fractions and concentrations in triplicate (at least). Adult horses had to be used as blood donors, as large volumes of blood were needed to isolate sufficient amounts of PBMCs for these first explorative experiments in the horse; withdrawing larger volumes of blood from neonatal foals is restricted by ethical concerns. From each horse, 250 ml of blood was collected by jugular venipuncture directly into sterile heparinised blood collection tubes (BD Vacutainer Systems, Plymouth, United Kingdom). Blood samples were kept cooled and PBMC isolation was started within 1 hour after collection. All experimental procedures were approved by the committee of ethical considerations regarding animal experiments of Utrecht University (DEC Utrecht).

PBMC isolation

Blood samples were diluted 1:1 in sterile PBS (Lonza, Basel, Switzerland) containing 2mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) and subsequently layered over Ficoll-Paque™ plus (GE Healthcare, Waukesha, WI, USA). After centrifugation (400*g*, 30 minutes at room temperature), PBMCs were pipetted from the Ficoll layer and washed twice in PBS/EDTA. PBMCs were resuspended in RPMI 1640 Medium (Lonza, Basel, Switzerland) supplemented with 2mM glutamine (Lonza, Basel, Switzerland), 100 IU/ml penicillin (Lonza, Basel, Switzerland), 100 µg/ml streptomycin (Lonza, Basel, Switzerland), and 10% horse serum (prepared in our own laboratory according to standard procedures). PBMCs were counted using trypan blue and resuspended to a density of $4*10^6$ cells/ml medium. Following storage overnight at 4 °C to attenuate possible stimulatory effects of the applied Ficoll, PBMCs were seeded in 24 well plates at a density of $4*10^6$ cells/ml medium/well.

Cell culture experiments

After seeding the PBMCs in 24 well plates, the plates were incubated for 2 hours at 37 °C and 5% CO_2. After this, the plates were centrifuged for 10 minutes at 400*g* before refreshing the medium without removing PBMCs. The experiments were started by pre-incubating the PBMCs for 2 hours with supplemented RPMI containing different concentrations of oligosaccharide fractions (including blank controls, i.e. supplemented RPMI without extra additions). After pre-incubation, plates were centrifuged again and the medium was replaced with medium containing 0 or 1 µg/ml LPS (*Escherichia coli* O111:B4; Sigma-Aldrich, St. Louis, MO, USA) combined with different concentrations of oligosaccharide fractions (including blank controls). Plates were placed in the incubator for another 4 hours, after which samples for ELISA were collected and stored at -80 °C. Thus, there was a total incubation time of 6 hours for all investigated conditions. All different incubations were performed in triplicate or in quadruplicate for each horse. Additional experiments were performed following the same protocol with different concentrations of glucose and lactose, to account for the fact that the oligosaccharide fractions- especially GOS- contain considerable amounts of these mono- and disaccharides.

We investigated the effects of three different standardised oligosaccharide fractions with known immunomodulatory properties *in vivo,* using oligosaccharide concentrations ranging from 0.5% to 2.0%. The exact composition of all 2% incubations is summarised in Table 1 (1% and 0.5% solutions were obtained by diluting 1:1 in supplemented RPMI). The applied GOS fraction (Vivinal GOS syrup; FrieslandCampina Domo, Zwolle, The Netherlands; dp 2-6) consisted of approximately 45% GOS, 14% glucose, 16% lactose and 25% water. GOS was investigated separately, combined with FOS (Raftiline HP; Orafti, Wijchen, The Netherlands; dp≥23, approximately 96.5% FOS

and 3.5% maltodextrine) in a 9:1 ratio, and combined with both FOS and AOS (Danone Research, Wageningen, The Netherlands; dp 1-20, approximately 85.5% AOS, 7.25% monomers and 7.25% moisture) in a 9:1:2 ratio. Ratio's and diluting factors were based on oligosaccharide contents of the used products. Thus, all 2% oligosaccharide solutions contained 20 mg/ml oligosaccharides and, in addition, defined amounts of mono- and disaccharides. The control incubations with glucose and lactose (both from Sigma-Aldrich, St. Louis, MO, USA) were chosen to correspond with the glucose and lactose concentrations of the GOS fraction, as this was the oligosaccharide fraction with the highest concentrations of mono- and disaccharides (see Table 1). The effects of glucose/lactose controls were investigated to eventually discriminate between effects of the oligosaccharide preparations and possible effects of glucose and lactose, which are present in the oligosaccharide fractions as well. All experiments in this study were performed using the same batch of GOS, FOS and AOS, of which the composition was analysed and stated by the manufacturers.

Incubation	Oligosaccharides	Glucose	Lactose
GOS 2%	200 mg GOS	62.22 mg	71.11 mg
GOS/FOS 2%	180 mg GOS 20 mg FOS (+0.73 mg maltodextrin)	56.00 mg	64.00 mg
GOS/FOS/AOS 2%	150 mg GOS 16.67 mg FOS (+0.60 mg maltodextrin) 33.33 mg AOS	46.67 mg (+2.83 mg monomers)	53.33 mg
Glucose/lactose, corresponding with GOS 2%	-	62.22 mg	71.11 mg

Table 1: Composition of the applied oligosaccharide fractions and glucose/lactose controls (per 10 ml of medium).

ELISA

Protein levels of tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10) were measured by means of ELISA on the cell culture supernatants, using Duoset® ELISA Development System for equine TNF-α and equine IL-10 (R&D Systems, Minneapolis, MN, USA). Standard operating procedures of the manufacturer were followed, applying all required buffers and solutions in the form provided by the manufacturer (R&D

Systems, Minneapolis, MN, USA). The lower limits of detection of the ELISAs were 15.6 pg/ml (TNF-α) and 156.3 pg/ml (IL-10), respectively.

Cell viability assessment

To investigate possible influence of the applied incubation mixtures on cell viability, CCK-8 viability assays were performed according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). Prior to viability assessment, cell culture experiments were carried out identical to the experiments which are described above, with the exception that cells were seeded and incubated in 96 well plates (at a density of $8*10^5$ cells/200 µl/well, similar to experiments in terms of cells per ml medium and cm^2 surface).

Endotoxin assay

All applied solutions (as described in Table 1) were tested for endotoxin contamination using a Limulus Ambocyte Lysate endotoxin assay according to manufacturer's instructions (Genscript, Piscataway, NJ, USA). The selected standard curve (determining the range of detection) ranged from 0.1-1 Endotoxin Unit (EU)/ml.

Data analysis and statistical methods

Data were analysed by means of Bayesian hierarchical linear regression, assuming a normal distribution of the log-transformed ELISA data. A hierarchical approach was taken to account for variation between horses. PBMC responses were allowed to vary between horses with regard to general PBMC reactivity (random intercept), and PBMC reactivity specifically against incubation with LPS, GOS, FOS, and AOS (random slopes). Analyses were performed in a Bayesian framework, using JAGS, a program for analysis of Bayesian models using Markov Chain Monte Carlo (MCMC) simulation (version 3.2.0; Plummer, 2012). Simulations in JAGS were set up and analysed in R (version 2.14.2; R Development Core Team, 2011), using packages *rjags* (version 3-5; Plummer, 2011) and *R2jags* (version 0.03-06; Su, 2011). Posterior distributions were simulated based on uninformative prior distributions (normal distributions with mean 0 and standard deviation 100 for parameter means; inverse gamma distributions with mean 1 and variance 10,000 for parameter variances; inverse scaled Wishart distribution for the variance-covariance matrix of random effects). Bayesian credible intervals (BCI) for parameter estimates were based on the 2.5% and 97.5% percentiles of posterior distributions. Posterior distributions were simulated by means of four Markov chains, each consisting of 1 million Monte Carlo samples (saving only every 50^{th} sample). The first half of all samples was discarded for burn-in, allowing the model to converge. Model convergence was assessed according to Gelman and Rubin's convergence diagnostic, the potential scale reduction factor (Gelman and Rubin, 1992).

Differences are stated significant, based on the calculated 95% BCI. In the results section, significant differences are quantified by percentages. Not all significant differences are quantified in the results section. An overview of all data and the corresponding 95% BCI's is given in an additional file, annexed to this chapter (annex I).

Results

Oligosaccharide fractions dose-dependently modulate TNF-α production in unchallenged PBMCs

In Table 2, TNF-α and IL-10 production are illustrated after incubation of PBMCs with oligosaccharide fractions (including blank controls). All fractions significantly increased TNF-α production by PBMCs compared to blank controls and did so at all concentrations. Dose-dependent effects were seen for all three oligosaccharide fractions. For GOS, the dose-dependent increase was only significant after incubation with 2% GOS compared to incubation with 1% GOS (43% increase). For GOS/FOS, the dose-dependent increase of TNF-α production was significant through the whole concentration range (1% vs. 0.5%: 110% increase, 2% vs. 1%: 101% increase). For GOS/FOS/AOS, a significant increase of TNF-α production was evident when comparing the 1% incubation with the 0.5% incubation (81% increase). In contrast, PBMCs incubated with 2% GOS/FOS/AOS produced significantly lower amounts of TNF-α compared to PBMCs incubated with 1% GOS/FOS/AOS (54% reduction).

Production of IL-10 in unchallenged PBMCs was not significantly influenced by oligosaccharide fractions in comparison with blank controls.

	TNF-α (pg/ml)			IL-10 (pg/ml)		
Incubation	**Point estimate**	**Lower bound**	**Upper bound**	**Point estimate**	**Lower bound**	**Upper bound**
Blank controls	**12.6**	9.8	15.7	**187.2**	156.2	222.5
GOS 0.5%	**19.7** *	16.5	23.3	**183.1**	152.8	217.7
GOS 1.0%	**24.9** *	21.1	29.3	**184.2**	153.5	219.2
GOS 2.0%	**35.5** * #	30.2	41.6	**179.8**	149.6	214.2
GOS/FOS 0.5%	**30.2** *	25.7	35.4	**185.5**	154.8	221.4
GOS/FOS 1.0%	**63.4** * #	54.1	74.2	**190.2**	160.0	225.7
GOS/FOS 2.0%	**127.5** * #	108.6	149.5	**186.0**	154.5	221.4
GOS/FOS/AOS 0.5%	**73.6** *	62.9	86.4	**205.2**	173.8	242.5
GOS/FOS/AOS 1.0%	**133.5** * #	113.7	156.6	**214.2**	181.6	252.4
GOS/FOS/AOS 2.0%	**61.4** * #	52.4	72.2	**186.8**	157.3	221.0

Table 2: Mean protein concentrations of TNF-α and IL-10 (pg/ml) and 95% Bayesian Credible Intervals for unchallenged PBMCs incubated with oligosaccharide fractions (including blank controls). Significant differences between oligosaccharide fractions and blank controls are marked with an asterisk (*). Significant dose-dependent differences (1.0% vs. 0.5% and 2.0% vs. 1.0%) are marked with a hash (#).

Oligosaccharide fractions dose-dependently modulate cytokine production in LPS-challenged PBMCs

Table 3 illustrates TNF-α and IL-10 production in LPS-challenged PBMCs after pre-incubation and co-incubation with the applied oligosaccharide fractions (including incubations with LPS alone). The TNF-α response in PBMCs incubated with LPS alone was significantly increased with 13,793% (138-fold) in comparison with blank controls. Incubation with 0.5% solutions of all three oligosaccharide fractions resulted in significantly higher TNF-α concentrations compared to incubation with LPS alone. A dose-dependent increase of TNF-α production was observed for GOS and GOS/FOS within the entire concentration range. When comparing 1% incubations with 0.5% incubations, the increase of TNF-α production was 36% for GOS and 53% for GOS/FOS. For GOS, incubation with 2% solutions resulted in an additional significant increase of TNF-α production compared to 1% solutions (55% increase), whereas for GOS/FOS this increase was not significant. In contrast to GOS and GOS/FOS, incubation with increasing concentrations of GOS/FOS/AOS resulted in a dose-dependent reduction of TNF-α production in LPS-challenged PBMCs (1% vs. 0.5%: 22% reduction; 2% vs. 1%: 69%

reduction). Moreover, TNF-α production in LPS-challenged PBMCs incubated with GOS/FOS/AOS 2% was significantly lower compared to incubation with LPS alone (41% reduction).

The IL-10 response in PBMCs incubated with LPS alone was significantly increased with 49% in comparison with blank controls. Whereas both GOS and GOS/FOS incubations did not significantly alter the IL-10 response following LPS stimulation, incubation with LPS and GOS/FOS/AOS 0.5% did increase IL-10 production significantly in comparison with incubation with LPS alone (35% increase). Incubation with higher concentrations of GOS/FOS/AOS resulted in a dose-dependent reduction of the LPS-induced IL-10 response in PBMCs (1% vs. 0.5%: 28% reduction, 2% vs. 1%: 33% reduction). Moreover, IL-10 production in LPS-challenged PBMCs incubated with GOS/FOS/AOS 2% was significantly lower compared to incubation with LPS alone (35% reduction).

	TNF-α (pg/ml)			**IL-10 (pg/ml)**		
Incubation	**Point estimate**	**Lower bound**	**Upper bound**	**Point estimate**	**Lower bound**	**Upper bound**
LPS 1 µg/ml	**1749.4**	1495.2	2050.8	**278.1**	236.0	328.7
LPS + GOS 0.5%	**3655.5** *	3115.0	4285.5	**293.8**	248.4	347.6
LPS + GOS 1.0%	**4989.0** * #	4251.4	5843.0	**268.8**	227.5	316.7
LPS + GOS 2.0%	**7754.3** * #	6607.8	9090.6	**265.6**	225.0	313.6
LPS + GOS/FOS 0.5%	**3601.1** *	3074.8	4221.7	**344.1**	290.9	407.5
LPS + GOS/FOS 1.0%	**5530.3** * #	4712.6	6483.4	**307.4**	260.6	362.9
LPS + GOS/FOS 2.0%	**6721.1** *	5733.0	7879.3	**264.0**	223.9	311.7
LPS + GOS/FOS/AOS 0.5%	**4242.9** *	3608.3	4969.1	**374.3** *	317.0	442.3
LPS + GOS/FOS/AOS 1.0%	**3317.6** * #	2832.7	3893.3	**269.6** #	227.9	318.3
LPS + GOS/FOS/AOS 2.0%	**1029.7** * #	874.8	1205.9	**180.7** * #	149.0	216.6

Table 3: Mean protein concentrations of TNF-α and IL-10 (pg/ml) and 95% Bayesian Credible Intervals for LPS-challenged PBMCs incubated with oligosaccharide fractions (including incubations with LPS alone). Significant differences between oligosaccharide fractions and LPS alone are marked with an asterisk (*). Significant dose-dependent differences (1.0% vs. 0.5% and 2.0% vs. 1.0%) are marked with a hash (#).

Oligosaccharide fractions affect the TNF-α response significantly more profoundly compared with mono- and disaccharide controls

To account for the mono- and disaccharide contents of the applied oligosaccharide preparations, effects of these oligosaccharide preparations were compared with effects of corresponding glucose/lactose concentrations in the same model. A comparison of the effects of oligosaccharide preparations and glucose/lactose controls on the TNF-α response in unchallenged PBMCs is given in Figure 1.

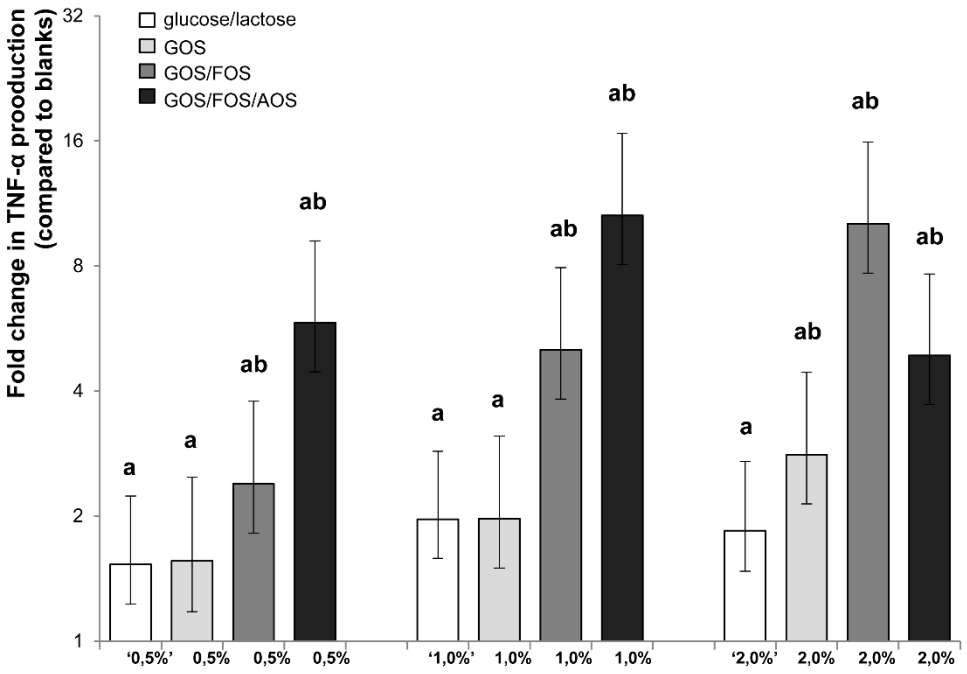

Figure 1: Comparison of the effects of oligosaccharide fractions vs. glucose/lactose controls on TNF-α response in unchallenged PBMCs. Relative TNF-α production compared to blank controls and 95% Bayesian Credible Intervals are given for all investigated incubations, including glucose/lactose controls (glucose/lactose 'x%' meaning corresponding with glucose/lactose concentrations of x% GOS). Significant differences are marked with lower case letters. a: Significant difference between oligosaccharide or glucose/lactose fractions and blank controls. b: Significant difference between oligosaccharide fractions and corresponding glucose/lactose controls.

Incubation of PBMCs with medium containing glucose and lactose resulted in significantly higher TNF-α production in comparison with blank controls (increase of 53%, 97%, and 84% for glucose/lactose solutions resembling 0.5%, 1% and 2% GOS, respectively).

However, no significant dose-dependent effects were detected among the glucose/lactose incubations.

A comparison with the GOS incubations revealed that incubation of unchallenged PBMCs with 2% GOS resulted in significantly more TNF-α production compared to the corresponding glucose/lactose controls (52% increase), whereas effects of the 0.5% and 1% GOS and corresponding glucose lactose incubations did not differ significantly. For GOS/FOS and GOS/FOS/AOS, all effects on TNF-α production were significantly more profound compared to the effects of corresponding glucose/lactose controls.

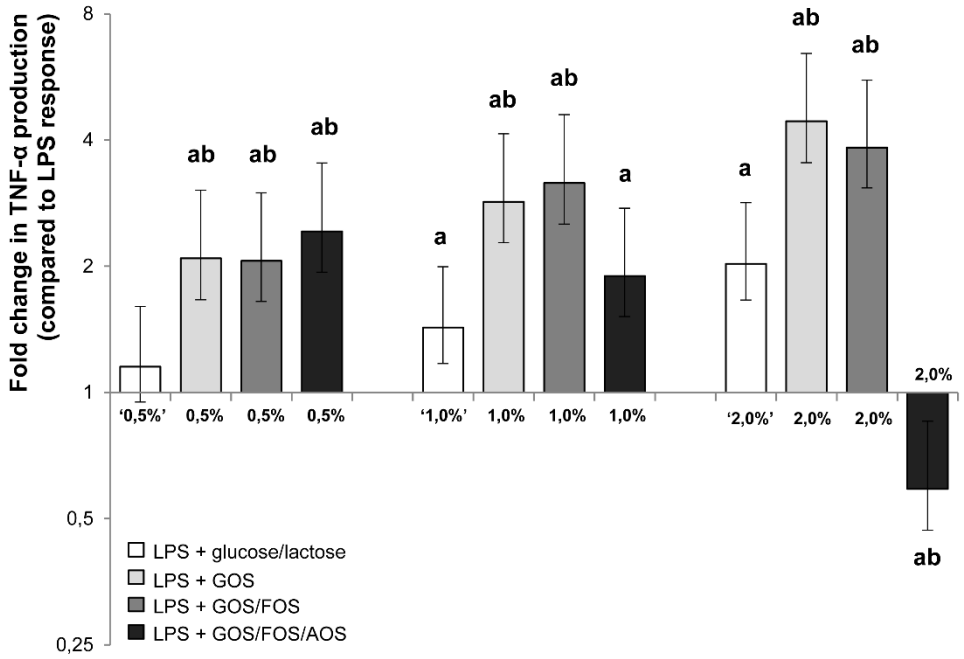

Figure 2: Comparison of the effects of oligosaccharide fractions vs. glucose/lactose controls on TNF-α response in LPS-challenged PBMCs. Relative TNF-α production compared to PBMCs incubated with 1 µg/ml LPS and 95% Bayesian Credible Intervals are given for all investigated incubations, including glucose/lactose controls (glucose/lactose 'x%' meaning corresponding with glucose/lactose concentrations of x% GOS). Significant differences are marked with lower case letters. a: Significant difference between oligosaccharide or glucose/lactose fractions and LPS alone. b: Significant difference between oligosaccharide fractions and corresponding glucose/lactose controls.

Effects of oligosaccharide preparations and glucose/lactose controls on the TNF-α response in LPS-challenged PBMCs are compared in Figure 2. Whereas incubation of PBMCs with glucose/lactose resembling 0.5% GOS did not alter LPS-induced TNF-α production significantly, the glucose/lactose solutions corresponding with 1% and 2% GOS enhanced the LPS-induced TNF-α production dose-dependently (1% vs. 0.5%: 24% increase; 2% vs. 1%: 42% increase).

Differences between effects of the applied oligosaccharide preparations and glucose/lactose controls were evident through the entire concentration range. For all investigated GOS and GOS/FOS concentrations, TNF-α production was significantly higher after incubation with the oligosaccharide fraction compared to the corresponding glucose/lactose concentrations. In contrast, for GOS/FOS/AOS a dose-dependent decrease in TNF-α production was evident. LPS-challenged PBMCs incubated with GOS/FOS/AOS 2% produced significantly less TNF-α compared to corresponding glucose/lactose controls (71% reduction).

A comparison of the effects of oligosaccharide preparations and corresponding glucose/lactose concentrations on IL-10 production could not be executed, as IL-10 concentrations which were measured in the glucose/lactose experiments were mostly below the detection limit of the ELISA.

Incubation of PBMCs with GOS/FOS/AOS significantly enhances cell viability

Results for the CCK-8 viability assays are illustrated in Figure 3. The investigated incubations did not significantly reduce cell viability compared to blank controls. However, all investigated GOS/FOS/AOS incubations significantly increased the apparent cell viability (ranging from 38-61% increase). Besides this, a mild (14%) but significant increase of cell viability was observed for glucose/lactose concentrations resembling 1% GOS.

Minor endotoxin contamination of the applied oligosaccharide fractions

According to the performed bioassay, all oligosaccharide solutions contained measurable concentrations of endotoxin. For the 2% solutions, the endotoxin concentrations (mean values ± standard deviation) were estimated at 0.24 ± 0.02 EU/ml (GOS), 1.06 EU/ml (GOS/FOS, based on measurements in 1% solution: 0.53 ± 0.07 EU/ml) and 1.92 EU/ml (GOS/FOS/AOS, based on measurements in 1% solution: 0.96 ± 0.07 EU/ml). The glucose and lactose solutions did not contain measurable amounts of endotoxin (the lower limit of detection of the assay was 0.1 EU/ml).

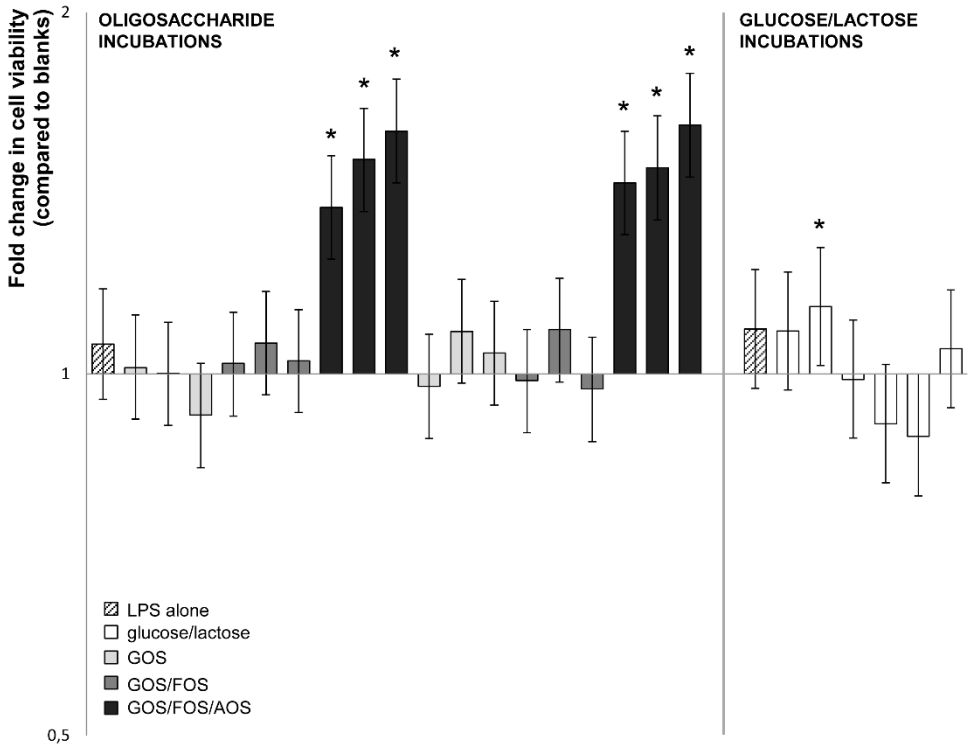

Figure 3: Results for the CCK-8 viability assay. Cell viability is given for all investigated incubations, relative to cell viability as determined in blank controls, including 95% Bayesian Credible Intervals. Significant increases of cell viability are marked with an asterisk (*).

Discussion

Individual oligosaccharides exhibit distinct immunomodulatory properties

Based on our results, we can conclude that both GOS and GOS/FOS fractions dose-dependently enhanced the pro-inflammatory response by LPS-challenged equine PBMCs. In contrast, after incubation with 2% GOS/FOS/AOS the LPS-induced inflammatory response was dose-dependently reduced. This indicates that with increasing the AOS concentration in the incubations, the stimulatory effects of both GOS and GOS/FOS were eventually overruled by the suppressive effects of AOS in this model. Suppression of the cytokine response was not caused by cytotoxic properties of AOS. On the contrary, PBMC viability was significantly increased in all GOS/FOS/AOS incubations compared to blank incubations, suggesting a protective or mitogenic effect. In conclusion, the reduced cytokine production by PBMCs incubated with GOS/FOS/AOS

was not the result of a reduced number of viable cells, as lower amounts of cytokines were produced by a higher number of cells.

We cannot exclude that a direct interaction between AOS and LPS influenced the LPS response in PBMCs incubated with GOS/FOS/AOS. Binding of LPS by AOS in the gut lumen might even contribute to the documented beneficial effects of oligosaccharide fractions containing AOS in *in vivo* studies.

Though we observed modulatory effects of mono- and disaccharides on the LPS-induced inflammatory response in our model, these effects were less pronounced and overshadowed by the immunomodulating effects of the investigated oligosaccharide fractions. To our knowledge, literature regarding the underlying mechanism of action of mono- and disaccharides, which enhance the LPS-induced inflammatory response, is lacking. However, our results are in line with a recently published study, which reported that high glucose conditions enhance the TNF-α and IL-6 response following LPS challenge (Chen et al., 2013).

Endotoxin contamination is not responsible for the enhancement of the LPS response by oligosaccharide fractions

As PBMCs are extremely sensitive to bacterial endotoxin, it cannot be excluded that the significant increase of TNF-α production in unchallenged PBMCs after incubation with the applied oligosaccharide fractions was caused by the low endotoxin concentrations in these natural products. Although it is not possible to give an exact concentration based on the results obtained with the Limulus assay, it can be assumed that the degree of endotoxin contamination ranged from 1.20-19.20 ng/ml for the 2% oligosaccharide solutions. This estimate is based on the information of the manufacturer of the applied LPS (Sigma-Aldrich, St. Louis, MO, USA; 1 ng LPS= 5-10 EU). We challenged the PBMCs with 1000 ng/ml LPS, as preliminary experiments with this PBMC model indicated that this concentration already results in a maximum TNF-α response after 4 hours of incubation. As 1.20–19.20 ng/ml is almost negligible compared to a concentration of 1000 ng/ml, it can be assumed that this low endotoxin contamination cannot be held accountable for the enhancement of the LPS response by the oligosaccharide fractions in our model. Moreover, the enhancement of the TNF-α response in LPS-challenged cells was not proportional to the mild stimulatory effects in unchallenged PBMCs.

However, endotoxin contamination might play a role in the reduction of the LPS response after incubation with 2% GOS/FOS/AOS. In this solution, the measured endotoxin concentration was the highest (approximately 10-19 ng/ml) and we cannot exclude that endotoxin tolerance was induced during the PBMC pre-incubation period, suppressing the response after the actual challenge (Frellstedt et al., 2012). On the other hand, the 1% solution of GOS/FOS/AOS (approximately 5-10 ng/ml endotoxin) already caused a significant reduction of the LPS response in comparison with the 0.5%

GOS/FOS/AOS solution, whilst the 2% GOS/FOS solution (approximately 6-11 ng/ml endotoxin) induced a significant increase of the LPS response compared with the 1% GOS/FOS solution. Hence, distinct modulatory effects of the AOS fraction remain plausible.

Direct effects of oligosaccharides

Previously performed *in vivo* studies often ascribe immunomodulatory effects of dietary oligosaccharides to their prebiotic properties. However, the results of this study support the hypothesis of direct modulatory effects of oligosaccharides, independent of the intestinal microbiota. Though this notion is also supported by other *in vitro* studies (Eiwegger et al., 2004; Eiwegger et al., 2010), the method of action behind direct immunomodulation by oligosaccharides remains unclear. Carbohydrate (glycan)- binding receptors expressed on the surface of intestinal epithelial cells and antigen presenting cells may be involved in modulation of the immune response by sugar molecules (de Kivit et al., 2011). In addition, next to modulation of Toll-like receptor (TLR) signalling in the gastrointestinal tract due to the changes in the gut flora, oligosaccharides may directly modulate TLR- signalling. For instance, hyaluronan oligosaccharides have been shown to directly mediate inflammatory responses via TLR-4 (Termeer et al., 2002; Campo et al., 2012). As known TLR-4 ligands such as LPS display structural similarity with the investigated oligosaccharides, other oligosaccharides may influence TLR-4 signalling as well.

As of yet, research with oligosaccharides mainly involved direct intervention studies in human infants or in experimental (rodent) animal species as surrogates for human infants (Moro et al., 2006; Arslanoglu et al., 2007; Vos et al., 2007a; Vos et al., 2007b; Arslanoglu et al., 2008; van Hoffen et al., 2009; Westerbeek et al., 2010; Gopalakrishnan et al., 2012; Schijf et al., 2012; Yasuda et al., 2012). One *in vivo* study in adult horses demonstrated beneficial effects of prebiotic short-chain fructo-oligosaccharides, preventing disruption of the intestinal flora in the equine hindgut in case of an abrupt change of diet composition (Respondek et al., 2008). Concerning immunomodulation by oligosaccharides in the horse, no data have been published to our knowledge. The data obtained from the present study confirm the activity of heterologous oligosaccharides in horses and are in line with the very few published *in vitro* studies with human cell culture models (Eiwegger et al., 2004; Eiwegger et al., 2010).

The most notable finding was the divergence between the immuno-activation of PBMCs by GOS and GOS/FOS preparations and the apparent suppressive effect of AOS. This was an unexpected outcome and indicates that forthcoming experimental protocols should include conditions with AOS only. In the current experiments we applied the (commercially available) combinations of oligosaccharides used in previous studies, and hence AOS was not tested separately.

Conclusion

This study provides evidence that oligosaccharides from different sources have distinct direct immunomodulatory properties. We showed that both GOS and FOS dose-dependently stimulated the LPS-induced inflammatory response in equine PBMCs, and that AOS dose-dependently suppressed the production of both TNF-α and IL-10 following an LPS challenge. Such an activation or suppression of the immune system could be beneficial *in vivo*, depending on the clinical context. Future research into dietary supplementation of specific oligosaccharide fractions in foals and underlying immunomodulatory mechanisms could lead to the development of oligosaccharide supplements in foals, to support a rapid maturation of the immune competence in the first phase of life, hence protecting them from early, often life-threatening infections.

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Annex I: Model parameter estimates and 95% Bayesian Credible Intervals

Significant differences are marked grey (Tables b, c, d, and e)

Abbreviations used in tables:

AOS: acidic oligosaccharides
FOS: fructo-oligosaccharides
GLU: glucose
GOS: galacto-oligosaccharides
LAC: lactose
LPS: lipopolysaccharide
rel.: relative

a. Cytokine protein levels

	TNF-α (pg/ml)			IL-10 (pg/ml)		
Interpretation	***Point Estimate***	***Lower Bound***	***Upper Bound***	***Point Estimate***	***Lower Bound***	***Upper Bound***
Blank controls	**12.62**	9.87	15.74	**187.17**	156.18	222.52
LPS 1 µg/ml	**1751.10**	1490.70	2054.94	**278.11**	236.04	328.65
GOS 0.5%	**19.69**	16.54	23.31	**183.09**	152.78	217.67
GOS 1.0%	**24.85**	21.09	29.25	**184.20**	153.55	219.20
GOS 2.0%	**35.45**	30.20	41.55	**179.83**	149.61	214.22
GOS/FOS 0.5%	**30.17**	25.71	35.41	**185.49**	154.78	221.41
GOS/FOS 1.0%	**63.43**	54.11	74.37	**190.19**	159.97	225.65
GOS/FOS 2.0%	**127.36**	108.64	149.46	**186.05**	154.47	221.41
GOS/FOS/AOS 0.5%	**73.63**	62.74	86.40	**205.20**	173.82	242.50
GOS/FOS/AOS 1.0%	**133.49**	113.75	156.02	**214.22**	181.64	252.40
GOS/FOS/AOS 2.0%	**61.37**	52.25	72.02	**186.79**	157.28	220.96
LPS + GOS 0.5%	**3655.54**	3118.17	4289.82	**293.83**	248.39	347.58
LPS + GOS 1.0%	**4989.05**	4251.38	5848.85	**268.81**	227.47	316.71
LPS + GOS 2.0%	**7754.28**	6614.37	9099.73	**265.60**	224.98	313.56
LPS + GOS/FOS 0.5%	**3604.72**	3068.67	4217.51	**344.12**	290.91	407.48
LPS + GOS/FOS 1.0%	**5535.85**	4707.91	6489.88	**307.35**	260.60	362.85
LPS + GOS/FOS 2.0%	**6721.05**	5727.30	7879.34	**264.01**	223.86	311.69
LPS + GOS/FOS/AOS 0.5%	**4238.65**	3615.55	4974.10	**374.28**	317.03	442.31
LPS + GOS/FOS/AOS 1.0%	**3317.61**	2832.74	3885.47	**269.62**	227.92	318.30
LPS + GOS/FOS/AOS 2.0%	**1028.65**	877.43	1205.92	**180.73**	149.01	216.59

B. TNF-α; RELATIVE PROTEIN LEVELS

Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*
Dose-response effect GOS 1.0% rel. to 0.5%	**1.26**	1.00	1.60
Dose-response effect GOS 2.0% rel. to 1.0%	**1.43**	1.14	1.79
Dose-response effect GOS/FOS 1.0% rel. to 0.5%	**2.10**	1.68	2.63
Dose-response effect GOS/FOS 2.0% rel. to 1.0%	**2.01**	1.60	2.52
Dose-response effect GOS/FOS/AOS 1.0% rel. to 0.5%	**1.81**	1.45	2.27
Dose-response effect GOS/FOS/AOS 2.0% rel. to 1.0%	**0.46**	0.37	0.58
Dose-response effect LPS + GOS 1.0% rel. to LPS + 0.5%	**1.36**	1.09	1.71
Dose-response effect LPS + GOS 2.0% rel. to LPS + 1.0%	**1.56**	1.24	1.95
Dose-response effect LPS + GOS/FOS 1.0% rel. to LPS + 0.5%	**1.54**	1.23	1.93
Dose-response effect LPS + GOS/FOS 2.0% rel. to LPS + 1.0%	**1.21**	0.97	1.53
Dose-response effect LPS + GOS/FOS/AOS 1.0% rel. to LPS + 0.5%	**0.78**	0.63	0.98
Dose-response effect LPS + GOS/FOS/AOS 2.0% rel. to LPS + 1.0%	**0.31**	0.25	0.39
Response in LPS + GOS 0.5%, rel. to LPS	**2.09**	1.67	2.61
Response in LPS + GOS 1.0%, rel. to LPS	**2.85**	2.28	3.57
Response in LPS + GOS 2.0%, rel. to LPS	**4.43**	3.54	5.55
Response in LPS + GOS/FOS 0.5%, rel. to LPS	**2.06**	1.64	2.58
Response in LPS + GOS/FOS 1.0%, rel. to LPS	**3.16**	2.52	3.96
Response in LPS + GOS/FOS 2.0%, rel. to LPS	**3.84**	3.06	4.81
Response in LPS + GOS/FOS/AOS 0.5%, rel. to LPS	**2.42**	1.93	3.04
Response in LPS + GOS/FOS/AOS 1.0%, rel. to LPS	**1.89**	1.52	2.37
Response in LPS + GOS/FOS/AOS 2.0%, rel. to LPS	**0.59**	0.47	0.74
Response in LPS alone, rel. to blank controls	**138.93**	105.53	185.49
Response in LPS + GOS 0.5%, rel. to blank controls	**1.56**	1.18	2.10
Response in LPS + GOS 1.0%, rel. to blank controls	**1.97**	1.50	2.64
Response in LPS + GOS 2.0%, rel. to blank controls	**2.81**	2.14	3.77
Response in LPS + GOS/FOS 0.5%, rel. to blank controls	**2.39**	1.82	3.21
Response in LPS + GOS/FOS 1.0%, rel. to blank controls	**5.03**	3.83	6.73
Response in LPS + GOS/FOS 2.0%, rel. to blank controls	**10.10**	7.69	13.49
Response in LPS + GOS/FOS/AOS 0.5%, rel. to blank controls	**5.84**	4.45	7.80
Response in LPS + GOS/FOS/AOS 1.0%, rel. to blank controls	**10.58**	8.07	14.17
Response in LPS + GOS/FOS/AOS 2.0%, rel. to blank controls	**4.87**	3.71	6.50

c. TNF-α; relative protein levels (glucose/lactose comparisons)

Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*
Dose-response effect GLU/LAC 1.0% rel. to 0.5%	**1.29**	1.06	1.57
Dose-response effect GLU/LA 2.0% rel. to 1.0%	**0.94**	0.77	1.15
Dose-response effect LPS + GLU/LAC 1.0% rel. to LPS + 0.5%	**1.24**	1.02	1.51
Dose-response effect LPS + GLU/LAC 2.0% rel. to LPS + 1.0%	**1.42**	1.17	1.73
Response in LPS + GLU/LAC 0.5%, rel. to LPS	**1.15**	0.95	1.40
Response in LPS + GLU/LAC 1.0%, rel. to LPS	**1.43**	1.17	1.74
Response in LPS + GLU/LAC 2.0%, rel. to LPS	**2.03**	1.67	2.46
Response in GLU/LAC 0.5%, rel. to blank controls	**1.53**	1.23	1.93
Response in GLU/LAC 1.0%, rel. to blank controls	**1.97**	1.58	2.48
Response in GLU/LAC 2.0%, rel. to blank controls	**1.84**	1.47	2.34
Response in GLU/LAC 0.5%, rel. to GOS 0.5%*	**0.98**	0.68	1.41
Response in GLU/LAC 1.0%, rel. to GOS 1.0%*	**1.00**	0.69	1.42
Response in GLU/LAC 2.0%, rel. to GOS 2.0%*	**0.66**	0.45	0.94
Response in GLU/LAC 0.5%, rel. to GOS/FOS 0.5%*	**0.64**	0.44	0.91
Response in GLU/LAC 1.0%, rel. to GOS/FOS 1.0%*	**0.39**	0.27	0.56
Response in GLU/LAC 2.0%, rel. to GOS/FOS 2.0%*	**0.18**	0.13	0.26
Response in GLU/LAC 0.5%, rel. to GOS/FOS/AOS 0.5%*	**0.26**	0.18	0.37
Response in GLU/LAC 1.0%, rel. to GOS/FOS/AOS 1.0%*	**0.19**	0.13	0.27
Response in GLU/LAC 2.0%, rel. to GOS/FOS/AOS 2.0%*	**0.38**	0.26	0.54
Response in LPS + GLU/LAC 0.5%, rel. to LPS + GOS 0.5%**	**0.55**	0.41	0.74
Response in LPS + GLU/LAC 1.0%, rel. to LPS + GOS 1.0%**	**0.50**	0.37	0.68
Response in LPS + GLU/LAC 2.0%, rel. to LPS + GOS 1.0%**	**0.46**	0.34	0.62
Response in LPS + GLU/LAC 0.5%, rel. to LPS + GOS/FOS 0.5%**	**0.56**	0.41	0.75
Response in LPS + GLU/LAC 1.0%, rel. to LPS + GOS/FOS 1.0%**	**0.45**	0.33	0.61
Response in LPS + GLU/LAC 2.0%, rel. to LPS + GOS/FOS 1.0%**	**0.53**	0.39	0.71
Response in LPS + GLU/LAC 0.5%, rel. to LPS + GOS/FOS/AOS 0.5%**	**0.48**	0.35	0.64
Response in LPS + GLU/LAC 1.0%, rel. to LPS + GOS/FOS/AOS 1.0%**	**0.75**	0.56	1.02
Response in LPS + GLU/LAC 2.0%, rel. to LPS + GOS/FOS/AOS 1.0%**	**3.45**	2.57	4.65

**both rel. to blank controls*

***both rel. to LPS*

D. IL-10; RELATIVE PROTEIN LEVELS

Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*
Dose-response effect GOS 1.0% rel. to 0.5%	**1.01**	0.78	1.29
Dose-response effect GOS 2.0% rel. to 1.0%	**0.98**	0.76	1.26
Dose-response effect GOS/FOS 1.0% rel. to 0.5%	**1.03**	0.80	1.31
Dose-response effect GOS/FOS 2.0% rel. to 1.0%	**0.98**	0.76	1.25
Dose-response effect GOS/FOS/AOS 1.0% rel. to 0.5%	**1.04**	0.82	1.32
Dose-response effect GOS/FOS/AOS 2.0% rel. to 1.0%	**0.87**	0.69	1.11
Dose-response effect LPS + GOS 1.0% rel. to LPS + 0.5%	**0.91**	0.72	1.16
Dose-response effect LPS + GOS 2.0% rel. to LPS + 1.0%	**0.99**	0.78	1.25
Dose-response effect LPS + GOS/FOS 1.0% rel. to LPS + 0.5%	**0.89**	0.71	1.13
Dose-response effect LPS + GOS/FOS 2.0% rel. to LPS + 1.0%	**0.86**	0.68	1.08
Dose-response effect LPS + GOS/FOS/AOS 1.0% rel. to LPS + 0.5%	**0.72**	0.57	0.91
Dose-response effect LPS + GOS/FOS/AOS 2.0% rel. to LPS + 1.0%	**0.67**	0.52	0.86
Response in LPS + GOS 0.5%, rel. to LPS	**1.06**	0.84	1.34
Response in LPS + GOS 1.0%, rel. to LPS	**0.97**	0.76	1.22
Response in LPS + GOS 2.0%, rel. to LPS	**0.95**	0.76	1.21
Response in LPS + GOS/FOS 0.5%, rel. to LPS	**1.24**	0.97	1.57
Response in LPS + GOS/FOS 1.0%, rel. to LPS	**1.11**	0.87	1.40
Response in LPS + GOS/FOS 2.0%, rel. to LPS	**0.95**	0.75	1.20
Response in LPS + GOS/FOS/AOS 0.5%, rel. to LPS	**1.35**	1.06	1.70
Response in LPS + GOS/FOS/AOS 1.0%, rel. to LPS	**0.97**	0.76	1.22
Response in LPS + GOS/FOS/AOS 2.0%, rel. to LPS	**0.65**	0.50	0.83
Response in LPS alone, rel. to blank controls	**1.49**	1.17	1.90
Response in LPS + GOS 0.5%, rel. to blank controls	**0.98**	0.76	1.25
Response in LPS + GOS/FOS 0.5%, rel. to blank controls	**0.99**	0.77	1.28
Response in LPS + GOS/FOS/AOS 0.5%, rel. to blank controls	**1.10**	0.86	1.40

E. CCK-8 VIABILITY ASSAY

Interpretation	*Point Estimate*	*Lower Bound*	*Upper Bound*
Viability assays oligosaccharide solutions			
LPS, rel. to blank controls	**1.06**	0.95	1.18
GOS 0.5%, rel. to blank controls	**1.01**	0.92	1.12
GOS 1.0%, rel. to blank controls	**1.00**	0.91	1.10
GOS 2.0%, rel. to blank controls	**0.92**	0.84	1.02
GOS/FOS 0.5%, rel. to blank controls	**1.02**	0.92	1.13
GOS/FOS 1.0%, rel. to blank controls	**1.06**	0.96	1.17
GOS/FOS 2.0%, rel. to blank controls	**1.03**	0.93	1.13
GOS/FOS/AOS 0.5%, rel. to blank controls	**1.38**	1.25	1.52
GOS/FOS/AOS 1.0%, rel. to blank controls	**1.51**	1.36	1.66
GOS/FOS/AOS 2.0%, rel. to blank controls	**1.59**	1.44	1.76
LPS + GOS 0.5%, rel. to blank controls	**0.98**	0.88	1.08
LPS + GOS 1.0%, rel. to blank controls	**1.08**	0.98	1.20
LPS + GOS 2.0%, rel. to blank controls	**1.04**	0.94	1.15
LPS + GOS/FOS 0.5%, rel. to blank controls	**0.99**	0.89	1.09
LPS + GOS/FOS 1.0%, rel. to blank controls	**1.09**	0.98	1.20
LPS + GOS/FOS 2.0%, rel. to blank controls	**0.97**	0.88	1.07
LPS + GOS/FOS/AOS 0.5%, rel. to blank controls	**1.44**	1.31	1.59
LPS + GOS/FOS/AOS 1.0%, rel. to blank controls	**1.48**	1.34	1.64
LPS + GOS/FOS/AOS 2.0%, rel. to blank controls	**1.61**	1.46	1.78
Viability assays glucose/lactose solutions			
LPS, rel. to blank controls	**1.09**	0.97	1.22
GLU/LAC 0.5%, rel. to blank controls	**1.09**	0.97	1.22
GLU/LAC 1.0%, rel. to blank controls	**1.14**	1.02	1.27
GLU/LAC 2.0%, rel. to blank controls	**0.99**	0.88	1.11
LPS + GLU/LAC 0.5%, rel. to blank controls	**0.91**	0.81	1.02
LPS + GLU/LAC 1.0%, rel. to blank controls	**0.89**	0.79	1.00
LPS + GLU/LAC 2.0%, rel. to blank controls	**1.05**	0.94	1.17

Chapter 9

Effects of orally administered galacto-oligosaccharides on immunological parameters in foals

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Abstract

Newborn foals are highly susceptible to bacterial infections, due to both immature innate and adaptive immune responses, and the strong dependence on the transfer of passive immunity through colostral immunoglobulins. Next to strategies to optimise maternally acquired immunity in individual foals, other options to modulate immune responses in foals are explored. During the past decades, oligosaccharide supplements were developed to mimic beneficial properties of the oligosaccharides, which are present in colostrum and milk. In human infants and experimental animal species, dietary supplementation with galacto-oligosaccharides (GOS) has been shown to result in prebiotic and immunomodulating effects, with long-term beneficial consequences for both defensive and allergic immune responses. As of yet, no studies are published concerning effects of GOS in horses, neither in vitro nor in vivo. The current study was designed as a pilot study to investigate the effects of an orally applied, commercially available GOS product in a group of foals. Foals were treated during the first 4 weeks of life and subsequently followed up for another 10 weeks. In peripheral blood mononuclear cells derived from GOS-treated foals at day 28, a standardised lipopolysaccharide challenge resulted in significantly lower relative mRNA expression levels of the pro-inflammatory cytokines interferon-γ and interleukin-6 compared with PBMCs of control foals. In the 98 day period of investigation, no significant effects of the GOS supplement were observed on clinical and blood parameters for immunity and general health in these foals. Based on these first results, we can conclude that this dose regimen of GOS was well accepted by the foals and did not result in any detectable undesirable side effects. More extensive research is required to confirm the attenuating effects of GOS treatment on equine immune responses and to implement this into practice. Possibly, dietary supplementation with GOS can lead to long-term beneficial effects in the horse with regard to bacterial infections and immune-mediated inflammatory disorders, in line with the published literature with regard to other mammalian species.

Introduction

With birth, the immune system of the newborn foal is subjected to a range of environmental challenges. The process of bacterial colonisation of epithelial surfaces, including the gut, is one of the major events in early life, which will determine the immune responses in later phases of life (Martin et al., 2010). As the foal is born without sufficient plasma concentrations of immunoglobulins, the transfer of passive immunity through the uptake of colostral immunoglobulins on the first day of life is an essential part of the foal's defence during the first weeks of life (LeBlanc et al., 1992). When maternal immunity fades, active immunity of the foal itself becomes pivotal to prevent pathogens from causing disease (Giguere and Polkes, 2005). For example, the relatively late onset of the foal's endogenous synthesis of IgG subtypes has been considered to contribute to the increased susceptibility of foals to bacterial infections between the age of 2-4 months (Holznagel et al., 2003).

In addition to the basic concept of protection by maternal antibodies that are present in the colostrum, recent interest focuses on the non-protein components in colostrum, such as oligosaccharides. In human infants and in experimental animals, dietary supplementation with commercially produced galacto-oligosaccharides (GOS) has been shown to be beneficial for health in several ways (Sangwan et al., 2011). In most studies, short chain GOS are applied, which are oligomers (degree of polymerisation 2-6) constructed from lactose, derived from cow's milk, using β-galactosidase enzymes (Rijnierse et al., 2011). Next to the published prebiotic effects of supplementation of GOS alone (Ben et al., 2008; Fanaro et al., 2009; Davis et al., 2010), immunomodulatory properties of GOS and combinations of GOS with fructo-oligosaccharides (FOS) and/or acidic oligosaccharides (AOS) have been documented *in vivo*. Dietary supplementation with these oligosaccharide fractions was shown to be beneficial for both allergic immune responses (Moro et al., 2006; Vos et al., 2007b; Arslanoglu et al., 2008; van Hoffen et al., 2009; Yasuda et al., 2012) and defensive immune responses following viral or bacterial challenges (Vos et al., 2007a; Gopalakrishnan et al., 2012; Schijf et al., 2012). Furthermore, there is evidence that early supplementation with GOS/FOS (with or without AOS) lowers the incidence of infections in human infants (Arslanoglu et al., 2007; Westerbeek et al., 2010).

The exact mechanisms involved in the immunomodulatory properties of dietary oligosaccharides are not yet well understood. In most studies, the effects on the intestinal microbiota are discussed as the primary mode of action. Next to these effects on bacterial colonisation, there is increasing evidence for direct immunomodulatory effects of oligosaccharide fractions. Eiwegger et al (Eiwegger et al., 2004; Eiwegger et al., 2010) reported that human milk-derived oligosaccharides and plant-derived oligosaccharides (low-molecular-weight fucoidan) affect the cytokine production and activation of

unchallenged cord blood derived T cells *ex vivo*. Furthermore, with an *in vitro* model, they provided evidence for epithelial transport of oligosaccharides, enabling direct contact between oligosaccharides and cells of the immune system (Eiwegger et al., 2010).

Given their immunomodulatory properties, oligosaccharides possibly also play an important role in the development of immunity in foals. Up to now, a very limited amount of research into this topic has been conducted in the horse. One study in adult horses confirmed prebiotic properties of short-chain FOS in the horse, reporting that this supplement prevented disruption of the intestinal flora in the equine hindgut in case of an abrupt change of diet composition (Respondek et al., 2008). Furthermore, we have previously demonstrated *in vitro* that the carbohydrate fraction of equine colostrum elicits suppressive effects on the lipopolysaccharide-induced inflammatory response in equine peripheral blood mononuclear cells (PBMCs) (Vendrig et al., 2012).

The present study was designed as a pilot study to investigate the *in vivo* effects of a commercially available GOS product in a group of foals. Foals were orally supplemented with GOS twice daily during the first 4 weeks of life, and blood parameters for immune status and general health were determined up to 98 days of age. The aims of the study were (I) to assess the safety of this dose regimen of GOS in young foals, and (II) to investigate if the chosen treatment protocol elicited any beneficial effects on immunological and general health parameters in these foals. In addition, after the 4 weeks of treatment, PBMCs were isolated from each foal and the immune responses of PBMCs after a standardised lipopolysaccharide (LPS) challenge were compared.

Materials and Methods

Foals

Twelve warmblood pony foals (crossbreed New Forest*Arabian) were included in the study, all born at a horse dairy farm in The Netherlands, where the experiment was carried out. All mares and foals were housed together as a group under identical and stabile conditions. Foals were born within a period of 10 weeks and were allocated to the treatment group and the control group at random. In total, six foals were supplemented with GOS and six foals were included in the control group. All experimental procedures were approved by the committee of ethical considerations in animal experiments of Utrecht University (DEC Utrecht, Permit Number: 2012.III.05.053).

Dietary supplementation

Foals were orally supplemented with the GOS product or with equal amount of glucose/lactose (control) for 28 days, twice daily, starting at the day of birth. The applied GOS fraction (Vivinal GOS syrup, FrieslandCampina Domo, Zwolle, The Netherlands; degree of polymerisation 2-6) consisted of approximately 45% GOS, 16% lactose, 14%

glucose, and 25% water. An amount of 15 g GOS syrup was supplemented twice daily. Foals in the control group were supplemented with 2.4 g lactose and 2.1 g glucose, resembling the amounts of glucose and lactose that the GOS treated foals received through the GOS syrup.

Sampling and output parameters

At day 0, 1, 7, 14, 21, 28, 42, 56, 70, 84, and 98, serum and EDTA blood was collected by jugular venipuncture directly into sterile blood collection tubes (BD Vacutainer Systems, Plymouth, United Kingdom). At all time points, samples were sent to the University Veterinary Diagnostic Laboratory in Utrecht to determine red and white blood cell parameters (including haematocrit, leukocyte count, and leukocyte differentiation) and the total protein concentrations including a protein electrophoresis (albumin and differentiation of globulins). In addition, aliquots of the serum samples were stored at -80 ºC to perform several ELISAs, quantifying serum concentrations of IgG(a), IgG(b), IgG(T), IgM and IgA using commercial ELISA kits according to the manufacturer's instructions (Bethyl Laboratories Inc., Montgomery, AL, USA). Colostrum of the concerning mares was also sampled within 12 hours postpartum to investigate the concentrations of immunoglobulin subtypes, by means of the same ELISA kits (Bethyl Laboratories Inc., Montgomery, AL, USA).

PBMC experiments

At day 28, the last day of GOS application, peripheral blood mononuclear cells (PBMCs) were isolated from each foal. Blood was collected by jugular venipuncture directly into sterile heparinised blood collection tubes (BD Vacutainer Systems, Plymouth, United Kingdom). Blood samples were kept cooled and PBMC isolation started within 1 hour after collection. In brief, blood samples were diluted 1:1 in fresh PBS (Lonza, Basel, Switzerland) containing 2mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) and subsequently layered over Ficoll-PaqueTM plus (GE Healthcare, Waukesha, WI, USA). After centrifugation (400*g*, 30 minutes at room temperature) PBMCs were pipetted from the Ficoll layer and washed twice in PBS/EDTA. PBMCs were resuspended in RPMI 1640 Medium (Lonza, Basel, Switzerland) containing 2mM glutamine (Lonza, Basel, Switzerland), 100 IU/ml penicillin (Lonza, Basel, Switzerland), 100 µg/ml streptomycin (Lonza, Basel, Switzerland) and 10% horse serum (prepared in our own laboratory according to standard procedures). PBMCs were counted using trypan blue and resuspended to a density of $4*10^6$ cells/ml medium. Following storage overnight at 4 ºC to attenuate possible stimulatory effects of the applied Ficoll, PBMCs were seeded in 24 well plates at a density of $4*10^6$ cells/ml medium/well.

After seeding the PBMCs in 24 well plates, the plates were incubated for 2 hours at 37 ºC and 5% CO_2. After this, the plates were centrifuged for 10 minutes at 400*g*

before refreshing the medium without removing PBMCs. The experiments were started by replacing the medium with medium containing 0 or 1 µg/ml LPS (*Escherichia coli* O111:B4; Sigma-Aldrich, St. Louis, MO, USA). Plates were placed in the incubator and samples for qPCR and ELISA were collected after 4 hours and stored at -80 °C. All individual incubations were performed in triplicate for each foal. For the ELISAs, supernatants were stored at -80 °C. For qPCR analyses, the PBMCs were lysed using RNA lysis buffer (Promega, Madison, WI, USA) and stored at -80 °C until RNA isolation was resumed.

Protein levels of tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10) were measured by means of ELISA on the cell culture supernatants, using Duoset® ELISA Development System for equine TNF-α and equine IL-10 (R&D Systems, Minneapolis, MN, USA). Standard operating procedures of the manufacturer were followed, applying all required buffers and solutions in the form provided by the manufacturer (R&D Systems, Minneapolis, MN, USA). The lower limits of detection of the ELISAs were 15.6 pg/ml and 156.3 pg/ml for TNF-α and IL-10, respectively.

RNA was isolated from PBMCs using SV Total RNA isolation system according to the manufacturer's instructions (Promega, Madison, WI, USA). Isolated fractions were dissolved in 50 µl ribonuclease free water and stored at -80 °C. Quality and quantity of RNA was determined spectrophotometrically (Nanodrop). cDNA was generated using iScriptTM cDNA Synthesis Kit (Biorad, Hercules, CA, USA) according to the manufacturer's protocol. For reverse transcriptase reaction, 1000 ng RNA was applied per sample. Expression of mRNA was assessed by real-time PCR using a Biorad iQ5 Multicolor Real-time PCR detection system and iQTM SYBR® Green Supermix (Biorad, Hercules, CA, USA). Specific primer pairs were designed and tested for efficiency and accuracy, after having checked their specificity using the NCBI-BLASTN search program. Primer pairs were synthesised commercially (Eurogentec Nederland B.V., Maastricht, The Netherlands). For this study, mRNA expression of interferon-γ (IFN-γ), IL-4, IL-6, IL-10, IL-13, IL-17, transforming growth factor-β1 (TGF-β1), TNF-α, TLR-2, TLR-4, TLR-9, β-actin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was determined using the following primer pairs:

IFN-γ:	F 5'-GATCTGAAGGTCCAGCGCAA-3'
	R 5'-TCCGGCCTCGAAATGGATTC-3'
IL-4:	F 5'-GCCCGAAGAACACAGATGGA-3'
	R 5'-CAGTACAGCAGGTCCCGTTT-3'
IL-6:	F 5'-TGGCTGAAGAACACAACAACT-3'
	R 5'-GAATGCCCATGAACTACAACA-3'
IL-10:	F 5'-GAGAACCACGGCCCAGACATCAAG-3'
	R 5'-GACAGCGCCGCAGCCTCACT-3'

IL-13:	F 5′-AGCAGTCATTGCTCTCGCTT-3′
	R 5′-TGGGTGATGTTGACCAGCTC-3′
IL-17:	F 5′-GCTGAGTCTGGTGGCTATCG-3′
	R 5′-TTCTTGTCCCCAGTGTTCGG-3′
TGF-β1:	F 5′-TGTCCACCTGCAAGACCATC-3′
	R 5′-CCGCAACTTGGACAGGATCT-3′
TNF-α:	F 5′-TCCAGACGGTGCTTGTGC-3′
	R 5′-GGCCAGAGGGTTGATTGACT-3′
TLR-2:	F 5′-TGCTGCCATTCTCATTCTTC-3′
	R 5′-GGGCCACTCCAGGTAGGT-3′
TLR-4:	F 5′-CCCTTTCAACTCTGCCTTCACT-3′
	R 5′-GGGACACCACGACAATAACTTTC-3′
TLR-9:	F 5′-GACTGGCTACCTGGCAAGAC-3′
	R 5′-GAAGCTGGCACGCAAGAG-3′
β-actin:	F 5′-CAAGGCCAACCGCGAGAAGATGAC-3′
	R 5′-GCCAGAGGCGTACAGGGACAGCA-3′
GAPDH:	F 5′-TGGCATGGCCTTCCGTGTCC-3′
	R 5′-GCCCTCCGATGCCTGCTTCAC-3′

Statistical data analysis

Data on blood parameters (day 0–98) and PBMCs (day 28 only) were analysed by means of Bayesian linear regression. To account for variation between horses, all models contained a random intercept for each horse. Similar to previous analyses (Vendrig et al., 2012), PBMC responses were allowed to vary between horses with regard to incubation with LPS (random slope). To stabilise the variance, all blood and PBMC data were log-transformed, except for data on haematocrit, which were analysed on the unit scale. For blood data, time was entered into the model as a continuous variable. To account for the sometimes non-linear association between time and blood data, time was transformed by means of fractional polynomials, such that the model best fitted the data. To minimise bias in blood data due to colostrum intake and colostral globulin content, parts of the data were excluded from analysis such as for β-2 and γ-globulins (up to day 42), IgA and IgM (up to day 28), IgG(a) (up to day 42), and IgG(b) and IgG(T) (up to day 56). Exclusion was based on visual inspection of the data, and was made such that at worst, estimates of the difference between the control and GOS group would be conservative.

For each data type, the best fitting fractional polynomials were determined in R (version 2.15.3; R Development Core Team, 2011), using the package mfp (version 1.4.9; Ambler and Benner, 2010), not yet accounting for variation between horses. Final models, including random intercepts and fractional polynomials, were analysed in JAGS, a program for analysis of Bayesian models using Markov Chain Monte Carlo (MCMC)

simulation (version 3.3.0). Simulations in JAGS were performed using R packages rjags (version 3-10; Plummer, 2011) and R2jags (version 0.03-08; Su, 2011). Posterior distributions were estimated based on uninformative prior distributions (normal distributions with mean 0 and standard deviation 100 for parameter means; inverse gamma distributions with mean 1 and variance 10,000 for variance of measurement error; uniform distributions with range 0 to 100 for standard deviation of random intercepts). Differences between control and GOS groups were stated significant, based on 95% Bayesian credible intervals (BCI), which were defined as the 2.5% and 97.5% percentiles of the posterior distributions. Posterior distributions were simulated by means of four Markov chains, each consisting of 10,000 Monte Carlo samples. The first 5,000 samples were discarded for burn-in, allowing the model to converge. Model convergence was assessed by means of Gelman and Rubin's convergence diagnostic, the potential scale reduction factor (Gelman and Rubin, 1992).

Results

Course of the study

During the 98 day period of investigation, no effects of the treatment on the apparent clinical health parameters were observed, neither in the GOS-treated foals nor in the control group. Eventually, two foals in the control group were excluded from the study. One foal was stillborn, and another foal was not treated on the first two days of life due to colic.

Blood parameters

Figures 1, 2, and 3 present the data on the investigated blood parameters in GOS-treated and control foals. None of the studied blood parameters differed significantly between the two groups of foals.

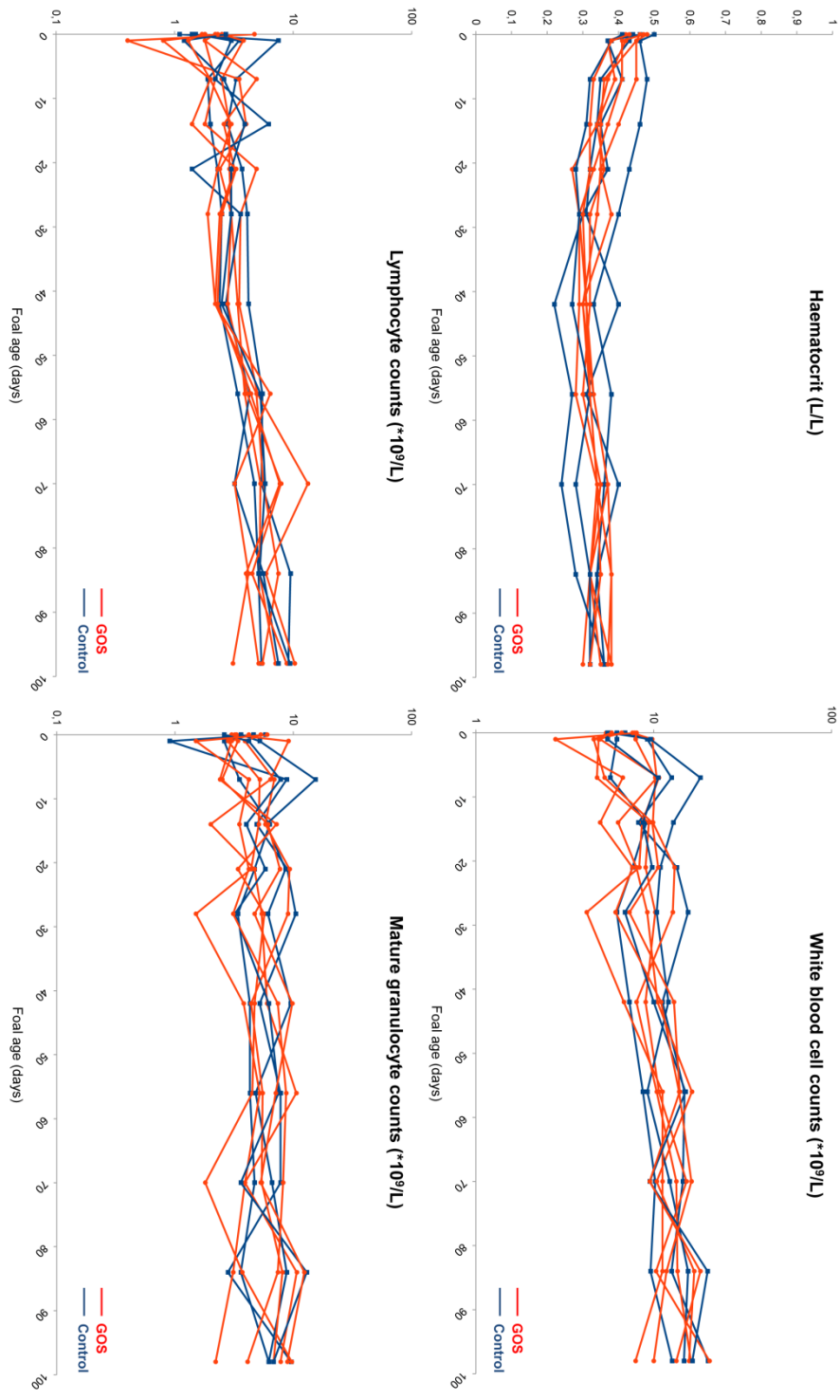

Figure 1: Parameters for red and white blood cells in individual foals, supplemented with GOS (in red) or glucose/lactose (in blue).

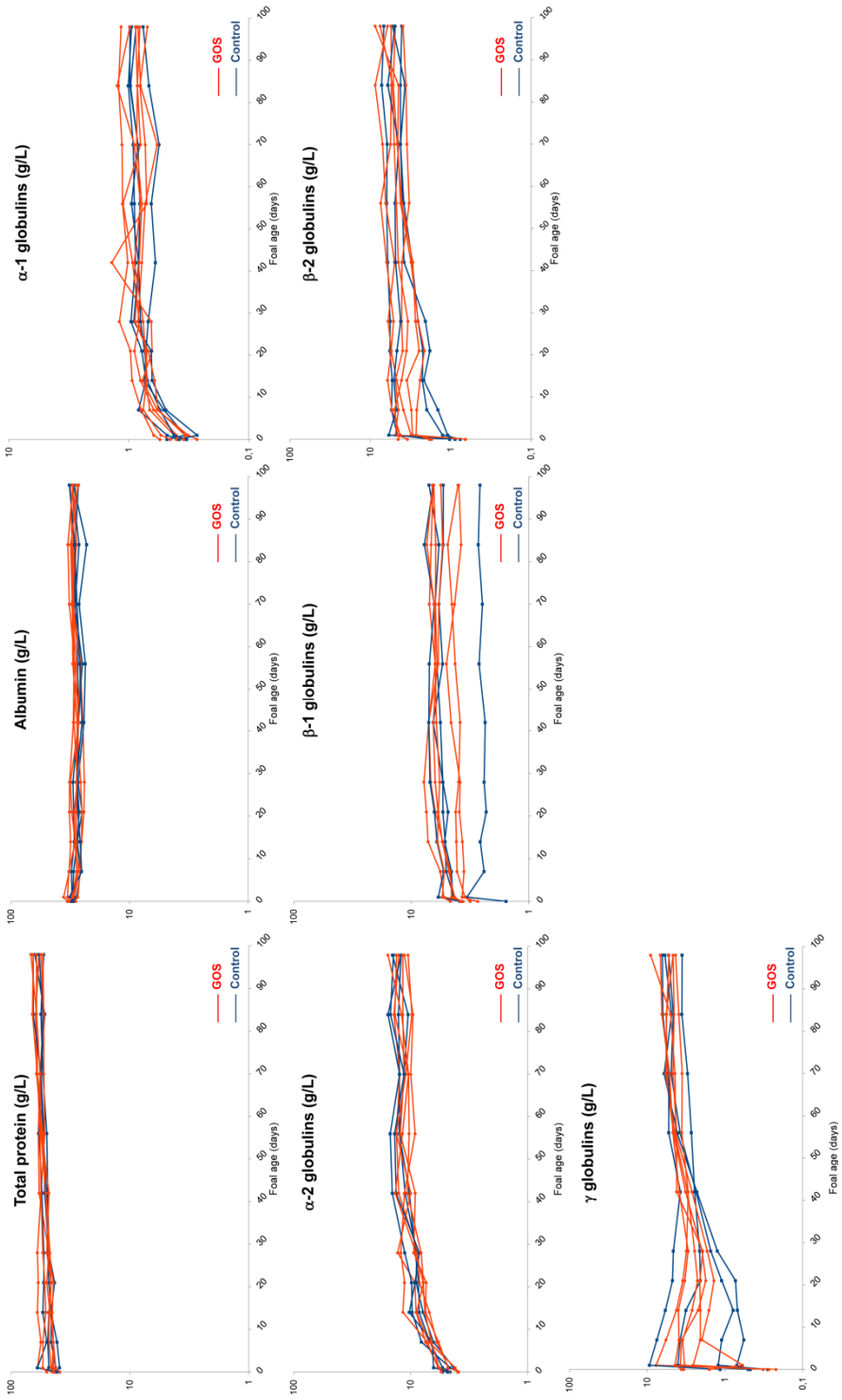

Figure 2: Results for total protein values and the protein electrophoresis in individual foals, supplemented with GOS (in red) or glucose/lactose (in blue).

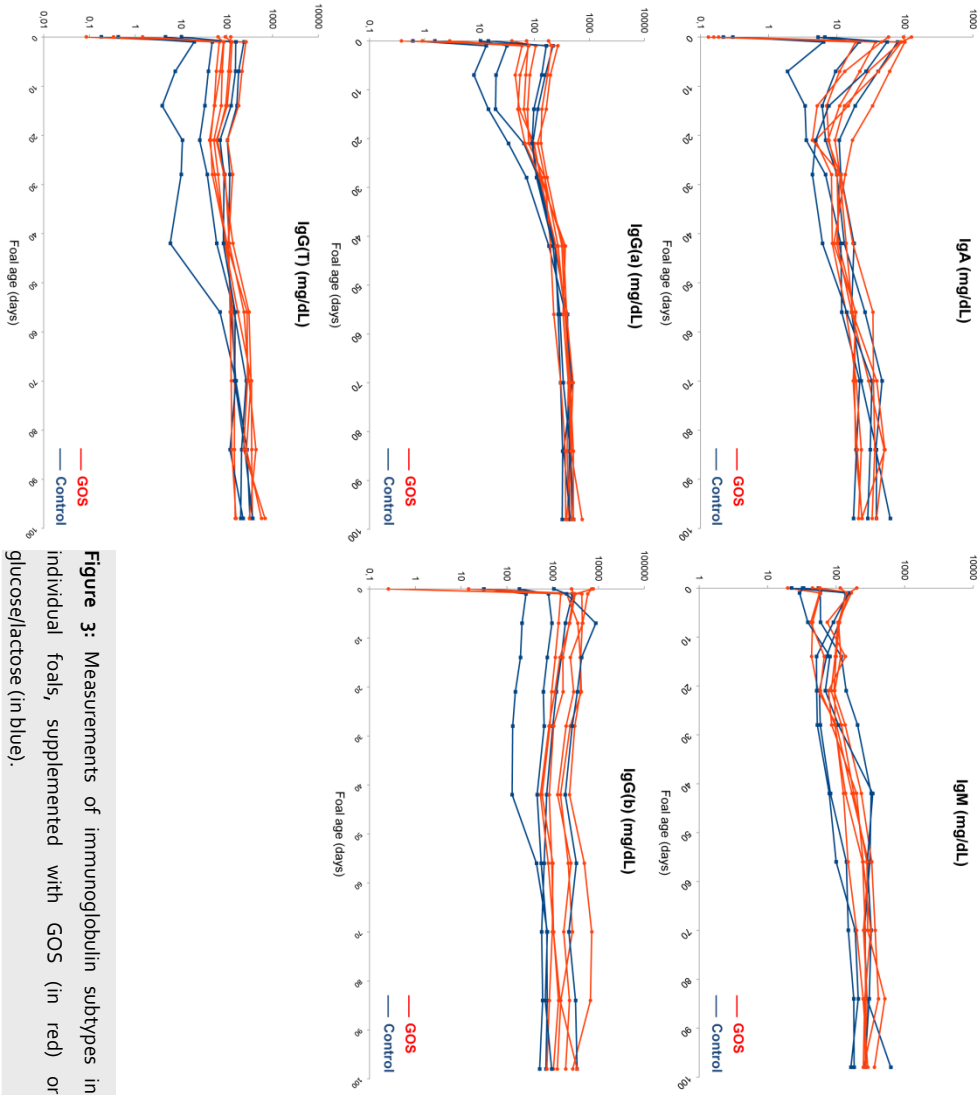

Figure 3: Measurements of immunoglobulin subtypes in individual foals, supplemented with GOS (in red) or glucose/lactose (in blue).

PBMC experiments

Figure 4 illustrates supernatant concentrations of TNF-α for unchallenged and LPS-challenged PBMCs. No significant difference in TNF-α production was observed between the two groups. The data for supernatant concentrations of IL-10 were mostly below the detection limit of the applied assay. Hence, analysis of these data was not possible.

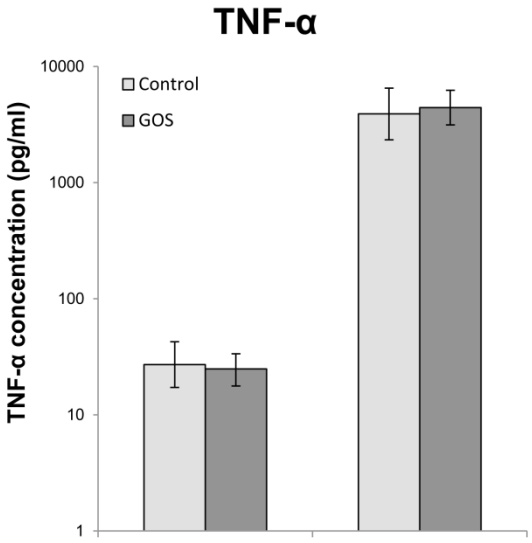

Figure 4: Mean supernatant TNF-α concentrations including 95% Bayesian credible intervals for unchallenged and LPS-challenged PBMCs, derived from GOS-treated and control foals.

Relative cytokine expression levels following LPS stimulation in PBMCs are presented in Figure 5. In both groups, *ex vivo* LPS stimulation resulted in a significant up-regulation of mRNA expression for all investigated cytokines, except IL-4 and TGF-β1. IL-4 expression was not significantly influenced by the LPS challenge in neither of the groups, and the expression of TGF-β1 was significantly down-regulated in both groups. For IFN-γ and IL-6, the relative mRNA expression levels following LPS challenge were significantly lower in PBMCs derived from GOS-treated foals compared with the control group (80% and 40% reduction for IFN-γ and IL-6, respectively). No other significant differences were detected among the two groups.

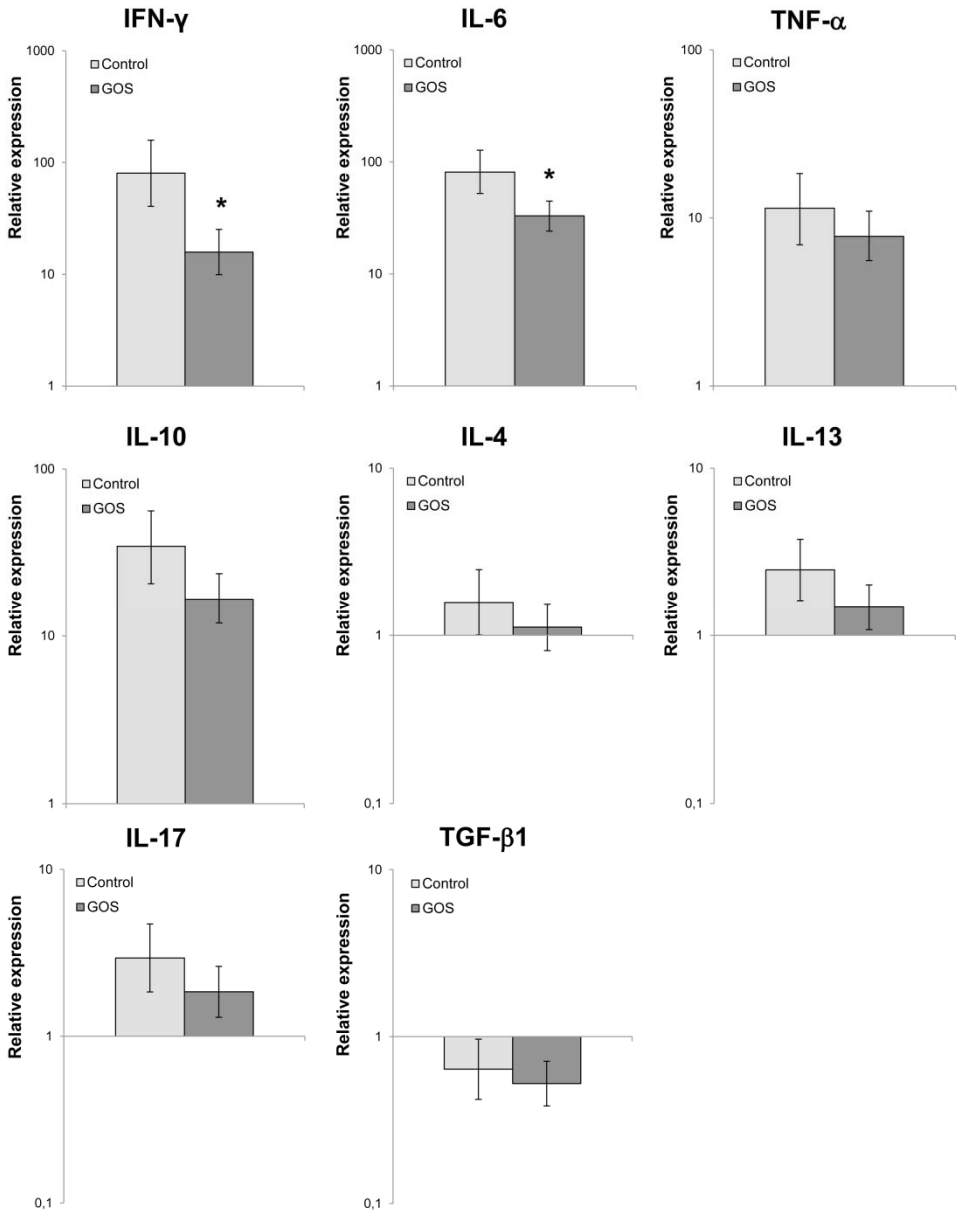

Figure 5: Mean relative cytokine mRNA expression levels including 95% Bayesian credible intervals in LPS-challenged PBMCs, relative to blank controls. Significant differences between PBMCs derived from GOS-treated or control foals are indicated with an asterisk (*).

Relative TLR expression levels in LPS-challenged PBMCs are illustrated in Figure 6. For both groups, LPS stimulation resulted in a significant increase of TLR-2 expression levels and a significant reduction of TLR-9 expression levels. Expression levels of TLR-4 were not significantly altered in neither of the groups. No significant differences in TLR expression were detected between PBMCs derived from GOS-treated or control foals.

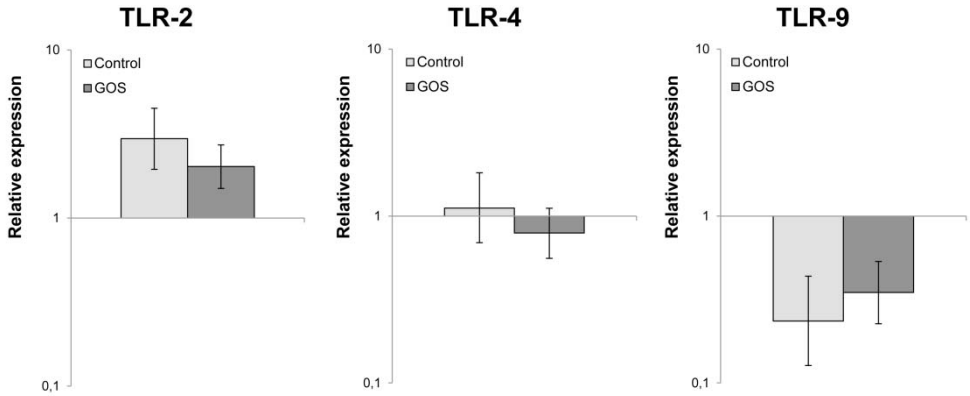

Figure 6: Mean relative TLR mRNA expression levels including 95% Bayesian credible intervals in LPS-challenged PBMCs, relative to blank controls. No significant differences were detected between PBMCs derived from GOS-treated or control foals.

Variation of mRNA expression could be excluded as a confounding factor, as no significant differences in either β-actin or GAPDH expression were detected amongst control samples and the incubations with LPS or amongst the two groups.

Discussion

Parameters for animal health and immunity

In this pilot study, we found that GOS was tolerated well by newborn foals, causing no observable side effects. Furthermore, we did not find any significant differences between GOS-treated and control foals with regard to the investigated blood parameters. The serum globulin contents varied highly during the first weeks of life, most likely due to differences in the uptake of maternal globulins by individual foals. This was not caused by insufficient quality of the mares' colostrum, as the colostral immunoglobulin levels for all mares exceeded the minimal quality standards for equine colostrum (for instance 3000 mg/dl for IgG (Reed et al., 2010)). Moreover, the colostrum of the dams of individual foals with relatively low serum globulin concentrations during the first weeks did not contain lower amounts of immunoglobulins relative to the colostrum of dams belonging to foals with high serum globulin concentrations. Despite variation in the level of maternally acquired immunoglobulins, all foals eventually reached comparable and sufficient immunoglobulin levels through endogenous production of immunoglobulins during the experimental period.

PBMC functionality

Our results show that the LPS-induced mRNA expression levels of IFN-γ and IL-6, which are both related to type 1 T helper cell (Th) responses, were significantly lower in PBMCs derived from GOS-treated foals compared with the control group. However, the LPS-induced production of TNF-α in PBMCs did not differ between the two groups; similarly, there was no significant difference in mRNA expression of TNF-α. These findings should be further investigated with a more extensive setup for PBMC experiments, including more horses, additional samples at different time points, and the investigation of more functional parameters.

Young foals have previously been shown to display lower expression levels of Th1 related cytokines, including IFN-γ and IL-6, compared with older individuals (Breathnach et al., 2006; Liu et al., 2009; Merant et al., 2009). Immature lymphocyte responses in neonates, with a bias towards Th2 responses, are thought to play a role in the increased susceptibility to infections in early life. Therefore, immunomodulation in young individuals is generally focused on the enhancement of defensive cytokine responses instead of reducing them (Diesner et al., 2012). On the other hand, a reduced immune response to bacteria that colonise epithelial surfaces, including the intestines, during the first period of life, is a prerequisite to establish bacterial colonisation and concurrently maintain homeostasis. Exaggerated immune responses to endogenous bacteria in the phase of colonisation would cause tissue damage and decreased epithelial barrier function, possibly leading to bacterial infections and sepsis. If indeed GOS treatment in

foals would result in a lower responsiveness of PBMCs to LPS (or other challenges), this could be an interesting finding with regard to the eventual implementation of oligosaccharide supplements in practice. Depending on the clinical context, next to disease prevention through enhancing defensive immune responses, the suppression of exaggerated cytokine responses to bacterial compounds could prevent pathology in early life, particularly in the gastrointestinal tract.

In addition, as beneficial effects of oligosaccharide supplements on allergic immune responses have been reported in other mammalian species (Moro et al., 2006; Vos et al., 2007b; Arslanoglu et al., 2008; van Hoffen et al., 2009; Yasuda et al., 2012), forthcoming research in horses could focus on potential long-term effects of orally applied oligosaccharides on the incidence and severity of relevant immune-mediated inflammatory diseases, such as recurrent airway obstruction, inflammatory airway disease, and insect hypersensitivity (summer eczema).

Conclusion

The results from this pilot study indicate that the chosen dose regimen of GOS was well accepted by the foals, and that no undesirable side effects were encountered. At the same time, no differences in the investigated haematological parameters, including serum immunoglobulins, could be detected between GOS-treated foals and the control group. GOS treatment appeared to reduce the pro-inflammatory responses in PBMCs following an *ex vivo* LPS challenge. With this knowledge, additional experiments with the GOS preparation can be performed in larger groups of foals, perhaps with the appliance of higher dosages and longer periods of treatment. Moreover, a longer follow up of GOS-treated foals may reveal long-term beneficial effects on the incidence and severity of immune-mediated inflammatory diseases, in line with published studies in other mammalian species.

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Chapter 10

Preliminary results: Identification and comparison of equine colostral oligosaccharides in four different horse breeds

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Abstract

The non-digestible carbohydrate fraction of colostrum, in particular the colostral oligosaccharide fraction, is known to exhibit prebiotic properties. Next to modulating effects on the composition of the intestinal microflora, there is increasing evidence for immunomodulatory properties of both naturally occurring and commercially synthesised oligosaccharides. As it is well established that the composition of oligosaccharides in colostrum is species specific, and equine colostrum has been reported to contain unique oligosaccharides, this study aimed to obtain an overview of the specific oligosaccharide patterns in equine colostrum. For this purpose, colostral samples of four different horse breeds were investigated. Besides the detection of three individual oligosaccharides, which are known as human milk oligosaccharides as well, we reported the occurrence of at least eight specific equine colostral oligosaccharides, which have not been identified before. Moreover, distinct oligosaccharide patterns in different horse breeds were observed, and variation of the relative amounts of specific oligosaccharides among the different breeds.

Introduction

Many beneficial properties have been ascribed to colostrum throughout published literature. During the past decades, there is increasing evidence for advantageous effects of the non-digestible carbohydrate fraction of colostrum, in particular the colostral oligosaccharides. Next to known prebiotic properties, modulating the composition of the intestinal microflora, there is increasing evidence for direct immunomodulatory properties of both naturally occurring and commercially synthesised oligosaccharides. Human milk-derived oligosaccharides and plant-derived oligosaccharides (low-molecular-weight fucoidan) have been reported to affect the cytokine production and activation of unchallenged cord blood derived T cells *ex vivo* (Eiwegger et al., 2004; Eiwegger et al., 2010). Bovine colostrum has been shown to modulate immune responses in several *in vitro* models (Biswas et al., 2007; An et al., 2009; Jenny et al., 2010; Jorgensen et al., 2010). We have previously documented suppressive effects of equine colostral carbohydrates on the lipopolysaccharide-induced inflammatory response in equine peripheral blood mononuclear cells *ex vivo* (Vendrig et al., 2012).

The composition of oligosaccharides in colostrum is known to be species specific (Urashima et al., 2001; Fukuda et al., 2010), and previously published studies have documented the presence of unique oligosaccharides in equine colostrum next to oligosaccharides, which are also present in milk or colostrum of other species (Urashima et al., 1989; Urashima et al., 1991; Nakamura et al., 2001; Urashima et al., 2001). The present study aimed to obtain an overview of the specific oligosaccharide patterns in equine colostrum. Moreover, differences in occurrence and quantity of specific equine colostral oligosaccharides (ECOs) were investigated among four different horse breeds.

Materials and Methods

Colostrum samples were collected within 12 hours postpartum, from four different horse breeds: Dutch Warmblood horses (K.W.P.N.), Shetland ponies, Crossbred Arabian/New Forest ponies (N.R.P.S.) and Friesian horses. Oligosaccharides were extracted according to Nakamura et al (2001). After removal of the excess of lactose using Size Exclusion Chromatography, the Equine Colostral Oligosaccharides (ECOs) were labelled with a fluorescent label (APTS) and analysed by means of Capillary Electrophoresis (CE) with Laser Induced Fluorescence (LIF) detection as reported by Albrecht et al (Albrecht et al., 2011).

Results and discussion

The CE-LIF electropherograms of colostral extracts are shown in Figure 1, with one representative sample for each breed (differences within breeds will be discussed in the full manuscript, which is in preparation for publication). A detailed profile of major and minor peaks was obtained within a separation window of 5.3 and 7.5 minutes. The peak marked with a hash symbol (#) represents the internal standard and mobility marker xylose. At least 15-20 different peaks can be recognised in the different ECO samples. Up to now, we have identified 11 peaks by means of CE-mass spectrometry (CE-MS) and Hydrophilic Interaction Liquid Chromatography-MS, using available standards. The detected peaks are illustrated and numbered in Figure 1, and the concerning structures are presented in Figure 2.

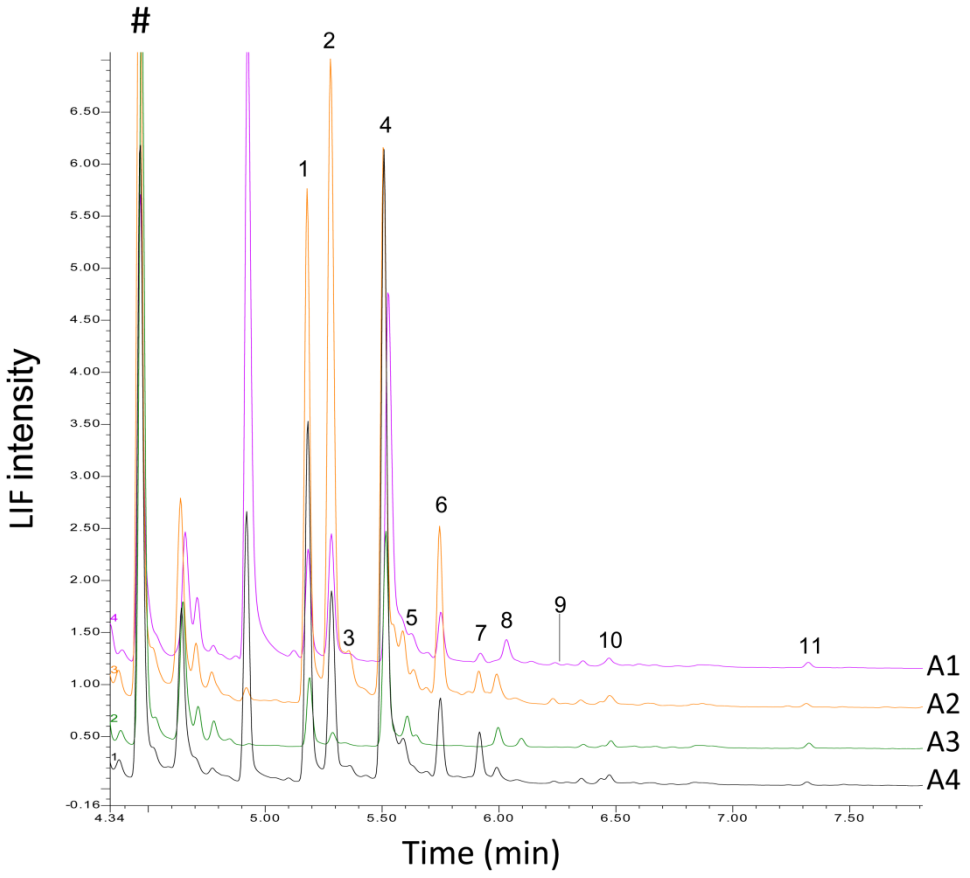
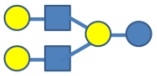
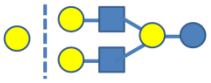


Figure 1: CE-LIF electropherograms of APTS-derivatised oligosaccharides extracted from horse colostrum. A1: Dutch Warmblood horse (K.W.P.N.), A2: Shetland pony, A3: Crossbred Arabian/New Forest pony (N.R.P.S.), A4: Friesian horse. 1-11: peaks identified by CE-MS (Table 1). #: Internal standard xylose.

Peak CE-LIF (Figure 1)	Name	Structure
#	Xylose	#
1	Lactose	
2	Hex-NAc-Hex	
3	Hex-NAc-Hex isomer	
4	3-Sialyllactose	
5	4-Galactosyllactose	
6	Galactosyllactose isomer	
7	Hex-NAc-Hex-Hex	
8	Disialyllactose	
9	Sialyllacto-N-tetraose	
10	Lacto-N-pentaose	
11	Lacto-N-hexaose	

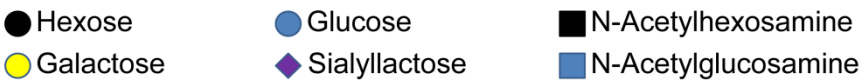


Figure 2: Overview of structures detected in CE-MS of equine colostral oligosaccharide extracts.

In the ECO sample of the Dutch Warmblood horse (A1 in Figure 1), both acidic and neutral oligosaccharides were identified. Next to the lactose (peak 1 in Figure 1), seven other neutral oligosaccharides were identified. Peak 2 and 3 (Figure 1) represent isomers of a dimer containing a Hexaose and an N-Acetyl-Hexosamine unit. In addition, three different trimers were identified: 4-Galactosyllactose, an isomer of galactosyllactose and HexNAc-Hex-Hex (Figure 1, peak 5, 6, 7 respectively; Hex-Hexose; HexNAc-N-Acetylhexosamine). Lacto-N-pentaose and Lacto-N-hexaose, respectively a pentamer and a hexamer, were also identified (Peak 10 and 11 in Figure 1). Three acidic oligosaccharides were detected in the Dutch Warmblood horse sample: 3-Sialyllactose, Di-sialyllactose and Sialyllacto-N-tetraose (Figure 1, peak 4, 9, 12).

Having a closer look at the ECO profiles from different breeds, differences were observed concerning both the presence and relative abundance of the different oligosaccharides. The 3-Sialyllactose was one of the most abundant oligosaccharides in all four horse breeds, although it was present in different levels depending on the breed. Colostrum derived from Friesian horses (A4) contained the highest amounts of 3-Sialyllactose, while NRPS pony colostrum (A3) contained the lowest amounts. Sialylated oligosaccharides are reported to have a wide spectrum of bioactivity (Devaraj et al., 1994; Stins et al., 1994). Following 3-Sialyllactose, Galactosyllactose (peak 6) was the second most abundant oligomer in the samples of all tested breeds, except for the NRPS pony sample (A3), in which it was not present. Similar observations were done for peak 3, 6, and 8 (Figure 1). While these three oligomers were not present in the NRPS pony sample, an additional compound could be recognised in the colostrum sample (Figure 1, time 6.1 min). A component migrating at 6.0 minutes (not identified yet) is present in colostrum derived from all horse breeds except for the Dutch Warmblood horse. From the same figure, it is also clear that the relative amounts of the different oligosaccharides varied significantly for the different breeds. Interestingly, three of the detected ECOs are known to be present in human milk and colostrum as well: Lacto-N-Hexaose, 3-Sialyllactose, and Sialyllacto-N-tetraose. Of the other oligosaccharides, which were detected, only a part was identified before in equine milk or colostrum (Urashima et al., 1989; Urashima et al., 1991; Urashima et al., 2001).

Conclusion

These preliminary results demonstrate that equine colostrum contains a variety of oligosaccharides, of which only a part was identified before in equine milk or colostrum. In addition to the previously identified structures, we now report the presence of HexNAc-Hex, HexNAc-Hex isomer, 4-Galactosyllactose, HexNAc-Hex-Hex, Disialyllactose, Sialyllacto-N-tetraose, Lacto-N-pentaose and Lacto-N-hexaose. Identification of other (unknown) ECOs is ongoing. To our knowledge, this is the first report of distinct oligosaccharide patterns in different horse breeds, and variation of the relative amounts of specific oligosaccharides amongst different breeds.

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Chapter 11

General discussion

Introduction

The described research in this thesis aimed to provide further insight into options for immunomodulation in the horse, particularly in the foal. Foals are born with a very incomplete protection by maternal antibodies and are strongly dependent on the colostral uptake of immunoglobulins. The foal's immune system is challenged within the first hours of life by numerous bacterial antigens, including bacteria that colonise the intestines. The initial phase of intestinal colonisation with the endogenous microflora is considered to be one of the most important factors to control immune homeostasis and immune competence, not only in the neonate, but also in later phases of life (Martin et al., 2010; Vyas and Ranganathan, 2012). The presented investigations focused on the recognition of bacterial antigens by TLR-2 and TLR-4, and subsequent induced immune responses. TLRs are a crucial link between innate and adaptive immune responses, regulating both tolerance to commensal bacterial flora as well as defensive immune responses to potentially harmful pathogens. Excessive activation of TLRs results in inflammatory responses and related pathologies.

The primary colonisation of the intestines is suggested to be influenced by constituents of colostrum and milk, particularly non-digestible carbohydrates. Such complex carbohydrates may act as decoy binding sites for pathogens while at the same time serve as prebiotics facilitating the enrichment of beneficial commensal bacteria (Westerbeek et al., 2006; Newburg, 2009; Rijnierse et al., 2011). Conventionally, equine colostrum is mainly regarded as an essential source of maternal antibodies, protecting the newborn in the first phase of life, until its own immune system is able to provide sufficient amounts of immunoglobulins. However, the additional role of colostral oligosaccharides could to be of equal importance, influencing the early immune tolerance to the intestinal microbiota and priming the immune system for later stages in life.

The aim of the current investigations was to contribute to the understanding of the effects of various ligands for TLR-2 and TLR-4 receptors in terms of inflammatory responses, measured by cytokine expression and release profiles, and to identify a potential crosstalk between TLRs, as TLR-2 recognises mainly cell surface markers of Gram-positive bacteria, while TLR-4 is activated by lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria. For the assessment of TLR responses prototypical ligands such as Pam3-Cys-Ser-Lys4 (PCSK; a synthetic specific TLR-2 agonist) and LPS (specific for TLR-4) were used as well as natural colostrum from horses, and defined fractions of commercially synthesised oligosaccharides, derived from natural products. .

While a considerable amount of experimental models and clinical data are available from laboratory animal species and even human infants, the published literature concerning this topic in the horse is nearly absent. Moreover, there is a lack of established equine *in vitro* models, allowing the rapid assessment of individual substances that are

candidates for immunomodulatory intervention. Hence, in our first approaches to study immunomodulation in the horse, we mainly used *in vitro* models with immune cells derived from adult horses as a proof of principle. While developing research models with *ex vivo* cultured equine immune cells, we chose to investigate some specific effects of the compounds of interest in established mouse models as well. The data derived from the murine models (included in **Chapters 3 and 4**) together with the evaluation of previously published findings and our preliminary results with several equine models (amongst others described in **Chapter 5**), led to more extensive experiments into immunomodulation in the horse with the use of equine peripheral blood mononuclear cells (**Chapters 6, 7, 8, and 9**). In these studies not only PBMCs obtained from adult horses were included, but also cells derived from neonatal foals to assess possible differences associated with an incompletely developed immune system. Sampling of neonatal foals is restricted due to both ethical considerations and a limited availability of neonatal foals for research purposes, which explains the limited number of animals that could be involved in the presented studies. Next to the fundamental studies *in vitro*, we set up an *in vivo* experiment in foals as a pilot study to assess the effects of dietary galacto-oligosaccharides on diagnostic parameters of the developing immune system. (**Chapter 9**).

Main findings of this thesis

The main findings of this thesis can be summarised as follows:

- In both equine and murine models, activation of TLR-2 and TLR-4 with PCSK and LPS, respectively, resulted in an induction of pro-inflammatory cytokine responses. TLR-4 activation resulted in a marked induction of pro-inflammatory cytokine responses, whereas activation of TLR-2 led to a mild pro-inflammatory response (**Chapter 3, 4, 5, and 6**).
- Concomitant incubation with the TLR-2 ligand PCSK did not influence the LPS-induced pro-inflammatory response in murine intestinal epithelial cells or equine peripheral blood mononuclear cells (PBMCs), derived from neonatal foals and adult horses (**Chapters 4 and 6**).
- Following TLR-2 and TLR-4 activation, relatively high TLR mRNA expression levels with concurrently lower functional cytokine responses were found in PBMCs derived from neonatal foals compared with PBMCs derived from adult horses (**Chapter 6**).
- In equine peritoneal macrophages and PBMCs, activation of either TLR-2 or TLR-4 resulted in up-regulation of TLR-2 mRNA expression levels, whereas TLR-4 mRNA expression levels remained unchanged, and TLR-9 mRNA expression levels were down-regulated (**Chapters 5, 6, and 9**).
- Equine colostral carbohydrates dose-dependently suppressed the LPS-induced inflammatory response in murine intestinal epithelial cells and equine PBMCs (**Chapters 4 and 7**).
- Both galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) dose-dependently enhanced the LPS-induced cytokine response in equine PBMCs, whilst acidic oligosaccharides (AOS) dose-dependently suppressed the LPS response (**Chapter 8**).
- *In vivo* experiments in foals provided no convincing evidence that within the period of investigation the chosen dose regimen of GOS influenced clinical and blood parameters indicative for general health and immune status in the included group of foals. In PBMCs derived from GOS-treated foals after four weeks of treatment, a standardised LPS challenge resulted in significantly lower relative mRNA expression levels of the pro-inflammatory cytokines interferon-γ and interleukin-6 compared with PBMCs of control foals (**Chapter 9**).
- Preliminary results concerning the structural analysis of equine colostral oligosaccharides in four different horse breeds revealed distinct oligosaccharide patterns in horses and in different horse breeds. Moreover, specific oligosaccharides were detected, which have not been identified up to now (**Chapter 10**).

IN VITRO EFFECTS OF KNOWN TLR AGONISTS

The LPS-induced inflammatory response

LPS, derived from the cell wall of Gram-negative bacteria, is the most widely applied substance to activate TLR-4 and has been used in virtually all animal species as well as in experiments with human cells or tissues. As expected, our presented data derived from all included *in vitro* models consistently demonstrated marked pro-inflammatory responses as a result of LPS-mediated TLR-4 activation (**Chapters 3, 4, 5, 6, 7, 8, and 9**). Hence, valid investigations into significant modulatory effects of the compounds of interest on LPS-induced inflammatory responses were possible. The translation of direct modulation of LPS-induced inflammation *in vitro* into the clinical significance of such modulatory effects *in vivo*, either enhancing or mitigating the effects of LPS, remains a challenge.

Robust TLR-4 activation is critical for functional defence mechanisms against Gram-negative pathogens, including the initiation of adaptive immune responses (Testro and Visvanathan, 2009; Kawai and Akira, 2010; Noreen et al., 2012). On the other hand, unnecessary TLR-4 activation in response to endogenous bacteria could easily lead to inflammation and subsequent pathology (Testro and Visvanathan, 2009; Olivares et al., 2013).Therefore, positive and negative modulators of LPS-induced immune responses could indeed be promising candidates for immunomodulation *in vivo*, depending on the clinical context (Gioannini and Weiss, 2007; Diesner et al., 2012; Paul-Clark et al., 2012).

Modulators of TLR-2 signalling

In the included studies, PCSK is used to activate TLR-2. PCSK is a synthetic triacylated lipopeptide, which is recognised by TLR-2 in concert with TLR-1, or independent of TLR-1 (Buwitt-Beckmann et al., 2005; Buwitt-Beckmann et al., 2006). TLR-2 activation by PCSK has been confirmed throughout the published literature, and immunomodulating effects of PCSK were described in experimental animals *in vivo* (Cario et al., 2004; Cario et al., 2007). In our *in vitro* models, using monocultures of murine intestinal epithelial cells and PBMCs derived from newborn and adult horses, PCSK induced mild inflammatory responses, but did not influence LPS-induced immune responses. This could be due to indirect effects of PCSK in the *in vivo* studies on targets, which were not present in our model. For instance, a crosstalk between intestinal epithelial cells and immune cells residing in the gastrointestinal epithelial barrier could be of great importance. Future experiments with co-cultures of intestinal epithelial cells and immune cells, such as dendritic cells or PBMCs, could elucidate indirect immunomodulatory effects of PCSK *in vitro.*

Additional experiments into immunomodulation by other TLR-2 agonists than PCSK, including diacylated lipopeptides, which interact with TLR-2 and TLR-6, could reveal distinct properties of different TLR-2 agonists (Palsson-McDermott and O'Neill,

2007). Furthermore, as TLR-2 function is influenced by several other co-molecules and receptors, such as CD-14, scavenger receptors, and integrins, a broad range of additional targets can be identified as potential modulators of TLR-2 function and concerning immune responses (van Bergenhenegouwen et al., 2013).

TLR expression and signalling in neonates

In **Chapter 6**, effects of separate and concomitant incubation with LPS and PCSK were investigated in PBMCs derived from adult horses and newborn foals. The observed lower amplitudes of cytokine responses in neonates following bacterial challenge were in accordance with previously published studies (Adkins et al., 2004; Levy. 2007; Liu et al., 2009). In contrast, basal TLR mRNA expression levels tended to be higher in foal PBMCs than in adult PBMCs. These findings suggest that TLR signalling is impaired in newborn foals compared with adult horses. Moreover, our data illustrate that in foals, TLR mRNA expression levels were not influenced by a challenge with specific ligands, representing bacterial patterns of either Gram-negative or Gram-positive bacteria, in contrast to the situation in adults. Such a limited TLR function is essential in neonates, as the transition from the sterile womb into an environment full of micro-organisms and the concurrent colonisation of epithelial surfaces would lead to exaggerated inflammatory responses and subsequent pathology, if all TLR signalling pathways were fully operational. The relatively high TLR expression levels, which were found in foals, might facilitate more profound defensive responses towards specific pathogens, if necessary. For instance, *Rhodococcus equi* is a specific pathogen that elicits mature defensive responses in young foals (Jacks et al., 2007; Liu et al., 2011). The challenge for future research in this area is to find methods or ligands that enhance TLR functionality and bacterial defence mechanisms in young foals without interfering with the physiological process of bacterial tolerance and colonisation.

Crosstalk amongst TLR-2, TLR-4, and TLR-9

Our qPCR results demonstrate evident TLR crosstalk in both murine and equine models. In equine peritoneal macrophages and PBMCs, activation of either TLR-2 or TLR-4 resulted in up-regulation of TLR-2 mRNA expression levels and down-regulation of TLR-9 mRNA expression levels (**Chapters 5, 6, 9**). In contrast, in murine macrophages, increased mRNA expression levels of both TLR-2 and TLR-9 were found as a result of incubation with LPS or PCSK (**Chapter 3**). The latter results are in line with previously published results in murine dendritic cells, documenting up-regulation of TLR-2 and TLR-9 following LPS treatment (An et al., 2002). To our knowledge, no comparable studies are published in the horse. One study in horses demonstrated an up-regulation of TLR-9 mRNA expression in horse lungs as a result of LPS exposure (Schneberger et al., 2009). Hence, the consistent down-regulation of TLR-9 mRNA expression levels following TLR-2

and TLR-4 activation in our *in vitro* studies with equine immune cells is a new, unexpected finding, which requires further investigation. The up-regulation of TLR-2 mRNA expression by both LPS and PCSK was evident in all studies presented in this thesis, whereas TLR-4 mRNA expression levels remained unchanged (**Chapters 5, 6, 9**) or were even down-regulated (**Chapter 3**). We hypothesised that the observed shifts in the TLR-2/TLR-4 balance following bacterial challenge, could be a protective mechanism. Aberrant TLR-4 activation would lead to unnecessary inflammation and tissue damage. The up-regulation of TLR-2 expression could initially lead to more activation of NFκB or MAPK pathways with a concurrent mild inflammatory response, eventually resulting in a suppression of TLR-mediated inflammatory responses. Regulation of LPS-induced TLR-2 and TLR-9 expression by means of interference with NFκB or MAPK pathways was documented previously in murine dendritic cells (An et al., 2002). This hypothesis concerning a regulatory function of TLR-2 supports the existing literature on beneficial effects of TLR-2 ligands, and may even contribute to the immunomodulatory effects of pre- and probiotics, as bifidobacteria and lactic acid bacteria trigger TLR-2 as well.

Effects of defined oligosaccharide fractions

Distinct direct immunomodulatory effects of GOS, FOS, and AOS in equine PBMCs

Previously published research into oligosaccharide fractions mainly comprises *in vivo* studies aiming to identify beneficial effects of dietary oligosaccharides in experimental animals and humans. The documented immunomodulatory effects of dietary oligosaccharides have most often been ascribed to their prebiotic properties. However, our experiments with equine PBMCs support the hypothesis of direct modulatory effects of oligosaccharides, independent of the intestinal microbiota. These findings are in line with previously conducted experiments in human immune cells *ex vivo* (Eiwegger et al., 2004; Eiwegger et al., 2010). In **Chapter 8**, we demonstrated that the LPS-induced cytokine response in equine PBMCs was dose-dependently enhanced by both galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). In contrast, acidic oligosaccharides (AOS) were shown to dose-dependently suppress LPS-induced inflammation.

Though there is increasing evidence of direct immunomodulatory properties of oligosaccharides, the exact mechanisms of action remain to be elucidated. Carbohydrate (glycan)- binding receptors expressed on the surface of intestinal epithelial cells and antigen presenting cells may be involved in modulation of the immune response by sugar molecules (de Kivit et al., 2011). In addition, oligosaccharides may directly modulate TLR-signalling, next to the indirect modulation of TLR signalling in the gastrointestinal tract due to the changes in the gut flora. For instance, hyaluronan oligosaccharides have been shown to directly mediate inflammatory responses via TLR-4 (Termeer et al., 2002;

Campo et al., 2012). Recently, direct modulation of TLR-2 signalling by wheatgrass derived oligosaccharides was documented as well (Tsai et al., 2013).

As of yet, research with oligosaccharides mainly involved direct intervention studies in human infants or in experimental animal species as surrogates for human infants (Moro et al., 2006; Arslanoglu et al., 2007; Vos et al., 2007a; Vos et al., 2007b; Arslanoglu et al., 2008; van Hoffen et al., 2009; Westerbeek et al., 2010; Gopalakrishnan et al., 2012; Schijf et al., 2012; Yasuda et al., 2012). One *in vivo* study in adult horses demonstrated beneficial effects of prebiotic short-chain fructo-oligosaccharides, preventing disruption of the intestinal flora in the equine hindgut in case of an abrupt change of diet composition (Respondek et al., 2008). Concerning immunomodulation by oligosaccharides in the horse, no data have been published to our knowledge. The described results in **Chapter 8** confirm the activity of heterologous oligosaccharides in horses and are in line with the very few published *in vitro* studies with human cell culture models (Eiwegger et al., 2004; Eiwegger et al., 2010).

Effects of dietary supplementation with GOS in foals

The pilot study described in **Chapter 9** is a first approach to investigate effects of an orally applied defined GOS fraction in foals during the first period of life. Though we did not find evidence for beneficial effects of GOS on parameters for general health and immunity in this group of foals, we can state that the chosen dose regimen of GOS was well accepted by the foals, and that we did not encounter any undesirable side-effects. The acquired results from the experiments with PBMCs, derived from each foal after four weeks of treatment, suggested that GOS treatment reduced pro-inflammatory responses in PBMCs following LPS challenge.

Young foals have previously been shown to display lower expression levels of Th1 related cytokines, including IFN-γ and IL-6, compared with older individuals (Breathnach et al., 2006; Liu et al., 2009; Merant et al., 2009). Immature lymphocyte responses in neonates, with a bias towards Th2 responses, are thought to play a role in the increased susceptibility to infections in early life. Therefore, immunomodulation in young individuals is generally focused on enhancement of defensive cytokine responses instead of reducing them (Diesner et al., 2012). On the other hand, reduced immune responses to endogenous bacteria during the first period of life are a prerequisite to allow bacterial colonisation of epithelial surfaces. Exaggerated immune responses to endogenous bacteria in the phase of developing bacterial tolerance would cause tissue damage and decreased epithelial barrier function, possibly leading to bacterial infections and sepsis. If indeed GOS treatment in foals would result in lower responsiveness PBMCs to LPS (or other challenges), this could be an interesting finding with regard to the eventual implementation of oligosaccharide supplements in practice. Depending on the clinical context, next to disease prevention through enhancing defensive immune responses, the

suppression of exaggerated cytokine responses to bacterial compounds could prevent pathology in early life, particularly in the gastrointestinal tract.

Moreover, as beneficial effects of oligosaccharide supplements on allergic immune responses have been reported in other mammalian species (Moro et al., 2006; Vos et al., 2007b; Arslanoglu et al., 2008; van Hoffen et al., 2009; Yasuda et al., 2012), forthcoming research in horses could focus on potentially long-term effects of orally applied oligosaccharides on the incidence and severity of relevant immune-mediated inflammatory diseases, such as recurrent airway obstruction, inflammatory airway disease, and insect hypersensitivity (summer eczema).

Equine colostral carbohydrates as promising candidates for immunomodulation

Suppression of LPS-induced inflammatory responses in vitro

Up to now, limited research has been conducted with equine colostrum, despite the known immunomodulatory effects of colostrum of other species (Biswas et al., 2007; An et al., 2009; Jenny et al., 2010; Jorgensen et al., 2010) and the detection of unique oligosaccharide patterns in horse colostrum (Urashima et al., 1989; Urashima et al., 1991; Nakamura et al., 2001) In **Chapter 7**, we demonstrated that the carbohydrate fraction of equine colostrum dose-dependently suppressed the LPS-induced inflammatory response in equine PBMCs. As we expect that the oligosaccharides in this equine colostral carbohydrate fraction are mainly responsible for the documented immunomodulatory effects, our research focuses on the structural analysis of specific equine colostral oligosaccharides, as it is well established the composition of oligosaccharides in colostrum is species specific.

Preliminary results: distinct colostral oligosaccharide patterns in the horse

Our preliminary results concerning the structural analysis of equine colostral oligosaccharides in four different horse breeds revealed that equine colostrum contains specific oligosaccharides, which have not been identified up to now. Next to the detection of unique equine oligosaccharides, which are promising candidates for future immunomodulation in horses and other mammals, distinct oligosaccharide patterns were observed amongst four different horse breeds (**Chapter 10**). This study is in progress, and a more detailed and extensive overview of these analyses will be provided shortly in a manuscript, which is in preparation.

Recommendations for future research

Species-specific research
Improvement of gut health and immunity in foals is of increasing relevance in terms of disease prevention and animal welfare. We have demonstrated that previously documented effects of immunomodulatory agents in other species cannot easily be translated to the actual effects in horses. Moreover, there is a limited availability of published research into this topic in equines. Hence, in general, there is a need for further investigations into immunomodulatory agents with the use of equine experimental models, both *in vitro* and *in vivo*.

Dietary oligosaccharide supplementation in foals
Taking into account the results from our *in vitro* experiments and the pilot study into the effects of oligosaccharides, combined with the present load of evidence for beneficial effects of oligosaccharides in other species, oligosaccharide supplements are promising candidates to improve the immunocompetence in foals. With the current knowledge, several defined oligosaccharides could be tested *in vivo* in foals. With regard to GOS, we recommend additional experiments in larger groups of foals with longer periods of follow-up. Moreover, different dose regimens need to be investigated, as perhaps a higher dosage of GOS could induce more apparent effects *in vivo.* In the follow-up of foals, which were treated with oligosaccharides, it would be interesting to assess possible effects of treatment on immunoglobulin responses following vaccination as well.

Dietary oligosaccharide supplementation in adult horses
Oligosaccharide supplements have been proven to alter allergic immune responses in other mammals beneficially. Forthcoming research in horses could focus on potentially long-term effects of orally applied oligosaccharides on the incidence and severity of relevant immune-mediated inflammatory disorders in horses, such as recurrent airway obstruction, inflammatory airway disease, and insect hypersensitivity (summer eczema).

Fundamental research into oligosaccharides
As a mechanism-based application of dietary supplements or preventive medicine is required to optimise the chosen constituents for specific individuals or groups, continuous research into underlying mechanisms of immunomodulatory agents is a prerequisite. Evaluating our *in vitro* work, additional experiments with longer incubation periods, sample collection at multiple time points, and determination of additional (functional) parameters could help to elucidate underlying mechanisms. Further mechanistical studies could confirm the increasing evidence of direct modulation of TLRs by oligosaccharides, for example by blocking specific TLRs or TLR signalling pathways concurrently.

Immunomodulatory effects of specific equine oligosaccharides
After a characterisation of specific equine oligosaccharides, derived from horse colostrum and milk, further experiments will focus on the immunomodulatory properties of specific equine oligosaccharide fractions. As we have identified unique oligosaccharides, which haven't been described up to know, forthcoming research may reveal new agents with high immunomodulatory potential in both equines and other species.

TLR-2 agonists
Our experiments with PCSK did not confirm its previously described beneficial effects in experimental animals *in vivo.* However, modulation of TLR-2 signalling and the TLR-2/TLR-4 balance in the gastrointestinal tract remains an interesting and relevant topic of investigation. The main reasons for continuous research into modulation of TLR-2 would be its importance in gut homeostasis and immunity, and the known beneficial effects of pre- and probiotics, which act as TLR-2 agonists as well (directly or indirectly). With regard to our *in vitro* work, we would recommend additional investigations into immunomodulatory potency of PCSK and other TLR-2 mediators, with the use of co-cultures of intestinal epithelial cells and immune cells, such as dendritic cells or PBMCs.

Conclusion

Our data provide the first evidence for efficacy of immunomodulatory agents in the horse, with positive future prospects for methods to improve immunocompetence in young foals. Our results concerning defined oligosaccharide fractions await further confirmation *in vivo*, as only one pilot study has been conducted in foals up to now. Specific and unique oligosaccharides, which were recently isolated from horse colostrum and milk, are promising future candidates for immunomodulation in horses and other species. Taken together, the different chapters in this thesis provide a valuable starting point for future research into the development of methods to improve immunocompetence in foals, in particular by modulating TLR signalling.

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Nederlandse samenvatting voor niet-ingewijden

Inleiding

Het doel van het onderzoek, dat beschreven is in dit proefschrift, is het ontwikkelen van methoden om de competentie van het immuunsysteem in veulens te verbeteren. De focus en nadruk ligt hierbij op bacteriële infectieziekten, waar het veulen met name gedurende de eerste maanden van het leven bijzonder gevoelig voor is. Behalve nieuwe ontwikkelingen op het gebied van therapie, is preventieve gezondheidszorg de laatste decennia steeds belangrijker geworden. Dit proefschrift beschrijft nieuwe invalshoeken die in de toekomst kunnen bijdragen aan de verbetering van gezondheid en welzijn in paarden, met als belangrijkste startpunt het veulen.

Context van het proefschrift

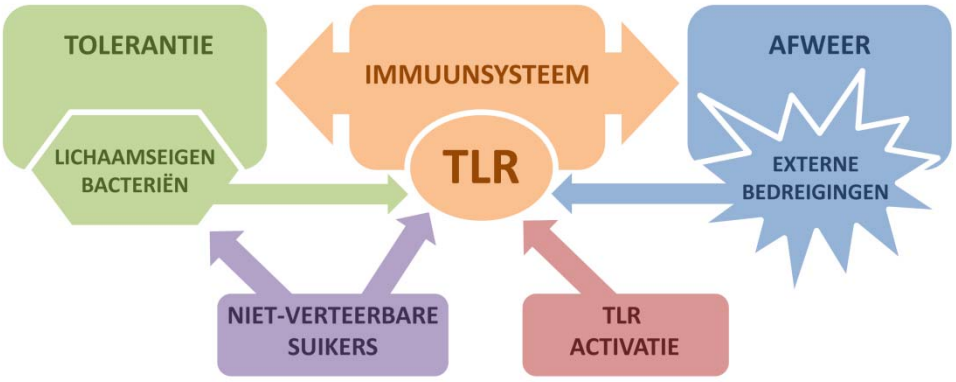
Mensen en dieren komen tijdens het leven voortdurend in aanraking met bacteriën. De aanwezigheid van lichaamseigen bacteriën in het maagdarmkanaal (en tevens op de huid en andere slijmvliezen), is belangrijk voor het goed functioneren van zoogdieren en hun algehele gezondheid. Aan de andere kant kunnen lichaamseigen bacteriën onder bepaalde omstandigheden voor problemen zorgen, bijvoorbeeld bij verstoringen van de barrièrefunctie van de darm, een afwijking in de balans tussen de verschillende darmbacteriën of een verstoorde functie van het immuunsysteem. Onder deze omstandigheden is het mogelijk dat normaal gesproken onschadelijke bacteriën het lichaam binnendringen en vervolgens wel ziekte veroorzaken. Ook kunnen bepaalde bacteriën vanuit de omgeving primair een bedreiging zijn voor de gezondheid van individuen. In het bijzonder in de eerste periode van het leven, als de kolonisatie van de darm door bacteriën plaats vindt en het immuunsysteem hierdoor beïnvloed wordt, kan een verstoring van deze processen gemakkelijk leiden tot *òf* een verminderde afweer tegen bacteriën *òf* overdreven ontstekingsreacties. Beide scenario's geven in de pasgeborene een verhoogd risico op het ontstaan van bacteriële infecties en sepsis, doordat bacteriën de kans krijgen om het lichaam binnen te dringen.

Het immuunsysteem heeft de belangrijke taak om lichaamseigen bacteriën te tolereren en tegelijkertijd voldoende afweer te bieden tegen bacteriën als dat nodig is, bijvoorbeeld bij primair bedreigende (pathogene) bacteriën of als lichaamseigen bacteriën dreigen problemen te geven. Cellen van het immuunsysteem en ook epitheelcellen op de oppervlakte van slijmvliezen en de huid kunnen bacteriën herkennen met behulp van zogenaamde '*Toll-like receptors*' (TLRs). Voor de herkenning van bacteriën zijn verschillende TLRs bekend, die geactiveerd kunnen worden als ze specifieke structuren van bacteriën tegenkomen. De functie van deze TLRs is bepalend voor de reactie van het immuunsysteem op bacteriën (zie tevens Figuur 1). Afwijkingen in het functioneren van

TLRs zijn dan ook in verband gebracht met ziekteprocessen, bijvoorbeeld bacteriële infecties en onstekingsprocessen in het maagdarmkanaal. Bovendien heeft onderzoek in proefdieren aangetoond dat het toedienen van synthetische stoffen die specifieke TLRs activeren, juist een vermindering kan geven van ontstekingsprocessen en sterfte.

Veulens zijn bijzonder vatbaar voor bacteriële infecties gedurende de eerste maanden van het leven. Net als bij andere dieren en ook mensen, is aangetoond dat het immuunsysteem van veulens in de eerste levensfase niet volledig ontwikkeld is en nog niet dezelfde afweerreactie kan geven tegen bacteriën als volwassen individuen. Bovendien is bij paarden- in tegenstelling tot andere diersoorten en de mens- de placenta niet of nauwelijks doordringbaar voor antilichamen van de moeder, waardoor het veulen vrijwel zonder antilichamen in het bloed wordt geboren. Aangezien het veulen zelf in de eerste maanden nog onvoldoende antilichamen kan produceren, is het volledig afhankelijk van de opname van voldoende antilichamen via de biest, de eerst gevormde melk van de merrie, op de eerste levensdag. Als veulens onvoldoende biest drinken of als de biest van onvoldoende kwaliteit is, zal dit zorgen voor een verminderde afweer van het veulen in de eerste maanden van het leven.

Behalve antilichamen blijkt de biest en moedermelk van zoogdieren ook andere stoffen te bevatten die essentieel zijn voor de gezondheid van de pasgeborene. In de afgelopen decennia is er veel onderzoek gedaan naar de suikerfractie van biest en melk, in het bijzonder naar oligosacchariden. Oligosacchariden zijn specifieke niet-verteerbare suikerstructuren die in hoge mate in biest en moedermelk aanwezig zijn. In mensen en proefdieren is aangetoond dat het toedienen van specifieke oligosacchariden aan pasgeborenen ervoor kan zorgen dat er een betere balans tot stand komt tussen de lichaamseigen bacteriën, met name in het maagdarmkanaal, en dat dit de afweer verbetert. Daarnaast heeft recent onderzoek het eerste bewijs geleverd voor een directe beïnvloeding van het immuunsysteem door specifieke oligosacchariden.



Figuur 1: Schematisch overzicht van de rol van '*Toll-like receptors*' (TLRs) in tolerantie en afweer en de ingangen voor beïnvloeding van het immuunsysteem, zoals voorgesteld in dit proefschrift.

In dit proefschrift worden methoden onderzocht om het immuunsysteem in veulens zo te beïnvloeden, dat de gevoeligheid voor bacteriële infecties en sepsis afneemt. Dit is van belang, omdat deze aandoeningen in jonge veulens nog regelmatig een slechte afloop hebben, ondanks optimalisatie van therapie- en managementprotocollen door paardenhouders en –dierenartsen. De eerder beschreven invalshoeken om het immuunsysteem te beïnvloeden zijn veelbelovend in andere diersoorten en de mens, maar tot nu toe is er nauwelijks onderzoek op dit gebied in paarden en/of veulens gedaan. Daarom bevat dit proefschrift zowel studies over stoffen die specifieke TLRs activeren als ook studies met biest van paarden en verschillende oligosacchariden. Behalve de indirecte beïnvloeding van TLRs door oligosacchariden door het effect op de samenstelling van lichaamseigen bacteriën, wordt ook de directe beïnvloeding van TLRs bediscussieerd als mogelijk werkingsmechanisme van oligosacchariden (zie tevens Figuur 1).

Kort overzicht van het proefschrift

In **hoofdstuk 1** van dit proefschrift worden het doel en de context van het proefschrift geschetst. **Hoofdstuk 2** geeft een overzicht van de afweermechanismen van het veulen tegen bacteriën, in het bijzonder toegespitst op de darmbarrière en de rol van TLRs hierin. Tevens worden potentiële methoden aangedragen om afweerreacties in het veulen te beïnvloeden, waaronder stoffen die specifieke TLRs beïnvloeden en oligosacchariden van natuurlijke oorsprong.

In verband met het gebrek aan bestaande laboratorium modellen voor het paard, hebben we ten behoeve van de ontwikkeling van een geschikt model voor onze eerste

studies in het paard, ook onderzoek gedaan in bestaande celkweek modellen voor de muis. De studies beschreven in de **hoofdstukken 3 en 4,** in respectievelijk immuuncellen en darmcellen van muizen, geven een indruk van de effecten van stoffen die specifieke TLRs activeren. Deze studies zijn mede de basis geweest voor de condities in de uiteindelijke experimenten met paardencellen. Tevens wordt in **hoofdstuk 4** aangetoond dat in darmcellen van muizen een in gang gezette ontstekingsreactie kan worden afgeremd door de cellen tegelijkertijd bloot te stellen aan de suikerfractie van paardenbiest. Het activeren van een specifieke TLR (TLR-2) met een synthetische stof gaf geen vermindering van de ontstekingsreactie in dit model, terwijl eerder beschreven is dat in levende muizen toediening van deze stof een gunstige invloed had op ontstekingsprocessen in de darm.

In de zoektocht naar een geschikt model om mechanistische studies te doen in ontstekingscellen van het paard, hebben we verschillende methoden onderzocht. **Hoofdstuk 5** beschrijft een van deze methoden, namelijk de isolatie van specifieke immuuncellen uit de buikholte van paarden. Na isolatie van deze ontstekingscellen en een optimalisatie van de kweekomstandigheden, hebben we onze eerste valide resultaten verkregen in het paard met betrekking tot stoffen die specifieke TLRs activeren. Met name omdat deze methode belastend is voor de paarden, alleen uitgevoerd kan worden onder algehele narcose, er een beperkt aantal cellen geoogst kan worden per experiment en de levensvatbaarheid van de cellen een beperkende factor is, hebben we deze methode niet toegepast voor uitgebreidere experimenten. De methode is wel bruikbaar voor specifieke vraagstukken bij het paard waar relatief weinig cellen nodig zijn (als '*proof of principle*').

De uitgebreidere mechanistische studies werden uitgevoerd in PBMCs, geïsoleerd uit het bloed van paarden (**hoofdstukken 6, 7, 8, en 9**). PBMCs, ofwel '*peripheral blood mononuclear* cells', zijn een selectie van witte bloedcellen die belangrijke functies hebben binnen het immuunsysteem. Dit model met PBMCs hebben we gekozen als beschikbare en haalbare methode voor valide studies in het paard en het veulen, aangezien ethisch gezien de bloedafname en het relatief kleine bloedvolume dat nodig was opwoog tegen de bewijsvoering die verkregen kon worden middels de experimenten. Na het optimaliseren van de kweekomstandigheden en het standaardiseren van een in de celkweken in gang gezette ontstekingsreactie, hebben we verschillende studies uitgevoerd om de effecten van specifieke stoffen op ontstekingsreacties te onderzoeken.

In **hoofdstuk 6** beschrijven we de effecten van stoffen die specifieke TLRs activeren in PBMCs van volwassen paarden en pasgeboren veulens. In overeenstemming met bestaande literatuur laten onze resultaten zien dat afweerreacties in pasgeboren veulens wel in gang gezet worden door bacteriën, maar milder zijn dan in het volwassen paard. Bovendien laten we zien dat specifieke TLRs voor de herkenning van bacteriën in hogere mate voorkomen in PBMCs van veulens dan in volwassen paarden. De

verminderde reactiviteit van immuuncellen van veulens in aanwezigheid van bacteriële structuren zou functioneel kunnen zijn in de eerste fase van het leven. Immers, tijdens de kolonisatie van de darm door bacteriën zou een overmatige afweer reactie op deze bacteriën onnodige ontsteking teweeg brengen. Aan de andere kant zorgt de verminderde reactiviteit van het immuunsysteem van het veulen wel voor een verhoogd risico op het binnendringen van lichaamseigen bacteriën, waaronder de darmflora, in het lichaam èn op problemen door primair pathogene bacteriën. In de ontwikkeling van methoden om de afweer van veulens te verbeteren, is het de uitdaging om de balans tussen tolerantie ten opzichte van lichaamseigen bacteriën en afweer tegen bacteriën die een mogelijke bedreiging vormen gunstig te beïnvloeden.

In **hoofdstuk 6** wordt tevens beschreven dat zowel in PBMCs van het veulen als van het volwassen paard, de ontstekingsreactie niet wordt beïnvloed door tegelijkertijd TLR-2 te activeren in deze cellen (in lijn met onze resultaten met de muizencellen in **hoofdstuk 4**). Mogelijk wordt de discrepantie tussen eerder gepubliceerde resultaten in levende muizen en onze mechanistische studies in celkweek modellen veroorzaakt door het feit dat de communicatie tussen verschillende celtypen in de darmbarrière essentieel is voor het beoogde effect.

In overeenstemming met de resultaten in **hoofdstuk 4** met muizencellen, laten we in **hoofdstuk 7** zien dat de suikerfractie van paardenbiest de ontstekingsreactie vermindert in PBMCs, geïsoleerd uit het bloed van paarden. In **hoofdstuk 8** wordt in hetzelfde model onderzoek gedaan naar effecten van commercieel beschikbare oligosaccharide preparaten. Drie verschillende oligosaccharide producten, waarvan eerder positieve effecten op zowel de afweer als allergische reacties zijn beschreven in andere diersoorten, werden onderzocht: galacto-oligosacchariden (GOS), fructo-oligosacchariden (FOS) en '*acidic*' oligosacchariden (AOS). GOS wordt geproduceerd met behulp van koeienmelk en FOS en AOS zijn van plantaardige oorsprong. Onze resultaten in **hoofdstuk 8** laten zien dat deze verschillende oligosaccharide specifieke effecten hebben in dit model. Terwijl GOS en FOS de ontstekingsreactie versterken, heeft AOS een remmend effect op de in gang gezette ontsteking in PBMCs van paarden. Beide scenario's zijn interessant voor de ontwikkeling van mogelijke methoden voor beïnvloeding van het immuunsysteem in het veulen en eventueel het volwassen paard, aangezien zowel een beperkte immuunrespons als een overmatige immuunrespons voor problemen kunnen zorgen, afhankelijk van de klinische context.

Hoofdstuk 9 beschrijft een eerste klinische studie naar de effecten van GOS in een groep veulens. Een commercieel verkrijgbaar GOS product werd in deze veulens 2 keer daags toegediend via de mond. Veulens uit de controle groep kregen 2 keer daags een controle preparaat toegediend, waarin net zoveel kleine suikermoleculen zaten als in het GOS product. De veulens werden vanaf de geboorte 4 weken behandeld en vervolgens nog 10 weken opgevolgd. Gedurende de totale periode van 14 weken werden

er, wat betreft zichtbare klinische parameters en gemeten bloedwaarden voor gezondheid en afweer, geen significante verschillen gevonden tussen de twee groepen veulens. Wel werd er een verschil gezien in de reactie van PBMCs, die geïsoleerd werden uit het bloed van de veulens na 4 weken (aan het einde van de behandeling). In de PBMCs van de veulens die met GOS behandeld waren, was de in gang gezette ontstekingsreactie over het geheel genomen milder ten opzichte van de controle groep.

In **hoofdstuk 10** hebben we de eerste resultaten opgenomen van een onderzoek wat nog niet geheel afgerond is. Van 4 verschillende paardenrassen werden biestmonsters verzameld en zijn de verschillende oligosacchariden uit deze biestmonsters gekarakteriseerd. De eerste analyses geven aan dat er unieke oligosaccharides voorkomen in de biest van het paard ten opzichte van de structuren die eerder zijn beschreven in de literatuur. Bovendien zijn er verschillen tussen de onderzochte rassen in het voorkomen van specifieke oligosacchariden en ook in de hoeveelheid waarin deze structuren voorkomen in de biest ten opzichte van elkaar. Samengenomen wijst deze studie nieuwe structuren aan met veelbelovende potentie voor het beïnvloeden van afweer reacties. Na isolatie van specifieke oligosacchariden kunnen deze in de nabije toekomst individueel getest worden op hun capaciteit om het immuunsysteem te beïnvloeden.

Hoofdstuk 11 is een samenvattende discussie van het gehele proefschrift, waarin de belangrijkste bevindingen op een rij gezet worden en kritisch geëvalueerd. De relevantie van de bevindingen wordt beargumenteerd en er worden aanbevelingen gedaan voor vervolgonderzoek.

Conclusie

De bevindingen in dit proefschrift geven een eerste aanzet voor methodes om afweer reacties te beïnvloeden in het paard, met het toekomstperspectief om bij te dragen aan de preventieve gezondheidszorg van jonge veulens. Onze resultaten met specifieke oligosaccharide producten zullen aangevuld en bevestigd moeten worden in toekomstige klinische studies, omdat tot nu toe slechts één klinische studie uitgevoerd is. Unieke, voor het paard specifieke oligosaccharides, die recentelijk uit paardenbiest werden geïsoleerd, zijn veelbelovende structuren voor vervolgonderzoek naar immunomodulerende stoffen in het paard en in andere diersoorten, inclusief de mens. Samengenomen verschaffen de verschillende hoofdstukken van dit proefschrift een waardevol startpunt voor toekomstig onderzoek naar de ontwikkeling van methoden om de afweer en gezondheid in veulens te verbeteren, in het bijzonder met betrekking tot natuurlijke stoffen, afgeleid uit melk of plantaardig materiaal.

Dankwoord

The oldest, shortest words - yes and no –

are those which require the most thought.

Pythagoras

(ca. 570-495 BC)

Dat het moeilijk is om ergens volmondig 'ja' of 'nee' op te antwoorden, had Pythagoras ruim twee en een half duizend jaar geleden al door. Behalve dat dit natuurlijk duidelijk doorwerkt in de wetenschap en het onderzoek in het algemeen, herken ik in deze uitspraak ook zeker mezelf in het algemeen. De keuze om dit promotietraject in te gaan, maakte ik met name vanwege de uitdaging die eraan vast zat en de mogelijkheid om mezelf verder te ontwikkelen. Een uitdaging is het zeker geweest, met als extra uitdaging de onderwijstaak die bij de functie hoorde en ook alle nevenactiviteiten. Nu de eindstreep in zicht is, kan ik zeggen dat het een best heftige periode is geweest, maar dat het me ook zeker heel veel heeft gebracht. Ik heb veel geleerd, inhoudelijk en praktisch over het vakgebied en het onderzoek, maar ook zeker over mezelf. Dit alles was me nooit gelukt zonder de steun en hulp van een veelheid aan mensen om me heen. Bij deze zou ik daarom mijn dank uit willen spreken aan alle mensen, die op werk- en/of persoonlijk gebied betrokken zijn geweest in deze periode. Daarbij wil ik een aantal mensen in het bijzonder noemen.

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About the author

Curriculum vitae

Dax Vendrig was born on the 18th of May, 1980, in IJsselstein. After finishing secondary school (Cals College, Nieuwegein), he started his studies at the Faculty of Veterinary Medicine of Utrecht University in 1998. During his studies, he developed a particular interest in the equine species, internal medicine, and pharmacology. Consequently, as a student, he spent a year at the division of Veterinary Pharmacology, Pharmacotherapy and Toxicology to participate in research into polymorphisms of the Toll-like receptor 4 complex associated with the responsiveness to lipopolysaccharide in horses. In 2006, he graduated with a clinical differentiation into equine medicine and started working as an equine veterinary practitioner. As from 2008, he has been working fulltime at the division of Veterinary Pharmacology, Pharmacotherapy and Toxicology as a teacher and a PhD candidate. Aside from this affiliation, he has continued to practice equine medicine in night- and weekend shifts at the Department of Equine Sciences (Faculty of Veterinary Medicine, Utrecht University) to this day. His research project concerning immunomodulation in foals was supervised by prof. dr. J. Fink-Gremmels, and resulted in this PhD-thesis, which will be publicly defended on the 29th of August, 2013.

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List of abbreviations

AOS	acidic oligosaccharides
APC	antigen presenting cell
CD-14	cluster of differentiation 14
CpG	cytosine-phosphate-guanine
Ct	threshold cycle
DC	dendritic cell
dp	degree of polymerisation
DAP	diaminopimelic acid
eCC	equine colostral carbohydrates
ECO	equine colostral oligosaccharide
FOS	fructo-oligosaccharides
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GOS	galacto-oligosaccharides
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IEL	intra-epithelial lymphocyte
IFN	interferon
IL	interleukin
IRAK	interleukin-1 receptor-associated kinase
JNK	c-jun N- terminal kinase
LPL	lamina propria lymphocyte
LBP	LPS binding protein
LPS	lipopolysaccharide
MAL	MyD88-adapter like
MAPK	mitogen-activated protein kinase
MDP	muramyl dipeptide
MD-2	myeloid differentiation protein 2
MIP	macrophage inflammatory protein
MLN	mesenteric lymph nodes
MP	macrophage
MyD88	myeloid differentiation primary response protein
NFκB	nuclear factor κB
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain
PBMC	peripheral blood mononuclear cell
PCSK	Pam3-Cys-Ser-Lys4
PAMP	pathogen associated molecular pattern
PIM	pulmonary intravascular macrophage
PMN	polymorphonuclear neutrophils
PRR	pattern recognition receptor
TAK-1	transforming growth factor-β-activated protein kinase 1
TJ	tight junction
TGF	transforming growth factor
Treg	T regulatory cell
Th	T helper cell
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain containing adapter protein
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAF	tumour necrosis factor receptor-associated factor
TRAM	Trif-related adapter molecule
Trif	TIR domain-containing adapter inducing IFN-β