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Immune response against *Giardia duodenalis* infection in cattle

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Promotors

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS	4
CHAPTER 1. GIARDIA A LITERATURE REVIEW	8
1.1 Taxonomy	8
1.2 Life cycle and transmission	9
1.3 Pathogenesis	13
1.4 Giardiosis	18
1.5 Diagnosis, treatment and prevention	19
1.6 Immunity	21
1.6.1 <i>Innate immune responses</i>	21
1.6.2 <i>Adaptive immune response</i>	30
1.6.3 <i>Immunity against G. duodenalis in cattle</i>	34
1.7 References	36
OBJECTIVES	52
References	54
CHAPTER 2 THE ROLE OF DENDRITIC CELLS IN THE IMMUNOLOGY AGAINST G. DUODENALIS IN CATTLE	56
2.1 Introduction	56
2.2 Materials and Methods	56
2.3 Results	64
2.4 Discussion	74
2.5 References	77
CHAPTER 3 KINETICS OF THE IMMUNE RESPONSE AGAINST G. DUODENALIS IN CATTLE	82
3.1 Introduction	82
3.2 Materials and Methods	82
3.3 Results	88
3.4 Discussion	96

3.5 References	99
CHAPTER 4 GENERAL DISCUSSION, FUTURE PROSPECTIVES AND CONCLUSION	104
4.1 The effect of <i>Giardia duodenalis</i> on bovine monocyte-derived dendritic cells	104
4.2 Development of immunity against <i>Giardia duodenalis</i> in cattle	105
4.3 Conclusions and future research	111
4.4 References	116
SUMMARY	124
SAMENVATTING	130
DANKWOORD	137

List of abbreviations

ADI	arginine deiminase
APC	antigen-presenting cells
ATP	adenosine triphosphate
cpg	cysts per gram of faeces
CD	cluster of differentiation
conA	concanavalin A
cpm	counts per minute
DC	dendritic cells
DNA	deoxyribonucleic acid
CCL	CC-chemokine ligand
cDNA	complementary DNA
EF1a	elongation factor 1- α
ELISA	enzyme-linked immunosorbent assay
ES	excretion/secretion material
FACS	fluorescence-activated cell sorting
FoxP3	forkhead box P3
FSC	forward scatter
GDH	glutamate dehydrogenase
GM-CSF	granulocyte macrophage – colony stimulating factor
HSP	heat shock proteins
IEL	intraepithelial lymphocyte
IFA	immunofluorescence assay
IFN	interferon
IKK	I kappa B kinase
IL	interleukin
ILC	innate lymphoid cells
ILT	immunoglobuline like transcript
Ig	immunoglobulin
IRAK	interleukine-1 receptor-associated kinases
IRF3	Interferon regulatory factor 3
LPS	lipopolysaccharide
MACS	magnetic cell sorting

M cell	microfold cell
MFI	median fluorescence intensity
MHCII	major histocompatibility complex II
MLR	mixed lymphocyte reaction
MoDC	monocyte-derived dendritic cell
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NALP	NACHT, LRR and PYD domains-containing protein
NF- κ B	Nuclear Factor- κ B
NK	natural killer cell
NO	nitric oxide
NOD-like receptor	nucleotide-binding oligomerization domain receptor
NOS	nitric oxide synthase
OPD	o-phenylenediamine dihydrochloride
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PD L1	programmed cell death ligand 1
p.i.	post infection
PI	propidium iodide
PPAR	peroxisome proliferator-activated receptors
PRR	pattern recognition receptor
RIG-like receptor	Intracellular pattern recognition receptor
RNA	ribonucleic acid
ROR γ	RAR-related orphan receptor gamma
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium 1640
SEM	standard error of the mean
SSC	side scatter
TCR	T-cell receptor
TIR	Toll/interleukine-1 receptor
TGF	transforming growth factor

TLR	Toll like receptor
TNF	tumor necrosis factor
tpi	triose phosphate isomerase
TRAF6	tumour-necrosis-factor-receptor-associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon beta
Treg	regulatory T-cell
vsp	variant-specific surface protein

Chapter 1

Literature review

Chapter 1. *Giardia* a literature review

1.1 Taxonomy

Giardia was first seen in 1681 by Antoni Van Leeuwenhoek, while examining his own stool. *Giardia* was fully described in 1859 by Vilem Lamble and Alfred Giard but it was only in 1915 that *Giardia* was linked to pathology. Nowadays *G. duodenalis* is included in the World Health Organisation's neglected disease initiative (Savioli et al., 2006) and is known to cause a disease called giardiasis. *Giardia duodenalis* is synonymous with *G. lamblia* and *G. intestinalis*. For consistency *G. duodenalis* will be used in this thesis.

G. duodenalis is a member of the kingdom Protozoa (unicellular eukaryotes). In the old taxonomic system, based on morphology, *Giardia* belonged to Phylum Sarcomastigophora, Subphylum Mastigophora (= Flagellata), Class Zoomastigophorea, Order Diplomonadida and Family Hexamitidae. According to the new taxonomic system, based on genetics and biochemistry, *Giardia* belongs to Phylum Metamonada, Subphylum Trichozoa, Superclass Eopharyngia, Class Trepomonadea, Subclass Diplozoa, Order Giardiida and Family Giardiidae (Plutzer et al., 2010).

Six species of *Giardia* have been distinguished, of which five species are host specific (Table 1). The sixth species, *G. duodenalis*, includes *Giardia* strains isolated from a large range of mammalian hosts, and is divided into seven well-defined assemblages (A-H). These assemblages are identified based on the analysis of conserved genetic loci. Mainly assemblage A and B are found in humans (Cacciò and Ryan, 2008) while assemblage A and E are found in cattle (Geurden et al., 2008).

Table 1. Overview of different *Giardia* species after Xiao and Fayer (2008).

Species	Major hosts
<i>G. duodenalis</i>	
assemblage A	Humans, primates, dogs, cats, cattle, rodents, wild mammals
assemblage B	Humans, primates, dogs, horses, cattle
assemblage C	Dogs
assemblage D	Dogs
assemblage E	Artiodactyls
assemblage F	Cats
assemblage G	Rats
assemblage H*	Marine vertebrates
<i>G. agilis</i>	Amphibians
<i>G. muris</i>	Rodents
<i>G. psittaci</i>	Birds
<i>G. ardeae</i>	Birds
<i>G. microti</i>	Voles and muskrats

* Lasek-Nesselquist et al., 2010

1.2 Life cycle and transmission

Morphology and life cycle

Giardia has two morphological stages, the parasitic stage; the trophozoite and the environmental stage; the cyst (Fig. 1). The trophozoite is motile and pear shaped, about 10-12 μm long and 5-7 μm wide. It is dorsoventrally flattened with a ventral sucking disk that enables the parasite to attach to the hosts enterocytes. The trophozoite has two identical nuclei, two median bodies and four pairs of flagella (Adam, 1991). The cyst form of *Giardia* is non-motile and egg shaped. The cyst has a thick, refractile wall and is 8-12 μm long and 7-10 μm wide. New formed cysts have two nuclei, while mature cysts possess four nuclei. The cyst is the infective form of the parasite and after excystation one cyst can give rise to two trophozoites.

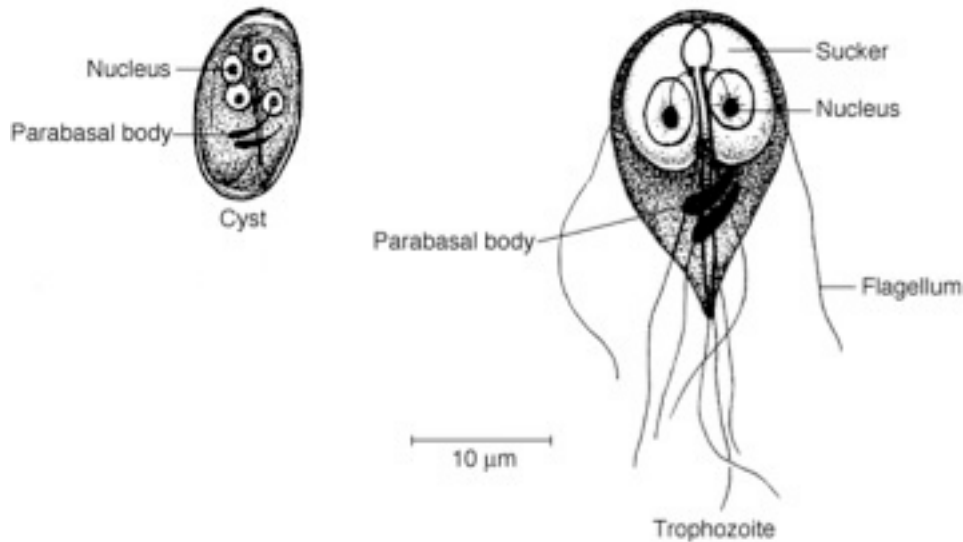


Figure 1. The two life stages of *Giardia*. Adapted from: <http://accessmedicine.net/popup.aspx>

Giardia has a direct and asexual life cycle (Fig. 2). The host gets infected after ingestion of cysts. When ingested cysts are exposed to stomach acid and bile salts, the release of trophozoites from the cysts is stimulated. The now motile trophozoites attach with their ventral sucking disk to the epithelium of the small intestine but do not invade the epithelium. Attachment of the trophozoites to the epithelium is necessary to maintain the infection. The apical surface of the intestinal epithelium is subsequently colonized by binary fission (Monis and Thompson, 2003). For growth *Giardia* requires biliary lipids and bile salts from the intestinal lumen (Farthing et al., 1985).

When trophozoites detach and pass through the small intestine, encystation is induced. This is most likely a result of exposure to bile salts, fatty acids and a more alkaline environment. The cysts passing with the faeces are immediately infectious and often excreted in high numbers. The minimum infection dose is established to be 10-100 cysts (Rendtorff, 1954). The prepatent period of *Giardia*, *i.e.* the interval between the start of the infection and ability to detect cysts, can differ from 4 days to 2 weeks (Xiao and Herd, 1994).

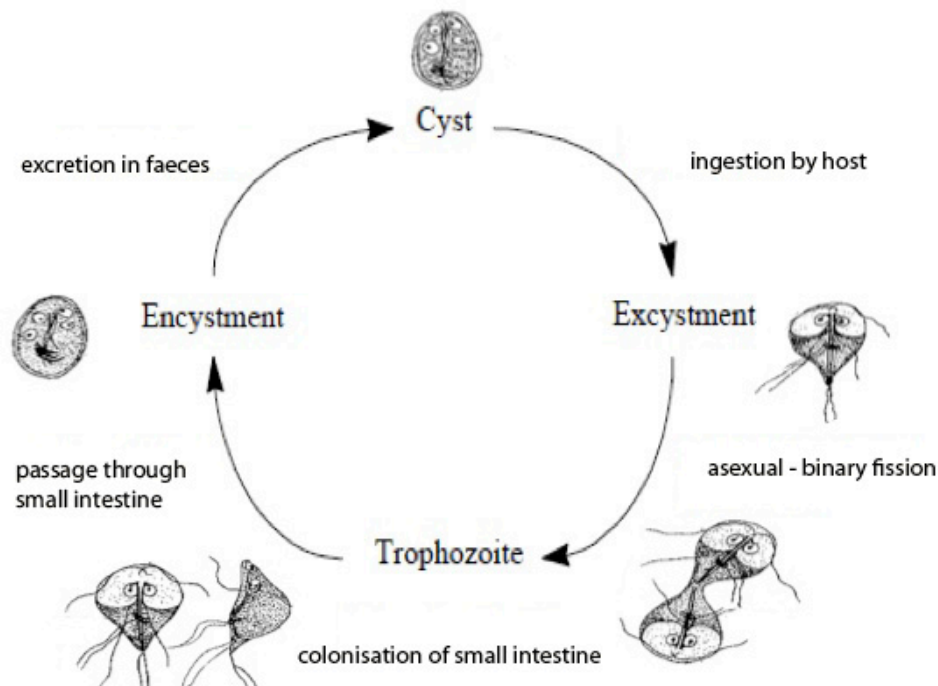


Figure 2. Life cycle of *Giardia*. Adapted from Monis and Thompson, 2003 and <http://www.soton.ac.uk/~ceb/Diagnosis/Vol3.htm>

Transmission

Transmission of *Giardia* occurs by the faecal-oral route through direct host-to-host contact or via contaminated materials, water or food. Due to the thick cyst wall, cysts can survive in the environment for several months, especially in a cool and moist matrix (Olson et al., 1999; Grit et al., 2012). The long cyst survival attributes to high contamination of the environment, which is often seen. This high environmental contamination by cysts facilitates re-infection (Hunter and Thompson, 2005). Water is an important vector in the transmission of *Giardia* and many waterborne outbreaks have been noted (Robertson and Ai Lian Lim, 2011). Most outbreaks are a result of bad drinking water treatment or contamination of surface water by sewage or agricultural runoff contaminated with animal faeces (Monis and Thompson, 2003).

G. duodenalis assemblage A and B are found in humans, while other assemblages are found in different animal species. Transmission from human to human is well described and different risk factors are known (Fig. 3): traveling, swimming in surface water, drinking water not coming from a main supply and contact with young children

(Hunter and Thompson, 2005). Few epidemiological studies have assessed the importance of zoonotic transmission in the occurrence of human giardiasis (Cacciò et al., 2005). Evidence for zoonotic transmission can be obtained from epidemiological studies identifying isolates of parasites from infected hosts in localized outbreaks or as a result of longitudinal surveillance and genotyping of positive cases (Thompson et al., 2008).

Farm animals can be infected with the zoonotic assemblage A or the livestock-specific assemblage E. *Giardia* has been reported in production animals worldwide, although prevalence data are mainly available for cattle, and to a lesser extent for other ruminants and pigs (Geurden et al., 2010b). Many studies demonstrated (cattle-) farm prevalences of 100% (Trout et al., 2004; Ralston et al., 2003 and Hunt et al., 2000). Considering that cyst excretion can be as high as 10^6 cysts per gram faeces (O'Handley et al., 2003), cattle can be proposed as a reservoir for zoonotic transmission.

Domestic pets have host-adapted *G. duodenalis* assemblages: assemblage C and D in dogs and assemblage F in cats. Numerous prevalence surveys demonstrated that dogs and cats may be infected with zoonotic as well as host-specific assemblages (Thompson and Monis, 2011). Although transmission between humans and dogs living in close contact is reported in different studies (Traub et al., 2009; Thompson et al., 2008), data on the frequency of zoonotic transmission is lacking and the importance of domestic pets as reservoir for zoonotic transmission is doubted (Feng and Xiao, 2011).

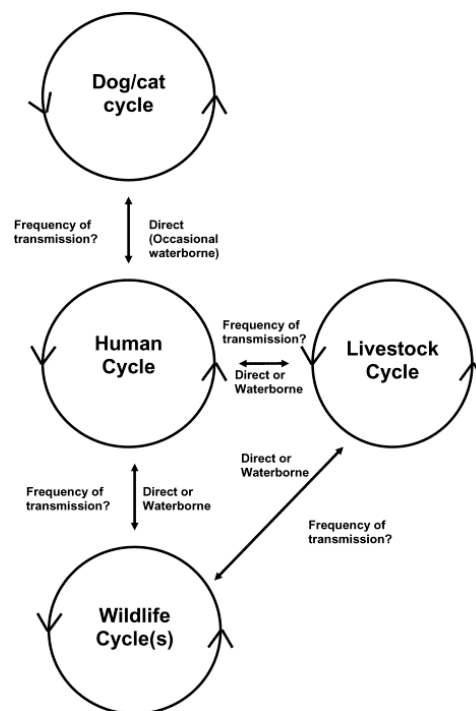


Figure 3. Different ways of *Giardia* transmission. From Thompson, 2004 with permission from Elsevier.

It was long thought that wild mammals are a potential source for waterborne infections. In North America the beaver has been implicated most often in water contamination (Appelbee et al., 2005), consequently giardiasis is commonly referred to as ‘beaver fever’. Next to beavers, *Giardia* has been reported in muskrats, moose and white tailed deer (Karanis et al., 1996; Trout et al., 2003; Robertson et al., 2007). Nevertheless, there is no evidence that wildlife plays an important role in zoonotic transmission and it is more likely that wildlife is infected by environmental contamination of cysts of human origin (Appelbee et al., 2005).

1.3 Pathogenesis

The *Giardia* pathogenesis is not fully understood and is controversial because 85% of *Giardia* infections in humans are asymptomatic (Muller and Von Almen, 2005). From individuals with *Giardia*-positive stool samples, 22-41% developed clinical giardiasis (Nash et al., 1987; Rauch et al., 1990; Soliman et al., 1998; Sahagùn et al., 2008). In one study in Peru, the presence of *Giardia* cysts in stool samples could not be correlated with symptoms at all (Hollm-Degado et al., 2008). It is suggested that the

development of clinical giardiasis depends on the host's immune response or on the virulence of different *G. duodenalis* isolates (Sahagùn et al., 2008).

Also the presence of intestinal injuries during *G. duodenalis* infection is controversial. Some researchers found no abnormalities in the intestine of symptomatic patients (Koot et al., 2009), others found abnormalities in 3.7-100% of the cases (Oberhuber et al., 1997; Bataga et al., 2004; Yakoob et al., 2005). In addition to human patients, damage to villi and microvilli and/or crypt hyperplasia was seen in mice (Farthing 1997; Scott et al., 2000), gerbils (Buret et al., 1992), cattle (Ruest et al., 1997; O'Handley et al., 2001) and goats (Koudela and Vitovec, 1998). These latter studies describe a pathophysiology cascade, which has similarities with a number of other intestinal diseases, such as cryptosporidiosis, Crohn's disease, bacterial enteritis, celiac disease and chronic intestinal anaphylaxis (Buret, 2005). The pathophysiology cascade of *Giardia* can be attributed to several mechanisms (Table 2).

Table 2. Overview of damage caused by *Giardia* and its consequences in relation to the symptoms

Damage	Consequence	Symptoms
Mechanical damage of intestinal surface	decreased absorption of electrolytes and nutrients	diarrhoea and weight loss
Loss of enterocyte barrier function	shift of fluid to the lumen	diarrhoea
Decreased activity of digestion enzymes	decreased digestion of sugars, proteins and fat.	weight loss, steatorrhoea
Increased mucus secretion	-	steatorrhoea
Damage by host immune response	loss of absorptive surface	diarrhoea and weight loss

Mechanical damage to intestinal surface

It is suggested that *Giardia* disturbs the intestinal function by acting as a mechanical barrier through colonisation of the intestinal absorptive surface (Farthing, 1997). Others think this is not possible, considering the small size of the trophozoites (7 x 12 µm) and the enormous intestinal surface (Röxstrom-Lindquist et al., 2006). Furthermore, the adherence of the trophozoite to the intestinal epithelium damages the intestinal surface. Parasite excretion/secretion products (ES), such as lectins and proteases induce apoptosis of enterocytes (Panaro et al., 2007; Buret, 2007). In 4% of the cases this resulted in villus flattening, villus atrophy, microvillus shortening, and crypt hyperplasia (Fig. 4) (Troeger et al., 2007; Oberhuber et al., 1997). All together these lesions cause a loss of brush border surface area or absorptive area and finally cause malabsorption and diarrhoea. The diffuse microvillus shortening is responsible for disaccharidase insufficiencies and malabsorption of glucose, sodium and water (Cotton et al., 2011). Besides the *Giardia*-induced villus atrophy, microvillus shortening is caused by CD8⁺ T-lymphocytes (Scott et al., 2000; 2004) (see below).

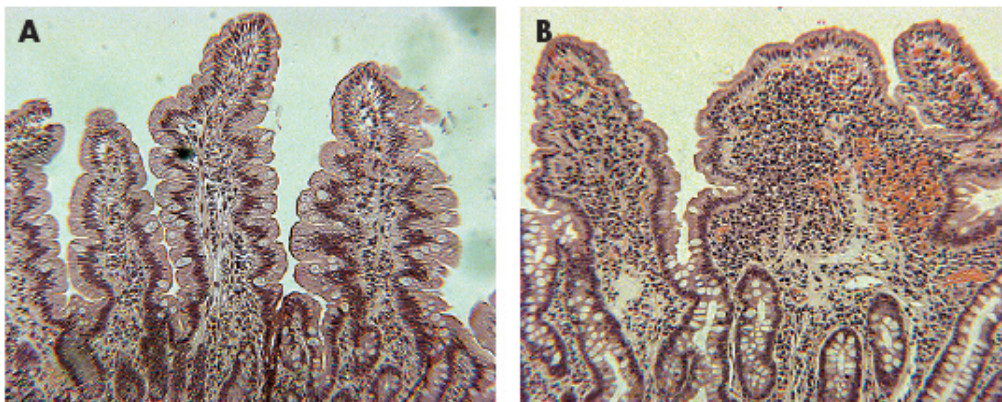


Figure 4. Section of human duodenum of a healthy control (A) and a patient with chronic giardiasis (B), stained with HE, magnification x 20. Villous flattening, microvillus shortening and crypt hyperplasia. (reprinted from Troeger et al., 2007 with permission from BMJ).

Loss of barrier function

In vitro experiments showed that contact of trophozoites with human intestinal monolayers induces apoptosis and disruption of tight junctions (Chin et al., 2002). Scott et al. (2002) demonstrated that *Giardia* induces reorganisation of the tight

junction proteins zona occludens-1 (ZO-1) and F-actin, which play key roles in regulating the permeability of the tight junctional complex. Moreover, *Giardia* may alter other tight junctional proteins, including the claudins (Troeger et al., 2007). Besides, activated T lymphocytes cause the brush border to retract. Several cytokines are possibly involved in this effect on the microvillus structure, but the exact mechanism is still unknown. (Scott et al., 2000). After breaking the epithelial barrier fluid is shifting from the tissue to the intestinal lumen and finally causes diarrhoea (Buret, 2007). The accumulation of intestinal fluid is enhanced by *Giardia* enterotoxins, inducing chloride hyper secretion by enterocytes (Shant et al., 2005). Besides this accumulation of fluid, the loss of the epithelial barrier function allows luminal antigens to cross the epithelial barrier, penetrate into the lamina propria and activate the host immune response. Therefore the loss of this barrier function is of great relevance in the parasite-host interaction.

Decreased digestion and absorption of nutrients

Absorption of nutrients and electrolytes occurs by the brush border microvilli, which harbour various digestive enzymes and transporters for nutrients and ions (Buret, 2005). Alterations of the brush border cause a reduced absorptive capacity and consequently reduced absorption of water, glucose and electrolytes (Troeger et al., 2007). During *Giardia* infection several disaccharidases, such as maltase, sucrase and lactase, display a reduced enzymatic activity (Scott et al., 2000; 2004). It is possible that high levels of undigested carbohydrates contribute to diarrhoea, following their conversion into short chain fatty acids by colonic microbiota (Robayo-Torres et al., 2006). Furthermore, it is shown that *Giardia* diminishes the activity of different pancreatic enzymes, including lipase, trypsin, chymotrypsin and amylase (Katelaris et al., 1991; Seow et al., 1993).

Finally an osmotic gradient is induced caused by the malabsorption of nutrients and electrolytes, and as a result water is drawn from the intestinal tissue into the lumen resulting in intestinal distension and rapid peristalsis (Cotton et al., 2011).

Increased mucus secretion and small intestine hypermotility

During *Giardia* infection the mucus production of the intestine is increased and increased goblet cell populations are found (Williamson et al., 2000). These goblet

cells produce mucins: glycoproteins, constituting the intestinal mucus layer. Likely, the mucus layer lowers the infection intensity and functions as a physical defence mechanism of the host preventing the adherence of the parasite (Roskens and Erlandsen 2002). In contrast, other *in vitro* studies found mucins to improve parasite adherence and growth (Muller and Von Allmen, 2005). It is also suggested that small intestinal hypermotility attributes to parasite clearance. Gerbils infected with *G. duodenalis* were shown to have increased intestinal transit rates (Li et al., 2006). It is likely that changes in intestinal transit during *Giardia* infections in humans contribute to symptoms, like abdominal cramps and diarrhoea (Li et al., 2006). The underlying mechanisms regulating this hypermotility remain unclear but seem to depend on the adaptive immune response, since no intestinal hypermotility was observed in mice lacking T- and B-cells (Andersen et al., 2006).

Damage by the host immune response

After disruption of the intestinal barrier, parasite antigens are able to trigger the host immune response. During *Giardia* infection an increased number of intraepithelial lymphocytes is seen (Oberhuber et al., 1997; Scott et al., 2000). Oberhuber et al. (1997) found a mild duodenitis with infiltration of neutrophils and intraepithelial lymphocytes (IEL) in a small number of patients. Hardin et al. (1997) observed an increased number of mucosal mast cells. It is known that in other intestinal disorders, such as celiac disease, Crohn's disease and bacterial enteritis, CD8⁺ T cells can cause villus atrophy. Also in giardiasis it is shown that microvillus shortening, crypt cell hyperplasia and the impaired enzymatic activity is mediated by activated CD8⁺ T-cells (Scott et al., 2004; Solaymani-Mohammadi and Singer, 2010). Besides, intestinal injuries could be induced after adoptive transfer of CD8⁺ T-cells of *G. muris*-infected mice (Scott et al., 2004). It is still unclear how *Giardia* induces the CD8⁺ T-cell response and how these cells cause damage to the intestinal tissue (Solaymani-Mohammadi and Singer, 2010). In contrast to Scott et al. (2004), Oberhuber et al. (1996) showed with an immunostaining that during *Giardia* infection the number of activated CD8⁺ T-cells was not increased and that IEL were mostly CD8⁺ T cells, negative for granzyme B, implying that these CD8⁺ cells are resting cytotoxic cells (Oberhuber et al., 1996).

1.4 Giardiasis

Human giardiasis

Giardia is one of the most common human intestinal parasites, about 280 million people in Asia, Africa, and Latin America have symptomatic infections. In Belgium the estimated incidence of giardiasis is 10.8 per 100,000 inhabitants (WIV, 2010) and *Giardia* is listed in the five most important infectious causes of diarrhoea (Geurden et al., 2009). Giardiasis exerts a significant public health impact, also in industrialised countries because of the high prevalence and disease burden of the infection, and due to its role in outbreaks in day-care centres and water-associated outbreaks, and its effects on growth and cognitive functions of infected children (Berkman et al., 2002; Robertson et al., 2010). *Giardia* is not related to a tropical climate but is often associated with developing countries where infection is linked to low socio-economic conditions and compromised hygiene infrastructure. Because of the social-economic impact giardiasis was included in the 'Neglected Disease Initiative' by the World Health Organisation (Savioli et al., 2006). Although *Giardia* infection generally is asymptomatic, *Giardia* can cause a broad spectrum of clinical symptoms. Clinical giardiasis is generally characterized by watery diarrhoea, which may turn into fatty (steatorrhoea) or mucous diarrhoea. Other clinical symptoms can be abdominal cramps, bloating, weight loss, and malabsorption (Robertson et al., 2010). Clinical symptoms, which usually appear 6-15 days post infection, are mostly seen in children and immunodeficient patients. The symptoms can last up from a few days to several weeks and in some cases the host fails to eradicate the parasite, which leads to a chronic infection (Feng and Xiao, 2011). A difference in clinical significance is described among different *Giardia* strains, although existing data are contradictory. Some studies found that assemblage A is found in patients with acute diarrhoea while chronic disease with less severe clinical symptoms was attributed to assemblage B (Homan and Mank, 2001; Read et al., 2002). On the other hand, by some researchers assemblage A was linked to intermittent/mild disease while assemblage B was detected in patients with more severe cases of diarrhoea (Muhsen and Levine, 2012). Again others found no clinical difference between patients infected with assemblage A and B (Kohli et al., 2008; Lebbad et al., 2011).

Giardiasis in domestic animals

Like human infections giardiasis in ruminants can be clinical or subclinical, acute or chronic. Clinical infection may exhibit chronic pasty diarrhoea, weight loss, lethargy and poor condition (Ralston et al., 2003). Experimental infections in young calves induced a weight loss of ± 100 g per day of infection (Geurden et al., 2010a). Experimental *Giardia* infection in lambs resulted in decreased weight gain, impaired feed conversion efficiency and reduced carcass weight (Olson et al., 1995; Geurden et al., 2011). Generally calves develop a chronic stage of *G. duodenalis* (O’Handley et al., 1999) and cysts can be excreted for a period of 112 days (Taminelli et al., 1989).

Symptoms in dogs and cats are similar to symptoms in other animals. Pets suffering from giardiasis can show intermittent diarrhoea, depression, anorexia, weight loss, poor condition and vomiting. In addition, the faeces turns soft, pale, steathorrhic and has an altered smell (Bianciardi et al., 2004).

1.5 Diagnosis, treatment and prevention

Diagnosis

Diagnosis of giardiasis is in the first place based on the presence of clinical symptoms (persistent diarrhoea), but must be confirmed by detection of the parasite in a faecal sample. However, especially in the chronic phase of infection the cyst excretion of *Giardia* is intermittent and hence faecal examination requires multiple sampling (O’Handley et al., 1999). *Giardia* can be diagnosed by microscopic examination, antigen detection or polymerase chain reaction (PCR). For the microscopic examination of stool samples two methods can be used: examination of a native smear to detect the presence of trophozoites or examination of enriched samples for the presence of cysts after flotation or staining with Lugol’s iodine (Chakarova, 2010). For the detection of parasite antigen immunofluorescence assays (IFA), enzyme-linked immunosorbent assays (ELISA) (Zimmerman and Needham, 1995) and rapid solid-phase qualitative immunochromatography assays (Garcia et al., 2003) are commercially available. Both IFA and ELISA use monoclonal antibodies against cyst wall proteins. IFA and real time qPCR can be used as a quantitative test (Zimmerman and Needham, 1995; Guy et al., 2003; Geurden et al., 2004). For identification of species and assemblages, PCR of the following genes can be used: small subunit

rRNA, glutamate dehydrogenase (GDH), triose phosphate isomerase (tpi), elongation factor 1- α (EF1a), β -giardin and variant-specific surface protein (vsp) (Liu, 2009). The detection limit of PCR is theoretically one cyst (Amar et al., 2002).

Treatment

Although giardiasis is generally self-limiting, it is recommended to treat patients with clinical symptoms. For therapeutic treatment different drugs are used: nitroimidazoles (metronidazole, tinidazole, ornidazole and secnidazole), albendazole, quinacrine, furazolidone, nitazoxanide and paromomycin (Gardner and Hill, 2001; Petri, 2003; Rossignol, 2010). Despite the efficacy of these compounds cyst excretion during treatment is not uncommon. It is possible that cyst excretion is suppressed during the treatment and increases when treatment is stopped (Hausen et al., 2003). But, failure of treatment with metronidazole in patients with immunodeficiency has also been described (Petri, 2003). These cases necessitate combined treatment of metronidazole and quinacrine to eradicate the parasite.

In cattle different compounds are effective to treat giardiasis: metronidazole, dimetridazole, fenbendazole, albendazole and paromomycin (O'Handley and Olson, 2006; Geurden et al., 2006), although the use of nitroimidazoles is no longer approved. The use of fenbendazole is currently allowed in many countries, *inter alia* Belgium, for treatment of helminth infections in companion animals and livestock and is proved to be efficacious against *Giardia* (BCFI diergeneeskunde; O'Handley et al., 1997; Xiao et al., 1996; Geurden et al., 2006). In companion animals fenbendazole is registered for the treatment of giardiasis, although other benzimidazoles such as oxfendazole and albendazole are also effective. Also a combination of pyrantel, febantel and praziquantel has been found to be efficacious against *Giardia* and the different components seem to have a synergistic effect (Geurden and Olson, 2011). This combination is also registered in Belgium for treatment of giardiasis in companion animals (BCFI diergeneeskunde). Despite the availability of registered drugs, *Giardia* infections in companion animals are often treated with nitroimidazoles (metronidazole, dimetridazole) (Bowman and Lucio-Foster, 2010).

Vaccination

Currently, a *Giardia* vaccine for dogs and cats is commercially available in the United States (GiardiaVax™, Fort Dodge Animal Health, Kansas, USA) and comprises sonicated trophozoites isolated from sheep. Clinical trials demonstrated that subcutaneous administration of this vaccine in young dogs and cats results in the reduction of *G. duodenalis* cysts in the faeces, the prevention of clinical disease, and significantly higher weight gains compared to non-vaccinated animals (Olson et al., 1996; 1997). Other studies in dogs showed that vaccination was not effective and the efficacy of the vaccine is questioned (Anderson et al., 2004; Lehmann en Lehmann, 2004). The vaccine was also tested in calves but no protection could be observed compared to the control group (Uehlinger et al., 2007).

1.6 Immunity

1.6.1 Innate immune responses

Introduction

Whether or not ingestion of *Giardia* cysts will lead to infection, depends on many parasite and host factors. Before trophozoites are able to adhere to the epithelium and colonize the small intestine the host immune response has to be evaded. The first line of defence is the innate immune system, acting in a non-specific way to many pathogens. The innate immunity is rapid (minutes) but provides only short-term protection. After a few days the adaptive immune system will be activated and will initiate a pathogen-specific defence mechanism consisting of a cellular and humoral immune response. The adaptive immunity is able to provide long-term protection by the induction of memory cells.

The cells involved in the mucosal innate immune response are phagocytic cells and antigen presenting cells, such as macrophages, neutrophils, dendritic cells (DC) and mast cells. Beside these well-defined immune cell populations, the newly identified innate lymphoid cells (ILC), cells which share characteristics with lymphoid cells, but lack antigen receptors, play key roles in innate immunity as they can produce a broad gamma of effector cytokines (Hwang and McKenzie, 2013). Natural Killer cells (NK) also belong to the innate lymphoid cell population (Spits and Cupedo, 2012). Innate immune cells detect and respond to conserved structures common on groups of

pathogens called pathogen-associated molecular patterns (PAMPs). These include cell wall components, flagella, lipoproteins and nucleic acids of bacterial, fungal or viral origin (Krishnaswamy et al., 2013). PAMPs are not expressed by the host but are also found on commensal microorganisms. PAMPs are recognized by pattern recognition receptors (PRRs) (Kaisho and Akira, 2006). Different PRRs are known; membrane bound receptors called Toll-like receptor (TLR) and C-type lectin receptor and cytoplasmic receptors called NOD-like receptor, NALP and RIG-like receptor (McGreal, 2009). The innate immune system has several major functions:

- providing a physical and chemical barrier
- recruiting immune cells to the place of infection: inflammation
- activation of the complement cascade
- identification and removal of pathogens/non-self substances
- activation of the adaptive immune system

Physical and chemical barrier

Giardia infection starts with ingestion of cysts followed by excystation and release of trophozoites in the small intestine. Several physical and chemical factors influence the adhesion and colonisation of the parasite (Fig. 5) and lower the infection intensity (Muller and Von Allmen, 2005). Increased peristaltic movements of the small intestine and increased secretion of mucus by goblet cells has been observed during infection. Mucus can prevent trophozoite adhesion *in vitro* and probably accelerates the parasites removal *in vivo* (Roskens and Erlandsen, 2002). Another factor is the commensal microbiota present in the small intestine, which compete with the trophozoites for nutrition (Muller and Von Allmen, 2005; El-Shewy and Eid, 2005) or can inhibit *in vitro* growth of the parasite (Perez et al., 2001), both resulting in inhibition of trophozoite proliferation. This influence of the intestinal microbiota can explain why *G. duodenalis* experimental infection in adult mice only proceeds upon treatment with antibiotics (Faubert, 2000).

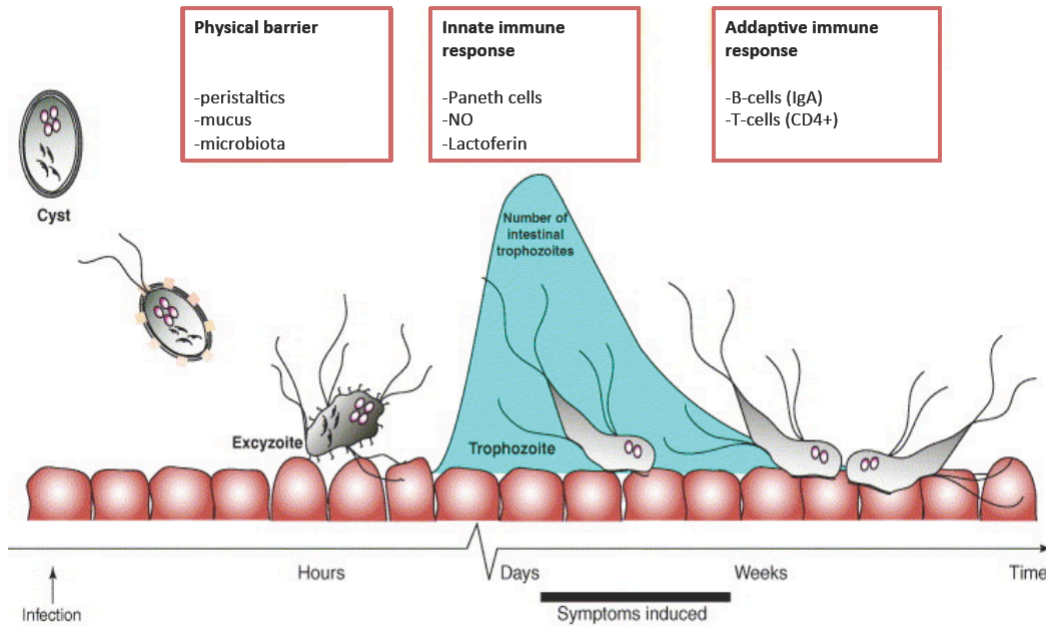


Figure 5. Kinetics of the host immune response against *Giardia* (Adapted from R oxstrom-Lindquist et al., 2006).

Epithelial factors

During *Giardia* infection ES products are produced as a result of cell-cell contact between the parasite and the intestinal epithelium. In contrast, epithelial cells and intestinal microbiota secrete antimicrobial products that contribute to the maintenance of the mucosal barrier and some of these products are known to possess anti-giardial activity. Paneth cells, epithelial cells based at the bottom of intestinal crypts, secrete defensins (also called cryptidins). Alpha defensins have been shown to kill *G. duodenalis in vitro* (Aley et al., 1994). However, the relevance of defensins in the host response is questioned as *Giardia* fails to induce defensin production in *ex vivo*-maintained small intestinal crypts and mice lacking active defensins do not show decreased protection against *G. muris* (Eckmann, 2003; Muller and Von Allmen, 2005). Some assume that the anti-giardial effect of defensins is an indirect consequence of changes in the intestinal flora induced by the down regulation of defensin secretion (Solaymani-Mohamadi and Singer, 2010; Muller and Von Allmen, 2005).

Besides defensins, enterocytes produce reactive oxygen species (ROS) and nitric oxide (NO). Both have cytotoxic and immune-modulatory activities during intestinal infection (Eckmann, 2003). NO has multiple other functions, including a role in

neurotransmission, regulation of the integrity of the mucosal barrier and vascular tone in the intestine. *In vitro*, NO inhibits growth, encystation and excystation of *G. duodenalis* but it has no effect on parasite viability (Eckmann et al., 2000), although some researchers did find a cytotoxic effect on *Giardia* (Fernandes and Assrey, 1997). NO is produced enzymatically from arginine by NO synthase (NOS), which exists as three different isoforms, NOS1 (neuronal NO synthase), NOS2 (inducible NO synthase), or NOS3 (endothelial NO synthase). It was long thought that NOS2 was the most important NOS in anti-*Giardia* defence, but Li et al. (2006) showed that NOS1 is required for parasite clearance in mice. The effect of NO *in vivo* is difficult to predict. It is known that NO production by host cells is inhibited by *Giardia*, since *Giardia* actively consumes arginine and reduces the availability of arginine for the host. Arginine is an essential amino acid, but is also a substrate for the host's production of NO (Muller and Von Allmen, 2005; Stadelmann et al., 2012). Some ES proteins produced by *Giardia*, *i.e.* arginine deiminase (ADI) and ornithinecarbamoyl transferase, actively metabolise arginine, thus depleting arginine for the host (Ringqvist et al., 2008).

Recruitment of innate immune cells to the place of infection: Inflammation

Although during *Giardia* infection little or no intestinal inflammation is seen (Oberhuber et al., 1997), endoscopic examination showed that 4.5-46% of the patients suffering from giardiasis developed duodenitis, characterized by influx of lamina propria and intraepithelial lymphocytes (Gómez et al., 1981; Bätaga et al., 2004; Yakoob et al., 2005). The pro-inflammatory cytokine IL-8 normally produced during bacterial infections and causing inflammation is not detected during *Giardia* infection (Röxstrom-Lindquist et al., 2006). Nonetheless, microarray analysis demonstrated that human intestinal epithelial cells co-incubated with *Giardia* trophozoites produced a distinct chemokine profile (CCL2, CCL20, CXCL1, CXCL2 and CXCL3) that would be expected to attract dendritic cells and lymphocyte populations (Röxstrom-Lindquist et al., 2005). Although a small number of leukocytes migrate across the epithelium to the intestinal lumen (Scott et al., 2004), IEL and lymphocytes from the lamina propria are not attracted to trophozoites or ES material in a migration assay. The increased numbers of IEL and lymphocytes in the lamina propria are probably

caused by proliferation, since proliferation could be induced after *in vitro* re-stimulation (Ebert, 1999).

In vitro studies showed that human monocytes and macrophages ingest trophozoites that are subsequently killed by an oxidative mechanism (Röxstrom-Lindquist et al., 2006). Ingestion is enhanced by the opsonisation of the trophozoites with serum antibodies (Hill and Pearson, 1987). Nevertheless, no increase in macrophage numbers was observed during infection in human intestinal mucosa (Oberhuber et al., 1997), suggesting that macrophages probably do not play an important role in the immune response against *Giardia* (Röxstrom-Lindquist et al., 2006).

During *Giardia* infection in gerbils and mice, mast cells accumulate in the small intestine (Li et al., 2004). Activation of mast cells in general occurs upon binding with IgE or IgA by its Fc receptor. As a result, a range of pro-inflammatory molecules are released, including histamine and several cytokines. Mast cells are a source of interleukin 6 (IL-6). IL-6 is known to help drive B-cells switching to production of IgA (Merluzzi et al., 2010) and to direct T-cell development towards Th17 cells (Kamda et al., 2012). It was seen that IL-6 is essential for clearance and early control of acute infections, since mice deficient for mast cells or IL-6 develop chronic giardiasis (Galli et al., 2005; Zhou et al., 2003). Moreover treatment with a histamine receptor antagonist results in prolonged infection, indicating that histamine release is important for parasite clearance (Li et al., 2004). In addition, recent data show that mast cells can influence the development of acquired immune responses by affecting dendritic cells, B-cells and/or T-cells (Galli et al., 2005; Kamda et al., 2012). Finally, mast cell degranulation (under influence of cholecystokinin) results in increased smooth muscle contractility in order to generate increased peristaltics and parasite clearance (Singer, 2011).

Activation of the complement cascade

In vitro, *Giardia* activates the complement cascade by the classical pathway after opsonisation of trophozoites with antibodies (Hill et al., 1984). Evans-Osses et al. (2010) showed involvement of the lectin pathway in complement killing of *G. duodenalis* trophozoites *in vitro*. Although the complement system is able to kill trophozoites, it is not likely that complement activation plays an important role *in vivo*, since *Giardia* is not penetrating the intestinal epithelium.

Activation of the adaptive immune system

Not much is known about the primary mucosal host defence mechanism against *Giardia*. It is known that microfold cells (M cells) play a role as antigen transporting cells. M cells endocytose and transport antigens from the lumen to the underlying Peyer's patches in the intestine. Adherent particles are taken up by endocytosis, whereas non-adherent antigens are internalised by fluid-phase endocytosis (Nicoletti, 2000). One study showed that *G. muris* trophozoites do not adhere or get transported by M-cells (Wolf and Bye, 1984). Other possible antigen-presenting cells are macrophages, but since no increased numbers of macrophages are seen during infection, macrophages probably play a minor role during *Giardia* infection (Oberhuber et al., 1997).

Dendritic cells

Dendritic cells (DC) are the first cells in line in the initiation of immune responses as these cells have the unique capacity to activate naïve T-cells. DC are present in all body tissues. In the intestine, different subtypes of DC are present in a steady state. They take care of the maintenance of oral tolerance but during infection they are able to induce immunity (Mazzini et al., 2014). DC sample the luminal content via the extension of dendrites between epithelial cells (Rescigno et al., 2001). Here, in the peripheral tissue, classic myeloid DCs are present in a so-called 'immature' state. These immature DC are well equipped to capture antigens and take-up particles and pathogens by phagocytosis. When the uptake of an antigen is combined with the presence of a danger signal the DC becomes activated (Kapsenberg, 2003). This danger signal can consist of molecules associated with cellular stress or tissue damage, such as uric acid or ATP, inflammatory tissue factors, or the recognition of a PAMP by a PRR (Kapsenberg, 2003; Akira and Takeda, 2004). In the absence of a danger signal, a small percentage of the DC can still present antigens and induce the differentiation of regulatory T cells, which contributes to oral tolerance (Mazzini et al., 2014).

After uptake of the antigen, it enters the endocytic pathway of the DC, subsequently antigens are processed and the DC starts to produce large amounts of MHCII, and antigen-MHCII complexes are formed and presented on the cell surface. Once a DC has captured an antigen, its potential to capture antigens rapidly declines. This is the

maturation state. During this maturation process, DC migrate to the draining lymph nodes, where the fully matured DC presents the processed antigen to naïve T-cells. When the DC is fully activated, co-stimulatory molecules, such as CD40, CD80, CD83 and CD86, necessary for the further activation of T-cells, are expressed on the cell surface. In addition, DC maturation is accompanied by changes in the cytokines and chemokine secretion profile, enabling the mature DC to dictate the polarization of T-cells.

Dendritic cells possess a repertoire of receptors, PRRs, such as TLRs, C-type lectins, NOD-like receptors, NALP and RIG-like receptors (see above), which are activated by molecular patterns (McGreal, 2009). Of these PRRs, TLR are the best characterized and are a key component in the innate immune system (Iwasaki and Medzhitov, 2004; Krishnaswamy et al., 2013). There are at least 13 TLR members in mammals, from which 10 in cattle and 11 in humans (Granucci et al., 2008). Each TLR recognises specific pathogenic components, such as lipoproteins, lipopolysaccharides or bacterial DNA. TLR not only recognize pathogens but also elicit a specific transcriptional response based on the type of pathogen encountered (Carmody and Chen, 2007). Sometimes a microorganism is recognized by more than one TLR (Dillon et al., 2004). For several protozoan parasites it is known by which TLRs they are recognized. For instance, *Cryptosporidium* is recognized by TLR2 and TLR4, *Trypanosoma* by TLR4, *Toxoplasma* is recognized by TLR11 and *Plasmodium* by TLR9 (Carmody and Chen, 2007; Kamda and Singer, 2009). However for *Giardia* it is not known which PRRs or TLR(s) are involved in the host-pathogen interaction. It is likely that *Giardia* can be recognised by TLRs since Kamda and Singer (2009) showed that *G. duodenalis* can regulate the response of DC to TLR2, TLR4 and TLR9 agonists.

DC do not only play a crucial role in the initiation of T-cell responses, they can also influence T-cell polarization. Distinct types of DC subsets can induce different polarized effector CD4⁺ T-cell populations (Dillon et al., 2004), which are the incentive of adaptive immune responses. To induce a T-cell response 3 signals are required. The first signal is T-cell receptor (TCR) triggering by the antigen-specific presentation of MHCII-associated peptides. Signal 2 is the co-stimulatory signal expressed on the surface of DC. In the absence of signal 2, T-cells can become anergic, which might lead to T-cell tolerance. Signal 3 is the polarizing signal that is

mediated by various soluble or membrane bound factors of the DC, such as interleukins or chemokines. The nature of signal 3 depends on the activation of the particular PRR by PAMPs (Kapsenberg, 2003). Signal 3 can also be provided by factors from the environment, for example antimicrobial proteins and peptides or cytokines, in response to infection or injury (McDonald and Maizels, 2008).

T-cell polarization is most likely not a result of one mechanism (McDonald and Maizels, 2008). However, various molecules have well documented polarizing capacities (Fig. 7); IL-12 and interferons (IFNs) are Th1-cell polarizing factors, the ligation of OX-40L, CC-chemokine ligand 2 (CCL2) and IL-4 are Th2-cell polarizing factors, TGF- β , IL-6 and IL-23 are Th17 polarizing factors and IL-10, TGF- β are regulatory T-cell factors (Kapsenberg, 2003; McDonald and Maizels, 2008). A less characterised Th3 population is also described. Naïve T cells differentiate into Th3 in the presence of TGF- β , IL-4, IL-10 and IL-12 while the regulatory Th3 cell produce TGF- β , IL-4 and IL-10 (Weiner, 2001).

DC that cause tolerance or suppress the downstream immune response, called tolerogenic DC, can be induced in several ways. First the DC can be activated by a tolerogenic signal such as apoptotic cells or cell debris. Second, it is hypothesised that in absence of signal 2, DC can be partially activated and persist in a state of semi-maturation (Tan et al., 2005). Third, tolerogenic DC can be induced under influence of cytokines, such as IL-10 and TGF- β , or by factors expressed or secreted by the intestinal epithelium (Rutella et al., 2006; Iliev et al., 2010). Tolerogenic DC are characterised by the expression of surface molecules such as programmed cell death ligand 1 (PD-L1) and immunoglobulin like transcript 3 (ILT-3) or ILT-4. Besides tolerogenic DC produce IL-10 and TGF- β but not IL-12 (Rutella et al., 2006).

Tolerance is induced by tolerogenic DC by the initiation of a regulatory T-cell response (see above) or by the induction of T-cell anergy or depletion. The latter typically occurs in case of chronic (viral) infections (Shortman et al., 2002).

Kamda and Singer (2009) showed that in combination with different TLR agonists, *G. duodenalis* extract caused a dose-dependent inhibition of the IL-12 production and increase of IL-10. This inhibitory effect is dependent on the activation of the phosphoinositide 3-kinase-pathway. Inhibition of DC by *G. duodenalis* was also shown after stimulation of human DC with *Giardia* ADI (Banik et al., 2013). ADI is

described as a virulence factor secreted by *Giardia*, and it competes with the host for arginine. ADI affects signalling via the rapamycin (mTOR) pathway, which in turn regulates the expression of co-stimulatory molecules on the cell surface of DC (Banik et al., 2013). Next to co-stimulatory molecules, ADI also affects NO synthesis as described above. This suggests that the parasite actively restricts the development of severe inflammation, which may contribute to the lack of pathology (Solaymani-Moammadi and Singer, 2010). It is suggested by Kamda and Singer (2009) that the immune response is suppressed by an inhibitory effect of *G. duodenalis* on DC, which initiate the adaptive immune response. This raised the question whether chronic infections seen in cattle can be caused by the effect of *G. duodenalis* on DC.

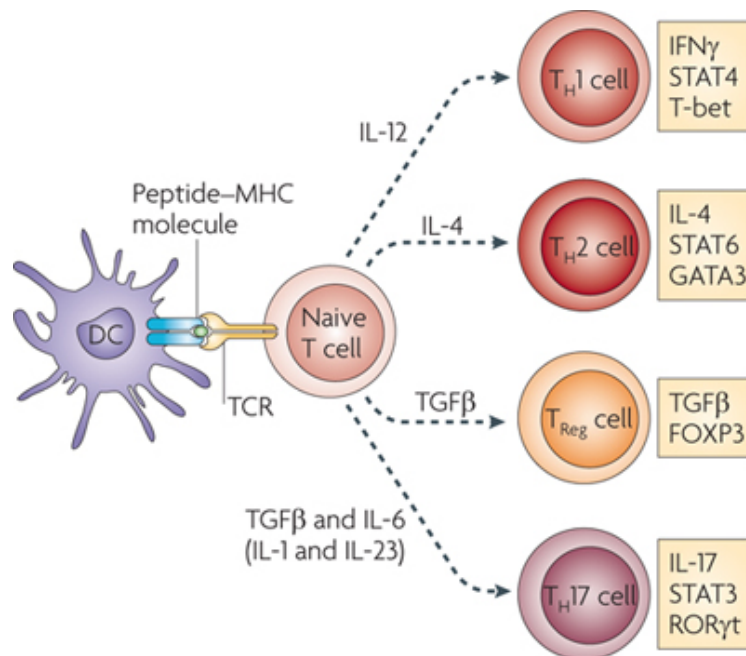


Figure 7. T-cell polarization by DC (from Zou and Restifo, 2010 with permission of Nature)

1.6.2 Adaptive immune response

Introduction

Giardiasis is self-limiting in 85% of the cases, however immunocompromised individuals often develop a chronic *Giardia* infection, suggesting that adaptive immune responses are required to control *Giardia* infection (Oberhuber et al., 1997). This presumption is strengthened by different *Giardia* outbreaks where locals, who were more likely to be previously exposed, had less symptoms compared to visitors (Gleason et al., 1970; Istre et al., 1984; Isaac-Renton et al., 1994). Current knowledge on the adaptive immune response is obtained from studies in mice and humans. *G. duodenalis* infections in mice are self-limiting and no cysts are found after a period of 14-17 days (Hill et al., 1983). In immunocompetent humans *G. duodenalis* infection is limited to a period of 2 days to a few weeks (Eckmann, 2003; Rendtorff, 1954).

Cellular immune response

The exact mechanism of the specific immune response against *Giardia* is not fully understood. In mice a few days after infection the specific or adaptive immune response is activated by the innate immune system. This specific response occurs in at least two distinct phases: the acute phase or cellular phase, which is T-cell dependent and the later B-cell dependent or humoral phase (Adam, 1991).

In mice, after 3-5 days post infection the number of intraepithelial lymphocytes (IEL) increases (Solaymani-Mohammadi and Singer, 2010). These IEL are mostly cytotoxic CD8⁺ T-cells as well as inducing or helper CD4⁺ T-cells (Scott et al., 2004). Since B-cell deficient mice eliminate the majority of parasites within two weeks (Li et al., 2004) antibodies are not necessary to clear infection in this acute phase. Mice lacking CD4⁺ T lymphocytes are unable to clear infection, and it was shown that especially CD4⁺ αβ T-cells are required to control *Giardia* infection (Singer and Nash, 2000; Zhou et al., 2007). CD4⁺ T cells are required at all time points post-infection (Solaymani-Mohammadi and Singer, 2010), probably because CD4⁺ T-cells play a role in the regulation of anti-*Giardia* IgA (Djamiatun and Faubert, 1998). The importance of CD4⁺ T-cell was also confirmed in human patients. In patients with clinical giardiasis CD4⁺ T-cell infiltration in the duodenum was detected (El-Shazly et al., 2003). Moreover, the activation of CD4⁺ memory cells after *in vitro* re-

stimulation was confirmed in human blood samples (Gottstein et al., 1991; Hanevik et al., 2011). In addition Ebert (1999) established that human intestinal CD4⁺ IEL and lamina propria lymphocytes could be activated by *in vitro* re-stimulation and subsequently produced IFN- γ .

Mice depleted of CD8⁺ cells show normal parasite eradication. In contrast, it is shown that during infection activated CD8⁺ T-cells are causing microvillus shortening and crypt cell hyperplasia (Scott et al., 2004) and interfere with the disaccharidase activity (Solaymani-Mohammadi and Singer, 2010). Moreover mice lacking thymic T-cells do not show intestinal villus injury (Buret, 2005), indicating that villus injuries are caused by $\alpha\beta$ T-cells. It has been shown that during the elimination phase the intraepithelial and lamina propria CD8⁺ T cell population declines while the CD4⁺ T-cell population increases (Vinayak et al., 1991). It is known that CD8⁺ and CD4⁺ T-cells regulate the proliferation and increased homing of lymphocytes into the intraepithelial compartment during *Giardia* infection (Scott et al., 2004).

Cytokine pattern and T-cell polarization

Based on *in vitro* studies in mice, CD4⁺ T-cell responses are divided into subsets characterised by the production of specific cytokines and the presence of subset-specific transcription factors. Th1 cells typically produce IFN- γ , Th2 are characterised by the production of IL-4, IL-5 and IL-13, Th17 cell by the production of IL-17 and Treg cells by the production of TGF- β (Fig. 7) (Zou and Restifo, 2010). However, no clear cytokine pattern was found in *Giardia* infections. Some researchers suggest that *Giardia* stimulates a mixed Th1/Th2 response (Jimenez et al., 2009; Venkatesan et al., 1996), while according to others neither Th1 nor Th2 is required for control of acute infections (Djamiatun and Faubert, 1998; Singer and Nash, 2000).

Elevated levels of IL-5, IL-6 and IFN- γ were measured in sera of infected humans (Matowicka-Karna et al., 2009; Long et al., 2010). Others found increased levels of IL-2, sIL-2R and TNF- α in human serum (Singer, 2011). These data indicate that humans produce a broad array of cytokines in response to *Giardia* infection, but it is unclear which cytokines contribute to parasite control (Singer, 2011).

No data are available on the *in vivo* production of cytokines during *Giardia* infection in the intestine (Roxström-Lindquist et al., 2006). *In vitro*, it was shown that stimulated mice spleen cells produced IL-4, IL-5, IL-10 and IFN- γ (Djamiatun and

Faubert, 1998; Jimenez et al., 2009). Cytokines probably play a role in controlling *Giardia* infection, but not all of them have an indispensable function since IFN- γ , IL-4 or STAT-6 deficient mice can still control *Giardia* infections (Zhou et al., 2003). The only cytokines known with a role in clearance of the parasite are IL-6 and TNF- α (Zhou et al., 2003). IL-6 or TNF- α deficient mice are not able to eradicate the infection even though normal *Giardia*-specific IgA production was observed (Roxström-Lindquist et al., 2006). The production of IL-6 and TNF- α by *G. duodenalis* stimulated mouse bone marrow DC was observed by Kamda and Singer (2009). Besides, Banik et al. (2013) measured the production of IL-6 by human DC after stimulation with the *Giardia* enzyme ADI. TNF- α is a pro-inflammatory cytokine. IL-6 plays a role in the switch of IgM to IgA and the development of the adaptive immune response. It is also known to be up-regulated after muscle contraction (see above).

Humoral immune response

If the infection persists beyond the acute stage, a second B-cell dependent phase can occur. In this phase specific anti-*Giardia* antibodies are produced by activated B-cells. The importance of the humoral response is supported by the fact that patients with hypogammaglobulinemia suffer from chronic giardiasis (Singer and Nash, 2000).

Antigenic variation is an evasion process used by several microorganisms to maintain chronic infection (Prucca et al., 2008). The variant *Giardia* antigens are called variant specific surface proteins (vsp) and cover the entire surface of the trophozoite. Furthermore vsp are excreted and/or secreted into the direct environment of the trophozoites. The *Giardia* genome encodes 190 vsp genes, switching the expression of another vsp once every 6 to 13 generations (Prucca et al., 2008). Vsp vary in size from 30-200kDa and possess a variable cysteine-rich amino-terminal region and a well-conserved carboxyl trans-membrane terminal with a cytoplasmic tail. It is thought that antigenic variation is driven by the immune system of the host since vsp expressed in athymic mice remain unchanged (Faubert, 2000). Antigenic variation of *G. duodenalis* is probably induced after the recognition by specific antibodies. On the other hand, it is suggested that the longer period required for antibody-dependent control of the infection is due to the ability of the parasite to undergo antigenic

variation (Li et al., 2004). Next to escaping the host's specific immune response vsp are thought to enable the trophozoite to survive in the intestinal environment of different hosts (Adam, 2001) or to expand the possible host range, since the selection of a single vsp is not similar in different host species, while it is in *in vitro* culture (Singer et al., 2001).

In humans or mice infected with *Giardia*, specific IgA, IgM and IgG were found in mucosal secretions and serum after a period of 7 and 9 days, respectively (Nash et al., 1990; Vinayak et al., 1991). *Giardia*-specific IgA is also detected in saliva and breast milk (Röxstrom-Lindquist et al., 2006). Mice deficient for IgM show normal parasite clearance, suggesting that IgM does not have any anti-giardia function in host defence (Langford et al., 2002). IgM is the first secreted isotype during infection, after B-cell activation IgM production switches into IgA production. The switching requires Th2 lymphocytes and mast cells, both secreting IL-5 and IL-6 (Faubert, 2000). The presence of specific IgA correlates with the clearance of the parasite (Langford et al., 2002). It has been shown that IgA and IgG antibodies coat the trophozoites but since IgA is reported not to kill trophozoites after *in vitro* co-incubation (Eckmann, 2003), it is likely that IgA is causing immobilization or detachment of the trophozoite from the epithelium (Langford et al., 2002).

IgA is shown to be the most important isotype in the clearance of *Giardia* and IgA is secreted in large quantities into the intestinal lumen (Stäger and Müller, 1997). It is known that IgA is indispensable for parasite clearance since B-cell deficient or IgA deficient mice could not eradicate either *G. muris* or *G. duodenalis* (Langford et al., 2002). The fact that IgA is crucial for clearance is strengthened by the fact that mice deficient for the poly Ig-receptor cannot clear infection, just as IgA deficient mice. The poly Ig-receptor transports IgA from the IgA producing cells in the lamina propria to the intestinal lumen (Solaymani-Mohammadi and Singer, 2010).

In the immunity against the mouse-specific species *G. muris*, B-cells appear to play a more important role in the early phase instead of the later phase (Li et al., 2004). This can explain why researchers using other animal models report that B-cells only play a limited role in the immunity. Besides, in the murine host the humoral immune response is predominantly directed against vsp while in human patients antibodies against invariant antigens are also produced during infection (Muller and Von Almen, 2005). In contrast to *G. muris* infection, *G. duodenalis* in mice could be controlled in the absence of antibodies (Solaymani-Mohammadi and Singer, 2010). This suggests

that mice have additional mechanisms, able to kill *G. duodenalis*, to which *G. muris* is resistant (Solaymani-Mohammadi and Singer, 2010).

1.6.3 Immunity against *G. duodenalis* in cattle

Most immunological data about *Giardia* infections is obtained from studies in mice and humans (Table 3). However, this data can possibly not be extrapolated to ruminants. Although it is plausible that animals, previously infected with *Giardia* develop resistance to subsequent infections, *G. duodenalis* infections in calves are in general chronic, in contrast to infections in mice. In calves, a peak prevalence of *Giardia* in animals aged between 1 and 6 months was observed and cysts are excreted over a period of more than 100 days (Xiao and Herd, 1994; O’Handley et al., 2003). Generally, calves suffer from different episodes of diarrhoea and during this chronic stage active immunity is developed (O’Handley et al., 1999; Chase et al., 2008). Due to this active immunity a decrease in prevalence is seen from the age of 6 months onwards (Xiao and Herd, 1994; Ralston et al., 2003). After 3 weeks of natural infection in calves, no histological changes in the jejunum were found by Dreesen et al., 2012. Microarray data suggested a decrease in inflammation, immune response, and immune cell migration in infected animals (Dreesen et al., 2012). However, after a period of 6 weeks, cellular infiltration of the mucosa and a lowered crypt/villus ratio in the jejunum has been observed in calves that were experimentally or naturally infected with *G. duodenalis* (Taminelli et al., 1998, Ruest et al. 1997).

In cattle only one study is performed on the presence of anti-*giardia* IgG in the serum of infected calves, but no anti-*Giardia* serum IgG was measured until 8 weeks post infection (O’Handley et al., 2003). Not much is known about the immune response in calves and further investigation about the host immune mechanism is necessary.

Table 3. Comparison of current knowledge of immune responses against *G. duodenalis* in mice, humans and cattle

	mice	human	cattle
Duration of cyst excretion	14-17	2 days - few weeks	>100 days
Innate immune response	mucus, microbiota, paneth cells, mast cells	?	?
Factors/ Cytokines produced by epithelium	defensins, ROS, NO	CCL2, CCL20, CXCL1, CXCL2 and CXCL3	?
Time until increase IEL	3-5 days	?	6 weeks
Time until T-cell activation	acute phase	?	?
T-cell subsets involved in protection	$\alpha\beta$ -CD4 ⁺	$\alpha\beta$ -CD4 ⁺	?
Cytokines important for protection	IL-6 and TNF- α	?	?
Cytokines produced by CD4 ⁺ T-cells	?	IFN- γ	?
Time until antibodies are measured in serum	9 days	7 days	?
Isotype of serum antibodies	IgA, IgG, IgM	IgA, IgG, IgM	? (no IgG)

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Objectives

Objectives

G. duodenalis occurs in human patients and animals, and is worldwide one of the most important parasitological causes of diarrhoea, ill thrift and weight loss. World wide high prevalences are reported in humans, companion animals and cattle. In cattle, *G. duodenalis* can cause diarrhoea and reduced weight gain (Geurden et al., 2010a; 2010b). In cattle and other natural hosts not much is known about the immune response against *G. duodenalis*. Most immunological data has been obtained from infection trials in mice and *in vitro* experiments. Since mice are not a natural host of *G. duodenalis*, it is uncertain if data regarding *G. duodenalis* or the related *G. muris* in mice can be extrapolated to *G. duodenalis* in ruminants. *G. duodenalis* infections in mice are hard to establish and are only inducible when intra-gastric inoculation of high numbers of trophozoites is used or when the infection is established in neonatal mice (Gottstein et al. 1990; Byrd et al., 1994). Hence in most studies the mouse-specific *G. muris* is used. While *G. duodenalis* infections in cattle are chronic, *G. muris* infection is self-limiting after 21-35 days (Robert-Thompson et al., 1976; Heyworth 1986; Singer and Nash 2000).

Recent data indicate that the immune response to *G. duodenalis* in calves is suppressed during the first weeks of infection (Dreesen et al., 2012). The question rose whether suppression of DC, as seen by Kamda and Singer (2009), could be the cause of the chronic *G. duodenalis* infections seen in cattle (Fig. 1). This regulatory effect on DC is seen with several protozoa, such as *Plasmodium*, *Leishmania*, and *Toxoplasma*. By manipulating DC they create an optimal environment to survive in the host (Terrazas et al., 2010).

Although *G. duodenalis* infections in cattle have a chronic character and calves can suffer from different episodes of diarrhoea, after several months protective immunity finally develops and cyst excretion declines (Fig 1). However, it is unknown by which immune mechanism this (protective) response is caused.

Therefore the objectives of this Ph.D. thesis were:

1. To investigate whether *G. duodenalis* influences the capacity of monocyte-derived dendritic cells (MoDC) to induce T-cell activation in cattle (Chapter 2)
2. To determine the kinetics of the systemic immune responses against *G. duodenalis* in calves and to investigate the role of antigen presenting cells in the cellular immune response (Chapter 3).

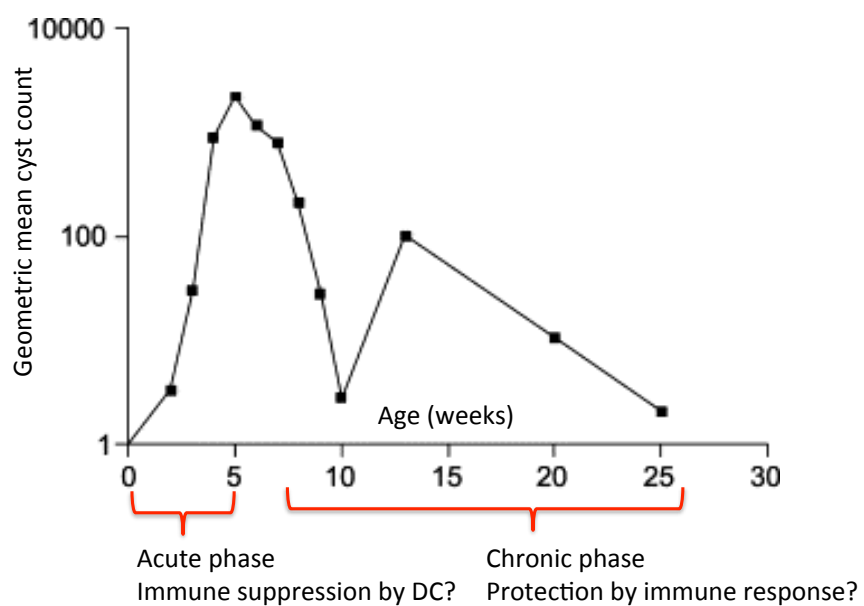


Figure 1. Geometric mean of cyst counts in 20 naturally infected beef calves (adapted from Ralston et al., 2003).

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Chapter 2.

The role of dendritic cells in the immunology against *G. duodenalis*

Based on: Grit G.H., Van Coppernolle S., Devriendt B., Geurden T., Hope J., Vercruyse J., Cox E., Geldhof P., Claerebout E., 2014. *Giardia duodenalis* stimulates partial maturation of bovine dendritic cells associated with altered cytokine secretion and induction of T-cell proliferation. *Parasite Immunology* (in press) doi: 10.1111/pim.12095

Chapter 2 The role of dendritic cells in the immunology against *G. duodenalis* in cattle

2.1 Introduction

In cattle, Dreesen et al. (2012) found no histological changes in the jejunum after 3 weeks of natural *G. duodenalis* infection. After a longer exposure period, cellular infiltration of the mucosa and a lowered crypt/villus ratio in the jejunum has been observed in calves that were experimentally or naturally infected with *G. duodenalis* (Taminelli et al., 1998, Ruest et al. 1997).

It has been shown that different protozoa actively interfere with DC to promote a more permissive environment for their own survival in the host (Terrazas et al., 2010). Since in some studies giardiasis was associated with little or no inflammation (Dreesen et al., 2012, Oberhuber et al., 1997) it may be hypothesized that *G. duodenalis* can cause inhibition of the immune response by actively influencing DC (Terrazas et al., 2010). Kamda and Singer (2009) established the effect of *G. duodenalis* on bone marrow-derived DC from mice and showed that *G. duodenalis* extract actively interferes with the host's innate immune system. Co-incubation of murine DC with *G. duodenalis* and the TLR4 ligand LPS resulted in enhanced production of IL-10 and reduced secretion of IL-12, which could be consistent with maintaining an anti-inflammatory environment in the gut (Kamda and Singer, 2009). In this study we assessed the effect of *G. duodenalis* on monocyte-derived DC (MoDC) from a natural host species, *i.e.* cattle, to investigate the potential of the parasite to influence the capacity of these MoDC to induce immune responses.

2.2 Materials and Methods

Animals and parasites

For MoDC maturation assays and for the allogeneic lymphocyte proliferation assay MoDC were obtained from blood samples from four healthy male Holstein Friesian calves, between one and four months of age. The calves were purchased from a farm with no history of giardiasis and their *Giardia*-negative status was confirmed by three negative faecal examinations during a nine days period. *Giardia* cyst excretion was determined by a direct immunofluorescence assay (Merifluor[®] Cryptosporidium/

Giardia kit, Meridian Diagnostics Inc.).

For the allogeneic lymphocyte proliferation assay, PBMC were obtained from one donor calf, that had previously tested negative for *Giardia* (see above), and which was unrelated to the animals used to isolate MoDC.

For the autologous lymphocyte proliferation assay, six calves were infected with 10^5 *G. duodenalis* (assemblage A + E) cysts at the age of 3 weeks and housed on straw bedding, allowing re-infection. The calves' infection status was monitored two times a week by a quantitative direct immunofluorescence assay, as described above. Blood samples were collected from the calves after 20 weeks of infection for the isolation of PBMC.

Generation of bovine monocyte-derived dendritic cells and stimulation of MoDC with Giardia

Venous blood was drawn from the vena jugularis using vacutainer tubes. Bovine monocyte-derived DC (MoDC) were generated from peripheral blood mononuclear cells (PBMC) as previous described (Hope et al., 2003; Werling et al., 1999). Briefly, PBMC were obtained using a Lymphoprep density gradient (Axis-Shield) and subsequently incubated with anti-human CD14 microbeads (Miltenyi Biotec). CD14⁺ monocytes were isolated using magnetic cell sorting (MACS, Miltenyi Biotec) according to the manufacturer's instructions. The purity of the cells was evaluated by flow cytometry and was always $\geq 95\%$. Monocytes were cultured (0.5×10^6 cells/ml) for seven days with 10ng/ml bovine rIL-4 (Sigma) and 37.5ng/ml porcine recombinant GM-CSF (provided by S. Inumaru from the Laboratory of Bioengineering, National Institute of Animal Health, Tsukuba, Ibaraki, Japan; Inumaru et al., 2000) in DMEM without phenol-red (Invitrogen) supplemented with 10% foetal calf serum (TCS biosciences), 500U/ml penicillin and 500 μ g/ml streptomycin. Every 3 days the cultures were supplemented with fresh cytokines. After 7 days the cells were stimulated with live *G. duodenalis* trophozoites or excretion secretion material (ES) for 40 hours (Fig. 1). Before adding live trophozoites to the cell cultures, the parasites were washed three times in PBS to remove the medium. To improve the survival of the trophozoites, 12mM L-cysteine (Sigma-Aldrich) was added to the cell culture medium. To measure the effect of ES, direct contact of live trophozoites with DC was prevented using 0.4 μ m cell culture

inserts for 24-well plates (BD biosciences) (Fig. 1). Different parasite/MoDC ratios were used: 1/1, 1/10 and 1/100. One $\mu\text{g/ml}$ LPS (*E. coli* 0111:B4 Invivogen) was used as a positive control (Ardehna et al., 2000). MoDC with only medium added were used as negative control.

To investigate the effect of *G. duodenalis* trophozoites or ES on LPS-induced MoDC responses, MoDC were stimulated with 1 $\mu\text{g/ml}$ LPS for 40h, followed by 40h incubation with medium, trophozoites or ES with a 1/1 trophozoite/DC ratio. Immature MoDC stimulated with only medium were used as a negative control.

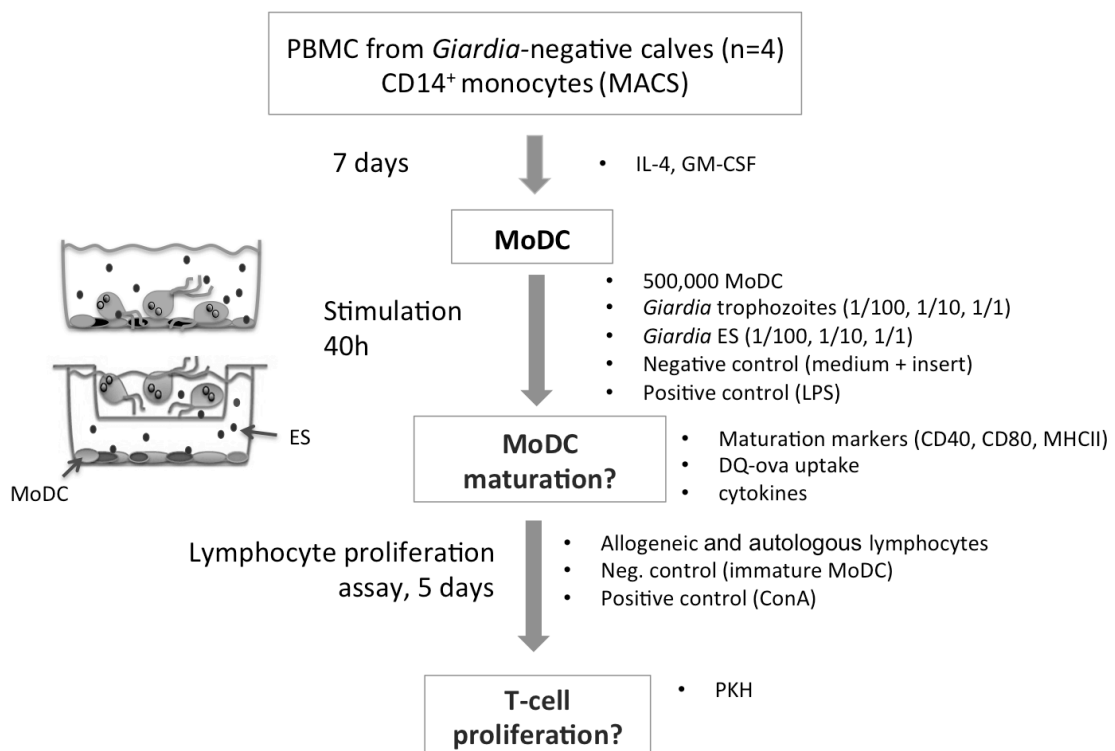


Figure 1. Scheme of study design.

Co-stimulatory molecules

Mouse anti-bovine antibodies were used to measure upregulation of the surface markers MHCII, CD40 and CD80. After 40h of stimulation MoDC were harvested and labelled with primary mouse anti-bovine antibodies recognising MHCII (CC158, IgG2a), CD40 (IL-A156, IgG1) and CD80 (IL-A159, IgG1). Bound monoclonal antibodies (mAb) were detected with isotype-specific goat anti-mouse IgG2a-PE (Invitrogen, molecular probes) and goat anti-mouse IgG1-FITC (BD biosciences). Cells stained with isotype-matched irrelevant mAbs were used as isotype control. Doublets were excluded from the analysis using FSC-H and FSC-A gating. Sytox red

dead cell stain (Invitrogen) was used to exclude dead cells from the analysis. At least 10.000 events were measured. Median Fluorescence Intensity (MFI) was measured by flow cytometry using a FACSCanto flow cytometer (BD biosciences) and analysed using FACSDiva software (Becton Dickinson). The final median fluorescence intensity (MFI) was calculated by subtracting the measured MFI values of the isotype controls from the MFI values measured in the stained samples.

Ovalbumin uptake

The ability of antigen uptake of *G. duodenalis*-stimulated MoDC was evaluated by DQ-ovalbumin uptake (DQ-OVA, Molecular Probes) in a well-established tracer molecule uptake assay. MoDC were incubated for one hour at 4°C and 37°C with 13µg/ml BODIPY-conjugated DQ-ovalbumin, the cells were then washed twice with PBS and the median fluorescence intensity (MFI) of the DQ-OVA uptake was measured by flow cytometry as described above. Doublets were excluded from the analysis. Sytox red dead cell stain (Invitrogen) was used to distinguish live cells from dead cells.

Enzyme-linked immune-sorbent assay (ELISA)

After 12, 24 and 40 hours of stimulation, the MoDC cell-free culture supernatant was harvested and stored at -80°C. Because of the limited number of available bovine antibodies, only a selection of cytokines was examined. Levels of IL-4, IL-6, IL-10, IL-12 and TNF- α in the supernatant were measured by ELISA. The following antibody pairs were used: IL-4 (CC314 and biotinylated CC313) (Hope et al., 2005), IL-10 (CC318 and biotinylated CC320) (Kwong et al., 2002) and IL-12 (CC301 and biotinylated CC326) (Hope et al., 2002). Recombinant cytokines were used as standards in every plate. IL-6 was measured using the Bovine IL-6 ELISA set (Thermoscientific, Pierce Protein) following the manufacturer's instructions. The TNF- α ELISA was carried out using the bovine TNF- α duo set (R&D systems) according to the manufacturer's instructions. To visualise the reaction, 67mU/ml of conjugate, streptavidin-POD (Roche) and 1mg/ml of o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) was used. The reaction was stopped by adding a 2.5M HCl solution. The reaction product was measured with a 492nm photo spectrometer. Results were analysed using DeltaSOFT JV 2.1.2 software

(BioMetallics) with a five-parameter curve-fitting algorithm. All conditions were performed in triplicate.

Quantitative Real-time PCR

MoDC were harvested after 12, 24 and 40 hours of stimulation and were washed twice in PBS. The supernatant was removed and the cell pellets were snap-frozen in liquid nitrogen and stored at -80°C until processing. Real-time quantitative PCR was carried out for the following cytokines: IL-2, IL-4, IL-6, IL-10, IL-15, IL-17, IL-23, TGF- β , IFN- γ and TNF- α , using a StepOnePlus Real-Time PCR system (Applied Biosciences). Additionally, the transcription of PPAR- α and PPAR- γ was measured, according to Dreesen et al. (2012). Four inhibitory molecules were evaluated: PD-L1, β catenin, ILT 3 and ILT4. These molecules are known to be upregulated in tolerogenic DC that are able to initiate Foxp3⁺ Treg cells (Schreiner et al., 2004; Atay et al., 2009; Penna et al., 2005). All primer sequences are listed in Table 1. RNA was extracted using the RNeasy kit (Qiagen) following the manufacturer's instructions. To remove contaminating genomic DNA, on-column DNase digestion was performed using the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. RNA concentrations were measured using a Nano-Drop 2000 spectrophotometer (Thermo Scientific) and first-strand cDNA was generated from 100ng RNA using the iScript cDNA synthesis kit (Bio-rad). The reaction was performed in a 20 μl volume, according to the manufacturer's instructions. Real-time PCR analysis was performed as described by Dreesen et al. (2012) using SYBR Green master mix (Applied Biosystems), 2 μl of single stranded cDNA (1ng of the input total RNA equivalent) and 400nM of each amplification primer in a 20 μl reaction volume. Primer efficiencies ranged between 1.88 and 2.05. All conditions were performed in duplicate. A relative quantification was carried out using the delta Ct method. Two control genes were chosen out of a panel of 4 candidate genes based on gene stability. The ribosomal protein S29 (RSP29) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were used for normalisation.

Allogeneic mixed lymphocyte reaction

Allogeneic PBMC were isolated from one *Giardia*-free donor as described earlier and were labelled with PKH using the PKH26 red fluorescent cell linker mini kit (Sigma-Aldrich) according to the manufacturer's instruction. Cells were cultured in cell culture medium (RPMI-1640 + Glutamax (Invitrogen) supplemented with 10% FCS, 50µg/ml gentamycin and 1/1000 β-mercapto-ethanol) in a culture flask (75 cm², NUNC) for 2 to 3 hours to allow the cells to attach to the plastic to deplete monocytes. Subsequently the non-adherent cells were harvested and 2 x 10⁵ cells/well were cultured in a 96-well round-bottom plate (Nunc) with the *G. duodenalis* stimulated MoDC at a MoDC/PBMC and trophozoite/PBMC ratio of 1/10. All conditions were performed in triplicate. As a positive control 5µg/ml Concanavalin A (Sigma-Aldrich) was used. After 6 days of co-culture, the cells were harvested and a quadruple staining was performed using the following mouse anti-bovine antibodies: CD3 (IgG1, MM1A VMRD), CD4 (IgG2a, MCA1653G, AbD Serotec), CD8 (IgM, BAQ111A VMRD) and TCRγδ (IgG2b, GB21A VMRD). Bound mAb were detected by anti-mouse IgG1-V450 (BD Biosciences), anti-mouse IgG2a-APC (Invitrogen), anti-mouse IgM-APC-Cy7 (Biolegend) and anti-mouse IgG2b-FITC (Southern Biotech). Sytox blue dead cell stain (Invitrogen) was added to distinguish live cells from dead cells. The PKH intensity was measured using the FACS Aria III (BD biosciences). Data were analysed using Flowjo software (Tree Star) and proliferation of different cell populations was quantified by calculating the stimulation index using ModFit LT software (Verity Software House). The different populations were gated and for each separate cell population a proliferation ratio was calculated based on the number of cell divisions. The proliferation ratios per cell population were then compared, medium vs. *Giardia* stimulated.

Table 1. Genes used in the RT-qPCR assay, indicating GeneBank accession number and primer sequences

Validation PCR	Accession number	Primer sequence
<i>RPS29</i>	<i>BC102702</i>	F: <i>GGAGCCATCCGAGAAAATTTCG</i> R: <i>CAACTTAATGAAGCCGATGTCCTT</i>
<i>HPRT1</i>	<i>NM_001034035.1</i>	F: <i>CACTGGGAAGACAATGCAGA</i> R: <i>ACACTTCGAGGGGTCCTTTT</i>
<i>RPLP0</i>	<i>NM_001012682.1</i>	F: <i>CTTCATTGTGGGAGCAGACA</i> R: <i>GGCAACAGTTTCTCCAGAGC</i>
<i>HDAC10</i>	<i>NM_001075460.1</i>	F: <i>CCGATGACGGGAGAAATCTA</i> R: <i>CTCAGGAACCCACCAGTTGT</i>
Cytokines	Accession number	Primer sequence
<i>IL-2</i>	<i>NM_180997.1</i>	F: <i>TCCAAGCAAAAACCTGAACC</i> R: <i>CAGCGTTTACTGTTGCATCATC</i>
<i>IL-4</i>	<i>NM_173921.2</i>	F: <i>GCGGACTTGACAGGAATCTC</i> R: <i>TCAGCGTACTTGTGCTCGTC</i>
<i>IL-6</i>	<i>NM_173923.2</i>	F: <i>TCCTTGCTGCTTTCACACTC</i> R: <i>CACCCCAGGCAGACTACTTC</i>
<i>IL-10</i>	<i>NM_174088.1</i>	F: <i>TGTTGACCCAGTCTCTGCTG</i> R: <i>GGCATCACCTCTTCCAGGTA</i>
<i>IL-15</i>	<i>NM_174090.1</i>	F: <i>AGAGGCTGGCATTTCATGTCT</i> R: <i>CTGCATCGCTGTTACTTTGC</i>
<i>IL-17</i>	<i>NM_001008412.1</i>	F: <i>GGACTCTCCACCGCAATGAG</i> R: <i>TGGCCTCCCAGATCACAGA</i>
<i>IL-23A</i>	<i>NM_001205688.1</i>	F: <i>CCCGTATCCAGTGTGAGGAT</i> R: <i>AGTATGGAGGCGTGAAGCTG</i>
<i>TGF-β1</i>	<i>NM_001166068.1</i>	F: <i>CTGCTGTGTTTCGTCAGCTCT</i> R: <i>TCCAGGCTCCAGATGTAAGG</i>
<i>IFN-γ</i>	<i>NM_174086.1</i>	F: <i>TTCTTGAATGGCAGCTCTGA</i> R: <i>TTCTCTTCGGCTTTCTGAGG</i>
<i>TNF-α</i>	<i>NM_173966.2</i>	F: <i>GCCCTCTGGTTCAGACACTC</i> R: <i>AGATGAGGTAAAGCCCGTCA</i>
<i>PPARA</i>	<i>NM_001034036.1</i>	F: <i>TCCCTCTTTGTGGCTGCTAT</i> R: <i>TCGTCAGGATGGTTGTTCTG</i>
<i>PPARG</i>	<i>NM_181024.2</i>	F: <i>GATCTTGACGGGAAAGACGA</i> R: <i>ACTGACACCCCTGGAAGATG</i>

<i>PD-L1</i>	<i>NM_001163412.1</i>	<i>F: GGG GGT TTA CTG TTG CTT</i> <i>R: GCCACTCAGGACTTGGTGAT</i>
<i>B catenin</i>	<i>NM_001076141.1</i>	<i>F: TCTATGGGTGGAACACAGCA</i> <i>R: GGGCTCCAGTACAACCTTCA</i>
<i>ILT3</i>	<i>XM_002695435.3</i>	<i>F: GGAGAATGTGACCCTGCTGT</i> <i>R: CTGTGCTGAGTGAGCCGTAG</i>
<i>ILT4</i>	<i>XM_002695438.3</i>	<i>F: ACCAGAGGTTCTCCCCAAAC</i> <i>R: AAGACCCCTGACACCAGATG</i>

Autologous mixed lymphocyte reaction

Blood samples were collected from 6 *G. duodenalis* infected calves and PBMC were collected and depleted for antigen presenting cells by plastic adhesion, as described above. Depleted PBMC were co-cultured with autologous MoDC, using the same conditions and MoDC/PBMC and trophozoite/PBMC ratios as described above. As a positive control 5µg/ml Concanavalin A was used. Depleted PBMC without MoDC were used as a control for the direct effect of trophozoites on the PBMC. Co-culture of depleted PBMC with unstimulated DC was used as negative control.

PBMC were labelled with PKH using the PKH26 red fluorescent cell linker mini kit (Sigma-Aldrich) according to the manufacturer's instruction. Cell cultures, identification of proliferating cell populations and calculation of proliferation ratios were performed as described above.

CD3⁻, TCRγδ⁺ and CD4⁺ cells were sorted from the co-culture of depleted PBMC and MoDC after 6 days of stimulation with *G. duodenalis* trophozoites. For the sorting a quadruple staining for CD3, CD4, CD8 and TCRγδ was done as described above and analysed using the FACS Aria III. Doublet exclusion was done based on FSC-H and FSC-A and subsequently, on SSC-H and SSC-A. Propidium iodide positive cells were excluded from the analysis. After sorting, cells were washed in PBS. The supernatant was removed and the cell pellets were snap-frozen in liquid nitrogen and stored at -80°C until processing. RNA extraction and real time quantitative PCR (RT qPCR) for a panel of cytokines and transcription factors was performed as described above.

Statistical analysis

Mean values of median fluorescence intensity, cytokine concentrations, Q values and proliferation indices of stimulated cells and un-stimulated cells were compared using a non-parametric Kruskal Wallis test with Dunn's *post hoc* test. P-values of less than 0.05 were considered statistically significant.

2.3 Results

Cultivation of MoDC and upregulation of co-stimulatory molecules

After 7 days of culturing the monocytes with IL-4 and GM-CSF, MoDC with typical dendrites were obtained (Fig. 2). Upregulation of co-stimulatory molecules is required for T-cell activation. Stimulation of MoDC with LPS induced a significantly increased CD40 expression and enhanced expression (NS) of CD80 and MHCII (Fig. 3). Stimulation of MoDC with *G. duodenalis* trophozoites or ES at low concentrations induced a small increase of CD40 transcription (NS). No difference in the expression of CD80 could be measured. Although MHCII expression was not significantly upregulated compared to unstimulated MoDC, MHCII levels in MoDC stimulated with diluted *G. duodenalis* trophozoites or with ES were comparable to MHCII expression induced by LPS (Fig. 3). This experiment was repeated with 3 different calves at slightly different conditions (3 days culturing of monocytes and 18h stimulation of MoDC with *Giardia* trophozoites and ES) and expression of co-stimulatory molecules was similar (results not shown).

Incubation of MoDC with *G. duodenalis* trophozoites or ES had no significant effect on LPS-induced expression of maturation markers. LPS-induced expression of CD40, CD80 and MHCII was not significantly reduced by trophozoites and ES (Fig. 4).

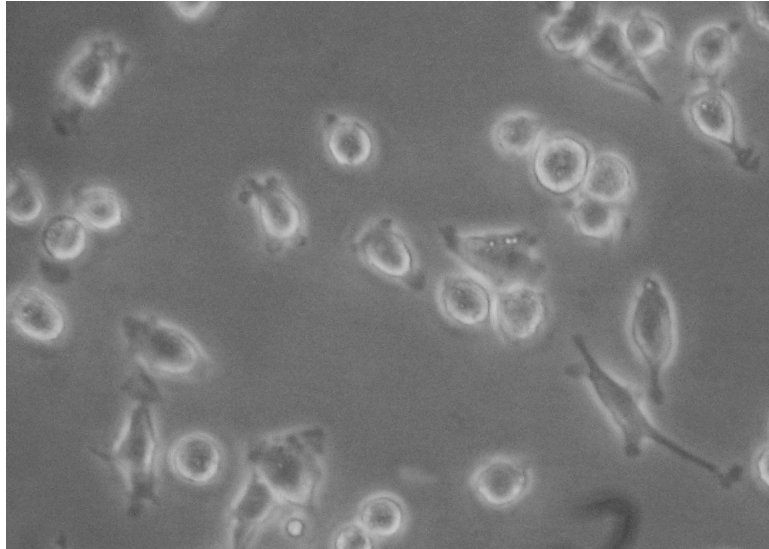


Figure 2. Light microscopic view of MoDC after 7 days of culture magnification 100x

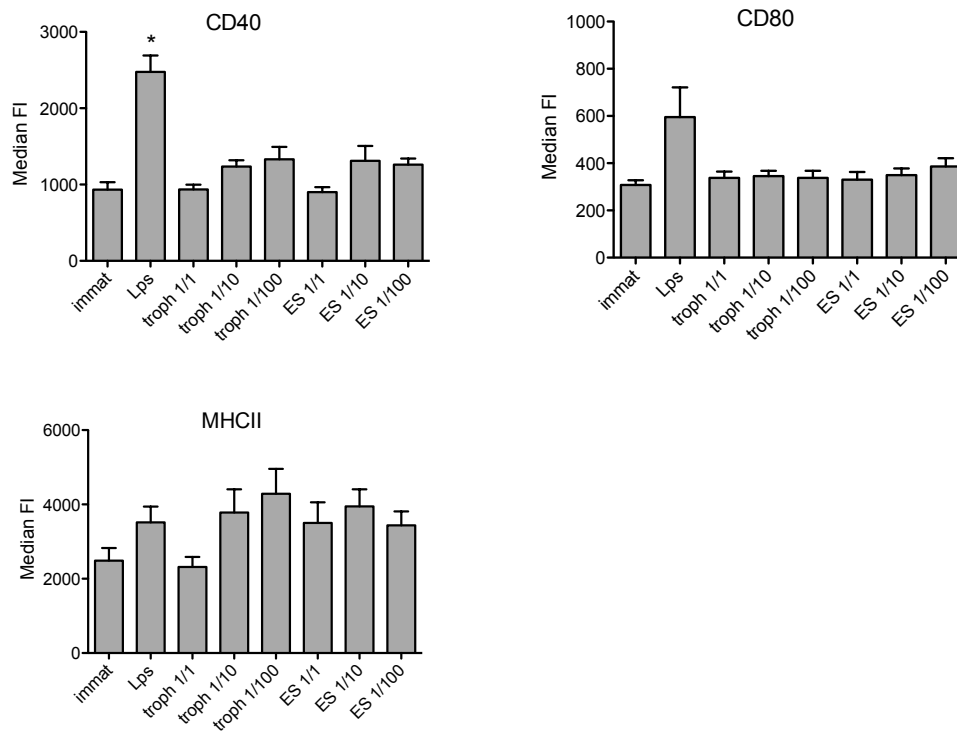


Figure 3. The effect of *G. duodenalis* on expression of bovine MoDC surface molecules. MoDC stimulated with LPS, *G. duodenalis* trophozoites or ES were compared to unstimulated MoDC. Arithmetic mean of Median Fluorescence Intensity of four animals \pm standard error of the mean are shown for unstimulated or immature MoDC (immat), for MoDC stimulated with LPS and for different parasite/MoDC ratios of live trophozoites (troph) and excretion secretion products (ES). * $P < 0.05$ to immature MoDC.

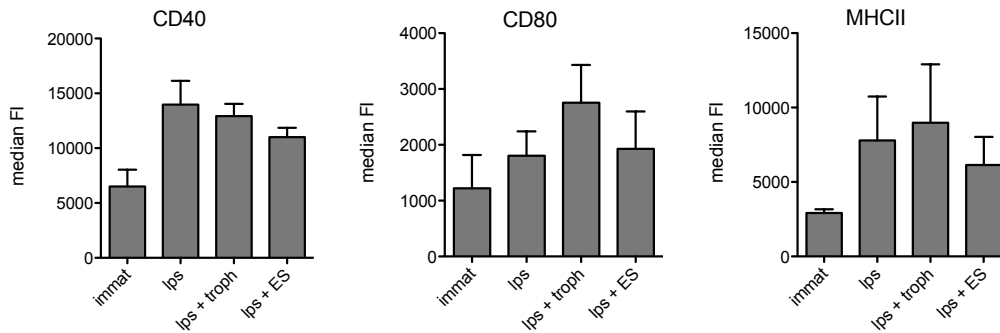


Figure 4. Marker expression of MoDC after 40h of stimulation with medium (immat), LPS and subsequently 40h with live trophozoites (troph + lps) or ES (ES + lps). MoDC stimulated with *Giardia* and LPS were compared to MoDC stimulated with LPS alone. Arithmetic mean of Median Fluorescence Intensity of four animals \pm standard error of the mean are shown. * $P < 0.05$ to MoDC stimulated with LPS alone.

Ovalbumin uptake

Immature DC are characterised by their high capacity for endocytosis. During maturation the endocytotic capacity and antigen uptake by DC is decreasing. Cells incubated with DQ-OVA at 4°C had a maximum MFI of 636, which was considered background fluorescence. LPS caused a decrease of DQ-OVA uptake compared with un-stimulated MoDC at 37°C. A dose-dependent decrease of DQ-OVA uptake was seen in MoDC stimulated with live trophozoites and ES at 37°C (Fig. 5). None of the observed changes were statistically significant.

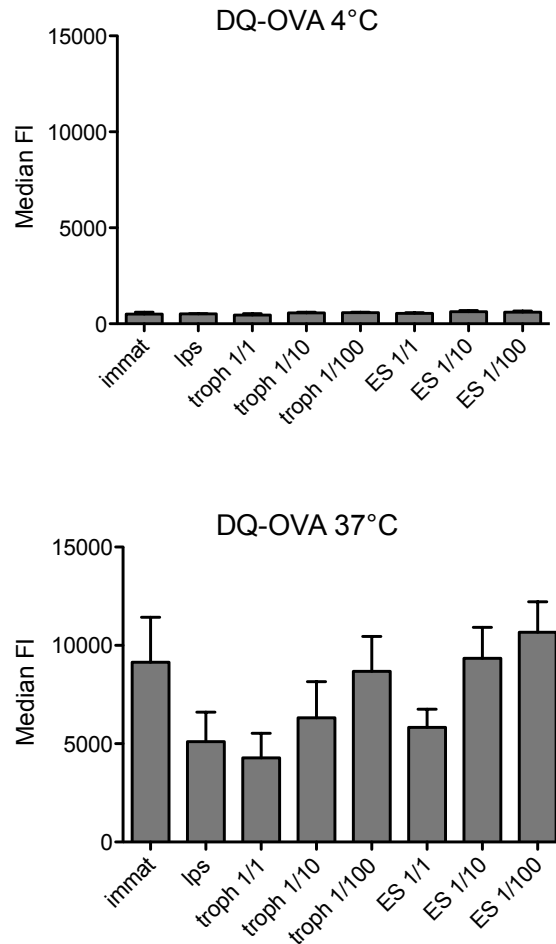


Figure 5. The effect of *G. duodenalis* on ovalbumin uptake by bovine MoDC. Stimulated MoDC were compared to immature MoDC. Arithmetic mean of Median Fluorescence Intensity of four animals \pm standard error of the mean is shown for MoDC incubated with DQ-OVA at 4°C and 37°C. * $P < 0.05$ to immature MoDC.

Cytokine production in stimulated MoDC

Stimulation with LPS significantly increased concentrations of IL-12 and TNF- α in the MoDC culture supernatant after 40h, but no significant levels of the cytokines tested (IL-4, IL-6, IL-10, IL-12 and TNF- α) could be detected by ELISA after stimulation with *G. duodenalis* trophozoites or ES (Fig. 6).

The mRNA transcription level for a panel of cytokines was evaluated by RT-qPCR. Mean fold changes of stimulated MoDC were compared to un-stimulated MoDC (Fig. 6). At time point 24h, one out of four samples stimulated with LPS contained no cDNA and hence some increases measured after stimulation with LPS were not statistically significant. Significantly increased transcription after stimulation with LPS was measured for IL-6 and IL-15. The transcription of TNF- α , IL-23, IL-10 and IL-4 was also increased, however not statistically significant (Fig. 6). Transcription of IL-15 was increased 2.5 fold ($p < 0.05$) and 2.2 fold (NS) after 24h stimulation with *G. duodenalis* trophozoites and ES, respectively. A 2.1 and 2.3 fold increase of IL-6 transcription was observed after 12h of incubation of MoDC with trophozoites or ES, an 8.3 fold increase of IL-4 was visible after 24h stimulation with ES and a 3.1 fold increase of TNF- α after 40h stimulation with trophozoites, but these apparent up-regulations were not statistically significant. The expression of several cytokines was more than two fold down-regulated after stimulation with *Giardia* (Fig. 6), but none of these changes were statistically significant. No significant differences in transcription levels of PPAR- α or PPAR- γ were found after 40h of stimulation with *Giardia* trophozoites or ES (results not shown).

G. duodenalis trophozoites and ES caused no significant changes in LPS-induced transcription of cytokines, although LPS-induced production of TNF- α seemed to be decreased (Fig. 7).

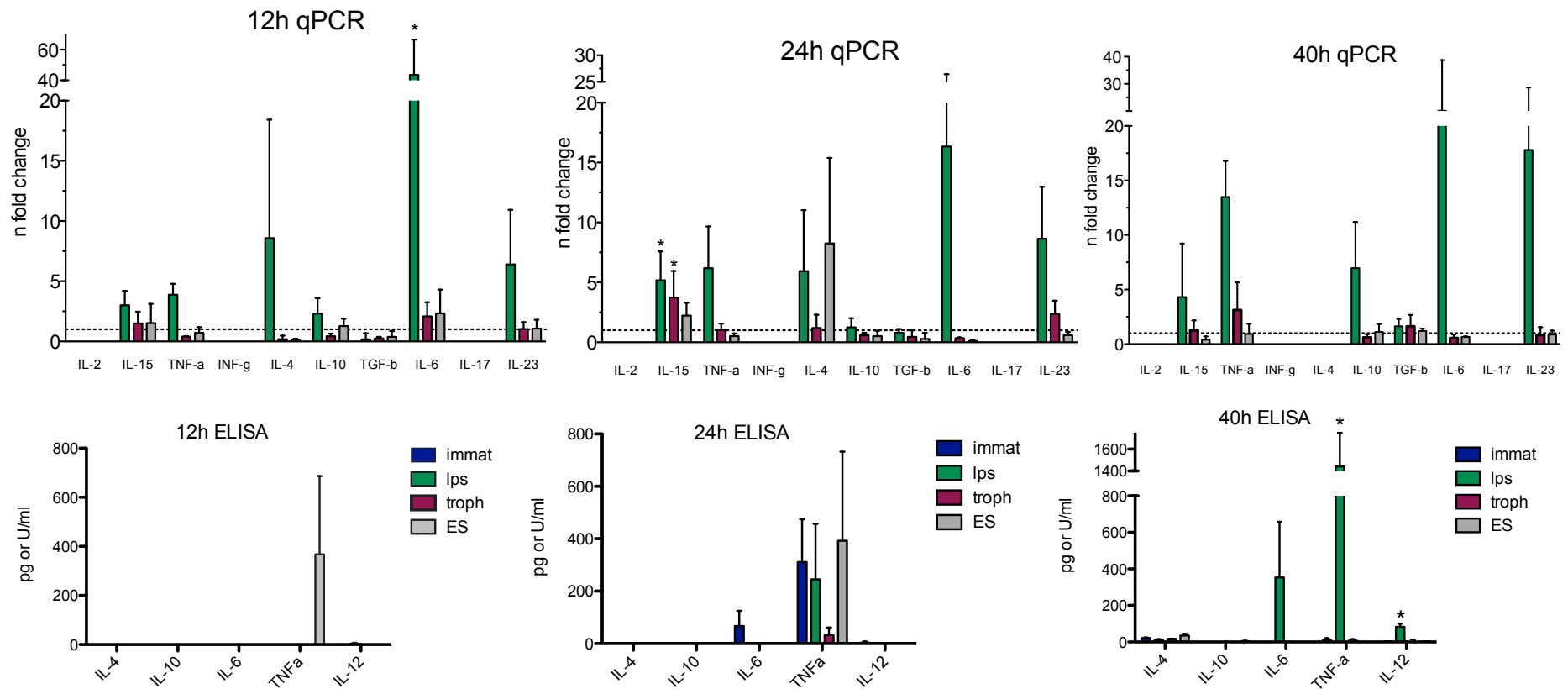


Figure 6. Cytokine (transcription) levels of MoDC from four calves after 12, 24 and 40h of culturing with medium (immat), LPS, live *G. duodenalis* trophozoites (troph) or ES. Mean fold changes in cytokine transcription levels (upper row) and cytokine concentrations (bottom row) of stimulated MoDC were compared to un-stimulated MoDC (n fold change =1, broken line) \pm standard deviation. * $P < 0.05$ to immature MoDC.

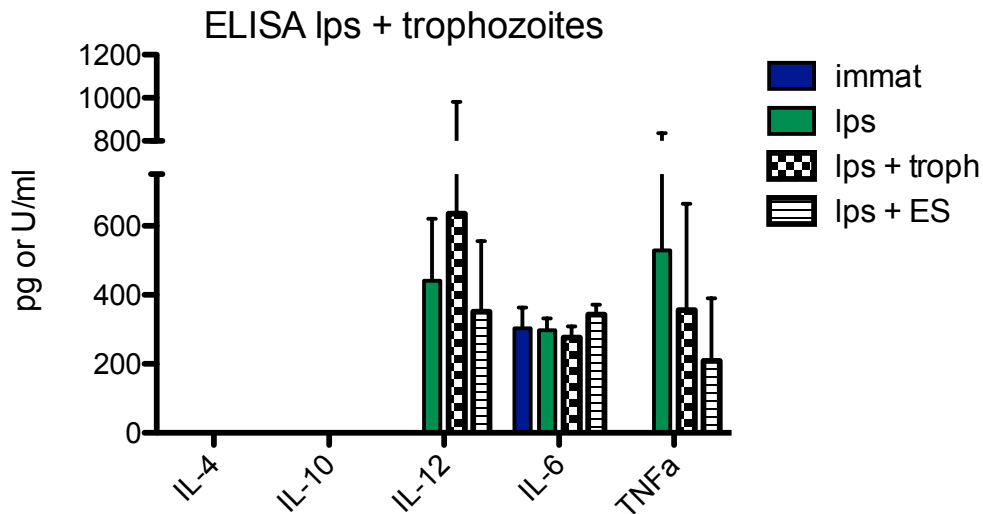


Figure 7. Cytokine concentrations (ELISA) after 40h of stimulation with LPS and subsequently with *G. duodenalis* trophozoites or ES. Mean of 4 animals is shown with sem. * $P < 0.05$ to LPS stimulated MoDC.

Inhibitory surface molecules

Transcription of the inhibitory surface molecules PD-L1, β catenin, ILT3 and ILT4 was evaluated by qPCR. Transcription levels of PD-L1, ILT3 and ILT4 were increased more than 2 fold (NS) after stimulation with LPS (Fig.8). The transcription level of β catenin was only increased (NS) after 24h of stimulation with LPS. After stimulation with *G. duodenalis* trophozoites or ES, the transcription levels were not significantly different from the medium control (Fig. 8).

Allogeneic mixed lymphocyte reaction

An allogeneic mixed lymphocyte reaction (MLR) was performed to assess the ability of *Giardia*-stimulated MoDC to induce T-cell proliferation. Co-culturing depleted PBMC with MoDC stimulated with *Giardia* trophozoites or ES caused proliferation of $CD3^+$, $TCR\gamma\delta^+$ and $TCR\alpha\beta^+ CD4^+$ and $CD8^+$ T-cells (Fig. 9), compared to cultures with unstimulated MoDC. None of these increases were statistically significant, except the proliferation of $\gamma\delta$ -T-cells and $CD4^+$ cells induced by ES stimulated MoDC.

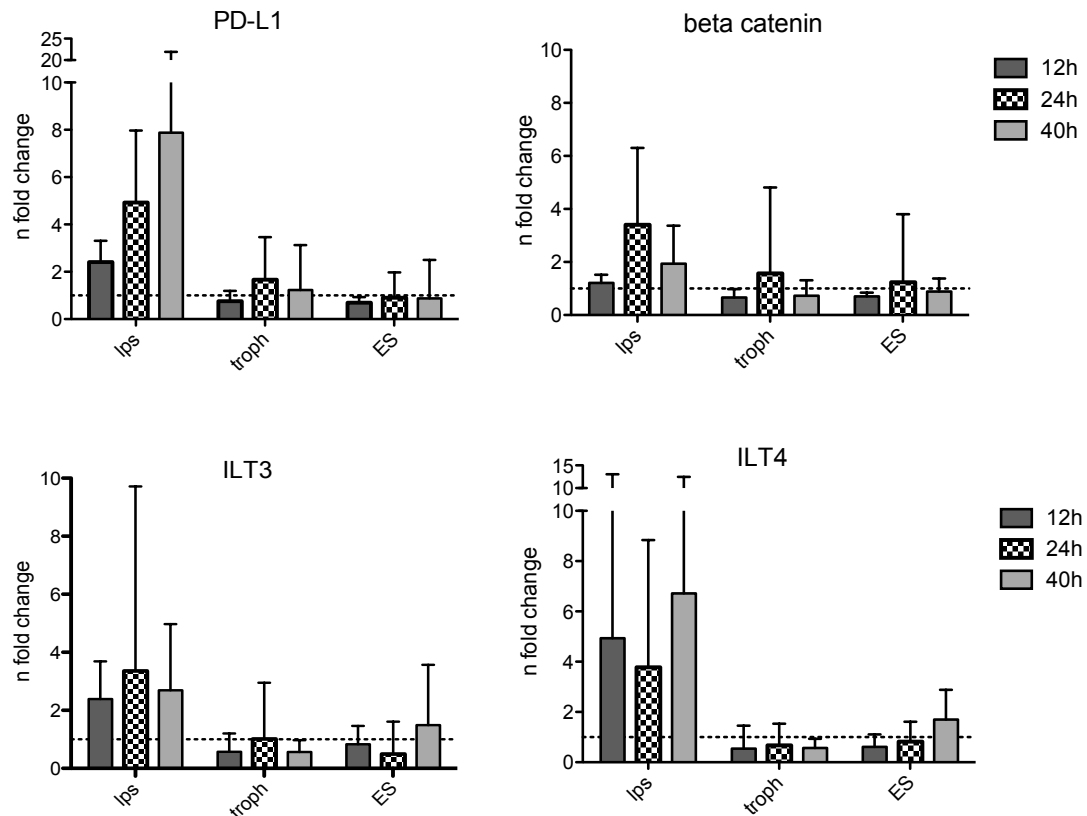


Figure 8. Transcription levels of inhibitory receptors on MoDC after 12, 24 and 40 hours of stimulation with *G. duodenalis* trophozoites (troph), *G. duodenalis* excretion/secretion products (ES) or LPS. Mean fold changes of stimulated MoDC (4 animals) were compared to un-stimulated MoDC (n fold change =1, broken line) \pm standard deviation. * P<0.05 to immature MoDC.

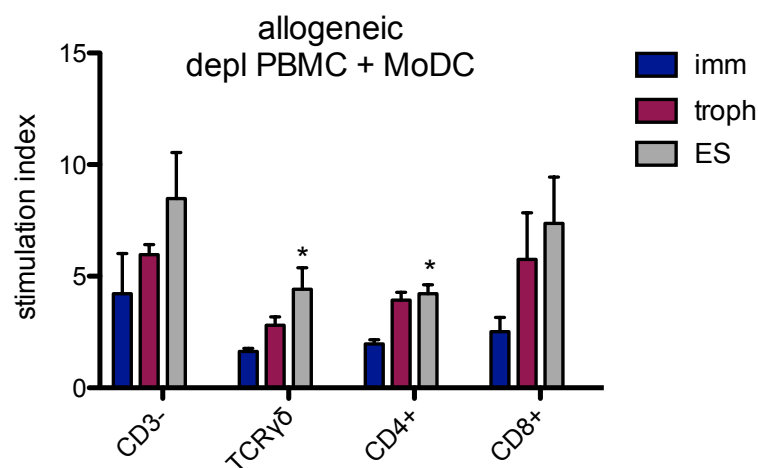


Figure 9. Stimulation index of different cell populations after co-culturing *G. duodenalis*-stimulated MoDC with depleted allogeneic PBMC. Arithmetic mean of four animals \pm standard error of the mean is shown. * P<0.05 to co-culture with immature MoDC.

Autologous mixed lymphocyte reaction

Co-culturing of MoDC stimulated with *Giardia* trophozoites with autologous depleted PBMC induced a significantly higher proliferation of CD3⁻ cells, $\gamma\delta$ -T-cells and TCR $\alpha\beta$ ⁺ CD4⁺ and CD8⁺ T-cells, compared to co-cultures with immature MoDC (Fig. 10).

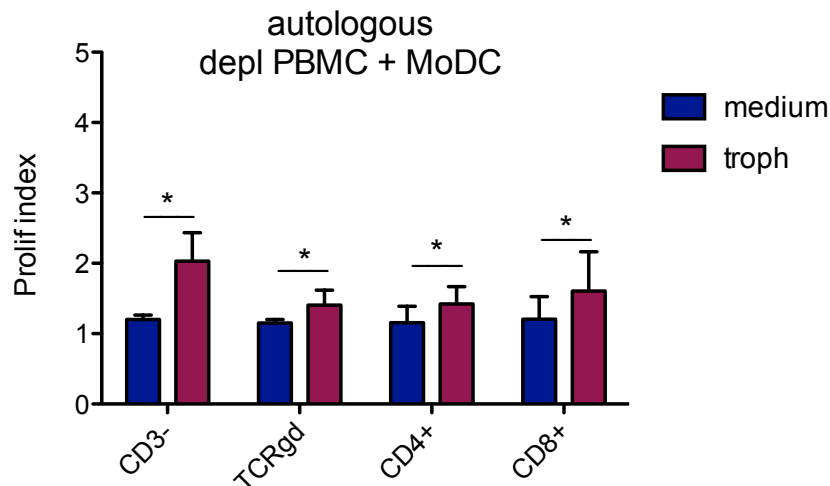


Figure 10. Proliferation index of different cell populations after co-culturing *G. duodenalis*-stimulated MoDC with autologous depleted PBMC. Arithmetic mean of six animals \pm standard error of the mean. * $P < 0.05$ to co-cultures with immature MoDC.

Cytokine production in co-culture of depleted autologous PBMC and MoDC

The transcription of different cytokines was measured with qPCR in cell populations that were sorted from a co-culture of depleted PBMC and MoDC. In the CD3⁻ cell fraction, no transcription of cytokines was upregulated. TNF- α and IL-4 were significantly downregulated after stimulation with *G. duodenalis* trophozoites (Fig. 11a). In the TCR $\gamma\delta$ ⁺ cell fraction TNF- α , TGF- β , IL-4 and IL-10 were highly upregulated (more than 10 fold), however, these differences were not statistically significant. Also the transcription of IL-17, IL-15, IL-6, PPAR α and PPAR γ was more than 2-fold upregulated (Fig. 11b). In the CD4⁺ T-cells, TGF- β ($P < 0.05$) and TNF- α (NS) were upregulated, while the transcription factor Foxp3 was significantly downregulated (Fig. 11c).

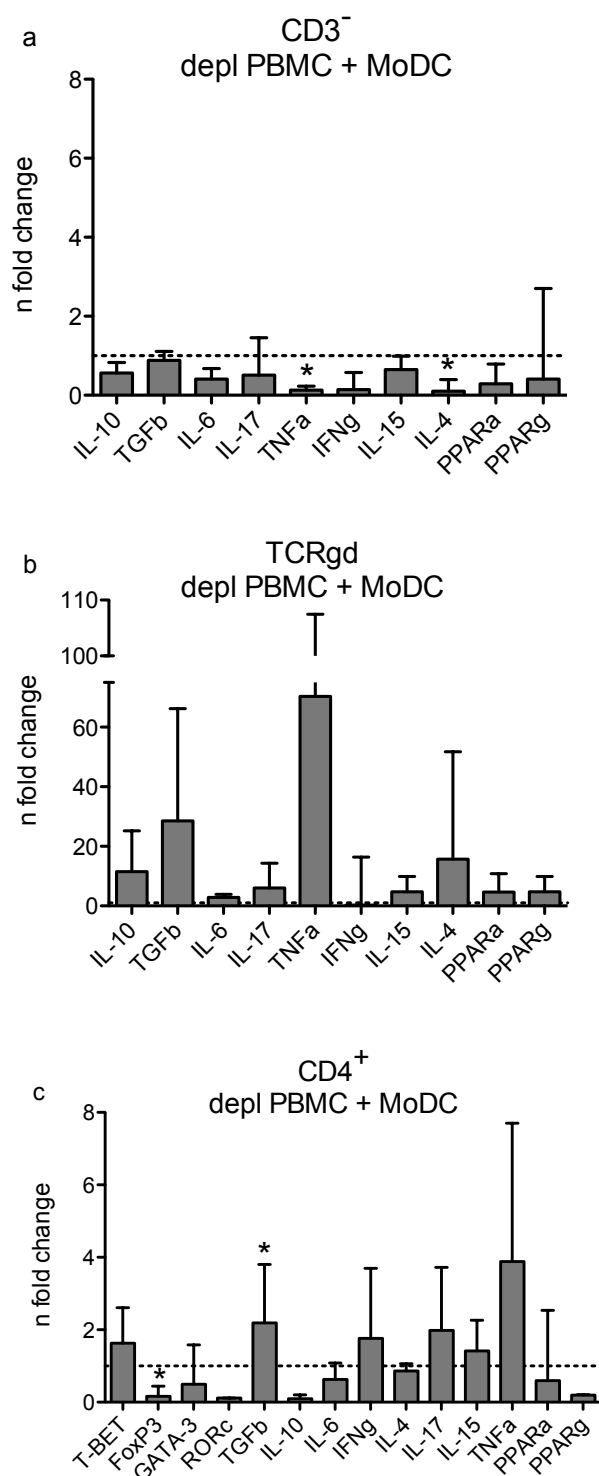


Figure 11. Cytokine transcription levels in different cell populations from co-cultures of MoDC with depleted autologous PBMC: CD3⁻ cells (a), TCRγδ⁺ cells (b) and CD4⁺ cells (c). Mean fold changes of six animals, PBMC cultured with trophozoite stimulated MoDC were compared to PBMC cultured with un-stimulated MoDC (n fold change = 1, broken line) ± standard deviation. *P < 0.05 to immature MoDC.

2.4 Discussion

Dendritic cells are the messengers between the innate and adaptive immunity and are required for the initiation of T-cell responses. It is known that several protozoa, such as *Plasmodium*, *Leishmania* and *Toxoplasma*, have a regulatory effect on DC, creating an optimal environment to survive in the host (Terrazas et al., 2010). Little is known about the effect of *Giardia* on DC function. R oxstrom-Lindquist et al. (2005) showed that human intestinal epithelial cells co-incubated with *Giardia* trophozoites produced a chemokine profile (CCL2, CCL20, CXCL1, CXCL2 and CXCL3) that is able to attract DC and lymphocyte populations. This suggested that DC play a role during *Giardia* infection. Kamda and Singer (2009) showed that mouse bone marrow-derived DC after stimulation with *G. duodenalis* extract had a weakly enhanced expression of CD80 and CD86. After incubation of these DC with both LPS and *G. duodenalis* extract the expression of CD80, CD86 and MHCII was downregulated by *Giardia* (Kamda and Singer 2009). In the present study, stimulation of MoDC with *G. duodenalis* trophozoites or ES resulted in upregulation of MHCII, which was not concentration dependent and little or no upregulation of CD40 and CD80 expression. In contrast to Kamda and Singer (2009) LPS-induced expression of CD40, CD80 and MHCII was not significantly downregulated after co-incubation with *G. duodenalis* trophozoites or ES. Moreover, transcription of the surface molecules PD-L1, β catenin, ILT3 and ILT4, expressed by tolerogenic DC, was not significantly increased in MoDC incubated with *G. duodenalis* trophozoites or ES.

The initiation of a response or the switch of tolerance to immunity depends on the T-cell priming and thus on the cytokine repertoire produced by DC and the maturation state of DC (Kapsenberg et al., 2003). Kamda and Singer (2009) reported that after co-incubation with the TLR-agonist LPS, *Giardia*-stimulated DC produced increased IL-10 and decreased IL-12, suggesting that *G. duodenalis* is inhibiting the immune response, as has been observed with other protozoa. In our study, LPS-induced production of TNF- α was decreased after co-incubation with *G. duodenalis*, but this was not statistically significant and IL-6 and IL-12 were not impacted. The dissimilarities found with the study of Kamda and Singer (2009) could be caused by the difference in *Giardia* assemblage used (assemblage B) or the fact that mice are not a natural host for *G. duodenalis*. In our study the hooved livestock-specific assemblage E was used, which is a commonly found genotype in calves (Geurden et

al., 2008). Furthermore, in the murine study 10^7 trophozoites per well were used, which is 200 times higher than the number of trophozoites used in this present study. Stimulation of bovine MoDC with *G. duodenalis* did not result in evidence of secretion of subsets of cytokines by DC that, at least in mice, have been demonstrated to drive differentiation of divergent T-cell responses, such as a Th1, Th2, Th17 or Treg response. The cytokine profile of MoDC was not significantly influenced by stimulation with trophozoites or ES, with the exception of a small increase of transcription of IL-15 at 1 time point (24h). In addition, a small but not significant upregulation of the transcription level of IL-6 (12h) was seen after stimulation of MoDC with both trophozoites and ES stimulation. It has been shown that IL-6 plays an important role in immunity against *Giardia*. IL-6 is essential for clearance and early control of acute infections, since mice deficient for IL-6 develop chronic giardiasis (Galli et al., 2005; Zhou et al, 2003). Low levels of produced IL-6 were seen after stimulation of murine bone marrow-derived DC with *G. duodenalis* extract, together with small amounts of TNF- α (Kamda and Singer, 2009). No upregulation of TNF- α transcription in MoDC was found in our study. A discrepancy was sometimes observed between cytokine transcription levels and cytokine levels measured by ELISA. The enhanced TNF- α concentration measured with ELISA at 12 and 24 hours was due to a high value in only one animal. No IL-10 could be measured in the culture supernatant of MoDC stimulated with LPS, although the transcription level of IL-10 was enhanced (8.1 fold). It is possible that the newly synthesized IL-10 cytokine was not yet present in detectable concentrations in the supernatant or that IL-10 mRNA in the LPS treated MoDC is subject to posttranslational modifications preventing the secretion of IL-10.

Stimulation of MoDC with trophozoites and ES resulted in a dose-dependent decreased antigen uptake. This implies that the cells were functionally active. Functionally active DC lacking enhanced expression of co-stimulatory molecules have previously been described as semi-mature or partially mature DC (Tan et al., 2005). Only little is known about this semi-maturation state. Where fully activated DC lead to immunogenicity, semi-mature but differentiated DC are thought to cause tolerance. The exact mechanism by which semi-mature DCs are induced is not known yet, but it is believed that the ability of DC to be either immunogenic or tolerogenic depends on the context and the antigen by which the DC are activated (Cools et al., 2007). Tolerance can be due to T-cell anergy or the induction of regulatory T-cells

(Shortman et al., 2007). In this study, *G. duodenalis* induced proliferation of PBMC in mixed lymphocyte reactions with allogeneic and autologous lymphocytes, showing that *Giardia* does not cause an anergic state of T-cells. MoDC stimulated with trophozoites and ES induced significant proliferation of autologous CD3⁻, $\gamma\delta$ -T-cells and TCR $\alpha\beta$ ⁺ CD4⁺ and CD8⁺ T-cells. Similarly, Werling et al. (2002) described proliferation of CD4⁺ T-cells after co-culturing bovine MoDC pulsed with bovine respiratory syncytial virus without enhanced upregulation of MHCII and CD80/86. It is possible that other surface molecules play a role in DC co-stimulation in cattle. Antigen-specific proliferation of CD4⁺ and CD8⁺ T-cells has previously been observed in human patients infected with *Giardia* (Scott et al., 2004; Hanevik et al., 2011). It was shown that CD4⁺ T-cells were activated by *Giardia* both *in vivo* and *in vitro* (Hanevik et al., 2011; Ebert 1999). Mice lacking CD4⁺ T-lymphocytes are unable to clear *G. duodenalis* infection, and it has been shown that especially CD4 $\alpha\beta$ ⁺ T-cells, are required to control *Giardia* (Singer and Nash 2000; Zhou et al., 2007). Proliferation of CD8⁺ T-cells was previously described but seems not to be important for the control of the infection. In contrast, activated CD8⁺ T-cells may contribute to the damage of the intestine during infection (Scott et al., 2004).

Although the proliferating CD4⁺ T-cells in the present study showed increased transcription levels of TGF- β mRNA, transcription of IL-10 and Foxp3 was downregulated, indicating that these cells are not regulatory T-cells. It has been suggested that in cattle, $\gamma\delta$ -T-cells rather than Foxp3⁺ T-cells have a regulatory function (Hoek et al., 2009). Proliferation of $\gamma\delta$ -T-cells was also observed in the present study and the transcription of the anti-inflammatory cytokines TGF- β and IL-10 was increased in these cells. However, transcription of the pro-inflammatory TNF- α was also high in both CD4⁺ and $\gamma\delta$ -T-cells and IL4, IL-6 and IL-17 were also upregulated in $\gamma\delta$ -T-cells. Both IL-6 and IL-17 have been associated with protective immune responses against *G. duodenalis* (Zhou et al., 2003; Dreesen et al., unpublished data).

In conclusion, despite the low expression of some of the co-stimulatory molecules, no evidence was found for the hypothesized inhibitory or regulatory effect of *G. duodenalis* on bovine MoDC. *G. duodenalis* did not induce transcription of regulatory surface molecules on MoDC and *G. duodenalis*-stimulated MoDC were functionally active, as shown by decreased antigen uptake and their capacity to induce PBMC

proliferation. Moreover, proliferating CD4⁺ and $\gamma\delta$ -T-cell populations had no unambiguous immune-regulatory cytokine profile.

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Chapter 3.

Kinetics of the immune response against *G. duodenalis* in cattle

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Chapter 3 Kinetics of the immune response against *G. duodenalis* in cattle

3.1 Introduction

In cattle little is known about the development of the immune response against *G. duodenalis*. It seems that during the infection the required adaptive immunity in calves is not complete and hence re-infections often occur. In mice antibodies play an important role during the infection; IgA is indispensable for protective immunity (Taherkhani et al., 2009). It is hypothesised that the development of immunity is complete when all *Giardia* variant specific surface proteins (vsp), which cover the entire surface of the trophozoite, are recognized by specific antibodies (Nash, 2011).

In this chapter we investigated the systemic immune response against *G. duodenalis* infection in a natural host, *i.e.* cattle. The immune response was monitored until decreasing cyst excretion indicated the development of protective immunity. We studied the kinetics and nature of circulating memory cells and identified the proliferating cells and their cytokine transcription levels. The role of antigen-presenting cells in the initiation of the cellular response was assessed. The kinetics of serum antibodies were evaluated and *G. duodenalis* trophozoites were stained with serum antibodies to investigate if the proportion of trophozoites that were recognised by serum antibodies increased with developing immunity.

3.2 Materials and Methods

Animals and infection

Six healthy male Holstein Friesian calves between 10 and 14 days old were confirmed to be free of *Giardia* and *Cryptosporidium* by faecal examinations on two consecutive days, using a direct immunofluorescence assay (Merifluor[®] Cryptosporidium/Giardia kit, Meridian Diagnostics Inc.). The calves were fed with colostrum in the first few hours after birth and were subsequently fed with a commercial milk replacer. The calves were housed two by two in boxes with straw. At the age of 3 weeks they were given an oral infection with 10^5 *G. duodenalis* cysts, isolated from 2 naturally infected calves. The cysts were confirmed to be a mixture of assemblage A and E by PCR, targeting the triose phosphate isomerase gene (Sulaiman et al., 2003; Geurden et al.,

2008). The calves were weaned at week 9 and housed together in a box with straw bedding. Fresh straw was added regularly but the boxes were never cleaned to allow reinfection from the environment.

During the experiment rectal faecal samples were collected two times a week and *Giardia* cyst excretion was monitored by a quantitative direct immunofluorescence assay (Merifluor[®] *Cryptosporidium/Giardia* kit, Meridian Diagnostics Inc.) (Geurden et al., 2004). Geometric mean cyst counts were calculated using Excel version 14.0.0. Faecal cyst counts were continued until repetitive cyst counts of ≤ 200 cyst per gram of faeces in all calves suggested that the animals had developed protective immunity.

Isolation of peripheral blood mononuclear cells

Every 2 weeks, venous blood was drawn from the *vena jugularis* using vacutainer tubes with heparin. Peripheral blood mononuclear cells (PBMC) were obtained using a Lymphoprep density gradient (Axis-Shield). The interphase was harvested and cells were washed 3 times in PBS. Cells were cultured in 96-well round-bottomed plates (Nunc) in cell culture medium (RPMI-1640 + Glutamax, Invitrogen) supplemented with 10% foetal calf serum, 50 $\mu\text{g/ml}$ gentamycin and 1/1000 β -mercapto-ethanol or were first depleted for antigen-presenting cells by plastic adhesion.

Cell proliferation assays

PBMC (2×10^5) were cultured with 2×10^4 live *G. duodenalis* trophozoites from an axenic *G. duodenalis* assemblage E culture (Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Belgium) in a 96-well round-bottom plate (Nunc) in modified TYI-S-33 medium (Keister, 1983). The trophozoites were viable and mobile for up to 36 h after the start of the co-incubation. Cells incubated with medium alone were used as negative controls. As a positive control 5 $\mu\text{g/ml}$ Concanavalin A (Sigma-Aldrich) was used. All conditions were performed in triplicate. PBMC depleted for either antigen-presenting cells or B-lymphocytes were stimulated with the same number of *G. duodenalis* trophozoites and in the same conditions as for full PBMC. After 5 days of culture, cells were pulsed with 1 μCi [³H] thymidine (Amersham ICN) per well and incubated for 18 hours. Subsequently cells were harvested on to glass fiber filters (Perkin Elmer, Life Sciences). The incorporated radioactivity was measured using a β -scintillation counter (Perkin

Elmer). The stimulation index was calculated by dividing the counts per minute of trophozoite-stimulated cells by the count per minute of the medium control.

PKH staining was performed to identify which cell populations from the PBMC were proliferating. PBMC were isolated as described above and labelled with PKH using the PKH26 red fluorescent cell linker mini kit (Sigma-Aldrich) according to the manufacturer's instruction. The same conditions and cell numbers were used as for the thymidine assay. After 6 days of culture, the cells were harvested and a quadruple staining was performed using the following mouse anti-bovine antibodies: CD3 (IgG1, MM1A VMRD), CD4 (IgG2a, MCA1653G, AbD Serotec), CD8 (IgM, BAQ111A VMRD) and TCR $\gamma\delta$ (IgG2b, GB21A VMRD). Bound mAb were detected by anti-mouse IgG1-V450 (BD Biosciences), anti-mouse IgG2a-APC (Invitrogen), anti-mouse IgM-APC-Cy7 (Biolegend) and anti-mouse IgG2b-FITC (Southern Biotech). Sytox blue dead cell stain (Live Technologies) was added to distinguish live cells from dead cells. The PKH intensity was measured using the FACSARIAIII (BD biosciences). Data were analysed using Flowjo software (Tree Star) and the proliferation of different cell populations was quantified by calculating the stimulation index using ModFit LT software (Verity Software House). To do so the different populations were gated and for each separate cell population a proliferation ratio was calculated based on the number of cell divisions. The proliferation ratios were then compared between cell populations stimulated with medium vs. *Giardia*.

Characterisation of proliferating CD4⁺ T-cells

On week 13 of infection, when CD4⁺ T-cell proliferation was the highest, CD4⁺ T-cells were sorted from stimulated full PBMC after 6 days of stimulation with *G. duodenalis* trophozoites and characterised by real time quantitative PCR (RT qPCR). For the sorting a quadruple staining for CD3, CD4, CD8 and TCR $\gamma\delta$ was done as described above and analysed using the FACSARIAIII. Doublet exclusion was performed based on FSC-H and FSC-A, and subsequently on SSC-H and SSC-A. Propidium iodide positive cells were excluded from the analysis. The CD3⁺ CD4⁺ T-cells were sorted. After sorting, cells were washed in PBS. The supernatant was removed and the cell pellets were snap-frozen in liquid nitrogen and stored at -80°C until processing. The RNeasy kit (Qiagen) was used to extract RNA following the manufacturer's instructions. To remove contaminating genomic DNA, on-column

DNase digestion was performed using the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. Obtained RNA concentrations were measured using a Nano-Drop 2000 spectrophotometer (Thermo Scientific) and first-strand cDNA was generated from 100ng RNA using the iScript cDNA synthesis kit (Bio-rad). The reaction was performed in a 20µl volume, according to the manufacturer's instructions.

Real-time quantitative PCR was carried out for a panel of selected cytokines and transcription factors, allowing differentiation of pro-inflammatory and anti-inflammatory or inhibitory cell functions. The transcription of the following cytokines and transcription factors was evaluated: T-Bet, Gata-3, FoxP3, RORc, IL-2, IL-4, IL-6, IL-10, IL-15, IL-17, TGF-β, IFN-γ, TNF-α and Peroxisome proliferator-activator receptor alpha and gamma (PPARα and PPARγ). All primer sequences are listed in Table 1 (Chapter 2). Real-time q PCR was performed according to Dreesen et al. (2012) using a StepOnePlus Real-Time PCR system (Applied Biosciences), using 10µl SYBR Green master mix (Applied Biosystems), 2µl of single stranded cDNA (1ng of the input total RNA equivalent) and 400nM of each amplification primer in a 20µl reaction volume. Primer efficiencies ranged between 1.90 and 2.09. All conditions were performed in duplicate. A relative quantification was carried out using the delta-delta Ct method. Out of a panel of 4 candidate control genes, the ribosomal protein S29 (RSP29) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) genes were selected for normalisation, based on gene stability.

Depletion of PBMC for antigen-presenting cells or B-cells

To determine the contribution of antigen-presenting cells and B-lymphocytes to the lymphocyte proliferation, proliferation assays were also performed with PBMC depleted for antigen-presenting cells or B-cells, respectively. PBMC were depleted for antigen-presenting cells by plastic adhesion. PBMC were cultured in a concentration of 2×10^6 cells/ml in cell culture medium in a cell culture flask (75cm², Novolab) for 2 to 3 hours to allow antigen-presenting cells to attach to the plastic. Afterwards the non-adhered cells were harvested and cultured. The efficiency of the plastic adhesion was measured by flow cytometry. Non-adherent cells were stained with mouse anti-bovine CD14 (IgG1, CCG33), and bound antibodies were detected

using anti-mouse IgG1-V450 (BD Biosciences). The maximum of remaining CD14⁺ cells measured in the depleted PBMC fraction was 3%.

PBMC collected 19-21 weeks post infection were depleted of CD21⁺ B-cells or MHCII⁺ cells (including all CD21⁺ cells). This experiment was performed using cells from 4 animals. A double staining was performed using primary anti-bovine CD21 (IgM, BAQ15A VMRD) and MHCII (IgG2a, CC158) antibodies. Bound antibodies were detected by anti-mouse IgG2a-APC (Invitrogen) and anti-mouse IgM-APC-Cy7 (Biolegend). Doublet exclusion was done based on FSC-H and FSC-A, and subsequently on SSC-H and SSC-A. Propidium iodide stain (Invitrogen) was added to distinguish live cells from dead cells, incubated for 3 minutes and unbound propidium iodide was then removed by washing the cells in PBS (BD biosciences). Propidium iodide positive cells were excluded from the analysis and MHCII^{low} and CD21⁻ cell fractions were gated and sorted. A post sort measurement was done and the purity of the MHCII⁻ population was at least 99%, while the purity for CD21⁻ cells was at least 98%. Depleted cell fractions were cultured with *G. duodenalis* trophozoites and cell proliferation assays were performed under the same conditions as described above.

To ensure that MHCII^{high} cells did not cause the proliferation seen in PBMC, a PKH26 staining of CD3⁻ (MHCII⁺) cells was also performed. On week 11, when the maximum proliferation was seen, PKH staining was performed as described above on full PBMC and CD3⁻ cells were analysed (Fig. 6).

Generation of bovine monocyte-derived dendritic cells and stimulation of MoDC with Giardia

In chapter 2 it was seen that *G. duodenalis*-stimulated bovine monocyte-derived DC (MoDC) induced proliferation of autologous PBMC. To test whether dendritic cells are the leading cells for induction of the cellular response, PBMC depleted for antigen-presenting cells were co-cultured with MoDC stimulated with *G. duodenalis* trophozoites. One week before PBMC were cultured, blood was drawn to generate MoDC. The MoDC were generated from PBMC as described by Werling et al. (1999). Briefly, PBMC were isolated as described above and subsequently, incubated with anti-human CD14 microbeads (Miltenyi Biotec). CD14⁺ monocytes were isolated using magnetic cell sorting (MACS, Miltenyi Biotec) according to the manufacturer's instructions. The purity of the cells was evaluated by flow cytometry

and was consistently $\geq 95\%$. Monocytes were cultured (0.5×10^6 cells/ml) for seven days with 10ng/ml bovine rIL-4 (Sigma) and 37.5ng/ml porcine recombinant GM-CSF (provided by S. Inumaru from the Laboratory of Bioengineering, National Institute of Animal Health, Tsukuba, Ibaraki, Japan; Inumaru et al., 1998) in DMEM without phenol-red (Invitrogen) supplemented with 10% foetal calf serum (TCS biosciences), 500U/ml penicillin and 500 μ g/ml streptomycin. Every three days the cultures were supplemented with fresh cytokines. After 7 days the cells were harvested using 2mM EDTA and brought in co-culture with PBMC. A total of 2×10^5 depleted PBMC were put in a 96-well round-bottomed plate (Nunc) without MoDC or with MoDC at a MoDC/PBMC ratio of 1/10. The cells were immediately stimulated with 2×10^4 live *G. duodenalis* trophozoites and a cell proliferation assay with depleted PBMC was performed as described above.

Enzyme-linked immune-sorbent assay (ELISA)

An ELISA was performed to detect *Giardia* specific IgG1, IgG2 and IgA in serum at different time points of the infection. PBS soluble extract was made of *G. duodenalis* assemblage A and E trophozoites by performing 3 freeze thaw cycles. The pellet was spun down by centrifuging 5 minutes at 350g and the supernatant containing the soluble proteins was collected and frozen at -70°C . The protein concentration in the supernatant was determined using a Pierce BCA protein assay (Thermo Scientific). The assay was performed following the manufacturer's instructions. Maxisorp 96 well plates (Nunc) were coated with 100 μ l per well of 5ng/ml *Giardia* extract for 12 hours at 4°C . The plates were washed with PBS with 0.05% Tween20 (PBST), and blocked using 2% bovine serum albumin (Invitrogen) in PBST solution (blocking buffer). The serum of all animals was diluted 1/50 in blocking buffer and 100 μ l was brought in each well. For detection, horseradish-peroxidase conjugated sheep anti-bovine IgG1 (AAI21P, AbD Serotec), sheep anti-bovine IgG2 (AAI22P, AbD Serotec) and sheep anti bovine-IgGA (AAI20P, AbD Serotec) were used at a concentration of 2 μ g/ml diluted in blocking buffer. To visualise the reaction, 67mU/ml of conjugate, streptavidin-POD (Roche) and 1mg/ml of o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) was used. The reaction was stopped by adding 50 μ l of a 2.5M HCl solution. The reaction product was measured with a 492nm spectrophotometer.

Results were analysed using DeltaSOFT JV 2.1.2 software (BioMetallics) with a five-parameter curve-fitting algorithm. All conditions were performed in triplicate.

Trophozoite staining with serum antibodies

Trophozoites from both assemblage A and E were harvested from axenic cultures and washed 3 times in PBS. From each assemblage 35,000 trophozoites were dried on a 3-aminopropyl tri-ethoxysilane (APES) coated slide for 5 minutes. After drying, the slides were incubated with 50µl of 1:2 diluted serum for 30 minutes at room temperature. The slides were washed and incubated with sheep anti-bovine IgG1-FITC (AAI21F, AbD Serotec) or sheep anti-bovine IgA-FITC (AAI20F, AbD Serotec) for 30 minutes at room temperature. The slides were washed and subsequently 50µl of 1µg/ml DAPI solution was brought on the slides for 5 minutes. The slides were examined using a fluorescence microscope at magnification of 200x. Overlays were made with Image J software.

Statistical analysis

For longitudinal data containing repeated measurements (Fig. 2, 3, 5 and 7), a non-parametric Friedman test was used with Dunn's post hoc test to compare mean values of stimulation index, proliferation index or optical density *pre infection* (week 0) and *post infection*. For the qPCR results (Fig. 4), Q values of unstimulated cells and stimulated cells were compared using a two-sided Wilcoxon signed-rank test. The mean stimulation index of six animals was compared between full PBMC and PBMC depleted for MHCII cells or B-cells (Fig. 6) using a Kruskal-Wallis test with Dunn's post-hoc test. P-values of less than 0.05 were considered statistically significant.

3.3 Results

Infection

Seven days after the oral infection excretion of cysts was observed. Geometric mean cyst counts reached a maximum of 62,936 cysts per gram faeces (cpg) at week 5 (Fig.1). From week 7 onwards a decrease was seen, with the lowest cyst excretion at week 10, probably due to change of housing. However, at week 11 the cyst number increased again and a second peak of 11,814 cpg was reached at week 13, followed by

a second decline. From week 15 onwards, the cyst excretion stayed below 200 cpg and the calves were considered to be immune. Throughout the experiment, all calves suffered short spells of mild to watery diarrhoea. During the whole period, the general condition of the calves remained good and the animals were vivid and alert.

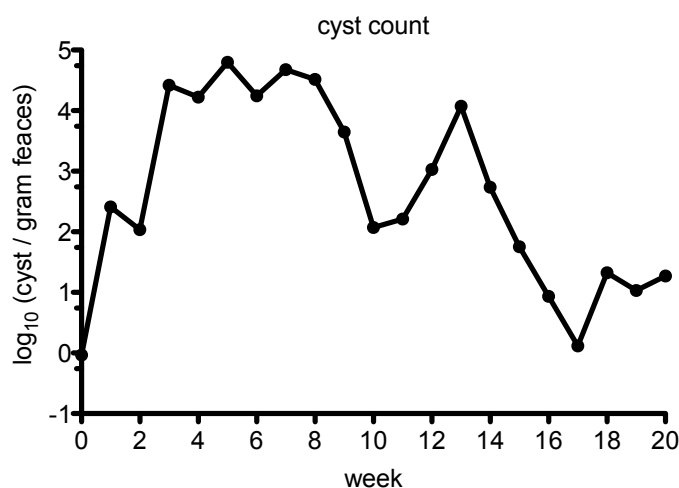


Fig. 1. Geometric mean cyst excretion (cysts per gram of faeces) during a period of 18 weeks after oral infection of six calves with 10^5 *G. duodenalis* cysts.

PBMC proliferation during infection

Proliferation of PBMC re-stimulated with *G. duodenalis* trophozoites was assessed every 2 weeks during 18 weeks by measuring the incorporation of thymidine in the DNA of dividing cells. Stimulated PBMC showed an increased proliferation from week 6 onwards with two peaks at week 7 and week 11 (Fig 2). The proliferation was significantly higher on week 7, 9 and 11 compared to pre-infection levels. The high cell proliferation between weeks 6 and 12 roughly corresponds with the period of high cyst excretion (Fig. 1). The maximum stimulation index (SI= 60.6) was measured at week 11 (Fig 2).

Every two weeks, proliferating cell populations were identified using PKH staining. At week 9 and week 13 proliferation of CD4⁺ αβ T-cells was significantly higher compared to pre-infection levels (Fig. 3). The maximum proliferation ratio (4.5) was observed at week 13. Strong proliferation of CD3⁻ cells was occasionally seen, but this proliferation was never significantly different from pre-infection levels (Fig. 3).

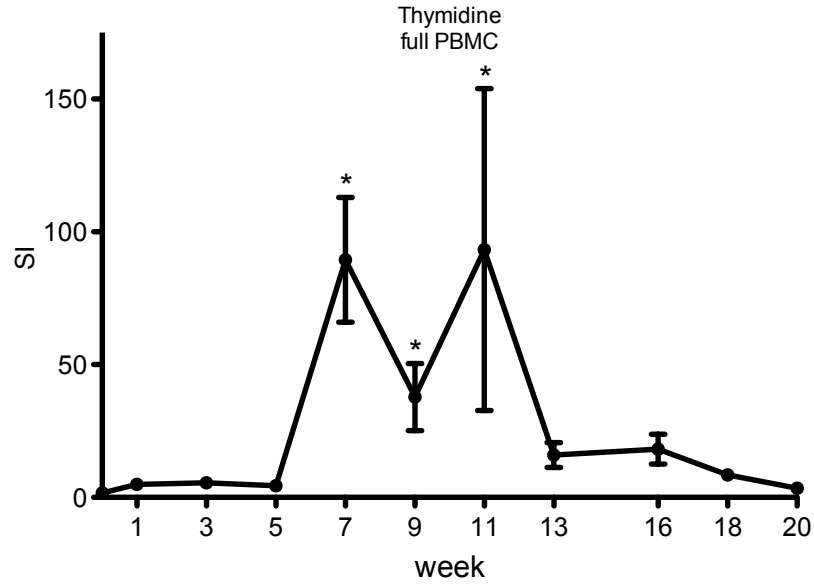


Figure 2. Arithmetic mean stimulation index (SI \pm st.dev) of PBMC stimulated with *G. duodenalis* from six *G. duodenalis* infected calves. * $P \leq 0.05$ between pre-infection (week 0) and post infection samples.

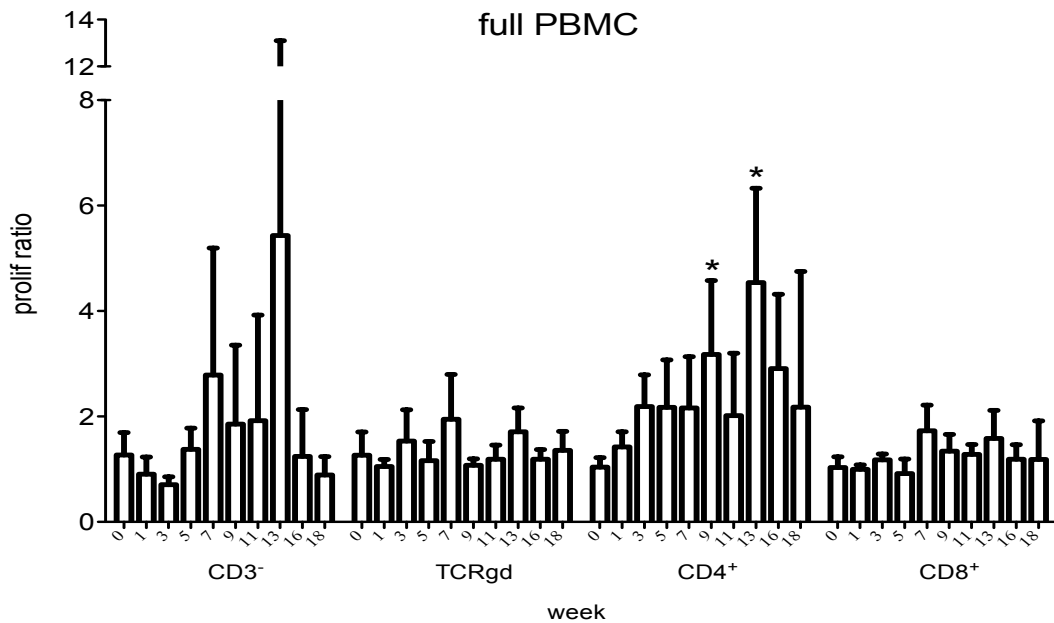


Figure 3. Arithmetic mean proliferation ratio (\pm st.dev) of PBMC stimulated with *G. duodenalis* from six *G. duodenalis* infected calves. T-cell receptor $\alpha\beta$ positive cells were divided in $CD4^+$ and $CD8^+$ T-cells. * $P \leq 0.05$ between pre-infection (week 0) and post infection samples.

Cytokine profile (qPCR)

The transcription level of mRNA for a panel of transcription factors and cytokines produced by CD4⁺ T-cells was evaluated by RT qPCR on week 13 when CD4⁺ proliferation was the highest. Mean fold changes of transcription levels of cytokines and transcription factors in CD4⁺ T-cells were compared to unstimulated cells (Fig. 4). *Giardia* stimulation resulted in significantly higher transcription of IL-17 (25.8 fold) and FoxP3 (6.4 fold). Transcription levels of TNF- α (13.5 fold), IL-15 (2.3 fold) and IL-2 (2.1 fold) were also increased after stimulation with *G. duodenalis*, but these differences were not statistically significant.

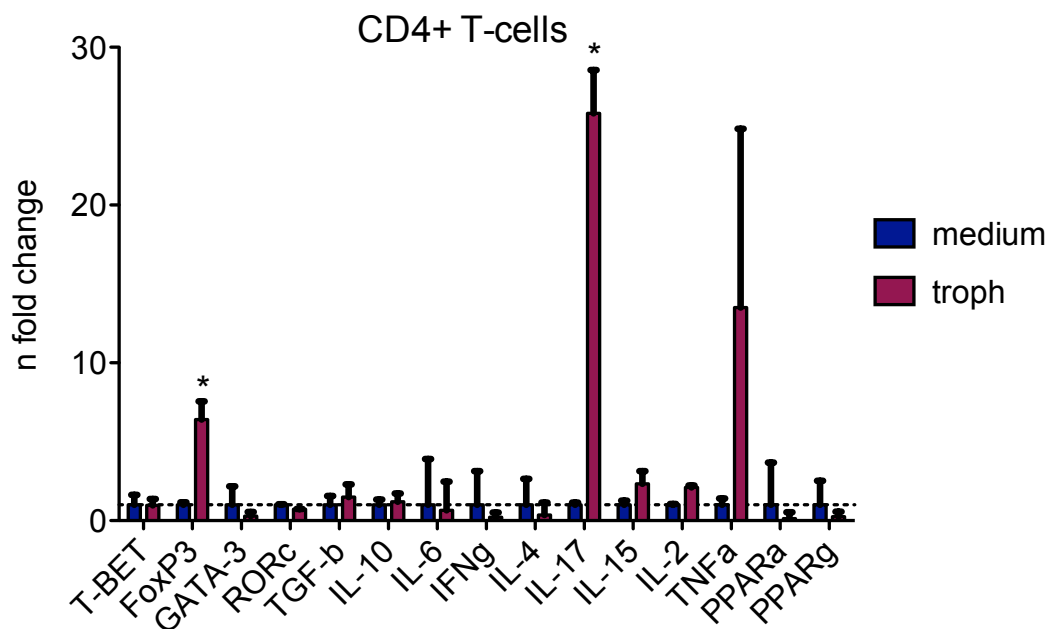


Figure 4. Effect of stimulation with *G. duodenalis* on transcription levels of a panel of cytokines and transcription factors in CD4⁺ T-cells from six *G. duodenalis* infected calves. The Q values of stimulated cells were compared to unstimulated cells. * $P \leq 0.05$.

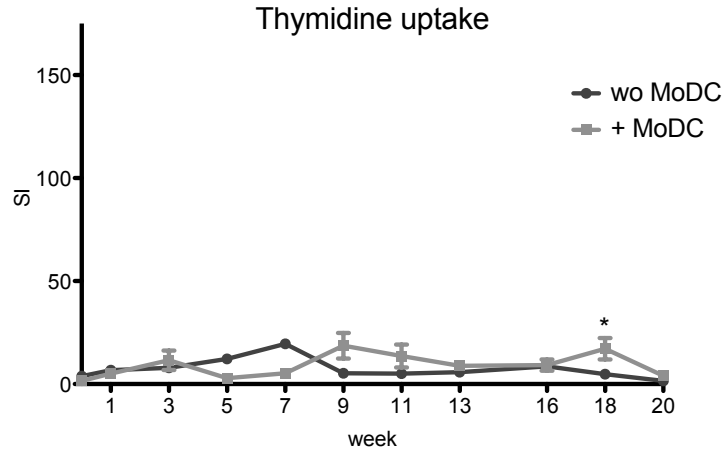


Figure. 5. Arithmetic mean stimulation index (SI \pm st.dev) of depleted PBMC from six *G. duodenalis* infected calves after stimulation with *G. duodenalis*: dark gray PBMC depleted for antigen-presenting cells, light gray depleted PBMC co-cultured with MoDC. * $P \leq 0.05$ between pre-infection (week 0) and post infection samples.

Thymidine proliferation assay with depleted PBMC

Proliferation of PBMC depleted from antigen-presenting cells, with and without MoDC stimulated with *G. duodenalis* trophozoites, was measured every 2 weeks during 20 weeks by the incorporation of thymidine in the DNA of dividing cells. No significant proliferation of depleted PBMC with or without MoDC was measured at several time points (Fig. 5), except in the co-culture of depleted PBMC with MoDC at week 18. The proliferation of depleted PBMC, either with or without MoDC, was much lower compared to the proliferation of PBMC (Fig. 2). A maximum stimulation index of depleted PBMC without MoDC (SI= 19.5) was measured at week 7. A maximum stimulation index of depleted PBMC with MoDC (SI= 18.6) was measured at week 9.

MHCII and CD21 depletion

PBMC collected at week 19-21 were depleted from MHCII⁺ cells or CD21⁺ B-cells and stimulated with *G. duodenalis* trophozoites. The stimulation index of PBMC depleted from MHCII⁺ cells was significantly lower (SI= 0.7) than the stimulation index of full PBMC (SI = 3.4) (Fig. 6a). Depletion of CD21⁺ cells had no influence on the proliferation, as the SI = 4.7 was not significantly different from the stimulation

index of full PBMC. No proliferation of CD3⁺ was seen at week 11 (Fig.6b), showing that PBMC proliferation was not due to proliferation of MHCII⁺ cells.

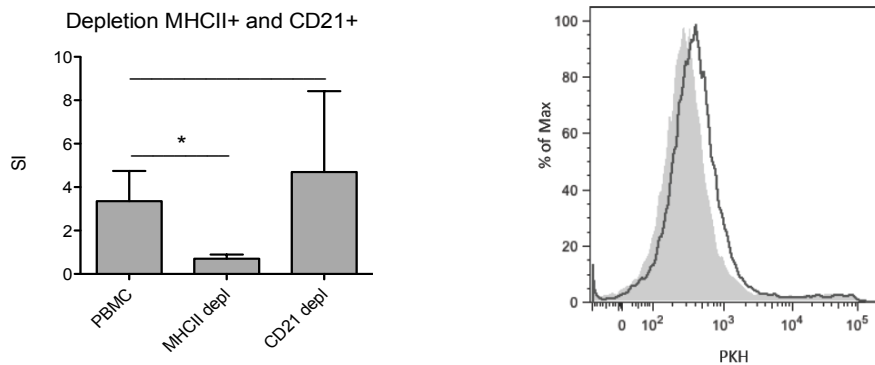


Figure 6. The effect of *G. duodenalis* trophozoites on proliferation of PBMC, MHCII depleted PBMC and CD21 depleted PBMC. A) The stimulation index (SI) and standard deviation of four animals is shown. B) Overlay of PKH staining of CD3 negative cells from week 11 of the infection, stimulated with medium (filled) or with *G. duodenalis* trophozoites (black line). One representative animal out of 6 is shown. * $P \leq 0.05$

G. duodenalis specific serum antibody responses

An ELISA was performed to determine the kinetics of the *Giardia*-specific serum antibody responses. *G. duodenalis*-specific IgG2 was not detected in serum (data not shown). A time-dependent increase was seen for IgG1 and IgA against both *G. duodenalis* assemblage A and E extract, with the highest response against assemblage E extract (Fig. 7). The IgA response was slightly higher compared to the IgG1 response, and the increase of IgG1 against assemblage A was not statistically significant (Fig. 7).

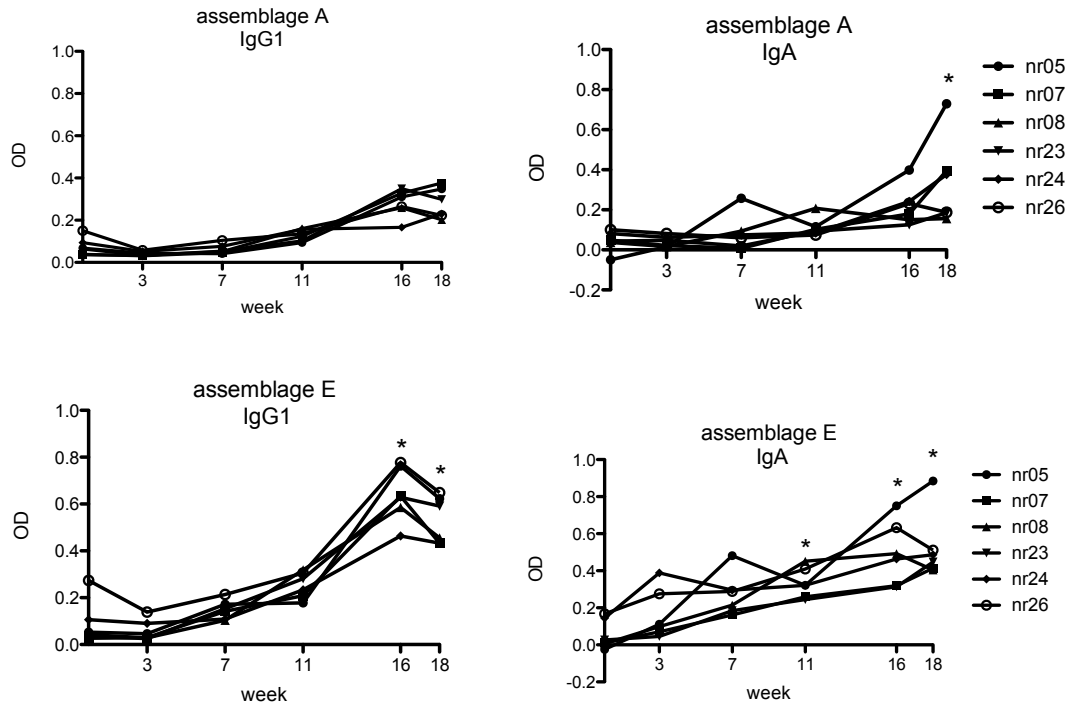


Figure 7. Optical density (OD) values for serum IgG1 and IgA antibodies against *G. duodenalis* extract of assemblage A and E, of 6 *G. duodenalis* infected calves at 6 time points, during 18 weeks. * $P \leq 0.05$ between pre-infection (week 0) and post infection samples.

Trophozoite staining with serum antibodies

Assemblage A and assemblage E trophozoites were stained with IgG1 and IgA from pooled sera obtained at week 0, 5, 11 and 18 of infection and detected using FITC. Nuclei of the trophozoites were stained with DAPI. On week 11 and week 18 there was visible staining of all assemblage E trophozoites with serum IgA, with the highest intensity of the staining on week 18 (Fig. 8). Similar results were obtained with trophozoites of assemblage A (data not shown). For IgG1 no difference could be seen between week 0 and week 18 of infection due to non-specific staining of the detection antibody (data not shown).

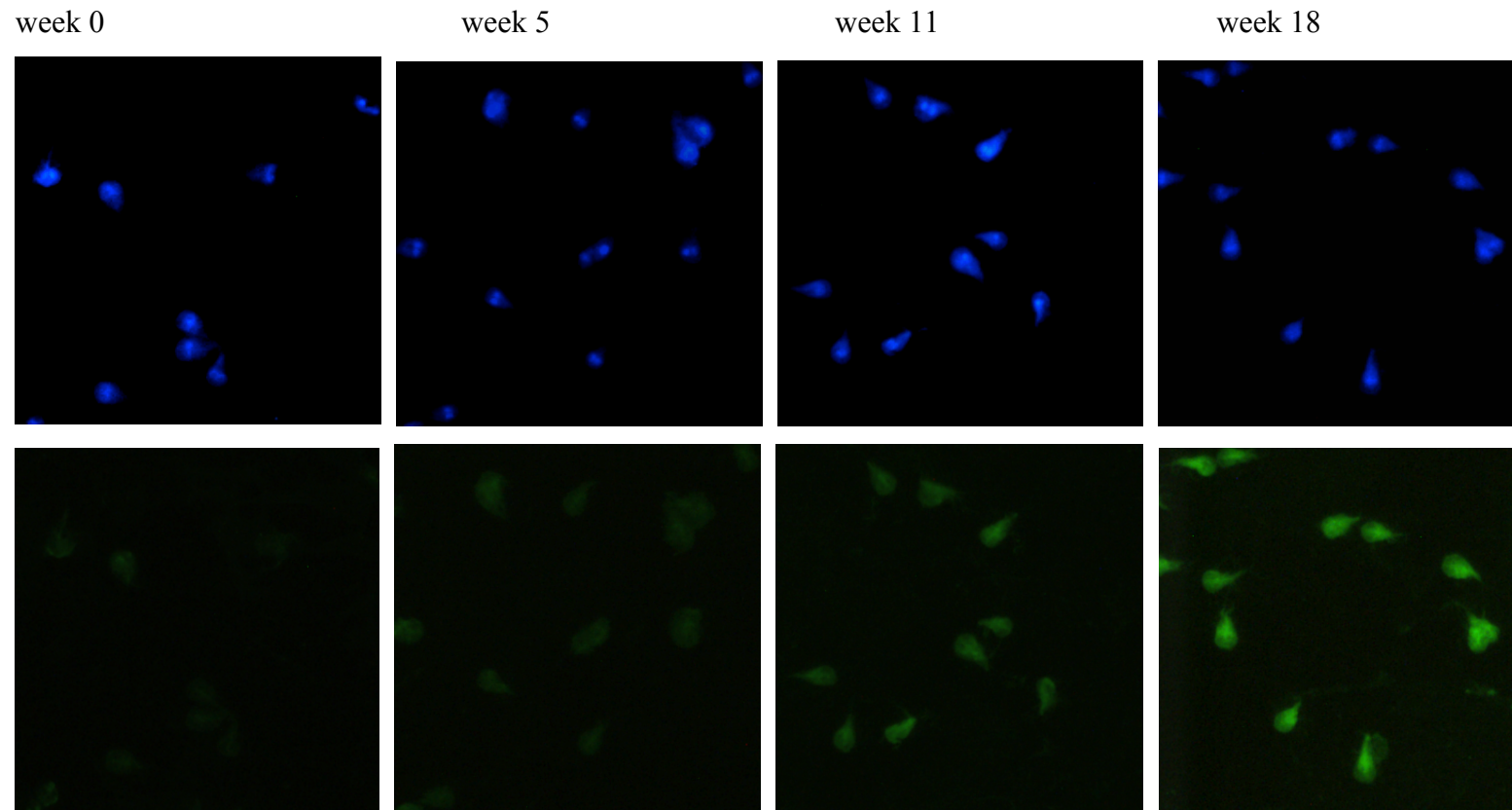


Figure 8. Trophozoites of assemblage E stained with DAPI (blue) and IgA (green) from sera collected pre-infection (week 0) and weeks 5, 11 and 18 post infection. Magnification 200x.

3.4 Discussion

In the present study *G. duodenalis* infected calves excreted cysts for a period of \approx 100 days, which is consistent with the observation of O'Handley et al. (1999) that *G. duodenalis* infections in cattle can last until day 112 post infection. The calves suffered from periods of mild to watery diarrhoea, but the general condition of the calves remained well. It is possible that the diarrhoea was caused by *G. duodenalis* infection, given the high cyst counts that were measured. Diarrhoea has previously been reported in naturally infected calves with similar *G. duodenalis* cyst counts (Geurden et al., 2006).

The chronic character of the infection might correlate with the slow immunological response seen in calves (O'Handley et al., 2003). In the present study systemic immune responses were only observed after several weeks. The first *in vitro* cell proliferation of re-stimulated PBMC was seen after 5 weeks, while *G. duodenalis* specific serum antibodies only increased significantly from week 11 post infection. This slow development of immunity is in agreement with previous observations, where no intestinal immune response was seen in calves after 3 weeks of infection (Dreesen et al., 2012).

After 15 weeks of infection, a repeatedly low cyst excretion indicated the development of protective immunity. This protective immunity may be associated with the strong cellular responses that were observed during the infection, although other mechanisms have a role in parasite clearance as well and it is possible that CD4⁺ T-cells play a role in the initiation of these additional mechanisms. A long-term cellular immune response against *G. duodenalis* has also been reported in exposed humans, and was largely driven by CD4⁺ T-cell activation (Hanevik et al., 2011). Similarly, the proliferation of re-stimulated PBMC in our experiment was mainly caused by CD4⁺ $\alpha\beta$ T-cells. CD4⁺ T-cells can actively direct immune responses and play an important role in immunity against *Giardia* in mice. Mice lacking CD4⁺ T lymphocytes are not able to clear infection, in particular CD4⁺ $\alpha\beta$ T-cells are required to control *Giardia* infection in mice (Singer and Nash, 2000; Zhou et al., 2007). Therefore the cytokine mRNA profile of CD4⁺ T-cells was evaluated. The fact that obvious up-regulation of some cytokines (e.g. TNF- α) was not statistically significant, may be due to the small sample size and the large variability between individual

animals' responses. Transcription of IL-17 was significantly (25.8 fold) upregulated. IL-17 is known for its role in immunity at epithelial and mucosal barriers. Th17 cells have a pro-inflammatory function and can induce production of pro-inflammatory cytokines and chemokines, causing recruitment of effector cells, such as neutrophils and macrophages (Gaffen, 2008). The production of IL-17 was described before in spleen cells and lymph node cells obtained from *G. duodenalis* infected mice (Solaymani-Mohammadi and Singer, 2011) and is essential for clearance of *G. muris* infections in mice (Dreesen et al., in press). Based on those studies and the present data, it can be hypothesized that IL-17 also plays an important role in the development of protective immunity against *G. duodenalis* in cattle. In most cases IL-17 is produced by ROR γ ⁺ Th17 cells under the influence of IL-6 and TGF- β . IL-6 is essential in *G. duodenalis* clearance in mice (Li et al., 2004). IL-6 causes upregulation of the expression of the IL-23 receptor and directs T-cell development away from Treg towards a Th17 response (Kamda et al., 2012). The potential role of IL-17 in parasite clearance would at least partially explain the necessity of IL-6. The fact that mRNA levels of IL-6 or TGF- β were not upregulated in this study may be due to the time of sampling of CD4⁺ T-cells, at 13 weeks post infection. It is likely that IL-6 or TGF- β responses occur earlier in the infection. The transcription factor FoxP3 was also significantly upregulated (6.4 fold). Although FoxP3 is typically expressed by regulatory T-cells, CD4⁺Foxp3⁺ IL-17 producing cells have been identified in human blood and lymphoid tissue. These cells display the functional features of both Treg and Th17 cells (Voo et al., 2009). Although it is possible that IL-17 induced by *G. duodenalis* is produced by Foxp3⁺ Treg cells, it is also possible that the CD4⁺ population consists of both Foxp3⁺ Treg cells and Th17 cells.

Dendritic cells (DC) are antigen-presenting cells that are able to initiate T- and B-cell responses. In chapter 2 it was seen that *G. duodenalis* stimulated bovine MoDC were able to cause proliferation of autologous PBMC. In contrast, *G. duodenalis*-stimulated bone marrow-derived DC from mice were suspected to actively interfere with the host innate immunity (Kamda and Singer, 2009). Since proliferation was strongly reduced when PBMC were depleted of monocytes by plastic adhesion in the present study, antigen presenting cells are required for the induction of the cellular response. Co-culturing depleted PBMC with autologous MoDC could not restore the reduced proliferation of depleted PBMC to the level of proliferation seen in full PBMC, implying that DC are not the leading cells in the induction of the cellular response.

Nor do B-cells play a role in *Giardia* antigen presentation since depletion of B-cells alone (CD21⁺) from PBMC did not result in decreased proliferation. However, the depletion of MHCII^{high} cells did cause significantly reduced proliferation of PBMC. Since the proliferating PBMC themselves were not MHCII^{high} cells, the results indicate that *Giardia* trophozoites have little or no direct effect on blood lymphocytes and that antigen-presenting cells, other than DC and B-cells are required to initiate proliferation. Which MHCII⁺ cells are able to induce lymphocyte proliferation remains unclear. Possible alternative antigen-presenting cells are monocytes, macrophages, innate lymphoid cells or mast cells.

Serum antibodies appeared shortly before cyst counts started to decline, indicating that antibodies may be involved in the development of protective immunity. In the study of O'Handley et al. (2003) no significant increase of serum IgG could be found in calves infected with *G. duodenalis* until 8 weeks post infection. In this study increasing *G. duodenalis*-specific serum IgG1 and IgA was measured from week 11 post infection and IgA responses seemed to be the most important. B-cells and IgA are also required for parasite clearance in mice. IgA was shown to be the most important isotype in the clearance of *Giardia* and is the major isotype present in intestinal mucosal secretions (Stäger and Müller, 1997; Langford et al., 2002). In infected humans and mice, serum IgG and IgA increased as soon as 2 weeks after infection (Daniels and Belosevic 1994; Soliman et al., 1998; Velazquez et al., 2005), which might explain why the eradication of the parasite in these hosts is much faster compared to cattle.

It was hypothesised that immunity is only developed when all various surface proteins (vsp) on the *Giardia* surface are recognized (Faubert, 2000), and that the slow development of immunity against *Giardia* is the result of this antigen variation (Müller and Von Allmen, 2005). However, in our study all trophozoites, and not only a subset, were recognized by serum IgG1 and IgA, and the number of binding antibodies increased over time during the infection. This suggests that a broad repertoire of antibodies is induced against all vsp or that the antibody response in cattle is at least partly directed against invariable surface antigens. These data implies that parasite clearance might depend on the vsp-specific antibody concentration.

In conclusion, the slow induction of cellular and humoral responses was in line with the chronic character of *G. duodenalis* infections in cattle. The IL-17 production by CD4⁺ T-cells might play a role in the development of protective immunity against *G.*

duodenalis. Further research is necessary to determine the role of IL-17 and to determine whether IL-17 is produced by FoxP3⁺ cells or by a different CD4⁺ T-cell population. *Giardia*-specific IgG1 and IgA in the serum increased from week 11 post infection and all trophozoites were stained by serum antibodies from week 11 p.i. onwards, suggesting that antibodies are directed against all variant-specific surface proteins or that the antibody response in cattle is at least partly directed against invariable surface antigens.

3.5 References

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Chapter 4

General discussion

Chapter 4 General discussion, future perspectives and conclusion

4.1 The effect of *Giardia duodenalis* on bovine monocyte-derived dendritic cells

In cattle *G. duodenalis* infections have a chronic character; calves excrete cysts for more than 100 days (Taminelli et al., 1989). When the intestinal immune response in calves was investigated after 3 weeks of infection, a gene pattern corresponding to decreased inflammation, decreased immune cell recruitment and immune response was measured (Dreesen et al., 2012). This implies that *G. duodenalis* is causing immune suppression during the acute period of infection. It is suggested by Kamda and Singer (2009) that the immune response is suppressed by an inhibitory effect of *G. duodenalis* on DC, which initiate the adaptive immune response. Several protozoa are capable of influencing DC functionality. *Trypanosoma cruzi* is able to inhibit DC maturation and the response caused by LPS stimulation (Van Overvelt et al., 1999; 2002). *Plasmodium falciparum* and *Toxoplasma gondii* are also able to suppress the production of pro-inflammatory cytokines by DC (Urban et al., 1999; Sanecka and Frickel, 2012).

Mouse bone marrow-derived DC produced increased levels of IL-10 and decreased levels of IL-12 after stimulation with both *G. duodenalis* and LPS. Similarly, LPS-stimulated human MoDCs incubated in the presence of *G. duodenalis* lysate produced less IL-12/23p40, IL-12p70, and IL-23, but more IL-10 than cells incubated in the absence of the parasite (Obendorf et al., 2013). Concomitantly, the expression of CD25, CD83, CD86, and HLA-DR was reduced on *G. duodenalis*-incubated DC as compared to control cells (Obendorf et al., 2013). A potential mechanism of DC inactivation by *Giardia* is depletion of arginine (Banik et al., 2013). Arginases and arginine deiminases are enzymes produced by *Giardia* with the ability to prevent the formation of nitric oxide by the host. Arginine depletion of human MoDC as a result of incubation with *G. duodenalis* arginine deiminases significantly increased TNF- α and decreased IL-10 and IL-12p40 secretion. It also reduced the upregulation of surface CD83 and CD86 molecules, which are involved in T-cell interactions (Banik et al., 2013).

Therefore, the effect of *G. duodenalis* on bovine monocyte-derived DC (MoDC) was assessed and the potential of the parasite to influence the capacity of these MoDC to induce immune responses was determined. In chapter 2, bovine MoDC were stimulated *in vitro* with *G. duodenalis* trophozoites or ES. Co-stimulatory molecules

were not or only slightly upregulated and no increased transcription of cytokines could be measured. However, the transcription of several molecules (PD-L1, β -catenin, ILT3 and ILT4), known to have an inhibitory or tolerogenic effect after ligation, was not increased. Moreover, *G. duodenalis* stimulated MoDC were able to activate allogeneic and autologous lymphocytes. This indicates that the MoDC were functionally activated by *G. duodenalis* and capable of inducing immune responses. In addition, co-incubation of LPS with *G. duodenalis* trophozoites did not influence the effect of LPS induced upregulation of the co-stimulatory molecules or cytokine production by MoDC. In contrast, in the study of Kamda and Singer (2009), the expression of the co-stimulatory molecules CD80, CD86 and MHCII was down-regulated after incubation of DC with LPS and *G. duodenalis* extract.

Based on the results of our study it cannot be concluded that *G. duodenalis* has a regulatory effect on the functionality of MoDC in cattle. It is not clear why these results are different from the results of Kamda and Singer (2009) and Obendorf et al. (2013). It is possible that DC from cattle react differently to *Giardia* than DC from mice or humans. Besides it is possible that the differences are caused by the different assemblages of *G. duodenalis* that were used. Kamda and Singer (2009) used *G. duodenalis* assemblage B for *in vitro* re-stimulation of DC and Obendorf et al. (2013) used assemblage A, while in our study the hoofed livestock-specific assemblage E was used.

Both Kamda and Singer (2009) and Obendorf et al. (2013) used 5 to 10 times higher concentrations of *Giardia* extract than the concentration used in our study, but the inhibitory effect on IL-12 was also seen by Kamda and Singer (2009) when a trophozoite/DC ratio of 1/1 was used, a similar concentration as used in our study. Finally, it is possible that the TLR pathways in cattle are different from mice and human. However, this is not likely, since Hope et al. (2003) showed that bovine DC react similarly to different TLR2 agonists as murine and human DC. To our knowledge there is no data on the stimulation of bovine (Mo)DC TLR4 with LPS or other TLR4 ligands.

4.2 Development of immunity against *Giardia duodenalis* in cattle

It remains unknown why *G. duodenalis* infections in cattle are chronic. Hence, in chapter 3 the systemic cellular and humoral response of calves during *G. duodenalis* infection was investigated.

Chronic infection pattern

In chapter 3 *Giardia*-naïve calves were experimentally infected with *G. duodenalis* at 3 weeks of age. Cyst excretion was highest when the calves were 5 weeks old and remained high until 16 weeks of age. This long period of cyst excretion is comparable with the observations of Taminelli et al (1989) and O’Handley et al. (2003), describing cyst excretion in infected calves over a period of at least 100 – 112 days. In our study a clear decrease in cyst excretion was seen when the calves were 16 weeks old. The cyst excretion remained low (< 200 cpg) until the end of the study. Although it is impossible to define whether the cysts excreted after week 16 were originating from intestinal passage of cysts taken up from the contaminated environment or from a persistent low level of infection. Nevertheless, the low cyst counts indicate that the calves had developed (partial) protective immunity. Persistent low cyst numbers have also been described in adult animals (O’Handley and Olson, 2006). Some researchers suggested that the number of excreted cysts in adult cows is low due to dilution by the increased faecal production (O’Handley et al., 2003). However, a peri-parturient rise in cyst excretion has been described in cattle, indicating that the immune status of the animals is important to keep the infection at a low level (O’Handley and Olson, 2006).

Serological response

The chronicity of *G. duodenalis* infections in cattle might be correlated with the weak or slow development of the immune response. Weak serological responses during *G. duodenalis* infection have been described in cattle (O’Handley et al., 2003) and in lambs (Yanke et al., 1998). Yanke et al. (1998) found a small increase of IgG and IgA in lambs from week 5 until week 11 of infection, after which the study ended. However, in our study IgG1 and IgA were only significantly increased from week 11 onwards. It is possible that Yanke et al. (1998) ended their study before the actual peak in serum antibodies was measurable. The time of appearance of IgA in the serum is possibly later than the time that IgA is produced in the intestine, this delay could have biased our results. However, this is unlikely since in mice serum IgA was measured 5-10 days after the start of the infection (Heyworth, 1989; Davids et al., 2005). In mice IgA is required for parasite clearance, as IgA-deficient mice are unable to eradicate *G. muris* or *G. duodenalis* (Langford et al., 2002).

A reason for the weak immune response probably lies within the variant-specific surface proteins (vsp) of *Giardia*, which allows the parasite to escape from the host's immune response (Singer et al., 2001). Antigenic variation is used by other microorganisms to maintain chronic infections under the continuous pressure of antibody recognition (Prucca et al., 2008). It is hypothesised that the shift of vsp results in chronic infection (Prucca et al., 2008). This would mean that immunity against *Giardia* could only be developed when all different vsp are recognised by host antibodies. Only one vsp is expressed per trophozoite (Roxström-Lindquist et al., 2006) and covers the entire surface of the trophozoite. *In vitro* each 6-13 generations a different vsp occurs on the surface even in the absence of antibodies (Li et al., 2013). To our knowledge it is not known whether *in vivo* different trophozoites express different vsp at the same time, *i.e.* whether in the intestine a mix of all different vsp is present. In that case antibodies against multiple vsp would be produced at the same time and might delay the host immune system by exhaustion, as can be seen for B-cells in patients with HIV, hepatitis C virus or malaria (Andrews and Wilson, 2010). In our study all trophozoites, obtained from axenic culture, were recognised simultaneously from the moment antibodies were measured in the serum (from week 5 onwards), which indicates that all different vsp were recognised from early on in the infection. This suggests that to obtain protection, all different vsp have to be recognised and the antibody concentration needs to be sufficiently high. Alternatively, it cannot be excluded that conserved surface proteins, such as taglin and GP49 (Adam 2001), were recognised by serum antibodies, causing a consistent staining of the trophozoites. Since taglin plays a role in the attachment of the parasite to the epithelium (Weiland et al., 2003) and GP49 is suggested to be important for parasite survival (Das et al., 1991), the antibody response against these proteins might be of great importance in parasite clearance as well.

Cellular response

Dreesen et al. (2012) observed no pathological or immunological changes in the jejunal mucosae in calves after 3 weeks of *G. duodenalis* infection. However, after 6 weeks of natural infection, pathological and immunological changes were observed by Ruest et al. (1997), including villus atrophy, distortion of the crypts, cellular infiltration and increased numbers of intra-epithelial leukocytes. These observations

are comparable to findings in mice and humans (Scott et al., 2004; Oberhuber et al., 1997), although in humans the presence of trophozoites could not be correlated to pathology in all cases (Hanevik et al., 2007). The presence of intestinal damage and the infiltration of inflammatory cells indicate that the immune system is activated at this time point of infection. This is in line with the results of this study: no proliferation of PBMC could be initiated after *in vitro* re-stimulation with *G. duodenalis* trophozoites or ES during the first weeks of infection (Chapter 3). Only after 5 weeks of infection, proliferation could be measured, meaning that memory cells were now present in the circulation. In general in intestinal infections, memory cells are 'primed' in the mesenteric lymph node after interaction with an antigen-presenting cell. After clonal expansion and differentiation of antigen-specific lymphocytes, some of these cells differentiate into memory cells (Mackay, 1999). A part of the memory B- and T-cells remain in the lymph node to provide central or reactive memory; these cells proliferate and differentiate into effector cells in response to antigenic stimulation. Next to this central memory, protective memory is provided by effector B-cells (long lived plasma cells) and by circulating or tissue resident effector memory T-cells (Sallustro et al., 2010). In cells obtained from the mesenteric lymph nodes, *in vitro* proliferation was over 70 times higher jejunum compared to cells obtained from blood (PBMC) (data not shown), confirming that memory cells mainly resided in the local lymph nodes to provide central memory and that only a small number of the memory cells occurred in the circulation.

Within the proliferating PBMC, in particular CD4⁺ T-cells were proliferating. In knock-out mice it could be shown that CD4⁺ T-cells are indispensable for parasite clearance. Also in humans the importance of CD4⁺ T-cells was confirmed and proliferation after *in vitro* re-stimulation was seen in PBMC (Gottstein et al., 1991; Hanevik et al., 2011), intestinal intra-epithelial lymphocytes and lamina propria lymphocytes (Ebert 1999; El-Shazly et al., 2003). Since CD4⁺ T-cells do not have the ability to kill trophozoites (Ebert, 1999), it is likely that CD4⁺ T-cells perform a role as 'helper cells' rather than effector cells. Besides CD4⁺ T-cells, IgA producing B-cells are crucial for parasite clearance in mice and it is plausible that B-cell activation is supported by CD4⁺ T-cells.

The transcription of both pro- and anti-inflammatory cytokines was increased in the proliferating CD4⁺ T-cells and no typical Th1 or Th2 cytokine pattern was measured. Some researchers found increased concentrations of the Th2 type cytokines IL-4 and

IL-5, but also IFN- γ , a typical Th1 cytokine, was detected in serum, spleen and *in vitro* CD4⁺ T-cell culture (Matowicka-Karna et al., 2009; Jimenez et al., 2009; Djamiantun and Faubert, 1998). However, none of these cytokines appeared to be crucial for parasite clearance (Zhou et al., 2003). In chapter 3, increased transcription of IL-17 could be measured in CD4⁺ T-cells at the peak of proliferation at week 13. IL-17 is a pro-inflammatory cytokine inducing the production of IL-6, TNF- α and GM-CSF (Miyazaki et al., 2010). The production of IL-17 was previously measured in mesenteric lymph nodes of *G. duodenalis* infected mice (Solomani-Mohammadi and Singer, 2001) and recent studies in IL-17 receptor (IL-17RA) knock-out mice proved that IL-17 plays a crucial role in clearance of *G. muris* (Dreesen et al., 2014). In IL-6 knock-out mice it was shown that IL-6 is also indispensable for parasite clearance in the acute phase of the infection (Galli et al., 2005). The crucial role of IL-17 could explain the importance of IL-6 in *Giardia* infections, since IL-6 is the major trigger in combination with TGF- β to initiate differentiation of CD4⁺ T-cell into Th17 cells (Korn et al., 2009). IL-17 has also been implicated in other protozoan infections. A role of IL-17 in protection against *Trypanosoma cruzi* was confirmed (Miyazaki et al., 2010) and during *Plasmodium vivax* infection IL-17 was highly increased (Bueno et al., 2012). On the other hand, IL-17 was associated with intestinal injuries in leishmaniosis and *Eimeria tenella* infection (Bacellar et al., 2009; Zhang et al., 2013). Also in other diseases, such as Crohn's disease, IL-17 was correlated with severe mucosal inflammation and injuries (Ahern et al., 2010).

Initiation of the cellular response

In a depletion experiment it was shown that MHCII⁺ cells are required to initiate cell proliferation (Chapter 3). It is generally accepted that DC are the most efficient APC in initiating T-cell responses. DC of the intestine are residing in the *lamina propria* of the mucosae and stick their dendrites through the tight junctions of the epithelial layer to sample the intestinal lumen. Since *G. duodenalis* is not an invading pathogen, DC are probably the first cells of the host immune system that have contact with the parasite. Although *Giardia*-stimulated MoDC were able to cause T-cell activation *in vitro* (Chapter 2), *Giardia*-stimulated MoDC were not able to restore proliferation of APC-depleted PBMC (Chapter 3), indicating that other APC were more efficient in

the induction of T-cell responses and that in the used *in vitro* setting DC seem of minor importance to induce T-cell activation. Possible other APC are discussed in 4.3.

Immune regulation

In cattle we found no role for DC in the suppression of the immune response against *G. duodenalis* (Chapter 2). Hence other mechanisms probably cause suppression. The anti-inflammatory response might be caused by the nuclear peroxisome proliferator-activated receptors PPAR α and PPAR γ acting as regulators (Dreesen et al., 2012). PPAR are ligand-activated transcription factors that control lipid and glucose metabolism and are able to regulate innate and adaptive immune responses (Pawlak et al., 2013; Wahli and Michalik, 2012). Both PPAR α and PPAR γ were upregulated in the jejunum of calves after 3 weeks of infection (Dreesen et al., 2012). Interestingly PPAR α was also induced in the early stage of *G. muris* infection in mice (Dreesen et al., 2014). Increased transcription of PPAR γ was also observed after incubation of an epithelial cell line of human colon cells with *G. duodenalis* (Roxström-Lindquist et al., 2005). PPAR α and PPAR γ can be expressed in T- and B-cells, DC, macrophages, epithelial cells and endothelial cells. After activation, PPARs inhibit the activity of transcription factors such as NF- κ B and activator protein 1, resulting in diminished release of pro-inflammatory cytokines and reduced recruitment of leukocytes (Dreesen et al., 2012). Transcription of PPAR was not increased in the proliferating CD4⁺ T-cells (Chapter 3) or in DC (Chapter 2), but it is possible that PPARs are expressed in other cell types during *G. duodenalis* infection.

An alternative mechanism to induce immunosuppression is the differentiation of CD4⁺ T-cells in regulatory T-cells (Treg). Treg are characterised by the nuclear transcription factor FoxP3 and are able to suppress T-cell proliferation by the production of the suppressive cytokines IL-10 and TGF- β . The transcription of FoxP3 was significantly upregulated in cultures of full PBMC (Chapter 3), but the typical immunosuppressive cytokines for Treg, IL-10 and TGF- β , were not. Conversely, in co-cultures with stimulated MoDC, the transcription of TGF- β was increased in proliferating CD4⁺ T-cells but FoxP3 was not (Chapter 2).

In cattle, it is described that the function of Treg can be taken over by $\gamma\delta$ ⁺ T-cells (Hoek et al., 2009). In cattle $\gamma\delta$ ⁺ T-cells are present in much higher numbers than in mice, and in calves $\gamma\delta$ ⁺ T-cells can comprise 50% of the PBMC (Price et al., 2006).

However, in our study no proliferation of systemic $\gamma\delta^+$ T-cells was measured (Chapter 3). This does not exclude a potential role for intestinal $\gamma\delta^+$ T-cells since intestinal $\gamma\delta^+$ T-cells might have different functions or consist of different subtypes.

4.3 Conclusions and future research

Some suggestions for future research are made in this paragraph. The suggestions are made in order of importance in my personal opinion.

Importance of cellular responses during G. duodenalis infection in cattle

The first weeks after the start of infection, *Giardia* possibly causes immunosuppression. How this suppression is caused remains unknown. Only 5 weeks after the start of the infection the cellular immune response is induced. When memory cells appeared in the circulation, the majority of the cells consisted of CD4⁺ T-cells, as also seen in mice. A mixed cytokine pattern was produced by these CD4⁺ T-cells as transcription of both pro- and anti-inflammatory cytokines was measured. Recently, an important role for IL-17 in parasite clearance in mice has been described (Dreesen et al., 2014). The observed increased transcription of IL-17 corresponds to the peak of the cyst excretion (Chapter 3), hinting at the potential key role of IL-17 in parasite clearance in cattle. This is the first time that IL-17 production could be confirmed in a *G. duodenalis* infection in a natural host. Because protection might be provided when an IL-17 response can be triggered, it is important that the role of IL-17 in the protection against *Giardia* in cattle is further investigated. The kinetics of IL-17 during the infection should be studied together with the number of trophozoites present in the intestine to be able to correlate IL-17 expression to protection. Herefore, the transcription of IL-17 should be investigated in lymphocytes from the *lamina propria* and the mesenteric lymph nodes of the jejunum on multiple time points during the infection, using real time qPCR and histological stainings. It is unknown whether this IL-17 was produced by Th17 or by FoxP3⁺ cell, or by both cell types. To identify the source of IL-17, IL-17 producing cells should be investigated by flow cytometry using an intracellular staining for IL-17, FoxP3 and ROR γ t.

Importance of humoral responses during G. duodenalis infection in cattle

In mice the humoral immune response is correlated with protection against *Giardia*. In cattle a significant concentration of *Giardia*-specific IgA and IgG1 appeared in the serum after 11 weeks of infection and the antibody concentration increased over time. Serum IgG1 was directed against all different vsp present in axenic culture and not only against a subpopulation. Hence, it is likely that parasite clearance in calves is based on the recognition of all different vsp and on the concentration of these vsp-specific antibodies. Further research is necessary to find a method to induce an early B-cell response that results in high concentrations of anti-vsp antibodies. A possible way to do so is to produce and isolate different vsp by transfection and to use these as a vaccine. A comparable approach was used by Rivero et al. (2010), who used genetically altered trophozoites, expressing multiple vsp at the same time. With this approach protection could be induced in gerbils.

Assemblage-specific approach

In this study livestock-specific *G. duodenalis* assemblage E trophozoites were used for *in vitro* re-stimulation of MoDC. To our knowledge there is no data on differences between the immune responses against different assemblages of *G. duodenalis*. However, it is unlikely that the differences between this study and the studies of Kamda and Singer (2009) or Obendorf et al. (2013) are caused by the use of different assemblages of *G. duodenalis*. Kamda and Singer (2009) and Obendorf et al. (2013) obtained similar results with assemblage B in mice and assemblage A in humans, respectively. Nevertheless no other data is available on the immune response against the different assemblages and further investigation is required. In cattle, the immune response in animals infected with *G. duodenalis* assemblage A could be compared with animals infected with assemblage E.

Initiation of the immune response during G. duodenalis infection in cattle

The question remains which cells initiate the immune response against *G. duodenalis* in cattle. In our study we found that MoDC and B-cells are not important in the initiation of the cellular response or at least have a minor role in the initiation and that other MHCII⁺ cells are required to initiate the cellular response (Chapter 3). Alternative APC might be monocytes or macrophages; it is shown that oxidative

products (nitric oxide and superoxide) of macrophages are able to kill *Giardia* trophozoites (Fernandes and Assreuy, 1997). Besides it was shown that human monocytes and macrophages are able to ingest and kill *Giardia* trophozoites (Hill and Pearson, 1987) and it might be plausible that ingested *Giardia* antigens are presented on the cell surface of monocytes or macrophages. Although the number of macrophages in the intestinal mucosae is not increased during infection (Roxström-Lindquist et al., 2006), it is possible that small numbers of macrophages are of high biological relevance.

Possible other APC candidates during *G. duodenalis* infection are mast cells. Mast cells can also express MHCII and can influence T-cell responses. Interestingly, the activation of mast cells by LPS induces Treg proliferation (Kambayashi et al., 2009). Although it is generally accepted that mast cells are indispensable for parasite clearance (Zhou et al., 2003) the exact function of mast cells during *Giardia* infections is not known and requires further investigation. Another possible APC is the M-cell, although this is less likely, since in experiments in mice it was shown that *G. muris* did not adhere to M-cells or could penetrate the epithelium by M-cells (Wolf and Bye, 1984). Some pathogens adhere to M-cells and use them as a portal to cross the barrier function of the epithelium (Nicoletti, 2000). A final alternative APC is the innate lymphoid cell, but to our knowledge there is no data on the influence of *Giardia* on innate lymphoid cells.

Although bovine *Giardia* stimulated MoDC showed no clear immune stimulating (Chapter 3) or immune modulating capacities (Chapter 2), it cannot be excluded that other cells are important in the interaction between *Giardia* and DC. *Giardia* adheres to the epithelial cells and has intensive contact with these cells. Cytokine production by epithelial cells can influence the downstream immune responses (Saenz et al., 2008). The role of epithelial cells in *G. duodenalis* infection was investigated previously (Roxström-Lindquist et al., 2005) and *G. duodenalis* was able to alter the gene expression of epithelial cells. The induced gene expression indicated that epithelial cells produce molecules to attract other immune cells and hence, have a pro-inflammatory function. Besides it was demonstrated that epithelial cells produced anti-giardial substances, such as defensins, reactive oxygen species (ROS) and nitric oxide after incubation with trophozoites (Eckmann et al., 2003). On the other hand Stadelmann et al. (2012; 2013) showed that *G. duodenalis* was able to reduce

proliferation of epithelial cells and its production of NO by the secretion of the enzyme arginine deiminase. It should however be kept in mind that these studies were performed on a human colon (caco-2) cell line, while *G. duodenalis* does not occur in the colon. Nevertheless, it is plausible that epithelial cells influence the function of intestinal DC. Gene expression and cytokine production of epithelial cells should be further investigated using *ex vivo* epithelial cells, isolated from the jejunum of infected animals at different time points of infection or by *in vitro* stimulation of a jejunal explant with live trophozoites.

MoDC as a model for intestinal DC

The data of this study and many other studies are based on findings in MoDC. It can be questioned whether MoDC obtained from blood monocytes are a good model for intestinal DC. *In vivo*, intestinal DC consist of a heterogeneous population. DC precursors develop in the bone marrow, but development is only completed in lymphoid and peripheral tissue. Here several factors act to promote and maintain the differentiation of specific DC subsets. During inflammation, circulating monocytes are attracted to the intestinal mucosae and are able to differentiate into inflammatory MoDC. These inflammatory MoDC are known to produce pro-inflammatory cytokines and are thought to correspond to the MoDC generated *in vitro* after culturing monocytes in the presence of GM-CSF and IL-4 (Schreurs et al., 1999). Since different DC subsets can have different functions it is likely that intestinal DC act differently to *Giardia* than MoDC or bone marrow derived DC. Hence, the use of intestinal DC would give results that would mimic the *in vivo* situation more closely. However, the isolation of intestinal DC is very difficult, only small numbers of DC can be isolated from one animal and several subtypes of DC are present in the small intestine of calves, each with different surface markers (Fries et al., 2011). Multiple factors can influence the outcome of an *ex vivo* experiment. Each DC subtype requires a different isolation method, which in turn can also influence the functionality of the DC (O'Neill and Wilson, 2004; Elkord et al., 2005). Also the presence of several other cells and cell secretions in the direct surrounding of the DC might have an influence on the DC functionality, *i.e.* other immune cells, structural cells, cytokines etc., as well as removing the DC from this environment. Additional knowledge on

intestinal cells is needed and it would be desirable to use intestinal DC instead of a MoDC- or bone marrow-derived DC model in the future.

Possible applications: manipulation of the immune response

Understanding the immune response against *G. duodenalis* is of importance for the development of new therapeutics or vaccines. Because treatment of *G. duodenalis* is difficult and reinfections often occur, an approach to gain immunological protection against *G. duodenalis* is desirable. Since a memory response can be induced against *G. duodenalis*, it is likely that the immune response can be manipulated in such a way that protection for the host is provided. Ideally CD4⁺ T-cells should be activated and subsequently, induce memory B-cells without the activation of CD8⁺ T-cells, since CD8⁺ T-cells can cause intestinal injuries.

A first holdback is the inhibition of the immune response in the acute phase of the infection. Effective protection might be feasible when this inhibitory response could be avoided. If it is known how immune suppression is caused by *G. duodenalis*, it might be possible to develop a way of activation of the protective response without triggering the inhibitory response.

It was shown by Kamda and Singer (2009) and by Obendorf (2013) that DC responses to a TLR4 agonist were inhibited by *Giardia*, while human DC stimulated with a TLR2 agonist were not. This raised the hypothesis that when *Giardia* antigens would be administered together with a TLR2 agonist, a protective, pro-inflammatory response could be initiated. Nevertheless, this approach includes a risk, *i.e.* the induction of intestinal inflammation that might possibly enhance mucosal injuries.

Transcription of the nuclear receptor PPAR α was increased early in *G. duodenalis* and *G. muris* infections in calves and mice. Activation of PPAR was also seen in other parasite infections (*Plasmodium*, *Toxoplasma* and *Leishmania*) and is thought to suppress inflammation to allow invasion of the parasite and avoid host destruction. Although further investigation is necessary, the use of antagonists of intestinal PPAR α might be able to prevent the suppression of the immunity by *Giardia*. Several synthetic PPAR ligands are approved as therapeutic drugs directed against diseases in the cholesterol and glucose metabolism. In experimental parasitic models PPAR ligands have proven to alleviate host tissue destruction (Chan et al., 2010). However, dependent on the type of stimulus, stimulation of PPAR α can also provide a pro-

inflammatory response. Dreesen et al. (2014) have shown that oral administration of a PPAR α agonist to mice resulted in significantly decreased infection levels compared to control animals. Since PPAR α has a role in the lipid metabolism it is most likely that this effect on the infection depends on a change in the levels of cholesterol, bile acids and bile salts in the intestinal lumen.

4.4 References

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Summary

Summary

Giardia duodenalis is an intestinal parasite in humans and animals. High prevalences are reported in humans, companion animals and livestock worldwide. In cattle *G. duodenalis* can cause diarrhoea and reduced weight gain. *G. duodenalis* infections in cattle have a chronic character and infected calves excrete cysts for several months, suggesting that *Giardia* is able to suppress or evade the immune system. Although *G. duodenalis* is known since long in both human and veterinary medicine, the knowledge on immunity against *G. duodenalis* is limited.

Chapter 1

Chapter 1 first provides a general overview of *Giardia*, including the transmission, the pathogenesis and symptoms of giardiasis, the diagnosis and treatment. In addition, the current knowledge on the immune response against *Giardia* is reviewed. The majority of the existing data on the immune response against this parasite has been generated by infection trials in mice and *in vitro* studies.

Because *Giardia* is an extracellular parasite that resides in the intestinal lumen, the innate immune system is of great importance. As part of the innate immune system the epithelium and its barrier function play an important role. Besides defensins, epithelial cells produce reactive oxygen species (ROS) and nitric oxide (NO) that may play a role in anti-*Giardia* defence. From studies in knock-out mice it is known that mast cells are also important for parasite clearance. During *Giardia* infections an accumulation of mast cells is seen in the small intestine. Activation of mast cells in general happens after binding of the Fc receptor with IgE or IgA. As a result a range of pro-inflammatory molecules are released, including histamine and several cytokines. Mast cells are also a source of interleukin 6 (IL-6). IL-6 is known to help drive B-cells switching to the production of IgA. It was demonstrated that IL-6 is essential for clearance of *Giardia* and early control of acute infections.

In the first days post infection the number of intraepithelial lymphocytes (IEL) is increasing, in particular cytotoxic CD8⁺ T-cells and helper CD4⁺ T-cells. While CD8⁺ T-cells are associated with tissue damage, CD4⁺ αβ T-cells are important for protection. The CD4⁺ T-cells do not produce a typical Th1 or Th2 cytokine pattern

during *Giardia* infections. IL-6, IL-17 and TNF- α are the only cytokines known to be indispensable for parasite clearance.

If the infection persists beyond the acute stage, a B-cell dependent phase occurs. IgA is the most important isotype in the clearance of *Giardia* and is the major isotype present in the mucosal secretions. IgA is indispensable for parasite clearance since B-cell deficient, IgA deficient mice or mice deficient for poly Ig-receptor could not eradicate *G. muris* or *G. duodenalis*.

As mentioned before, the current knowledge on immunity against *Giardia* is largely derived from mice models. The mouse, however, is not a natural host for *G. duodenalis*, and therefore, results from murine models cannot readily be extrapolated to natural host species, such as humans or cattle. The mouse-specific parasite *G. muris* induces a rapid immune response and is cleared from the intestine after a short period. In contrast, in natural hosts of *G. duodenalis*, infections often persist and can evolve to a chronic, intermittent infection, suggesting an immune-evasive or modulating effect of the parasite. An immune-suppressive effect of *G. duodenalis* in cattle was supported by the fact that after three weeks of infection, no influx of inflammatory cells was observed and immune regulating pathways were not upregulated (Dreesen et al., 2012). Therefore, the main objective of this thesis was to investigate the immune response against *G. duodenalis* in cattle, in an attempt to elucidate potential mechanisms that could explain the chronic nature of giardiasis in this natural host.

Chapter 2

In mice *G. duodenalis* suppressed the response of dendritic cells (DC) to Toll-like receptor ligands, suggesting an immune-modulating effect of *G. duodenalis* on DC. Therefore, we assessed the effect of *G. duodenalis* on monocyte-derived DC (MoDC) from a natural host species, *i.e.* cattle, and determined the potential of the parasite to influence the capacity of these MoDC to induce immune responses. The effect of *G. duodenalis* trophozoites and excretion/secretion products on bovine MoDC maturation and cytokine production was investigated. In addition, the ability of *G. duodenalis* stimulated MoDC to take up antigen and to induce lymphocyte proliferation was assessed.

The transcription of several molecules (PD-L1, β -catenin, ILT3 and ILT4) known to have an inhibitory or tolerogenic effect after ligation, was not increased in MoDC

after incubation with *G. duodenalis*, and *G. duodenalis* did not affect the response of MoDC to the TLR-4 ligand LPS. Although little or no upregulation of the maturation markers CD40 and CD80 was measured, MHCII expression was increased after stimulation with low parasite concentrations. A dose-dependent decrease of ovalbumin uptake was observed in *G. duodenalis* stimulated MoDC, suggesting functional maturation. Transcription of IL-15 was significantly increased after 24h of incubation with *G. duodenalis* trophozoites and excretion/secretion products and stimulated MoDC induced proliferation of CD3⁻ cells, $\gamma\delta$ -T-cells and TCR $\alpha\beta$ ⁺ CD4⁺ and -CD8⁺ T-cells. Increased transcription of TGF- β was shown in CD4⁺ T-cells, but transcription of IL-10 and Foxp3 was down regulated, indicating that these cells were not regulatory T-cells. It has been suggested that in cattle, $\gamma\delta$ -T-cells rather than Foxp3⁺ Treg cells have a regulatory function. However, in $\gamma\delta$ -T-cells transcription of both pro- and anti-inflammatory cytokines (TNF- α , TGF- β , IL-10 and IL-4) was observed after incubation with *G. duodenalis*.

In conclusion, we found no evidence that *G. duodenalis* has a regulatory or inhibitory effect on bovine MoDC. MoDC stimulated with *G. duodenalis* were functionally active and able to induce proliferation of T-cells that produce both pro- and anti-inflammatory cytokines.

Chapter 3

The objective of this chapter was to investigate the kinetics of the systemic immune response against *G. duodenalis* in cattle, in an attempt to identify immune mechanisms that could explain the chronic nature of *G. duodenalis* infections in cattle.

Six calves were experimentally infected with *G. duodenalis* assemblage A and E and cyst excretion was monitored twice a week, until decreased cyst counts indicated the development of protective immunity. Blood was collected every two weeks. The kinetics of the circulating memory cells and serum antibodies were followed up throughout this period. Cyst excretion started 1 week post infection and remained high until week 14. Thereafter, consistently low cyst counts indicated the development of protective immunity. From week 5 post infection memory cells appeared in the circulation and significant proliferation of CD4⁺ $\alpha\beta$ T-cells was observed after *in vitro* re-stimulation with *G. duodenalis* trophozoites, as also seen in mice and humans. Characterisation of the proliferating CD4⁺ T-cells using real time

qPCR showed that at the peak of antigen driven PBMC proliferation the majority of cells were expressing IL-17 and to a lesser extent FoxP3. IL-17 might play an important role in parasite clearance, as was recently confirmed in mice (Dreesen *et al.*, 2014).

The cell proliferation was strongly reduced after plastic adhesion of the PBMC, suggesting a role for antigen-presenting cells. Failure to restore proliferation of depleted PBMC with *Giardia*-stimulated monocyte-derived dendritic cells (MoDC) and unchanged proliferation after depletion of CD21⁺ B-cells showed that other antigen-presenting cells than MoDC and B-cells were important for T-cell proliferation.

Serum concentrations of IgG1 and IgA against *G. duodenalis* assemblage A and E increased significantly from week 11 post infection. From the start of the antibody response, all trophozoites obtained from axenic culture stained positive in an immunofluorescence assay with serum antibodies, indicating that a broad repertoire of antibodies was produced against all variant-specific surface proteins or against conserved surface proteins of *G. duodenalis*.

Chapter 4

In the general discussion, immune mechanisms that may be important in the development or suppression of immunity against *G. duodenalis* in cattle are discussed.

The hypothesis that the immune modulating activity of *Giardia* acts through dendritic cells (DC) could not be confirmed (Chapter 2). No obvious reasons for the discrepancy between these results and results from studies in mice (Kamda and Singer, 2009) and humans (Obendorf *et al.*, 2013) could be identified.

The results of Chapter 3 confirm that *Giardia* infections in cattle have a chronic nature, as shown by the long-lasting excretion of high numbers of cysts. Immune responses only developed after several weeks of infection. This was in agreement with an earlier study, where no signs of inflammation were observed in calves after 3 weeks of infection (Dreesen *et al.*, 2012).

When cellular immune responses were finally activated, the proliferation of re-stimulated PBMC was mainly caused by CD4⁺ αβ T-cells. CD4⁺ T-cells can actively direct immune responses and play an important role in immunity against *Giardia* in mice and humans. The transcription of IL-17 was significantly upregulated in

proliferating CD4⁺ αβ T-cells. Together with data obtained from studies in mice (Soleymani-Mohammadi and Singer, 2011; Dreesen et al., in press) these data suggest that IL-17 plays an important role in the development of protective immunity against *G. duodenalis*. The transcription factor FoxP3 was also significantly upregulated. Although FoxP3 is typically expressed by regulatory T-cells, CD4⁺Foxp3⁺ IL-17 producing cells have been identified in human blood and lymphoid tissue. Further work is needed to investigate whether IL-17 and FoxP3 are expressed by the same cells or by different CD4⁺ subsets. Alternative immune regulatory mechanisms are discussed, including the nuclear receptor PPARs acting as regulators, the induction of regulatory T-cells and/or regulation by γδ T-cells.

The kinetics of the antibody responses (IgG1 and IgA) suggested that antibodies were correlated with a protective immune response. Serum antibodies were directed against all different variant surface proteins (vsp) from the start, and not against successive vsp subpopulations, indicating that protection not only depends on recognition of all variant surface proteins, but also on antibody concentration.

Although MHCII⁺ cells were required for the initiation of the cellular response, DC and B-cells did not play a role in this initiation. A possible role of other antigen-presenting cell candidates such as monocytes, macrophages, mast cells, M-cells and innate lymphoid cells needs further investigation.

Further research should focus on manipulation of the host's immune response, to interfere with the immune-modulating effects of a *Giardia* infection and to stimulate a protective inflammatory response without inflicting tissue damage.

Samenvatting

Samenvatting

Giardia duodenalis is een parasiet van de dunne darm die voorkomt bij mensen en dieren. De prevalentie is hoog onder mensen, gezelschapsdieren en rundvee. In runderen kan *G. duodenalis* gewichtsverlies veroorzaken en infecties in runderen zijn meestal chronisch, waarbij kalveren gedurende meerdere maanden cysten kunnen uitscheiden. Dit suggereert dat *Giardia* in staat is om het immuunsysteem van de gastheer te onderdrukken of te ontwijken. Ondanks dat *G. duodenalis* al lang gekend is in de geneeskunde en de dierengeneeskunde is de kennis over de immuniteit tegen *G. duodenalis* beperkt.

Hoofdstuk 1

In hoofdstuk 1 wordt eerst een overzicht gegeven van *Giardia*, onder andere van de transmissie, pathogenese en symptomen van giardiosis, de diagnose en behandeling. Daarnaast wordt de huidige kennis over de immunrespons tegen *Giardia* besproken. De meeste data die beschikbaar zijn over de immunrespons tegen de parasiet zijn verkregen door het uitvoeren van infectieproeven en *in vitro* studies in muizen.

Omdat *Giardia* een extracellulaire parasiet is die in het lumen van de darm verblijft, is het aspecifieke immuunsysteem erg belangrijk. Het darmepitheel, dat als eerste barrière fungeert, heeft als deel van dit aspecifieke immuunsysteem een belangrijke functie. Epitheelcellen produceren behalve defensines ook reactieve zuurstofmoleculen en stikstofdioxide, die een rol spelen in de afweerreactie tegen *Giardia*. Door studies in 'knock-out' muizen is geweten dat mestcellen ook belangrijk zijn voor de eradicatie van de parasiet. Tijdens *Giardia* infecties werden verhoogde aantallen mestcellen geobserveerd in de dunne darm. Activatie van mestcellen gebeurt meestal door binding van IgE of IgA op de Fc receptoren van de mestcel, met als gevolg dat een waaier van pro-inflammatoire moleculen wordt vrijgesteld, waaronder histamine en verscheidene cytokines. Mestcellen zijn o.a. een bron van interleukine 6 (IL-6). IL-6 speelt een rol in de omschakeling van B-cellen voor het produceren van IgA. Er werd aangetoond dat IL-6 onmisbaar is voor de eradicatie van *Giardia* en belangrijk is in de vroege controle van acute infecties.

Tijdens de eerste dagen na de start van de infectie is het aantal intra-epitheliale lymfocyten (IEL) verhoogd, met name cytotoxische CD8⁺ T-cellen en CD4⁺ T-helper

cellen. Terwijl CD8⁺ T-cellen bijdragen aan de afbraak van het darmweefsel tijdens de infectie, zijn CD4⁺ T-cellen juist belangrijk voor bescherming tegen *Giardia*. De CD4⁺ T-cellen produceren geen cytokines die passen in het typisch T-helper 1 of 2 profiel. IL-6, IL-17 en TNF- α zijn de enige cytokines waarvan bekend is dat ze onmisbaar zijn voor de eradicatie van de parasiet.

Wanneer de infectie het acute stadium voorbij is, begint een B-cel afhankelijke fase. IgA is het belangrijkste isotype voor de eradicatie van de parasiet, IgA is ook het voornaamste isotype dat aanwezig is in de mucosale secreties. IgA is cruciaal voor de eradicatie van *Giardia* omdat muizen waarvan de B-cellen gedepleteerd zijn of waarvan de IgA productie of poly Ig-receptor verstoord zijn, de parasiet niet kunnen uitschakelen.

Zoals hiervoor vermeld werd, is de huidige kennis over de immuniteit tegen *Giardia* voornamelijk gebaseerd op muis-modellen. Nochtans is de muis geen natuurlijke gastheer voor *G. duodenalis*, daarom kunnen de resultaten bekomen met muizen niet zomaar geëxtrapoleerd worden naar natuurlijke gastheren zoals mensen en runderen. De muis-specifieke parasiet *G. muris* induceert een snelle immuunrespons en wordt binnen een korte tijd geëradiceerd. In natuurlijke gastheren daarentegen, kunnen *G. duodenalis* infecties persisteren en zich ontwikkelen tot een chronische, intermitterende infectie. Dit suggereert dat de parasiet het immuunsysteem kan ontwijken of manipuleren. Deze hypothese wordt ondersteund door het feit dat in kalveren 3 weken na de start van *G. duodenalis* infectie geen influx van ontstekingscellen waargenomen werd en een opregulatie van immuun-regulerende pathways is gemeten (Dreesen et al., 2012). Daarom is de doelstelling van deze thesis om de immuunrespons tegen *G. duodenalis* te onderzoeken in runderen en om potentiële mechanismen te identificeren die zorgen voor het chronische verloop van giardiosis in de natuurlijke gastheer.

Hoofdstuk 2

In muizen onderdrukt *G. duodenalis* de respons van dendritische cellen (DC) op Toll-like receptor liganden. Dit suggereert dat *G. duodenalis* een immuun modulerend effect heeft op DCs. Daarom werd het effect van *G. duodenalis* onderzocht op monocyt-afkomstige dendritische cellen (MoDC) afkomstig van een natuurlijke gastheer: het rund. Ook werd na gegaan of de parasiet in staat is om de capaciteit van MoDC om immuunresponsen te induceren, te beïnvloeden.

Het effect van trofozoieten en excretie/secretie materiaal op de maturatie en cytokineproductie van runder MoDC werd onderzocht. Daarnaast werd de capaciteit van *Giardia*-gestimuleerde MoDC om lymfocytproliferatie te induceren onderzocht. De transcriptie van verschillende moleculen (PD-L1, β -catenine, ILT3 en ILT4), met een gekend inhibitorisch of telerogeen effect, was niet verhoogd in MoDC na stimulatie met *G. duodenalis*. Daarnaast was *G. duodenalis* niet in staat om de respons van de MoDC na stimulatie van TLR4 door LPS, te beïnvloeden. Er werd weinig of geen toename in expressie van de maturatiemarkers CD40 en CD80 gemeten, alhoewel de MHCII expressie wel toegenomen was na stimulatie met lage parasiet concentraties. Een dosisafhankelijke daling van ovalbumine-opname werd echter waargenomen, wat aangeeft dat de MoDC toch functioneel geactiveerd zijn door *G. duodenalis*. Transcriptie van IL-15 was significant verhoogd na 24 uur incubatie met *G. duodenalis* trofozoieten en excretie/secretie materiaal. Gestimuleerde MoDC induceerden proliferatie van autologe CD3⁻ -cellen, $\gamma\delta^+$ T-cellen en TCR $\alpha\beta^+$ CD4⁺ en CD8⁺ T-cellen. Hoewel verhoogde transcriptie van TGF- β werd gezien in de prolifererende CD4⁺ T-cellen, was de transcriptie van IL-10 en FoxP3 juist gedaald, wat betekent dat deze prolifererende cellen waarschijnlijk geen regulerende T-cellen zijn. Uit voorgaand onderzoek is bekend dat in runderen de regulerende functie van FoxP3⁺ regulatoire T-cellen wordt overgenomen door $\gamma\delta^+$ T-cellen. Echter, in deze studie was de transcriptie van zowel pro- als anti-inflammatoire cytokines (TNF- α , TGF- β , IL-10 en IL-4) verhoogd in de prolifererende $\gamma\delta^+$ T-cellen.

In conclusie, we vonden geen bewijs dat *G. duodenalis* een regulerend of inhiberend effect heeft op runder MoDC. MoDC gestimuleerd met *G. duodenalis* waren functioneel actief en in staat om proliferatie van T-cellen te induceren, die zowel pro- als anti-inflammatoire cytokines produceren.

Hoofdstuk 3

De doelstelling van dit hoofdstuk is om de kinetiek van de systemische immuunrespons tegen *G. duodenalis* te onderzoeken in runderen, om het mechanisme te identificeren dat het chronische karakter van *G. duodenalis* infecties in runderen kan verklaren.

Zes kalveren werden experimenteel geïnfecteerd met *G. duodenalis* assemblage A en E, waarna de cystenexcretie twee keer per week werd onderzocht, totdat een blijvende

lage cystenexcretie aantoonde dat de kalveren een beschermende immuniteit ontwikkeld hadden. Elke twee weken werden bloedstalen genomen. Tijdens deze hele periode werd de kinetiek van de circulerende geheugencellen en serum antilichamen gevolgd. De cystenexcretie startte een week na de infectie en bleef hoog tot week 14, waarna een continue lage uitscheiding aangaf dat de dieren immuniteit ontwikkeld hadden. Vijf weken na de start van de infectie werden geheugencellen waargenomen in het bloed. Er werd significante proliferatie van CD4⁺ αβ T-cellen gemeten na *in vitro* her-stimulatie met levende *G. duodenalis* trofozoieten, zoals eerder ook werd aangetoond in muizen en mensen. De prolifererende CD4⁺ T-cellen werden geïdentificeerd met behulp van real-time qPCR. Tijdens de piek van de proliferatie werd door de meerderheid van de cellen IL-17 tot expressie gebracht en in mindere mate FoxP3. IL-17 heeft waarschijnlijk een belangrijke functie tijdens de eradicatie van de parasiet, dit werd voorheen al bevestigd in muizen.

De waargenomen celproliferatie na *in vitro* her-stimulatie van PBMC was sterk gedaald nadat plastic adhesie op de PBMC werd uitgevoerd, wat aantoont dat antigeen-presenterende cellen een belangrijke rol spelen in de inductie van proliferatie. Het feit dat *Giardia* gestimuleerde MoDC niet in staat waren om de proliferatie te herstellen en dat depletie van CD21⁺ cellen geen effect had op de proliferatie toont aan dat andere antigeen-presenterende cellen dan MoDC en B-cellen belangrijk zijn voor de inductie van T-cel proliferatie.

Serum concentraties van anti-*G. duodenalis* IgG1 en IgA stegen significant vanaf week 11. Al vroeg in de infectie konden alle trofozoieten van een *in vitro* cultuur aangekleurd worden met serum antilichamen in een immunofluorescentietest. Dit duidt aan dat een breed spectrum van antilichamen tegen alle variant-specifieke oppervlakte eiwitten of tegen geconserveerde oppervlakte-antigenen van *G. duodenalis* geproduceerd wordt door de gastheer.

Hoofdstuk 4

In de algemene discussie wordt besproken welke immuunmechanismen belangrijk kunnen zijn in de ontwikkeling of onderdrukking van de immuniteit tegen *G. duodenalis* in rundvee. De hypothese dat *Giardia* via DCs een suppressief effect heeft op de immuniteit kon niet bevestigd worden (hoofdstuk 2). Er zijn geen duidelijke redenen gevonden voor de verschillende resultaten tussen onze studie en voorgaande studies in muizen (Kamda en Singer, 2009) en mensen (Obendorf et al., 2013). De

langdurige hoge cystenxcretie beschreven in hoofdstuk 3 bevestigt dat *Giardia* infecties in rundvee een chronisch karakter hebben. Immune reacties ontwikkelden zich pas na verscheidene weken van infectie. Dit is overeenkomstig met voorgaande studies waar geen tekenen van ontsteking werden waargenomen in kalveren 3 weken na infectie (Dreesen et al., 2012).

Op het moment dat de cellulaire respons geactiveerd was, werd proliferatie van gestimuleerde PBMC vooral veroorzaakt door CD4⁺ αβ T-cellen. CD4⁺ T-cellen kunnen actief immunereacties sturen en spelen een belangrijke rol in de immuniteit tegen *Giardia* in muizen en mensen. De transcriptie van IL-17 was significant opgeregeerd in de prolifererende CD4⁺ T-cellen. Samen met voorgaande resultaten uit studies met muizen (Solaymani-Mohammadi and Singer, 2011; Dreesen et al., 2014), suggereren deze resultaten dat IL-17 een belangrijke rol speelt in de ontwikkeling van beschermende immuniteit tegen *G. duodenalis*. De transcriptiefactor FoxP3 was ook significant opgeregeerd. Hoewel de expressie van FoxP3 typisch is voor regulatoire T-cellen, werden humane CD4⁺Foxp3⁺ IL-17 producerende cellen beschreven in bloed en lymfoïd weefsel. Verder onderzoek is nodig om na te gaan of IL-17 en Foxp3 in runderen tot expressie gebracht worden door dezelfde cellen of door verschillende CD4⁺ T-cel subpopulaties. Alternatieve immuneregulerende mechanismen worden besproken, waaronder de nucleaire receptor PPAR, welke werkt als een regulator, de inductie van regulatoire T-cellen en/of suppressie door γδ T-cellen.

De kinetiek van de antilichaam respons (IgG1 en IgA) duidt er op dat antilichamen gecorreleerd zijn met een beschermende immunerespons. Serum antilichamen waren vanaf de start van de infectie gericht tegen alle verschillende variant-specifieke oppervlakte eiwitten (vsp) en niet enkel tegen bepaalde subpopulaties. Dit duidt er op dat bescherming niet alleen gebaseerd is op de herkenning van alle verschillende variant-specifieke oppervlakte eiwitten maar ook op de antilichaamconcentratie.

MHCII⁺ cellen zijn nodig voor het induceren van een cellulaire respons, echter bleken DC en B-cellen hierin geen rol te spelen. Om na te gaan of er hierin een mogelijke rol is voor andere antigeen-presenterende cellen, zoals monocyten, macrofagen, mestcellen, M-cellen en 'innate lymfoïde' cellen, is verder onderzoek nodig.

Toekomstig onderzoek zal zich moeten richten op de manipulatie van de immunerespons van de gastheer, om te kunnen interfereren met het modulerende effect van

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