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DISSERTATION

Titel der Dissertation

Immunological aspects of *Isospora suis* infections in suckling piglets

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To the piglets

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1. ABBREVIATIONS

APC	antigen presenting cells
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
cpm	counts per minute
CTL	cytotoxic T-lymphocytes
ELISPOT	enzyme-linked immunosorbent spot
FACS	fluorescence activated cell sorting
FCM	flow cytometry
FCS	fetal calf serum
Foxp3	forkhead box p3
IFN	interferon
IL	interleukin
mAb	monoclonal antibodies
MACS	magnetic activated cell sorting
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
mRNA	messenger ribonucleic acid
NK cells	Natural Killer cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SWC	swine workshop cluster
TcR	T-cell receptor
T_H cells	T-helper cells
TNF	tumor necrosis factor
T_{regs}	regulatory T cells

2. INTRODUCTION

Isospora suis, a protozoan parasite of swine, belongs to the apicomplexan class Coccidea and is closely related to the genera *Toxoplasma, Neospora, Sarcocystis* and *Eimeria* (Franzen *et al.* 2000). It is the causative agent of neonatal porcine coccidiosis, a disease which affects suckling piglets predominantly in the first three weeks of life. This disease is spread worldwide and affects pig breeding units independently of the farm management system (Lindsay *et al.* 1992; Meyer *et al.* 1999). *I. suis* was first described in 1934 (Biester and Murray 1934) but before the 1970ties it was not broadly recognised as one of the most important causes of diarrhoea of young piglets, maybe due to an increasing importance of pig breeding since that time (Harleman 1977; Stuart *et al.* 1979; Stuart *et al.* 1980).

Coccidiosis in livestock is of great economic importance and causes significant losses, especially with eimeriosis in poultry and cattle as well as isosporosis in pigs. *I. suis* infections are common, with prevalences of more than 70 % on the farm level in Germany, Austria and Switzerland (Mundt *et al.* 2005). Effective treatment is achieved with toltrazuril (Baycox® 5 %), an anticoccidial agent which inhibits parasite development and consequently abrogates oocyst excretion and effects on animal health such as diarrhoea, enteritis and weight loss (Bach *et al.* 2003; Mundt *et al.* 2007). It is currently the only licensed drug for the treatment of neonatal porcine coccidiosis. A study from Sardinia (Italy) demonstrated that treatment with toltrazuril leads to an increase in profit per animal of 8.6 % (Scala *et al.* 2009). With an estimated number of 5 million piglets reared per year (based on annual lifestock counting data, Statistik Austria, www.statistik.at) and 1.8 million doses of Baycox® 5 % oral suspension sold each year (personal communication: Mag. Tamara Pieringer, Bayer Austria GmbH, Vienna, Austria) nearly every third piglet in Austria might be treated against porcine neonatal coccidiosis. Therefore, the economical impact of this disease for pig breeding facilities in Austria can be considered as significant.

Due to its zoonotic risk, the broad host spectrum and the availability of appropriate murine and *in vitro* models, the immune response mechanisms to *Toxoplasma gondii* are the one most intensively studied among the coccidia (e. g. Kasper *et al.* 2004; Lang *et al.* 2007; Miller *et al.* 2009). Also *Eimeria* spp. of poultry were investigated in detail regarding the immune response and possible vaccination strategies due to their huge impact on production. The research on the immune response to non-cystforming

mammalian coccidia was restricted to infections of laboratory animals for a long time. In the last years an increasing number of immunological studies was performed on eimeriosis in cattle and also in rabbits (e. g. Hermosilla *et al.* 1999; Renaux *et al.* 2003; Hermosilla *et al.* 2006; Alcala-Canto and Ibarra-Velarde 2008; Behrendt *et al.* 2008; Pakandl *et al.* 2008). The risk of opportunistic infections with coccidia in immunocompromised human patients (Ong 2008) also led to intensive studies on the immune response in this particular host (reviewed in Blader and Saeij 2009). However, despite the economic importance of porcine isosporosis, the immunological knowledge in this infection – which is essential for the development of new prophylactic and treatment strategies – is marginal. Therefore, the aim of this work was to obtain primary data about the cellular immune responses to *I. suis* in its original host to provide a basis for detailed immunological research of this important pathogen.

2.1. *Isospora suis* and neonatal porcine coccidiosis

I. suis has a direct life cycle (Fig. 1) with the pig as its sole host. It starts with the ingestion of sporulated oocysts, from which the sporozoites are released in the small intestine and actively invade enterocytes, mainly of the villi in the jejeunum and

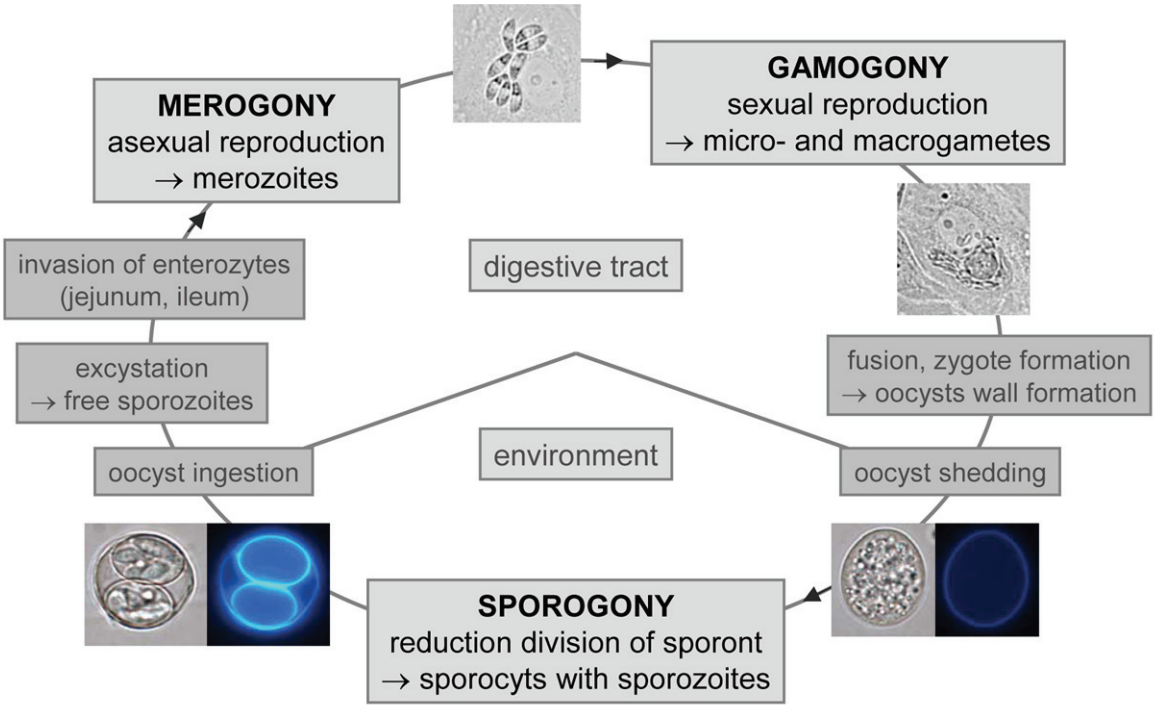

Fig. 1 Life cycle of *I. suis*. Unsporulated and sporulated oocysts are shown in bright field and epifluorescent microscopy. Merozoites and gametes are shown in phase contrast microscopy.

ileum. The intracellular stages of *I. suis* are found in a parasitophorous vacuole. During asexual reproduction in the enterocytes (merogony) merozoites are formed and released by destruction of the host cell and can invade additional enterocytes. Merozoites can be found 24h after infection. Four days after infection the sexual reproduction (gamogony) is initiated which leads to the production of micro- and macrogametes. These stages fuse to a zygote and after formation of the oocyst wall the unsporulated oocysts containing one sporont each are released from the enterocytes and shed with the faeces. Depending on the environmental conditions (temperature, humidity) the immature sporont divides (sporogony) within 1-3 days and forms two sporocysts with four sporozoites each. This fully developed environmental stage initiates further infections, so that the whole life cycle is completed within 6-7 days. After an excretion period of 3-4 days oocyst shedding declines and a second replication cycle is initiated which leads to a biphasic oocyst excretion pattern with a second peak five days after the first. The reasons for this are unknown; extraintestinal stages have been proposed but not been demonstrated yet (Lindsay *et al.* 1980; Matuschka and Heydorn 1980; Lindsay *et al.* 1982; Stuart *et al.* 1982a; Harleman and Meyer 1984).

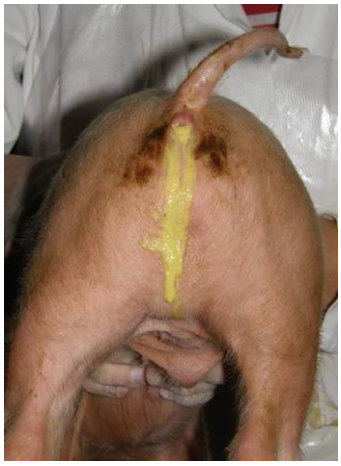

Fig. 2: Non-haemorrhagic, creamy to watery diarrhoea during isosporosis.

Both meronts (Niestrath *et al.* 2002) and gamonts (Vítovec and Koudela 1987) are described as the pathogenic stages. Parasite development within the porcine gut cells is accompanied by extensive sloughing of the epithelial lining with necrotic enteritis, villous atrophy and fusion as well as crypt hyperplasia, which are most pronounced around the time of asexual development. The damage of the epithelium leads to transient non-haemorrhagic diarrhoea (Fig. 2). Due to the damaged gut mucosa the uptake of nutrients and fluids is reduced and piglets can show dehydration, and reduced and uneven weaning weights (Fig. 3) are characteristic for this disease (Lindsay *et al.* 1985; Mundt *et al.* 2007).

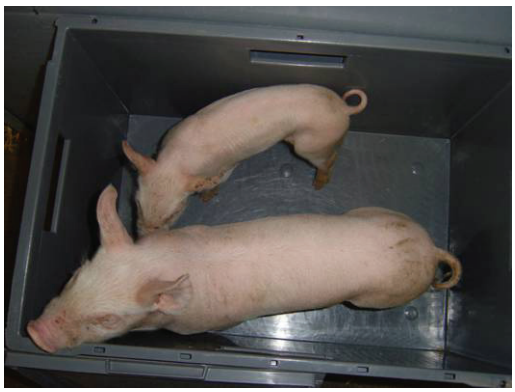

Fig. 3: Uneven weaning weights in littermates (3 weeks of age).

Pigs are mostly affected at the suckling age when non-haemorrhagic diarrhoea develops 4-5 days after infection in parallel with oocyst excretion and high morbidity in the litter. Although mono-infected piglets usually recover within 2 weeks weight gain is often severely reduced and secondary infections with other enteric pathogens can lead to increased mortality (Vítovec and Koudela 1990; Lindsay *et al.* 1992; Mundt *et al.* 2007).

The mechanisms of host-parasite interactions in *I. suis* infections are still poorly understood. Earlier studies lead to the belief that the parasite induces immunity (Stuart *et al.* 1982), and it is assumed that this immunity is based on cellular components, since supernatant from lymphocytes sensitised with *I. suis* antigen induce chemotaxis in leukocytes. Antibodies on the other hand do not seem to be protective (Taylor 1984). It has been speculated that the ability to mount an IgA antibody response is poorly developed in the first weeks of life (as reviewed in Baker *et al.* 1994), but this remains to be demonstrated. The enlargement of the mesenteric lymph nodes during the infection is an indication for a parasite-specific immune response (Harleman and Meyer 1985; Vítovec and Koudela 1990).

A reinfection of previously infected piglets results in reduced oocyst excretion; an age-related resistance to isosporosis in piglets has also been described (Stuart *et al.* 1982b; Koudela and Kučerová 1999). This effect could interfere with the development of innate as well as antigen-specific immune responses or also with the accelerated cell turnover in the gut mucosa of older piglets.

2.2. Selected aspects of the porcine immune system

In lifestock production animal and consumer health as well as responsible use of antimicrobials (to delay the development of drug resistance in pathogens) are some of the most important issues. Therefore, new treatments and prophylactic strategies including vaccines need to be developed. Additionally, the use of pigs as a large animal model for infectious diseases and developmental immunology is recently becoming more important (Rothkötter *et al.* 2002; Hein and Griebel 2003; Butler *et al.* 2009). Consequently, basic and applied research on the porcine immune system – which is characterised by several peculiarities in comparison to the murine and human systems – is of increasing importance.

To understand immune responses and the mechanisms of immunopathogenicity in porcine infectious diseases a lot of fundamental research was performed in the last decades. This was possible due to the development of monoclonal antibodies (mAb) against surface proteins characteristic for distinct leukocyte populations and a growing immunological toolkit for pigs. Some surface molecules have homologies to the system of human differentiation antigens and are therefore specified with the same CD (cluster of differentiation) nomenclature (Lunney 1993; Saalmüller 1996). For molecules without those homologies a distinct nomenclature was introduced, using swine workshop cluster (SWC) designations (Saalmüller *et al.* 1996; Saalmüller *et al.* 1998). Regarding several peculiarities of the porcine immune system compared to other mammalian species, the following paragraphs will give a short overview on selected extrathymic subsets of porcine lymphocytes with relevance for this study. Moreover, the involvement of the respective cell populations in infections with non-cystforming coccidia of other hosts will be outlined.

2.2.1. CD4 and CD8 as differentiation markers for T lymphocytes in pigs

CD4 and CD8 are co-receptor molecules which are required for the interaction of professional and non-professional antigen-presenting cells (APC) and effector cells (typically T cells) by antigen presentation via the major histocompatibility complex (MHC). Peptides are presented by MHC-molecules on the cell surface, especially on APC. MHC-molecules interact with the T-cell receptor (TcR) of the responding cell and need co-stimulatory signals for the initiation of a response. MHC-I is expressed on surfaces of all somatic cells and is predominantly used for the presentation of intracellular antigens like viruses, intracellular bacteria and (apicomplexan) protozoa. For the efficient recognition an interaction of the peptide-loaded MHC-I molecule with the TcR, CD8 is needed. Classically, CD8 is expressed on cytotoxic T-lymphocytes (CTL), which are able to initiate the lysis of infected cells and to produce IFN-γ. MHC-II on the other hand is mainly expressed by professional APC and used for presentation of extracellular antigens to cells with the co-receptor CD4. This co-receptor is found on T-helper (T_H) cells which are able to activate or help macrophages, B cells and neutrophils, and also on regulatory T cells (T_{regs}). The secretion of IFN-γ favours the development of T_H cells into Th_1 cells. IFN-γ produced by Th_1 cells activates

macrophages and directs the isotype-switch of B cells (reviewed in Billiau and Matthys 2009).

In swine the functions of T-cell subpopulations defined by CD4 and CD8 are similar to those of other species but there are some peculiarities. In the majority of immunologically investigated species CD8 molecules consist of an α and a β chain and are expressed as heterodimers. In swine a substantial proportion of cells expressing CDα monomers and also homodimers exist. In contrast to other mammals, pigs show a high percentage of extrathymic $CD4^+CD8\alpha^+$ T lymphocytes and also $CD4^-CD8\alpha^-$ lymphocytes – mostly with a coexpression of TcR-γδ – in addition to $CD4^+CD8\alpha^-$ and $CD4^-CD8\alpha^+$ lymphocytes (Saalmüller *et al.* 1987; Saalmüller *et al.* 1989; Summerfield *et al.* 1996; Saalmüller *et al.* 2002).

Classical MHC-I restricted CTL show the phenotype $CD2^+CD3^+CD4^-CD5^{high}CD6^+CD8\alpha^{high}CD8\beta^+$. The phenotype of memory-CTL is still unknown (reviewed in Gerner *et al.* 2009). T_H in pigs are defined as $CD3^+CD4^+$ cells with or without the co-expression of CD8α. The $CD8\alpha^-$ population seem to comprise resting T_H cells ($CD4^+CD8\alpha^-CD25^-CD45RC^+MHCII^+$) which acquire CD8α and upregulate MHC-II upon activation. Therefore, the $CD4^+CD8\alpha^+$ population consists of activated T_H cells ($CD4^+CD8\alpha^+CD25^+CD45RC^-MHCII^+$) but also of memory-$T_H$ cells ($CD4^+CD8\alpha^+CD25^-CD45RC^-MHCII^+$) (Saalmüller *et al.* 2002; Gerner *et al.* 2009).

In the immune response to coccidia $CD4^+$ and $CD8^+$ lymphocytes play different roles depending on the parasite species. During infections with *E. bovis* Th_1-like $CD4^+$ cells seem to be of particular importance (Hermosilla *et al.* 1999); in the primary immune response to *E. vermiformis* in mice and *E. tenella* in chicken $CD4^+$ lymphocytes were shown to be essential (reviewed in Lillehoj and Trout 1996). The involvement of $CD8^+$ in the immune response to coccidiosis and especially their need for the development of a protective immunity is reported for infections with *E. tenella* and *E. acervulina* in chicken (Lillehoj and Trout 1996; Swinkels *et al.* 2006), but also for infections with *E. intestinalis* in rabbits (Pakandl *et al.* 2008) and for *E. bovis* in calves (Hermosilla *et al.* 1999). Besides their importance for eradication and control of *Eimeria* infections, $CD8^+$ cells were also shown to act as transporters for sporozoites to the site of development after an active invasion of the lymphocytes by the parasites (Lillehoj and Trout 1996).

2.2.2. TcR-γδ T cells

In addition to cells with a TcR formed by a heterodimer of an α and a β chain there is a subset of T cells expressing TcR with a γ and a δ chain. These cells show in some cases a cytolytic activity independent of MHC-presentation and are able to recognise unprocessed non-peptide antigen, like it is found in parasites, fungi and plant extracts. For example alkylamine can induce proliferation and IFN-γ production of human TcR-γδ$^+$ cells (Saalmüller *et al.* 1994; Tanaka *et al.* 1994; Saalmüller *et al.* 1999; Kamath *et al.* 2003). TcR-γδ$^+$ cells are involved in epithelial wound repair, the induction of tolerance and they are able to produce pro- and anti-inflammatory cytokines and may be able to present antigen by MHC-II. The production of IFN-γ by TcR-γδ T cells in the gut mucosa attracts macrophages and induces the production of nitric oxides by enterocytes as a defence mechanism against pathogens. Moreover, TcR-γδ$^+$ cells are able to express a variety of pattern recognition receptors associated with the adaptive as well as the innate immune system. Porcine TcR-γδ T cells express cytokines and chemokines similar to that of humans and mouse and might therefore have the same functional properties (Takamatsu *et al.* 2006). In pigs, γ TcR-γδ T cells are able to proliferate in the intestines and recirculate to peripheral blood; therefore they seem to be an important pool of circulating effector cells (Thielke *et al.* 2003). The proportion of porcine TcR-γδ T cells (15-30 %) among circulating lymphocytes of adult swine is considerably higher than in mice and men and is even higher in piglets (Takamatsu *et al.* 2006).

TcR-γδ T cells where shown to be involved in the immune response to several coccidian species. They seem to be essential in the down-regulation of immune response in murine coccidiosis and are therefore protective against immunopathogenic effects (Roberts *et al.* 1996; Smith and Hayday 2000b). An example for TcR-γδ T cells as the first line of defence is the infection of young mice with *E. vermiformis.* In this case they are essential for the primary immune response but not responsible for the development of an immunological memory (Ramsburg *et al.* 2003). Also in coccidial infections of calves and lambs they seem to play an important role ((Aleksandersen *et al.* 1995; Hermosilla *et al.* 1999).

2.2.3. Regulatory T cells

Regulatory T cells (T_{regs}) with the phenotype CD4$^+$CD25$^+$Foxp3$^+$ show a variety of functions which were investigated mainly in mice and humans. They are defined as a

population of T cells with an inhibitory function and are regulating immune response by suppression to prevent allergy, autoimmunity and immunological reactions against non-pathogenic microorganisms. CD25 is a part of the IL-2 receptor and is expressed on T cells upon activation. Forkhead box p3 (Foxp3) is a transcription factor and crucial for the suppressive function and the development of T_{regs}. The expression of Foxp3 is involved in the suppression of proliferation and activation of e. g. T cells and NK cells (reviewed in Sakaguchi and Powrie 2007). Especially their important role in controlling intestinal inflammation (reviewed in Izcue and Powrie 2008) highlights them as a target for immunological research on an intestinal infection such as porcine isosporosis.

Their existence und suppressive functionality in pigs was demonstrated, showing that in general only small amounts (2-9 % of the $CD4^+$ lymphocyte subset in adult animals) of $CD4^+CD25^{high}$ cells can be found (Käser *et al.* 2008b).The primary phenotypic marker for regulatory T cells, the transcription factor Foxp3, is found in the majority of $CD4^+CD25^{high}$ porcine lymphocytes but only in a small number of $CD4^+CD25^{dim}$ cells (Käser *et al.* 2008a). The $CD4^+CD25^{high}$ lymphocyte subset is able to suppress the proliferation of activated T cells (Käser *et al.* 2008b). This functional property has not yet been demonstrated for $CD4^+CD25^{high}$ cells isolated from newborn piglets and therefore their existence does not implicate full functionality when considering the immature status of other cells of the immune system in these animals. To date no data are available about the involvement of T_{regs} in infections with non-cystforming coccidia.

2.2.4. Natural killer cells

NK cells are cells of the innate immune system which are able to kill infected or damaged cells as well as cancer cells by secretion of perforin without the need for prior sensitisation. In the course of the development of immunity against a pathogen they are active long before the adaptive response starts to be protective, therefore NK cells act as a first line of defence against pathogens. Moreover, NK cells were postulated to be essential for generation of *in vivo* T-cell responses and they are able to inhibit adaptive immune responses; IFN-γ produced by NK cells is needed for the activation of naïve T cells; besides IFN-γ, NK cells secrete a variety of pro-inflammatory cytokines and chemokines; they are able to kill activated T cells and TNF-α producing cells in general to terminate adaptive immune responses (reviewed in Denyer *et al.* 2006; Andoniou *et al.* 2008). In pigs NK cells are defined as lymphocytes with

the phenotype perforin$^+$CD2$^+$CD3$^-$CD4$^-$CD5$^-$CD6$^-$CD8α$^+$CD8β$^-$CD11b$^+$CD16$^+$ and were shown to actively kill virus-infected cells. They produce IFN-γ, TNF-α and perforin and are able to respond to cytokines such as IL-2, IL-12, IL-18 and IFN-γ (Denyer *et al.* 2006; Pintaric *et al.* 2008; Gerner *et al.* 2009).

So far, an essential role of NK cells for the immune response against *Eimeria* has only been demonstrated for the infection of mice with *E. papillata*. In that case neither T cells nor B cells appeared to be responsible for host protection (Schito and Barta 1997). They are also known to be essential in the early immune response to other intracellular protozoan pathogens like *Toxoplasma gondii*, *Leishmania* spp. and *Trypanosoma cruzi* (reviewed in Korbel *et al.* 2004).

2.2.5. Development of the immune system in the neonatal pig

Pigs have a syndesmochorial placenta prohibiting transmission of antibodies and lymphocytes before birth. Therefore, newborn piglets can be stated as immunodeficient reflected by a limited ability to react to pathogens via T cells or B cells. They receive large amounts of antibodies and also lymphocytes via the colostrum and milk in the first two days of life (Stokes *et al.* 2004). As isosporosis is an intestinal infection in neonatal pigs in this case the development of the mucosal immune system in the small intestine is of special interest. Almost no lymphocytes are found in the gut mucosa of healthy piglets at birth except clusters representing precursors of the Peyer's patches. The first cells to enter the intestinal villi in the first two weeks of life are lymphocytes expressing CD2 but not CD4 or CD8α. A majority of these cells is co-expressing the TcR-γδ at that time. Between the second and fourth week of life CD4$^+$ cells migrate into the gut mucosa followed by the majority of CD8α$^+$ lymphocytes from the fifth week on. In contrast to intraepithelial lymphocytes and lamina propria lymphocytes scattered throughout the mucosa, Peyer's patches develop an organised lymphoid structure until the twelfth day, harbouring B cells as the dominant lymphocyte population. The composition of lymphocytes in the gut mucosa representative for adult pigs is reached with six weeks but the absolute number of lymphocytes still increases afterwards. Active immune response to viruses can be detected from the age of three weeks on (Bailey *et al.* 2001; Stokes *et al.* 2004). The composition of lymphocyte subsets in blood, spleen and lymph nodes also shows significant changes during the first three weeks of life, including a decrease of CD4$^+$ cells in all three com-

partments and of TcR-γδ$^+$ cells in the blood. In contrast, the population of TcR-γδ T cells is expanding in the mesenteric lymph nodes and also the proportion of activated T_H cells (CD8α$^+$) increases. Furthermore, the population of B cells show a clear tendency to expand in the first three weeks of life (Solano-Aguilar *et al.* 2001; Grierson *et al.* 2007; Stepanova *et al.* 2007). Functionally, lymphocytes do not reach the full potential of responding to mitogens until the fourth week of life (Becker and Misfeldt 1993; Schwager and Schulze 1997).

2.3. Aims and hypotheses

This study had three aims: (a) The adaptation and establishment of experimental methods to investigate the immune responses during neonatal porcine coccidiosis in a model of experimental infections; (b) the phenotypical characterisation of lymphocyte populations from different lymphoid compartments during primary infections with *I. suis* in conventional suckling piglets in comparison with non-infected animals; and (c) the identification of lymphocyte subsets responsible for the immune response by functional characterisation of involved cells. The work was driven by the hypotheses that NK cells and T cells are involved in the immune response against *I. suis* including the T-cell subpopulations of T_H cells, TcR-γδ T cells and CTL; and that reactive cell populations can be found in the blood, the spleen, the MLN and the gut mucosa.

3. RESULTS

3.1. Publication 1

Changes in lymphocyte populations in suckling piglets during primary infections with *Isospora suis*

Worliczek H. L.[*], **Buggelsheim M., Alexandrowicz R., Witter K., Schmidt P., Gerner W., Saalmüller A. and Joachim A.** (2010). Changes in lymphocyte populations in suckling piglets during primary infections with *Isospora suis*. *Parasite Immunology* **doi 10.1111/j.1365-3024.2009.01184.x**.

* corresponding author

Own contribution:

- substantial contribution in project planning
- project management
- accomplishment of all laboratory work except immunohistochemistry and white blood cell count
- analysis and graphical editing of data
- draft of the first version and correction of the revised version of the manuscript

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Changes in lymphocyte populations in suckling piglets during primary infections with *Isospora suis*

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SUMMARY

Isospora suis*, a common intestinal parasite of piglets, causes neonatal porcine coccidiosis, which results in reduced and uneven weaning weights and economic losses in pig production. Nevertheless, there are no detailed studies available on the immune response to* I. suis*. The aim of this study was to carry out phenotypical characterization of lymphocytes during primary infections on day 3 after birth. Infected and non-infected piglets were investigated between days 7 and 16 after birth. Lymphocytes from the blood, spleen and mesenteric lymph nodes (flow cytometry) and of the jejunal mucosa (immunohistochemistry) were analysed. A decrease in T cells, especially with the phenotype of resting T-helper cells, T-cell receptor-γδ-T cells, and regulatory T cells in the blood, spleen and mesenteric lymph nodes was noticeable. An increase in cells with the phenotype of natural killer cells in the spleen of infected animals was found, and the subset of TcR-γδ-T cells was strongly increased in the gut mucosa. Our findings suggest an accelerated migration of those cells into the gut. This study provides a strong indication for the involvement of adaptive and innate immune response mechanisms in the primary immune response to* I. suis*, especially of TcR-γδ-T cells as a linkage between innate and adaptive immunity.*

Keywords *flow cytometry, immunohistochemistry,* Isospora suis*, neonatal coccidiosis, pig, primary immune response, T cells*

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INTRODUCTION

Isospora suis is a common intestinal parasite of piglets and the causative agent of neonatal porcine coccidiosis, a disease, which affects piglets up to the age of 3 weeks. *Isospora suis* belongs to the Apicomplexa and is spread worldwide with high prevalences in pig-breeding facilities independent of the farm management system (1,2).

Piglets are infected by ingestion of sporulated oocysts, and villous epithelial cells of the small intestine are invaded by sporozoites. The intracellular development (merogony followed by gamogony) takes place in a parasitophorous vacuole. The unsporulated oocysts are released from the enterocytes and shed with the faeces. The infectious stages develop in the environment within 1–3 days, depending on the temperature. The life cycle is completed within 5–6 days (3–6). Intracellular multiplication of the parasite leads to crypt hyperplasia and fusion, and atrophy and necrosis of the villi, resulting in diarrhoea and reduced uptake of nutrients by the mucosa (6–9).

Clinically, the infection is characterized by nonhaemorrhagic diarrhoea with yellow-greyish, creamy or liquid faeces, mostly in the second week of life. The disease shows a low mortality but weight gain can be severely compromised. As not all piglets are equally affected, the disease results in reduced and uneven weaning weights and, therefore, in economic losses for pig-breeding facilities (10,11).

As isosporosis is a neonatal infection in piglets, the development of the porcine immune system has to be taken into account for immunological studies. Besides various T-cell populations with similar function in other mammalian species – $CD4^+CD8^-$ T-helper cells (T_H cells) and cytotoxic T lymphocytes (CTL) with the phenotype $CD4^-CD8^+$ – there are some peculiarities in the porcine

immune system regarding the phenotype of reactive cells. Pigs have a high percentage of extrathymic $CD4^+CD8\alpha^+$ cells, a population which comprises activated and putative memory T_H cells, which is unique for the porcine immune system (12–15). In addition, T cells with the phenotype $CD4^-CD8\alpha^-$ exist, which represent in their majority T-cell receptor-γδ positive (TcR-$\gamma\delta^+$) cells. This population is found especially in the first 2 weeks of life in the intestinal villi (16). TcR-$\gamma\delta^+$ cells can also show the phenotype of $CD4^-CD8\alpha^+$ (17). In the small intestine, two major compartments are responsible for immune defence: jejunal Peyer's patches (PP) and one continuous ileal PP, consisting of organized lymphoid tissue, and lymphocytes scattered throughout the mucosa; the latter being the focus of this study. At birth, hardly any T cells can be found in the intestinal villi. They begin to enter this compartment in the first 2 weeks of life. At this time, mainly $CD4^-CD8\alpha^-$, and to a lesser extent $CD4^-CD8\alpha^+$ T cells, often with expression of TcR-γδ, are found. Other T cells start to enter the mucosa within the second week of life. After 6 weeks, the distribution of lymphocytes in the villi reaches values similar to those in adult animals, but the absolute number continues to increase (16). In other lymphoid compartments, including the blood, spleen and lymph nodes, the composition of lymphocyte populations also changes in the first 3 weeks of life. The proportion of γδ-T cells decreases in blood, but shows an increase in the mesenteric lymph nodes (MLN) without dominant changes in the spleen. $CD4^+$ cells, including the population of T_H cells, decrease during that time in the blood, spleen and MLN (18,19). $CD4^+$ cells, which express $CD8\alpha$ after activation (13), are rare at birth, but increase constantly during the first weeks of life (18–20). B cells show a strong increase in the MLN; their development in the blood seems to vary, but mostly they tend to increase (19–21). The immature status of the immune system in young piglets is also reflected by the ability of lymphocytes to respond to mitogens. This ability is not fully developed until the fourth week of life (21,22). In suckling rabbits, furthermore, it was shown that the reactivity of the immune system to coccidial infections increases with age (23). In relation to the situation in piglets, the incomplete development of the immune system may be a reason for the high susceptibility to *I. suis* infections in young piglets and the age resistance in older piglets (24–26). For coccidial infections in other hosts, more or less detailed phenotypical and functional studies of the involved leucocyte populations were carried out, especially for infections in chicken and mice, and also in rats, calves, sheep and rabbits. These studies revealed an involvement of various cell types in the immune response to coccidial infection including T_H cells, CTL, TcR-γδ-T cells and natural killer cells (NK cells) (27). Although porcine neonatal coccidiosis is of considerable economic importance for animal health, the immune response to *I. suis* has not yet been investigated in detail.

The aim of this study was to carry out phenotypical characterization of lymphocyte populations from different lymphoid compartments during primary infections with *I. suis* in conventional suckling piglets in comparison with noninfected piglets.

MATERIALS AND METHODS

Animals

A total of 34 conventional crossbred piglets (15 infected and 19 noninfected control animals) were used in this study as blood and tissue donors for immunological investigations. In addition, littermates of the donor piglets were used for monitoring the infection with *I. suis* or the *I. suis*-free status respectively (for details see samples). All piglets were reared conventionally with the sow in a farrowing crate on straw at the animal husbandry facilities of the Institute of Parasitology, University of Veterinary Medicine Vienna, Austria. Water was available *ad libitum* for the sow and the piglets. Piglets were vaccinated against *Mycoplasma hyopneumoniae* (Stellamun® One, Pfizer Pharma GmbH, Karlsruhe, Germany; 2 mL i.m.) and supplemented parenterally with iron dextrane (Myofer®, Intervet, Vienna, Austria, 2 mL s.c.) and vitamins (Vitasol AD_3E, Richter Pharma, Wels, Austria; 1 mL i.m.) 2 days after birth. After the first week of life, starter feed was available for the piglets *ad libitum*. The animals were stunned with a captive bolt pistol followed by exsanguination. All procedures were approved by the Austrian Federal Ministry of Science and Research and the Ethics Committee of the University of Veterinary Medicine Vienna according to the Austrian Animal Protection law.

Experimental infection

To avoid the exposure of control animals to highly infectious oocysts of *I. suis* shed by infected littermates, infected and control animals were taken from different litters (five litters for each group). The day of birth was defined as study day zero. On study day 3, piglets from the infection group were inoculated orally with 1000 sporulated oocysts of *I. suis* suspended in tap water. Oocysts for infection doses were previously isolated from experimentally infected piglets and sporulated in 2% potassium dichromate (28). To account for individual differences in the maturation of the immune system in suckling piglets, and possible temporal differences in the immune reaction

to infection, piglets were killed sequentially over the whole acute phase of infection (study day 7–16). During that period, individual faecal samples were taken daily from infected animals or on study days 7, 8, 9, 10, 12, 14 and 16 from noninfected animals. Oocyst shedding was screened qualitatively by autofluorescence microscopy, and quantitatively by McMaster counting when more than 10 oocysts were detected in the autofluorescence (29). Faecal consistency was scored as 1 (normal), 2 (pasty), 3 (semi-liquid) or 4 (liquid). Littermates of infected and noninfected animals were kept until study day 22 to monitor oocyst excretion and faecal consistency.

Blood and tissue

Blood was collected in heparinized bottles for the isolation of peripheral blood mononuclear cells (PBMC). Blood samples for white blood cell count were collected immediately post-mortem from the vena cava into EDTA tubes (Greiner Bio-one GmbH, Kremsmuenster, Austria) with a vacutainer system. Spleen and mesenteric lymph nodes were removed directly post-mortem and kept in cold PBS (without Ca^{2+}/Mg^{2+}, PAA Laboratories, Pasching, Austria) until isolation of lymphocytes. The small intestines were removed and divided into five sections from duodenum to ileum. The proximal part of the fourth section was defined as jejunal tissue. For immunohistochemistry, samples from the jejunum were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands), snap frozen and stored at −80°C until further processing.

Blood samples in EDTA tubes were used for an automated white blood cell count using an ADVIA® Laser flow cytometer with the Multi-Species-Software MS-V1·1·07 (Siemens Healthcare Diagnostics, Deerfield, IL, USA), followed by microscopic differentiation. Blood, tissues and isolated cells were kept on ice during the whole isolation process. Peripheral blood mononuclear cells were isolated by gradient centrifugation using lymphocyte separation medium (PAA Laboratories, Pasching, Austria) as described elsewhere (30). Mesenteric lymph nodes were separated from connective tissue and fat, strained through a metal sieve using a syringe plunge and rinsed with PBS. After centrifugation (8 min, 350*g*, 4°C), the suspension was filtered through cotton wool to remove remaining tissue fragments and dead cells, and rinsed again with PBS. After centrifugation, the pellet was treated with GEY's Solution (31) for 5 min at room temperature to remove any erythrocytes. Subsequently, the cells were washed with PBS twice, filtered through a nylon sieve (pore size approximately 70 μm) and were finally resuspended in PBS containing 10% (v/v) inactivated porcine plasma. The spleen was dissected into small pieces and lymphocytes were isolated as described for MLN. Cells were counted under trypan blue exclusion staining.

Flow cytometry

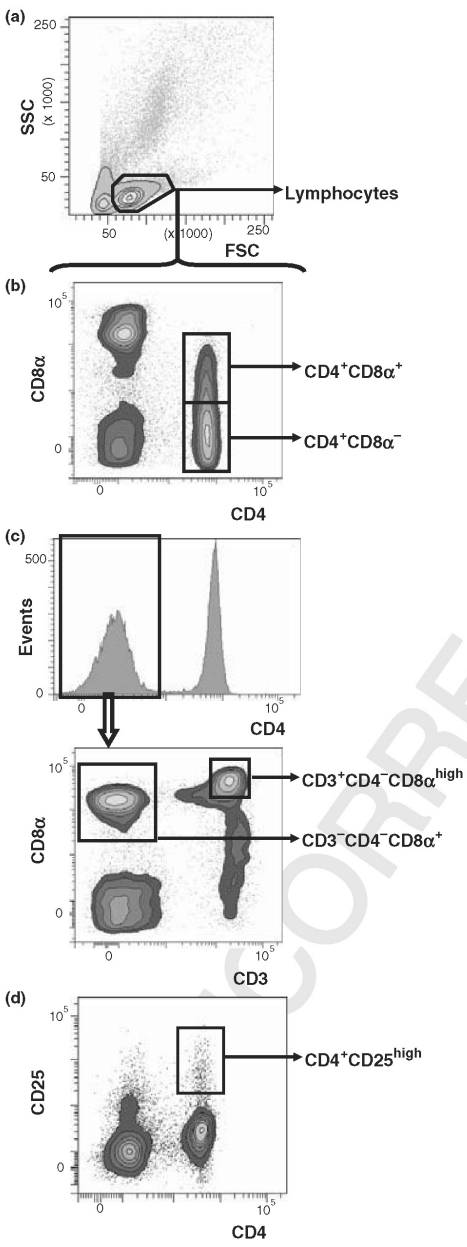
For phenotyping of lymphocytes by flow cytometry (FCM) after three-colour labelling, single cell suspensions of PBMC, splenocytes and lymphocytes from MLN were incubated with sets of primary antibodies (Table 1) against cell surface molecules in microtitre plates for 20 min on ice. After the first incubation, cells were washed twice with PBS containing 10% (v/v) inactivated porcine plasma. Then the cells were further incubated with fluorochrome-conjugated secondary antibodies (Table 1) for 20 min on ice and washed twice. Cells were fixed in fixation solution (32) containing 86% (v/v) sterilized deionized H_2O, 11% (v/v) 10× PBS (without Ca^{2+}/Mg^{2+}, PAA Laboratories, Pasching, Austria), 3% (v/v) formaldehyde stock solution (37% formaldehyde, Merck, Darmstadt, Germany) and stored in the dark at 4°C until analysis. For FCM analysis, labelled cells were washed and then resuspended in PBS containing 2% (v/v) FCS. Flow cytometry analyses were performed using a FACSAria with the FACSDiva Software Version 5·0·2 (BD Biosciences, San Jose, CA, USA) collecting at least 2×10^4 cells per sample.

Different lymphocyte subpopulations were chosen for analysis by phenotypes according to Gerner *et al.* Lym-

Table 1 Antibody combinations used for flow cytometry staining

Specifity	Clone name	Isotype	Labelling
Isotype control	NCG2B.01[a]	$mIgG_{2b}$	α-$mIgG_{2b}$-Alexa488[b]
	NCG2A.01[a]	$mIgG_{2a}$	α-$mIgG_{2a}$-Alexa647[b]
	NCG01[a]	$mIgG_1$	α-$mIgG_1$-PE[c]
CD4	74-12-4	$mIgG_{2b}$	α-$mIgG_{2b}$-Alexa488[b]
CD8α	11/295/33	$mIgG_{2a}$	α-$mIgG_{2a}$-Alexa647[b]
CD3	PPT 3	$mIgG_1$	α-$mIgG_1$-PE[c]
CD4	74-12-4	$mIgG_{2b}$	α-$mIgG_{2b}$-Alexa488[b]
CD8α	11/295/33	$mIgG_{2a}$	α-$mIgG_{2a}$-Alexa647[b]
CD25	3B2	$mIgG_1$	α-$mIgG_1$-PE[c]
CD4	74-12-4	$mIgG_{2b}$	α-$mIgG_{2b}$-Alexa488[b]
CD8α	11/295/33	$mIgG_{2a}$	α-$mIgG_{2a}$-Alexa647[b]
TcR-δ	PGBL22A[d]	$mIgG_1$	α-$mIgG_1$-PE[c]
CD21	B-ly4[e]	$mIgG_1$	α-$mIgG_1$-PE[c]

[a]Isotype controls from Dianova, Hamburg, Germany; [b]labelling antibodies from Invitrogen, Carlsbad, CA, USA; [c]SBA, Birmingham, AL, USA; [d]antibody from VMRD, Pullman, WA, USA; [e]antibody from BD Biosciences, San Jose, CA, USA; all other antibodies were provided by the Institute of Immunology, University of Veterinary Medicine Vienna, Austria.



phocytes were gated according to their light scatter signals (Figure 1a); T cells were identified by the expression of CD3, TcR-γδ-T cells by the expression of TcR-δ chain and B cells by the expression of CD21. $CD4^+$ cells were discriminated by their expression of CD8α in $CD4^+CD8α^-$ and $CD4^+CD8α^+$ cells (Figure 1b). Cells without expression of CD4 were further discriminated by the expression of CD3 and CD8α either as cells with the phenotype $CD3^-CD4^-CD8α^+$ or $CD3^+CD4^-CD8α^{high}$, representing in their majority the phenotypes of NK cells (33,34) and cytolytic T lymphocytes (35), respectively (Figure 1c). In addition, cells with the phenotype $CD4^+CD25^{high}$ – including the majority of Foxp3-positive porcine regulatory T cells in adult pigs (36) – were detected (Figure 1d).

The number of cells per microlitre of blood with the respective phenotypes was calculated according to the lymphocytes per microlitre of blood counted in the white blood cell count.

Immunohistochemistry

The snap frozen tissue samples were cut into sections of 4–6 μm thickness, which were mounted on SuperFrost®Plus slides (Menzel-Gläser, Braunschweig, Germany), fixed in chilled acetone (−20°C) and stored until staining at −20°C.

Single populations of lymphocytes were detected using a rabbit polyclonal anti-CD3 antibody (DakoCytomation, Glostrup, Denmark) detecting T cells, and the mouse monoclonal anti-TcR-δ chain antibody (clone PGBL22A, VMRD, Pullman, WA, USA) for TcR-γδ-T cells, which was also used for FCM.

Sections were rehydrated in PBS (pH 7·4). Endogenous peroxidase activity was blocked with 0·6% (v/v) H_2O_2 in methanol (H_2O was used instead of methanol for TcR-δ chain). Nonspecific binding was blocked with normal goat serum (DakoCytomation, 150 μL/10 mL PBS) at room temperature or with Background Terminator® (Biocare Medical, Concord, CA, USA) according to the instruc-

Figure 1 Analyses of lymphocyte subpopulations. Staining and selection strategies used for analyses of FCM data. (a) Lymphocytes were gated according to their light scatter signals. Relative numbers of lymphocyte subpopulations were calculated by the windows presented in (b)–(d). (b) By staining with antibodies against CD4 and CD8α, the subpopulations of $CD4^+$ cells ($CD4^+CD8α^-$ and $CD4^+CD8α^+$) were distinguished. (c) For the determination of $CD3^-CD4^-CD8α^+$ cells and $CD3^+CD4^-CD8α^{high}$ cells, only $CD4^-$ lymphocytes were analysed. (d) For the determination of the percentage of $CD4^+CD25^{high}$ cells, all lymphocytes were analysed and a specific window was set on the $CD4^+CD25^{high}$ subset.

tions of the manufacturer. Sections were incubated overnight with primary antibodies at 4°C. Primary antibody binding was detected with an avidin-biotin-peroxidase complex (Vectastain ABC Kit™, Vector Laboratories, Burlingame, CA, USA) after incubation of the sections with a secondary biotinylated anti-rabbit antibody (Vector Laboratories) for anti-CD3, or a biotinylated anti-mouse antibody (Vector Laboratories) for anti-TcR-δ chain according to the instructions of the manufacturer.

The reactions were visualized with diaminobenzidine (Sigma-Aldrich, Vienna, Austria) in 0·03% (v/v) H_2O_2 in Tris-buffered saline (pH 7·4). All sections were counterstained with Mayer's haematoxylin, dehydrated and mounted on a histological mounting medium soluble in xylene. For negative controls, either the primary antibody was omitted or a monoclonal mouse IgG1 (negative control for mouse IgG1 Ab-1, Clone NCG01, Dianova, Hamburg, Germany) was used instead of the primary antibody.

Quantification of lymphocyte populations in tissue sections

$CD3^+$ and TcR-$\gamma\delta^+$ cells were quantified in sections of the jejunum of healthy and *I. suis*-infected piglets using stereological counting rules. The area of positive $CD3^+$/TcR-$\gamma\delta^+$ cell profiles in relation to the area of epithelial or lamina propria profile within the section was assessed based on the Cavalieri principle (37).

A total section area of 2·9 mm^2 per sample was analysed. Twenty image fields (439·9 × 329·9 μm) per specimen and staining were photographed using systematic uniform random sampling (38). To assess the area of positive cell profiles and tissue profiles (epithelium or lamina propria), a counting point grid with 8 × 8 points and 4 × 4 points, respectively, was placed over the micrographs. The area per point (a/p) for the 8 × 8 point grid was 2267·3 μm^2, and 9069·2 $\mu m^2$for the 4 × 4 point grid.

Points hitting the desired structure were counted. The area of (i) $CD3^+$ cell profiles within the epithelium, (ii) $CD3^+$ cell profiles within the lamina propria, (iii) TcR-$\gamma\delta^+$ cell profiles within the epithelium, (iv) TcR-$\gamma\delta^+$ cell profiles within the lamina propria, (v) the profile of the epithelium and (vi) the profile of the lamina propria was estimated based on the number of hitting points:

$$A = \sum_{i=1}^{20} (P_i \times a/p) \quad (1)$$

(A, area of the profile of the studied structure; P_i, number of hitting points per image field; a/p, area per point).

The area of lymphocyte profiles was expressed as percentage of the area of the respective tissue profile.

Samples

Fifteen samples of blood, spleen and MLN from infected and 19 from noninfected animals were taken from five litters (L) for each group respectively: study day 7 $n_{infected}$ = 3 (L2, L4, L5), $n_{noninfected}$ = 4 (L9, L10); study day 8/9 $n_{infected}$ = 3 (L3, L4, L5), $n_{noninfected}$ = 5 (L6, L9, L10); study day 12/13 $n_{infected}$ = 4 (L2, L3, L4, L5), $n_{noninfected}$ = 4 (L9, L10); study day 15/16 $n_{infected}$ = 5 (L1, L2, L3, L4, L5), $n_{noninfected}$ = 6 (L7, L8, L10). One blood sample coagulated during recovery; therefore only 14 blood samples from infected piglets could be analysed for white blood cell count and by flow cytometry. Lymphocytes isolated from the spleen of one infected animal and stained with anti-TcR-δ chain antibody could not be analysed. Therefore, this phenotype in the spleen was analysed for 14 infected piglets only. Moreover, one sample from the MLN could not be analysed after staining with anti-CD25 antibody. Two samples of blood and three samples of spleen and MLN were not analysable after staining with anti-CD21 antibody, and therefore, only 12 samples from infected animals were analysed.

Gut samples were taken from seven infected and 18 noninfected animals of the following litters: study day 7 $n_{infected}$ = 1 (L2), $n_{noninfected}$ = 4 (L9, L10); study day 8/9 $n_{infected}$ = 1 (L3), $n_{noninfected}$ = 5 (L6, L9, L10); study day 12/13 $n_{infected}$ = 2 (L2, L3), $n_{noninfected}$ = 3 (L9, L10); study day 15/16 $n_{infected}$ = 3 (L1, L2, L3), $n_{noninfected}$ = 6 (L7, L8, L10). Sections stained with anti-CD3 antibody were analysable only from six infected animals because of technical reasons (high background staining in one animal). Sections stained with anti-TcR-δ chain antibody were analysable only from 17 noninfected animals because of the same reason.

Faecal samples were investigated from all piglets used as blood and tissue donors until they were slaughtered. In addition, identically treated littermates, which were not used as blood and tissue donors for this study, were screened for faecal consistency and oocyst shedding until study day 22 ($n_{infected}$ = 23, $n_{noninfected}$ = 24).

Statistical analysis

Statistical analyses were performed using the SPSS 14·0 (SPSS Inc., Chicago, IL, USA). Normal distribution of the data was investigated by the Kolmogorov–Smirnov (K–S) test. To compare infected with noninfected piglets, an analysis of covariance (ANCOVA) with the main effect infection was computed. The covariate study day was used to investigate and correct the influence of this parameter on the distribution of lymphocyte subpopulations, as piglets were investigated from study day 7 until 16. The

homogeneity of variances was tested by means of *Levene's* test. For data sets without normal distribution, Mann–Whitney (Wilcoxon) two-sample tests, and for data sets with nonhomogeneous variances, Welch tests were used instead of groupwise comparison. Significance was assumed for $P \leq 0{\cdot}05$.

RESULTS

Coproscopic examination

The noninfected piglets did not shed oocysts during the whole study period and no diarrhoea occurred. All infected piglets investigated after study day 7, which is still in the prepatent period, – shed oocysts. Oocyst excretion occurred from study day 8 to 18 with a typical reduction in excretion on study day 12. From study day 9 to 18, piglets showed nonhaemorrhagic diarrhoea (faecal score ≥2; Figure 2).

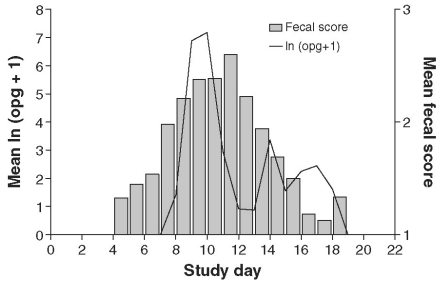


Figure 2 Intensities of oocyst excretion and consistency of faeces during infection of piglets with *Isospora suis* ($n = 23$). A faecal score ≥2 indicates diarrhoea. Piglets were infected with 1000 sporulated oocysts of *I. suis* on study day 3. Opg, oocyst per gram faeces.

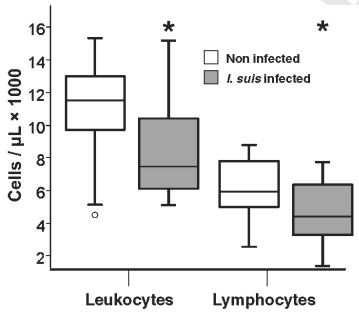


Figure 3 Absolute numbers of leucocytes and lymphocytes per microlitre of blood of noninfected and *Isospora suis*-infected piglets. Data were obtained by cell count in an ADVIA flow cytometer and by microscopic control. Each box plot spans the interquartile range of absolute cell numbers on the *y*-axis, with the inside line indicating the median. Whiskers extend to the minimum and maximum values excluding outside and far out values represented by circles. *Significant differences ($P \leq 0{\cdot}05$).

White blood cell count: alterations in leucocyte populations in the blood of *Isospora suis*-infected piglets

Leucocytes were significantly decreased in infected animals (Figure 3) with a mean of 8555·7 (SD 2821·4) per microlitre of blood compared with 10 945·8 (SD 2961) per microlitre of blood in noninfected piglets ($P = 0{\cdot}028$; d.f. = 1, 30; $F = 5{\cdot}297$).

The number of lymphocytes was also significantly lower in infected animals (Figure 3) with a mean of 4623·9 (SD 1980·1) per microlitre of blood in infected and 6081·3 (SD 1972·2) per microlitre of blood in noninfected animals ($P = 0{\cdot}044$; d.f. = 1, 30; $F = 0{\cdot}044$). Monocytes, neutrophils and eosinophils showed no differences between the groups (data not shown). The covariate study day was significant for segmented neutrophils ($P = 0{\cdot}027$; d.f. = 1, 30; $F = 5{\cdot}416$) only, indicating an influence of age, but not of infection, on the amount of these cells.

Influence of *Isospora suis* infections on the composition of lymphocyte subpopulations in the blood, spleen and mesenteric lymph nodes

For lymphocytes from spleen and MLN, percentages of the distinct phenotypes were used for statistical analyses. Percentages for PBMC were converted to absolute cell numbers per microlitre of blood according to counted lymphocytes in the white blood cell count.

In infected animals, T cells ($CD3^+$) significantly decreased in the blood, spleen and MLN (Figure 4a). The T-cell subpopulation of TcR-γδ-T cells was significantly reduced in the blood and MLN of infected animals, but showed a similar frequency in the spleen of infected and noninfected piglets (Figure 4b). $CD4^+CD8\alpha^-$ lymphocytes (phenotype of resting T_H cells (13)) were decreased in the blood, spleen and MLN (Figure 4c). $CD4^+CD8\alpha^+$ lymphocytes (phenotype associated with activated T_H cells and memory T_H cells (13)) were only reduced in the blood and MLN, but not in the spleen of infected piglets (Figure 4d). Unlike the T cells, $CD3^-CD4^-CD8\alpha^+$ cells (phenotype associated with NK cell function (39,40)) were strongly and significantly increased in the spleen of infected piglets without significant differences in the blood and MLN (Figure 4e). For cells with the phenotype $CD3^+CD4^-CD8\alpha^{high}$ (putative CTL phenotype (35)), no significant differences could be detected (Figure 4f). The subpopulation of

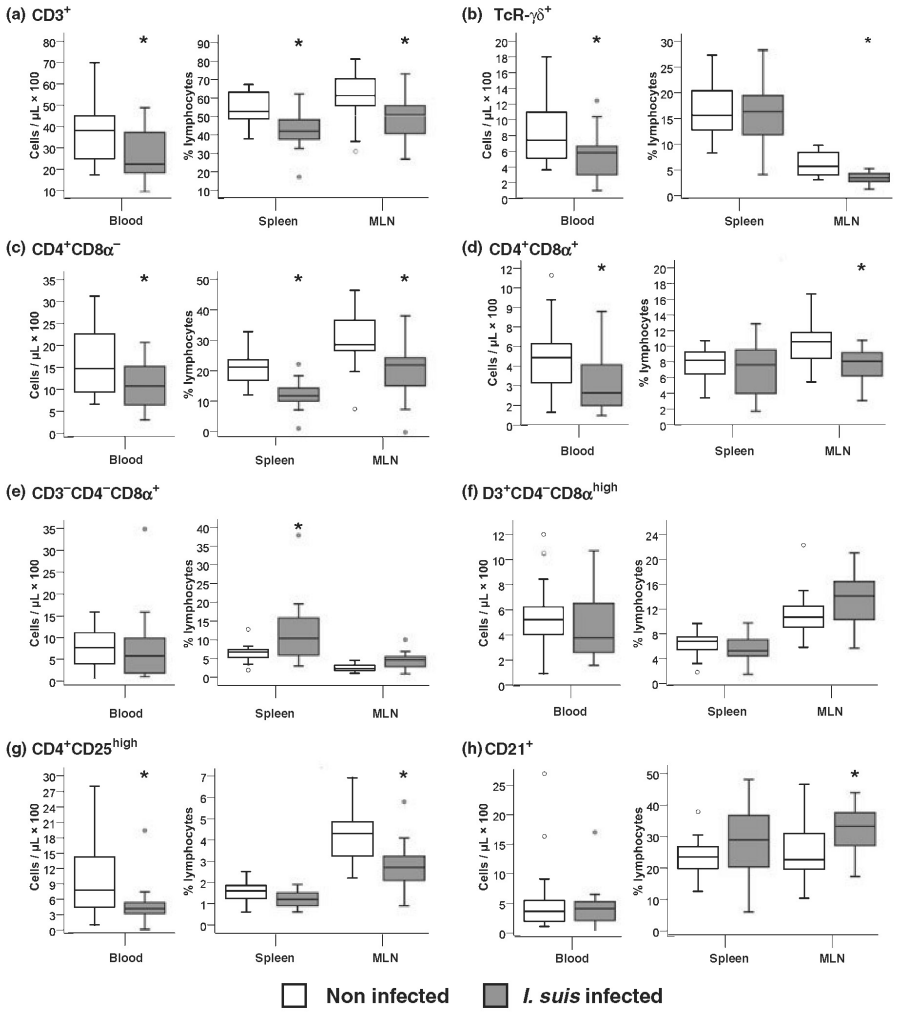


Figure 4 Distribution of indicated lymphocyte subpopulations in the blood, spleen and MLN of noninfected and *Isospora suis*-infected piglets. Data were obtained by flow cytometry (for selective analyses see Figure 1) in combination with the absolute cell number determined by lymphocyte counting (ADVIA). Each box plot spans the interquartile range of absolute (blood) or relative cell numbers (spleen, MLN) on the *y*-axes, with the inside line indicating the median. Whiskers extend to the minimum and maximum values excluding outside and far out values represented by circles. *Significant differences ($P \leq 0{\cdot}05$; see Table 2 and 3). MLN, mesenteric lymph nodes; TcR, T-cell receptor.

$CD4^+CD25^{high}$ (representing the phenotype of regulatory T cells) revealed a small, but significant decrease in the blood and MLN of infected piglets, but not in the spleen (Figure 4g). $CD21^+$ cells (a subpopulation of B lymphocytes) showed a significant increase in the MLN of infected animals (Figure 4h).

As the animals were investigated from study day 7 to 16, representing the acute phase of infection with oocyst

Table 2 Distribution of lymphocytes with specific phenotype in the blood measured by flow cytometry and between-subjects effects of ANCOVA comparing infected and noninfected piglets with *study day* as covariate

							ANCOVA between-subjects effects					
				Levene's test			Study day			Infection		
Phenotype	Infection	n[a]	Mean cells/μL (SD)	d.f.	F	P	d.f.	F	P	d.f.	F	P
$CD3^+$	–	19	3732·47 (1529·30)	1, 31	0·616	0·439	1, 30	0·057	0·812	1, 30	4·753	**0·037**
	+	14	2633·89 (1210·23)									
TcR-$\gamma\delta^+$	–	19	825·30 (382·44)	1, 31	0·931	0·342	1, 30	1·362	0·252	1, 30	4·512	**0·042**
	+	14	553·58 (326·97)									
$CD4^+CD8\alpha^-$	–	19	1622·04 (730·40)	1, 31	2·286	0·141	1, 30	0·065	0·800	1, 30	4·585	**0·040**
	+	14	1119·33 (542·61)									
$CD4^+CD8\alpha^+$	–	19	524·12 (285·31)	1, 31	1·172	0·287	1, 30	0·538	0·469	1, 30	5·643	**0·024**
	+	14	304·95 (214·29)									
$CD3^-CD4^-CD8\alpha^+$	–	19	791·55 (458·61)	1, 31	1·330	0·258	1, 30	2·294	0·140	1, 30	0·005	0·946
	+	14	816·42 (893·56)									
$CD3^+CD4^-CD8\alpha^{high}$	–	19	581·14 (281·24)	1, 31	0·004	0·951	1, 30	0·024	0·878	1, 30	1·926	0·175
	+	14	444·96 (261·96)									
$CD4^+CD25^{high}$	–	19	101·14 (73·52)	1, 31	4·580	**0·040**	–	–	–	–	–	**0·025**[b]
	+	14	52·21 (44·48)									
$CD21^+$	–	19	554·07 (629·54)	–	–	–	–	–	–	–	–	0·984[d]
	+	12[c]	456·44 (439·80)									

[a]One blood sample was not analysable, therefore only 14 samples from infected animals could be included; [b]results of Welch test as *Levene*'s test for equality of variances was significant; [c]two samples were not analysable after labelling with anti-CD21 antibody; [d]data were not normally distributed, the P-value from Mann–Whitney (Wilcoxon) two-sample test is shown. Significant results ($P \leq 0{\cdot}05$) are shown in bold characters. TcR, T-cell receptor; d.f., degrees of freedom.

excretion in infected animals, the ANCOVA was operated with study day as a covariate to investigate the influence of age on the distribution of lymphocytes.

In the blood, the covariate study day did not have a significant effect on any of the cell populations under investigation (Table 2). In the spleen, this covariate had a significant influence on the distribution of $CD3^+CD4^-CD8\alpha^{high}$ and $CD21^+$ lymphocytes (Table 3). In the MLN, it had a significant effect on $CD3^+$, $CD4^+CD8\alpha^-$, $CD4^+CD25^{high}$ and $CD21^+$ cells (Table 3). The group difference between infected and noninfected piglets was corrected for the influence of the study day. Therefore, only differences depending on the infection itself and not of age were detected when comparing infected and noninfected animals.

Influence of *Isospora suis* infections on the lymphocyte population in the gut

In parallel to the analyses of the composition and distribution of lymphocytes in the blood, spleen and MLN, we investigated the distribution of $CD3^+$ and TcR-$\gamma\delta^+$ cells in the mucosa of the jejunum by means of immunohistochemistry (Figure 5).

The area positive for CD3 was significantly increased in the epithelium as well as in the lamina propria of infected animals (Figure 6a). The area positive for γδ-T cells was also significantly increased in both compartments. Especially, γδ-T cells in the epithelium showed a more than 30-fold increase in *I. suis*-infected piglets (Figure 6b, Table 4) indicating their infiltration of the epithelium as a result of the infection.

A significant influence of the covariate study day on the distribution of T cells in the epithelium was noted (Table 4), which was removed before investigation of the group difference between infected and noninfected animals to exclude the effect of the age of the animals from this analysis.

Statistical analysis

Data of TcR-$\gamma\delta^+$ cells in the lamina epithelialis of the jejunum and of $CD21^+$ cells in the blood were not normally distributed and were therefore analysed with the Mann–Whitney (Wilcoxon) two-sample test. All other data were normally distributed. *Levene*'s test for equality of variances was significant for $CD4^+CD25^{high}$ cells in the blood (Table 2), $CD4^+CD8\alpha^+$ and $CD3^-CD4^-CD8\alpha^+$ cells in the spleen (Table 3), and for $CD3^+$ cells in the lamina propria of the gut (Table 4). As data with nonequal variances in the compared groups cannot be analysed by ANCOVA, these cell populations in infected and noninfected animals were compared by Welch tests.

Summarizing the results of this study, a decrease in T cells, including γδ-T cells, $CD4^+$ cells and $CD4^+CD25^{high}$

Table 3 Mean percentage of lymphocytes with specific phenotype in the spleen and mesenteric lymph nodes measured by flow cytometry and between-subjects effects of ANCOVA comparing infected and noninfected piglets with *study day* as covariate

							ANCOVA between-subjects effects					
				Levene's test			Study day			Infection		
Phenotype	Infection	*n*	Mean % of lymphocytes (SD)	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Spleen												
$CD3^+$	–	19	54·27 (9·06)	1, 32	0·097	0·758	1, 31	1·441	0·224	1, 31	11·390	**0·002**
	+	15	43·16 (10·67)									
TcR-$\gamma\delta^+$	–	18	16·01 (5·01)	1, 30	0·144	0·707	1, 29	0·070	0·794	1, 29	0·068	0·796
	+	14[a]	16·11 (5·86)									
$CD4^+CD8\alpha^-$	–	19	21·07 (5·66)	1, 32	0·502	0·484	1, 31	0·522	0·475	1, 31	23·434	**0·000**
	+	15	12·07 (5·00)									
$CD4^+CD8\alpha^+$	–	19	7·83 (2·05)	1, 32	10·378	**0·003**	–	–	–	–	–	0·388[b]
	+	15	6·89 (3·70)									
$CD3^-CD4^-CD8\alpha^+$	–	19	6·76 (2·97)	1, 32	13·000	**0·001**	–	–	–	–	–	**0·035**[b]
	+	15	12·28 (8·92)									
$CD3^+CD4^-CD8\alpha^{high}$	–	19	6·43 (1·91)	1, 32	1·075	0·308	1, 31	7·980	**0·008**	1, 31	2·230	0·145
	+	15	5·56 (2·34)									
$CD4^+CD25^{high}$	–	19	1·54 (0·53)	1, 32	1·191	0·283	1, 31	3·928	0·056	1, 31	4·075	0·052
	+	15	1·20 (0·39)									
$CD21^+$	–	19	23·05 (6·54)	1, 29	2·990	0·094	1, 28	9·483	**0·005**	1, 28	1·801	0·190
	+	12[a]	28·43 (11·91)									
Mesenteric lymph nodes												
$CD3^+$	–	19	61·00 (13·03)	1, 32	0·265	0·610	1, 31	5·728	**0·023**	1, 31	9·243	**0·005**
	+	15	47·93 (12·19)									
TcR-$\gamma\delta^+$	–	19	6·88 (4·67)	1, 32	3·962	0·055	1, 31	1·518	0·227	1, 31	7·165	**0·012**
	+	15	3·49 (1·13)									
$CD4^+CD8\alpha^-$	–	19	30·98 (8·42)	1, 32	0·000	0·984	1, 31	11·64	**0·002**	1, 31	15·739	**0·000**
	+	15	20·16 (8·87)									
$CD4^+CD8\alpha^+$	–	19	10·31 (2·75)	1, 32	0·007	0·933	1, 31	0·823	0·371	1, 31	9·674	**0·004**
	+	15	7·49 (2·54)									
$CD3^-CD4^-CD8\alpha^+$	–	19	3·02 (2·47)	1, 32	0·734	0·398	1, 31	0·153	0·699	1, 31	2·417	0·130
	+	15	4·39 (2·45)									
$CD3^+CD4^-CD8\alpha^{high}$	–	19	11·12 (3·69)	1, 32	1·689	0·203	1, 31	0·001	0·970	1, 31	2·161	0·152
	+	15	13·23 (4·56)									
$CD4^+CD25^{high}$	–	19	4·22 (1·40)	1, 32	1·200	0·281	1, 31	5·678	**0·023**	1, 31	11·012	**0·002**
	+	14[a]	2·75 (1·17)									
$CD21^+$	–	19	35·62 (11·90)	1, 29	0·190	0·666	1, 28	19·19	**0·000**	1, 29	5·579	**0·025**
	+	12[a]	46·37 (12·28)									

[a]Not all samples were analysable, therefore *n* varies; [b]results of Welch test are shown as *Levene*'s test for equality of variances was significant. Significant results ($P \leq 0{\cdot}05$) are shown in bold characters. TcR, T-cell receptor; d.f., degrees of freedom.

cells, was found in the blood, spleen and MLN in infected piglets. In contrast, T cells and their subpopulation of γδ-T cells were strongly increased in the jejunal mucosa of infected animals. For $CD3^-CD4^-CD8\alpha^+$ cells (NK cell phenotype) from spleens and for $CD21^+$ cells from MLN of infected animals, an increase was detected.

DISCUSSION

The infection dynamics of porcine isosporosis are well studied in experimental infections of conventional and gnotobiotic piglets (6,9,10,41,42). Excretion of oocysts and occurrence of diarrhoea depend on the infection dose, the age of the piglets and possibly on the pathogenicity of the *I. suis* strain used for infection. For studies on immunological parameters, the age of the infected piglets and infection doses should be selected with respect to both, low mortality and only moderate pathological changes at the site of infection. This helps to keep the influence of secondary infections by other enteric pathogens at a minimum. Furthermore, oocyst excretion and diarrhoea as signs of infection and clinical alterations should be present, similar to

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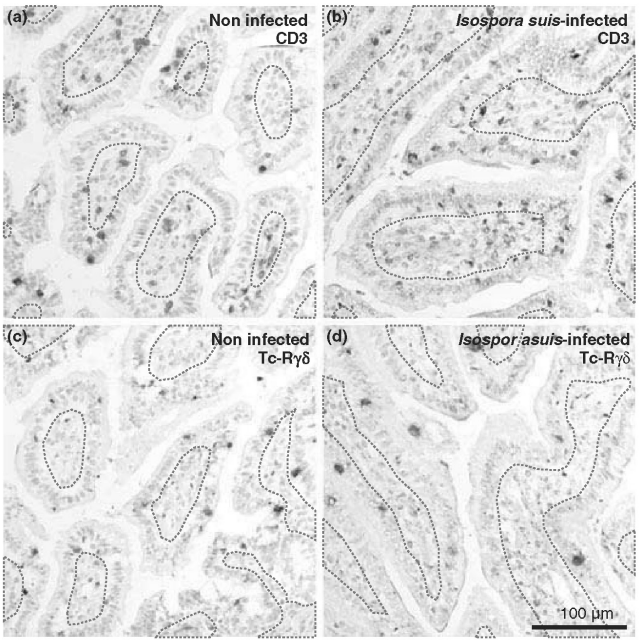

Figure 5 Distribution of T cells (a, b) and TcR-γδ-T cells (c, d) in the mucosa of the small intestine of noninfected (a, c) and *Isospora suis*-infected piglets (b, d). The red dotted lines mark the boundary between mucosal epithelium and lamina propria. Note that in the epithelium, T cells as well as TcR-γδ-T cells can be found, whereas the lamina propria contains T cells, but virtually no TcR-γδ-T cells in the shown image fields. Frozen sections, immunohistochemical detection of CD3 (a, b) and TcR-δ (c, d), detection system horseradish peroxidase/diaminobenzidine, counterstaining Mayer's haematoxylin. TcR, T-cell receptor.

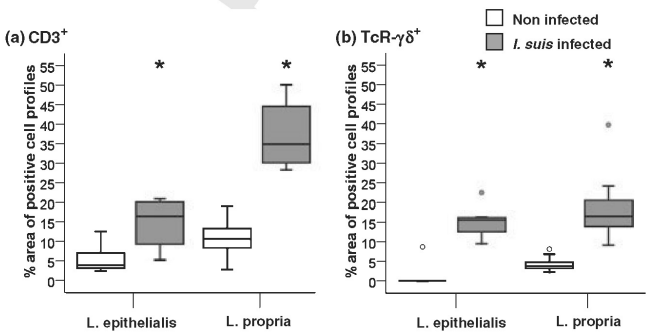

Figure 6 Distribution of lymphocyte subpopulations in the lamina epithelialis and lamina propria of the jejunum of noninfected and *Isospora suis*-infected piglets. Percentages of the areas of positive cell profiles in relation to the tissue profile area are shown. Data were obtained by stereology from histological sections stained immunohistochemically. Each box plot spans the interquartile range of data on the *y*-axis with the inside line indicating the median. Whiskers extend to the minimum and maximum values excluding outside and far out values represented by circles. *Significant differences ($P \leq 0{\cdot}05$; see Table 4). TcR, T-cell receptor.

Table 4 Distribution of lymphocytes in the lamina epithelialis and lamina propria of the jejunum. Mean percentage of the area of lymphocyte profiles positive for CD3 or TCR-γδ in relation to the respective mucosal area and between-subjects effects of ANCOVA comparing infected and noninfected piglets with *study day* as covariate

							ANCOVA between-subjects effects					
				Levene's test			Study day			Infection		
Phenotype	Infection	*n*[a]	Mean % area of positive cell profiles (SD)	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Lamina epithelialis												
$CD3^+$	–	18	5·21 (2·99)	1, 22	2·518	0·127	1, 21	5·622	**0·027**	1, 21	26·781	**0·000**
	+	6	14·62 (6·21)									
TcR-$\gamma\delta^+$	–	17	0·51 (2·11)	–	–	–	–	–	–	–	–	**0·000**[b]
	+	7	14·89 (4·36)									
Lamina propria												
$CD3^+$	–	18	10·87 (3·98)	1, 22	5·792	**0·025**	–	–	–	–	–	**0·000**[c]
	+	6	37·06 (8·55)									
TcR-$\gamma\delta^+$	–	17	5·37 (5·43)	1, 22	0·654	0·427	1, 21	3·379	0·080	1, 21	23·050	**0·000**
	+	7	19·06 (10·17)									

[a]Not all samples were analysable, therefore *n* varies; [b]data were not normally distributed, the *P*-value from Mann–Whitney (Wilcoxon) two-sample test is shown; [c]*Levene*'s test for equality of variances was significant, *P*-values from Welch test comparing infected and noninfected animals are shown. Significant results ($P \leq 0{\cdot}05$) are shown in bold characters. TcR, T-cell receptor; d.f., degrees of freedom.

the situation in the field. Therefore, an infection model for a laboratory *Isospora* strain with an infection dose of 1000 oocysts on study day 3 (day of birth = study day 0) with an expected result of oocyst shedding and nonhaemorrhagic diarrhoea was established (27).

Regarding the white blood cell counts, a decrease in leucocytes and lymphocytes in piglets infected with *I. suis* was found. As monocytes, neutrophils and eosinophils did not show significant differences between the groups, the differences in the quantity of leucocytes are presumably caused by changes in the lymphocyte population. As there were no differences detected in the amount of $CD21^+$ B cells in the blood of infected and noninfected animals, this reduction was presumably caused by T cells.

Indeed, a reduction in the number of T cells was noticeable in infected animals in the blood, spleen and MLN. In contrast, there was a strong increase in T cells in the epithelium and the lamina propria of the jejunum. This leads to the hypothesis that T cells migrate from the blood and secondary lymphoid organs to the site of infection, and that migration of lymphocytes to the gut mucosa is accelerated by the infection. In particular, the spleen is known to play an important role in the regulation of lymphocyte recirculation (43). This hypothesis of accelerated migration of lymphocytes is supported by the finding that from day 9 1 to 17 after birth, an increasing number of IEL is found in the jejunum, while no IEL can be detected in specific pathogen-free pigs in this interval (44). In addition, it was shown that lymphocytes derived from the spleen or MLN migrate to the gut mucosa (45). Therefore, it is likely that the increase in T cells in the jejunal mucosa during porcine coccidiosis is not only caused by proliferation, but also by migration. Interestingly, the majority of the T cells infiltrating the jejunal epithelium, and half of the T cells infiltrating the lamina epithelialis belonged to the TcR-γδ T-cell subpopulation. This result is noticeable, as healthy piglets are known to have almost no TcR-γδ-T cells in the epithelium and only a low number in the lamina propria of the gut mucosa (44). T cells expressing the TcR-γδ-T-cell receptor are able to recognize unprocessed nonpeptide antigens, and they are involved in mucosal epithelial wound repair, and the induction of tolerance. Furthermore, they are able to produce pro- and anti-inflammatory cytokines and may be capable of presenting antigen via MHC class II (46).

In the jejunal epithelium of infected piglets, they were found in a 30-fold higher frequency compared with healthy piglets. This increase is remarkable and supports the assumption of TcR-γδ-T cells as the first line of defence in piglets against coccidia. Representing a linkage between innate and adaptive immunity, they seem to play an essential role in the primary immune response to *I. suis*.

In the blood, spleen and especially MLN, $CD4^+CD8\alpha^+$ T lymphocytes with the phenotype of activated/memory T_H cells were found in a higher frequency than in other studies on piglets of that age group (18–20). This might be a result of vaccination against *M. hyopneumoniae* of piglets in our study, or of breeding and housing conditions. However, the present results correlate with a decrease in $CD4^+CD8\alpha^-$ cells in peripheral lymphoid organs, suggest-

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ing that the $CD4^+CD8\alpha^+$ derived from the $CD4^+CD8\alpha^-$ upon activation, as shown in *in vitro* experiments (15). On the other hand, it cannot be excluded that in infected piglets, $CD4^+CD8\alpha^-$ cells migrated from the periphery to the gut. This would support the hypothesis that, like in other coccidial infections (e.g. of chicken and mammals), $CD4^+$ T_H cells play a crucial role in the primary immune response to *I. suis* in suckling piglets (39,40). The role of $CD4^+$ cells during the infection with *I. suis* in the gut mucosa will be evaluated in a follow-up study.

With regard to other lymphocyte subpopulations, an essential role of NK cells in the immune response against *Eimeria* has been demonstrated for the infection of mice with *E. papillata* (47). In this study, we detected a significant increase in lymphocytes with the phenotype of NK cells ($CD3^-CD4^-CD8\alpha^+$, (33,34)) in the spleen of infected piglets. As there is currently no specific marker for porcine NK cells available for immunohistochemistry, we were not able to investigate changes in NK cell populations in the gut mucosa, but it cannot be excluded that this increase in the spleen reflects an involvement of those cells in the immune response to *I. suis*.

The results from lymphocyte subpopulations in the blood, spleen and MLN provide detailed data on the distribution of those cells in piglets suffering from porcine neonatal coccidiosis. They indicate an involvement of both innate and adaptive immune mechanisms in this disease. Although the number of investigated samples from the gut mucosa is small, the differences between infected and non-infected animals clearly lead to new hypotheses for future studies. The data presented suggest that TcR-γδ-T cells are the most important T-cell subpopulation involved in the local immune response in the epithelium of the jejunum and should therefore be investigated in more detail. Moreover, the possibility of the migration of lymphocyte populations from the periphery to the gut should be investigated in detail, including the detection of more subpopulations and possibly the use of labelled cells to track their circulation pathways in this model of a neonatal infectious disease. Furthermore, the antigen-specific involvement of various lymphocyte subpopulations has to be demonstrated in functional assays. This includes a determination of lymphocytes able to react *Isospora*-specific and to develop immunological memory. This approach might contribute to the verification of the hypothesis of age resistance to this coccidiosis being dependent on the development of the adaptive immune system. Moreover, the infection with *I. suis* provides an interesting system for the investigation of cellular immune response mechanisms in neonates and an immunological model for mammalian coccidiosis in its natural and economically important host.

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3.2. Publication 2

Faeces, FACS, and functional assays – preparation of *Isospora suis* oocyst antigen and representative controls for immunoassays

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* corresponding author

Own contribution:

- substantial contribution in project planning
- project management
- accomplishment of the majority of laboratory work
- analysis and graphical editing of data
- draft of the first version

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Stephen Phillips

Prof. Stephen Phillips

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Referee: 1
Comments to the Author
This interesting paper describes a process for the purification of I. suis oocysts for use in immunological studies. A key final step for optimal purification was FACS, although large numbers of parasites were lost. Antigen prepared from the purified oocysts stimulated IFN-gamma production from spleen cells derived from previously infected pigs.

Some points

1) I wonder if there are enough oocysts to excyst and purify sporozoites as done with Eimeria, which would reduce the potential problem of bacterial debris on the oocyst wall surface.

2) Have the immunological studies been carried out with oocysts at the stage prior to FACS isolation? Perhaps the contamination would not be so huge that it would greatly affect detecting antigen-specific activation of lymphocytes.

3) I think it would be advisable to include spleen cells from uninfected pigs as a control. This would be better than the control described here.

4) When writing percent values it would be better to write, for example, 85+/-14%, rather than 85% (+/-14) as in the manuscript.

5) The English needs a little attention in places

Minor points

1) P3L35 change spread to found
2)P4L78 gram
3) P9L92- Change to The percentage of sporulated oocysts varied between samples.....infection and different litters with values of 40-90%
4) various places Anorganic should be inorganic.
5) P11L261 in our...
2)

Any additional attached files can be viewed via the Author Center, as described above.

Faeces, FACS, and functional assays – preparation of *Isospora suis* oocyst antigen and representative controls for immunoassays

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Running title: *Isospora* antigen preparation

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SUMMARY

Highly purified antigen and appropriate controls are essential for antigen-specific immunoassays. In the case of *Isospora suis*, the causative agent of neonatal porcine coccidiosis, the only source of antigen are currently oocysts isolated from faeces. The aim of this study was to develop a procedure for high-grade purification of *I. suis* oocysts from piglet faeces to obtain antigen and representative controls suitable for *in vitro* restimulation of lymphocytes. This was achieved by filtration, density gradient centrifugation and fluorescence activated cell sorting (FACS). The feasibility for immunological studies was tested with IFN-γ ELISPOT assays after *in vitro* restimulation of lymphocytes from previously infected swine using the obtained antigen. The developed method allowed the production of highly purified antigen and representative controls from faeces with an oocyst recovery rate of 14%. Regarding the application of the obtained material it could be shown that lymphocytes from *I. suis* infected pigs react antigen specific in terms of an *in vitro* recall response by production of IFN-γ. This demonstrates the suitability of the developed method for the production of antigen and controls for sensitive immunological readout systems. Moreover, the detected specific IFN-γ response encourages further functional studies on the cellular immune response to *I. suis*.

Keywords: Isospora suis, recall response, antigen preparation, FACS, ELISPOT, cellular immune response, pig

INTRODUCTION

Porcine neonatal coccidiosis caused by *Isospora suis* is spread worldwide with high prevalences and has a substantial economic impact for pig breeding facilities (Lindsay *et al.* 1992; Scala *et al.* 2009). Despite its veterinary importance, knowledge on the immune response towards this parasite is limited (Taylor 1984; Worliczek *et al.* 2010). To investigate the antigen-specific immune response to *I. suis in vitro*, sufficient amounts of antigen with a high grade of purity are required. However, oocysts isolated from the faeces of piglets are currently the only source of antigen to be used for immunological studies on *I. suis* since there is no *in vitro* cultivation system available that allows propagation of this parasite in amounts necessary for such studies. In the case of piglets the purification of parasite material (oocysts of *I. suis*) is a challenge because of a high fat content in diarrhoeic faeces. This disturbs the commonly used purification protocols for oocysts by flotation methods. Therefore a method for purifying oocysts from faecal samples of piglets to retrieve antigen of high purity is needed.

Additionally, during the development of the protocol for oocyst isolation and purification the question arose whether observed responses in immunological assays were specific for *I. suis* since contaminating bacterial components may have been present in antigen preparations. Pakandl *et al.* (2008) measured lipopolysaccharides (LPS) concentrations in antigen preparations from *Eimeria* oocysts isolated from rabbit faeces and argued that a lack of LPS demonstrates specificity of observed responses towards the parasite. However, this strategy seems to be insufficient for *I. suis* antigen, since possible contamination with microorganisms additionally to gram negative bacteria has to be expected (Schulze 1978).

Therefore, a procedure that enables a high-grade purification of *I. suis* oocysts was developed, consisting of multiple purification steps including fluorescence activated cell sorting (FACS) to achieve this aim. Furthermore the feasibility of this procedure was demonstrated by

applying purified *I. suis* oocysts as antigen in Interferon-γ (IFN-γ) ELISPOT assays to identify recall responses in lymphocytes isolated from *I. suis* infected pigs.

MATERIALS AND METHODS

Infection model

For the production of oocysts, piglets (Landrace x Large White x Pietrain) were infected orally with 1000 sporulated oocysts of *I. suis* (field strain Wien I) on the third day of life. The piglets were reared conventionally with the sow in a farrowing crate on straw at the animal husbandry facilities of the Institute of Parasitology and Zoology, University of Veterinary Medicine Vienna, Austria, under standardised conditions. From day seven to 21 of life individual faecal samples were taken daily for oocysts counting and evaluation of faecal consistency. Piglets were reinfected with 1000 sporulated oocysts of *I. suis* after five months. Three weeks later the animals were stunned with a captive bolt pistol followed by exsanguination. Spleens were removed aseptically and kept on ice until further processing. All procedures were approved by the Austrian Federal Ministry of Science and Research and the Ethics Committee of the University of Veterinary Medicine Vienna according to the Austrian Animal Protection law.

Retrieval of sporulated oocysts

Oocysts per gramme faeces (opg) were counted according to a modified McMaster method (Worliczek *et al.* 2009). Oocysts of *I. suis* were obtained from faeces of infected animals between days six and fourteen post infection. The criterion for enrichment of oocysts from faecal samples was a minimum of 10,000 opg in loose faeces to obtain oocysts with as little debris as possible. Oocysts were enriched with a protocol modified according to Ruttkowski *et al.* (2001). Faecal samples were thoroughly suspended in tap water and sedimented by centrifugation at 1700 x *g* for 5 min. The pellet was resuspended in 25% Percoll® (GE

Healthcare Biosciences AB, Uppsala, Sweden) and centrifuged again to remove the majority of the fat from the samples. The pellet containing the oocysts was washed two times with tap water. For sporulation enriched oocysts were suspended in 2% potassium bichromate in tap water and incubated for two to three days at room temperature with daily aeration. Sporulated oocysts were filtered through a mesh with 100 µm pore size to remove debris and stored at 11°C until further use.

Purification of oocysts

For the establishment and evaluation of the whole purification procedure eight different batches of *I. suis* oocysts were used. Oocysts were further purified with a density gradient in 15 ml tubes containing 5 ml 2 M sucrose (ρ = 1.25 g/l) overlaid with 5 ml 1.25 M sucrose (ρ = 1.15 g/l). The latter was overlaid with a suspension of sporulated oocysts in water (2.5 ml) with 2% potassium bichromate. The gradients were centrifuged for 7 min, 1500 x *g* at room temperature. Both interfaces were screened microscopically for oocysts. If oocysts were present they were collected and washed once (5 min, 1700 x *g*, room temperature) by using tap water in order to dilute residual sucrose solution to a density of less than 1.02 g/l to allow oocysts to sediment. After a second washing step with tap water, the pellet was finally transferred to a sterile 15 ml tube and suspended in 5 ml sterile deionised water.

Disinfection of oocyst suspensions

To disinfect the oocyst suspension 100 µl of a freshly prepared NaOCl-solution (12% v/v, Carl Roth GmbH, Karlsruhe, Germany) were added to a final concentration of 0.24% of active chloride. The suspension was then incubated for 10 min at 4°C and washed four times with sterile PBS afterwards. Prior to the final washing step the suspension was filtered through a sterile CellTrics® 50 µm filter (Partec GmbH, Görlitz, Germany) to remove any coagulated debris.

To determine the absence of living bacterial and fungal contaminants and therefore the efficacy of the NaOCl-treatment, 1 ml of oocyst suspension (containing 1000 oocysts/ml) was tested in 10 ml BBL™ fluid thioglycollate medium (BD Diagnostics, Heidelberg, Germany) as well as in 10 ml tryptic soy broth (Merck, Darmstadt, Germany). After 24 h, 48 h, one and two weeks of incubation at 37°C aliquots of 100 µl were plated onto Difco™ Columbia agar (BD Diagnostics, Heidelberg, Germany) supplemented with 7% (v/v) sheep blood. Plates were incubated aerobically, anaerobically, and under microaerophilic conditions (7% CO_2) for 24 to 72 h at 37°C. The viability of oocysts was tested by excystation (Hermosilla *et al.* 2006) using porcine instead of bovine bile and microscopic control for motility of sporozoites during the excystation.

Fluorescence activated cell sorting (FACS)

To enrich oocysts in the suspensions FACS was performed on a FACSAria™ with FACSDiva™ Software Version 5.0.2 (BD Biosciences, San Jose, CA, USA). For this purpose oocysts were suspended in FACSFlow™ Sheath Fluid (BD Biosciences, San Jose, CA, USA). Besides excitation with the standard blue laser (488 nm) to determine scatter properties of the analysed sample, autofluorescent molecules of *I. suis* oocysts were excited with a violet laser at 407 nm and oocyst-autofluorescence was analyzed by using a bandpass filter with 450/40 nm and 530/30 nm, respectively. This autofluorescence was sufficient to discriminate between oocysts and bacteria or debris, and therefore enabled sorting with an electronic gate set on the oocyst fraction (P1, Fig. 1A). In parallel the sorting process was used to prepare a so-called mock control which is commonly used to test for the influence of by-products within an antigen preparation. Therefore, a second electronic gate containing all particles except oocysts (P2, Fig. 1A) was created and particles located in this gate were sorted into a separate tube. Collected objects from both electronic gates were suspended in PBS after sorting. To quantify the purity of the sorting process both samples with sorted objects from P1

(oocysts, Fig. 1B) and P2 (debris, bacteria, in the following called mock control, Fig. 1C) were analyzed by flow cytometry with the same threshold rate for 1 min. For the preparation of an adequate dilution of the mock control the counted events in P2 of the sorted oocyst (i.e. contaminating debris) were used to calculate the appropriate dilution of the mock control to achieve an equal concentration of debris/bacteria (particles in P2) in both samples. Dilution of the mock-control was done with PBS.

Oocysts were counted in a McMaster chamber diluted in sodium chloride/sucrose solution as described above. The oocyst suspension was then diluted with PBS to an oocyst concentration of 3 x 10^4 oocysts/ml. The mock control was diluted identically to the oocyst suspension in this second dilution step.

Production of oocyst homogenate

Oocyst suspensions and controls were subjected to three freeze-thaw cycles in liquid nitrogen and subsequently treated with a Sonoplus™ ultrasound homogenisator (Bandelin electronic, Berlin, Germany) at 65% power for 3 min on ice. Homogenisation of the oocysts was controlled microscopically. The antigen preparations were then stored at -20°C.

Isolation of lymphocytes

Splenocytes were isolated from homogenised spleen tissue as described previously (Worliczek *et al.* 2010). Lymphocytes were treated with GEY's Solution (Fang *et al.* 2000) for 5 min at room temperature to lyse any contaminating erythrocytes. Subsequently, the cells were washed with PBS twice and filtered through a sterile CellTrics® 50 µm filter (Partec GmbH, Görlitz, Germany). Cells were finally suspended in cell culture medium consisting of RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with 10% (v/v) heat-inactivated FCS, 100 IU/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories, Pasching, Austria). Cells were counted under trypan blue exclusion staining.

Stimulation of cells

Splenocytes were stimulated with *Isospora*-homogenate or mock control in the same dilution. For the ELISPOT-assays, cells ($4x10^5$/well) were stimulated with three dilutions of antigen and mock control, corresponding to $150x10^{-5}$, $37.5x10^{-5}$, and $9.4x10^{-5}$ sporulated oocysts per lymphocyte, respectively. Cell culture medium was used to dilute antigen and controls.

IFN-γ ELISPOT

The production of IFN-γ by splenocytes was measured in ELISPOT-assays. For this purpose, 96-well Multiscreen® IP plates (Millipore, USA) were coated with mouse anti-swine IFN-γ capture mAb (clone A151D5B8, Invitrogen, USA; 10 mg/ml in PBS) at 4°C over night and blocked with cell culture medium for 2 h at 37°C. Cells in a concentration of $4x10^5$ cells per well and respective dilutions of antigen and mock controls (see above) were added. Each dilution was tested in duplicates. Additionally, control cultures were stimulated either with cell culture medium only (negative control) or with Concanavalin A (ConA, positive control) at a final concentration of 3 µg/ml. The plates were incubated for 48 h at 37°C, 5% CO_2 and subsequently washed with washing buffer (PBS with 0.01% Tween 20 and 0.1% BSA). Bound IFN-γ was detected by incubation with biotinylated mouse anti-swine IFN-γ mAb (clone A151D13C5, Invitrogen, USA; 2.5 mg/ml in washing buffer) at room temperature for 1h followed by incubation with streptavidin-bound alkaline phosphatase (dilution 1:2000 in washing buffer, Invitrogen, USA). The binding of alkaline phosphatase was visualised with SIGMAFAST® BCIP/NBT substrate (Sigma-Aldrich, Vienna, Austria) according to the manufacturer's instructions. Plates were then thoroughly washed with tap water and dried over night at room temperature. Spots were detected with an AID ELISPOT reader (AID GmbH, Strassberg, Germany) (Pintaric *et al.* 2008). Results of the ELISPOT-assays were converted to spots per $1x10^6$ lymphocytes for graphical presentation.

RESULTS

Retrieval of oocysts

Oocysts were sporulated to obtain antigen from sporozoites as well as from oocysts walls. The majority of oocysts was usually sporulated after three days. The rate of sporulated oocysts differed between samples from different days post infection or different litters between 40-90%. Despite the use of 25% Percoll®, contamination with fat compounds and also the occurrence of debris, fibres, anorganic matter and bacteria was observed after the isolation of oocysts from faeces (Fig. 2A). Anorganic matter and the majority of fibres could be removed by the filtration step trough a mesh with 100 µm pore size (Fig. 2B). The recovery rate for the filtration was 86% (± 14).

Density gradient

As a second cleaning step oocysts were centrifuged on a density gradient made of tap water and sucrose. The majority of oocysts was found at the interface between water and 1.25 M sucrose; a significant cleaning effect could be observed here (Fig. 2C). Only a small proportion of oocysts was recovered from the interface between 1.25 M and 2 M sucrose. This proportion was not used for further processing since this interface was heavily contaminated with debris. The recovery rate from the first interface was 66% (± 18).

Sterility of oocyst suspension

All tested oocyst suspensions were bacteriologically sterile after treatment with NaOCl, even after 2 weeks of incubation under aerobical, anaerobical or microaerophilic conditions. The oocysts themselves were still viable, showing distinct sporozoite mobility during and after excystation.

Enrichment and production of mock control by FACS

Oocysts could easily be identified by their autofluorescence which was excited by a violet laser at 407 nm. They were discriminated from other particles by their fluorescence in the 450/40 nm bandpass filter against their fluorescence after blue excitation in the 530/30 bandpass filter (Fig. 1A) and had a mean proportion of 0.6% (± 0.4) on all events. The usage of these two filters revealed the best separation of oocysts from other particles and allowed a specific electronic gating. Due to low oocyst recovery rates during FACS only samples with at least 70% sporulation rate were used for FACS although it was possible to enrich sporulated oocysts only. The FACS-procedure enriched oocysts significantly (Fig. 1B) to a mean proportion of 70.8% (± 27.7) of all events (calculated by all sorted charges of oocysts) and to a high grade of purity (Fig. 2D). For a successful sort, filtration of the suspension through the 50 µm filter and also the suspension of oocysts in FACSFlow™ Sheath Fluid (BD Biosciences, San Jose, CA, USA), which contains detergents, was crucial. Without FACSFlow™, threshold rates during the sort were insufficient. Nevertheless, some oocyst batches could not be sorted as the threshold rate broke down continuously.

The proportion of oocysts in the recovered mock control was marginal (Fig. 1C) and in the final dilution of the mock control for immunological assays no oocysts were found. The mean recovery rate of oocysts by FACS was 21% (±6). The overall recovery rate in relation to sporulated oocysts isolated from the faeces was 14% (± 4).

Evaluation of in vitro application

To test purified oocysts and mock controls for their suitability in immunological restimulation assays, homogenates of both were used for stimulation of splenocytes from previously *I. suis* infected and challenged pigs. As a readout system IFN-γ ELISPOTs were performed to detect the number of cells producing this key effector molecule of the cellular immune response. The ELISPOT-assays were sensitive enough to detect the relatively low frequency of IFN-γ

secreting cells after 48h of incubation (Fig. 3). Moreover, there was a clear dose-dependent response of IFN-γ producing cells when oocyst homogenate was used for stimulation. In Fig. 3 the results of one animal are shown which are representative for the pattern of IFN-γ production among six investigated animals (data not shown). In contrast, frequency of cells producing IFN-γ after mock-treatment was only slightly higher than in cells cultivated solely in medium and showed no distinct dose-dependence. Stimulation with ConA induced a fivefold higher frequency of IFN-γ producing cells compared to *I. suis* stimulated cells. In summary, by using IFN-γ ELISPOTs an *Isospora suis*-specific recall response was detected.

DISCUSSION

In the present study a protocol to isolate, purify and enrich oocysts of *I. suis* to obtain material suitable for immunological research but also other parasitological *in vitro* studies was established.

In cases were oocysts are used as infection material of their natural hosts the purity of the oocyst suspension is not crucial; faeces with a sufficiently high oocyst content can be used for the recovery of parasite material regardless of the faecal consistency. Moreover, faeces with normal consistency often contain a high number of oocysts that can be enriched (Mundt *et al.* 2006). However, in the present study only faeces from swine showing diarrhoea were used to minimize contamination with fibres, anorganic matter or straw to obtain the cleanest material possible. Heavy contamination with smaller particles cannot be removed by density gradient centrifugation and to our experience disturbs subsequent FACS resulting in the breakdown of threshold rates during the sorting process. Some oocyst batches were not suitable for FACS most probably due to heavy contamination with such particles.

The treatment with NaOCl turned out to be a feasible method to produce infectious parasite material in the absence of viable bacterial contaminants. Such oocysts can be used for

instance in experiments where sterile material is necessary, such as infection of gnotobiotic piglets or cell cultures.

For antigen specific immunological assays bacteria killed by the NaOCl-treatment would either have to be removed completely from the antigen preparation to exclude stimulation by bacterial components, or a sufficient control has to be included. Such a control can be produced in an elegant manner by FACS as shown above, where the frequency of contaminating particles within purified oocysts was used to calculate and adjust the same frequency of such particles in a corresponding mock control.

It has previously been shown that coccidian oocysts can be isolated by FACS using fluorochrome labelling (Ferrari *et al.* 2000; Everson *et al.* 2002) or utilising the autofluorescence of oocysts in flow cytometry (Fuller and McDougald 1989; Everson *et al.* 2002). Since *I. suis* oocysts are also known to display autofluorescence (Daugschies *et al.* 2001) this was applied in an approach using FACS of this material for enrichment and for the retrieval of mock controls. With a recovery rate of only 14% after the whole purification procedure including FACS (with the highest losses being encountered during this step), the loss of oocysts during the whole process is considerable but seems acceptable when taking into account that a highly purified population of oocysts is obtained. Moreover, an appropriate negative control for *in vitro* stimulation assays of lymphocytes is also acquired with the procedure presented. Mock-treated cells showed a very weak response after stimulation which was only slightly stronger than the medium control but distinctly lower than the response to the *Isospora* antigen, allowing a clear differentiation between the *Isospora*-specific response and response to other antigens which may have contaminated the antigen-preparation. Especially in cases of a low frequency of reactive cells – as it was shown for *I. suis*-specific lymphocytes in this study – and therefore a possibly weak response in the applied readout systems, an appropriate control for the specificity of the stimulation is needed and can easily be provided by the method described in this paper.

In summary, material (antigen and controls) provided by the preparation of *I. suis* oocysts by centrifugation, sterilisation and FACS is suitable for sensitive immunological readout systems and therefore may also be applied to other coccidian species where faecal material is used for this purpose. Moreover, the detected *Isospora*-specific IFN-γ recall response encourages further functional studies on the cellular immune response to *I. suis*.

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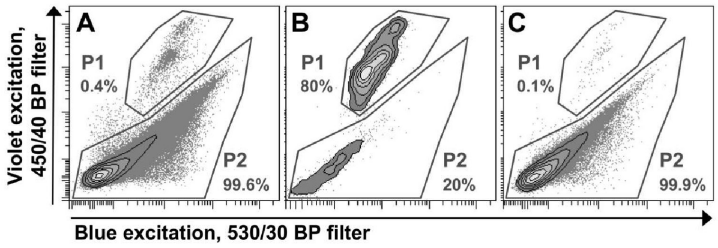
CAPTIONS TO FIGURES

Fig. 1 Flow cytometric analysis of one charge of *I. suis* oocyst suspensions and electronic gates for FACS-sort. P1 represents oocysts, P2 represents bacteria or debris. Percentages are proportions of events in the gates in relation to all events. (A) Before sort. (B) Sorted oocysts from P1 in (A). (C) Sorted mock control from P2 in (A).

Fig. 2 Oocyst suspensions of *I. suis* during purification procedure. (A) After isolation and sporulation in 2% potassium bi-chromate. (B) After filtration through mesh with 100 µm pore size. (C) After density gradient centrifugation. (D) After FACS-sort. Pictures were obtained by using differential interference contrast. For pictures (B)-(C) oocysts were suspended in the same volume as in (A) after treatment. Oocysts in (D) were suspended in 2 ml PBS after FACS.

Fig. 3 Results of IFN-γ ELISPOT of splenocytes after *in vitro* restimulation with homogenates of *I. suis* oocysts and mock control. The result for stimulation with ConA as positive control is given in numbers. Representative experiment out of six. Error bars extend to +/- 1 standard deviation.



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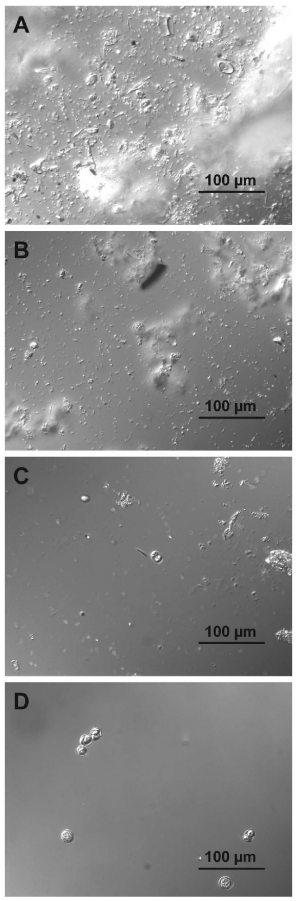

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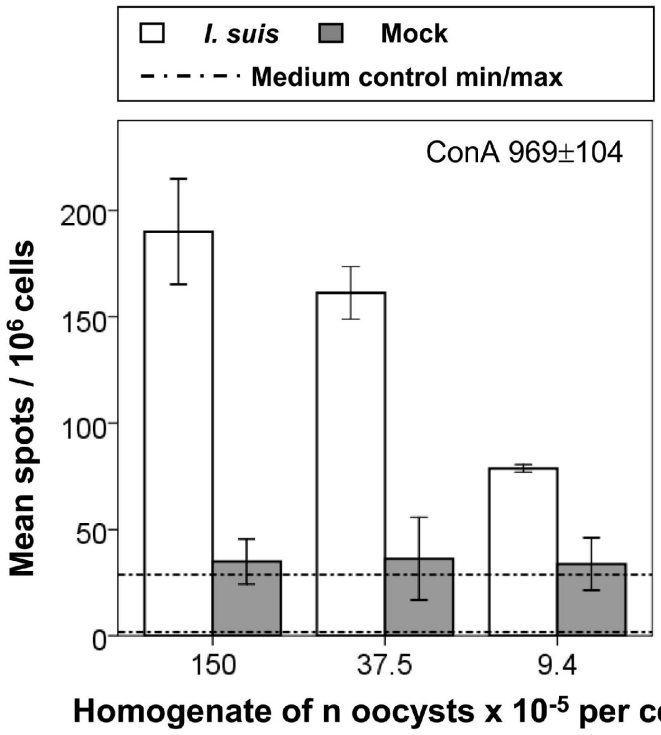
79x244mm (300 x 300 DPI)



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78x78mm (600 x 600 DPI)

3.3. Additional results – Cellular immune response against *I. suis*

The ELISPOT-results of splenocytes presented in *Publication 2* brought up two questions: question which lymphocyte populations are reacting specifically to *I. suis* in the sense of an *in vitro* recall response; and how do cells from blood and MLN react to the antigen preparation. Cells producing IFN-γ and also the proliferation in response to *I. suis* should be investigated. Therefore lymphocytes were isolated from blood (peripheral blood mononuclear cells, PBMC), spleen and MLN of three pigs previously infected and challenged with *I. suis*. The lymphocytes were stimulated *in vitro* with homogenates of sporulated oocysts and the respective mock controls. The response to the stimulation was measured by IFN-γ ELISPOT assays to determine the frequency of specific IFN-γ producing lymphocytes and by ^{3}H-thymidine incorporation assays to investigate specific proliferation. As a first approach a carboxyfluorescein succinimidyl ester (CFSE)-assay was performed with lymphocytes from MLN to identify the phenotype(s) of proliferating lymphocyte subsets. In addition to whole lymphocyte preparations selected subpopulations were removed by magnetic activated cell sorting (MACS®) from the splenocytes prior to stimulation to investigate the effect of the respective cell population. The depleted cell populations were defined by expression of the surface antigens CD3 (T cells), CD8β (CTL), CD4 (mainly T_H cells) and TcR-γδ (Gerner *et al.* 2009). After the depletion of the respective T-cell subpopulations splenocytes were stimulated and the IFN-γ production was measured in ELISPOT assays.

3.3.1. Materials and Methods

Experimental procedures (animal housing, experimental infection, isolation of lymphocytes, retrieval of antigen preparations for *in vitro* restimulation and ELISPOT-assay) were performed as described in *Publication 2*. Additionally, lymphocytes were isolated from blood and MLN. Cells isolated from blood, spleen and MLN were used for ^{3}H-thymidine incorporation assays. Splenocytes were sorted by MACS® and investigated by ELISPOT assays. With MLN-lymphocytes from one animal a CFSE-assay was performed.

Isolation of lymphocytes from blood and MLN

Blood was collected in heparinised bottles for isolation of PBMC. MLN were removed directly *post mortem* and kept in cold PBS (without Ca^{2+}/Mg^{2+}, PAA Laboratories, Pasching, Austria) until isolation of lymphocytes. Blood, MLN-tissue and isolated cells were kept on ice during the whole isolation process. PBMC were isolated by gradient centrifugation using lymphocyte separation medium (PAA Laboratories) as described elsewhere (Saalmüller *et al.* 1987). MLN were dissected from connective tissue and fat, strained through a metal sieve with a syringe plunge and rinsed with PBS. After centrifugation (8 min, 350×*g*, 4°C) the suspension was filtered through cotton wool to remove remaining tissue fragments and dead cells and rinsed again with PBS. After centrifugation the pellet was treated with GEY-Solution (Fang *et al.* 2000) for 5 min at room temperature to remove any erythrocytes. Subsequently the cells were washed with PBS two times, filtered through a nylon sieve (pore size app. 70 µm) and were finally suspended in cell culture medium consisting of RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with 10 % (v/v) heat-inactivated FCS, 100 IU/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories, Pasching, Austria). Cells were counted under trypan blue exclusion staining (*Publication 1*).

Magnetic activated cell sort (MACS®)

Additionally to ELISPOT assays of whole splenocytes, they were also performed with splenocytes-suspensions after removal of $CD3^+$ (T cells), $CD8\beta^+$ (CTL), $CD4^+$ (mainly T-helper cells) and TcR-$\gamma\delta^+$ cells (Gerner *et al.* 2009).

Cells were labelled with anti-CD3 mAb, anti-CD8β mAb, anti-CD4 mAb or anti-TcR-δ mAb (Table 1) as described in *Publication 1*. The labelled cells were washed with cold MACS buffer (PBS containing 2 mM EDTA and 2 % FCS) and were then labelled with MACS® rat anti-mouse magnetic particles (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 15 min at 4°C.

Cells were then washed and resuspended in MACS buffer. To remove cells with bound magnetic particles from the cell suspension MACS® LD depletion columns (Miltenyi Biotech GmbH) were used according to the instructions of the manufacturer. The purity of depleted splenocytes was controlled by FCM analysis before further use.

Table 1: Antibodies used for MACS® sort and reanalysis by FCM.

Specifity	Clone Name	Isotype	Isotype magnetic particles[b]	Labelling for reanalysis
CD8β	PG164A[a]	$mIgG_{2a}$	IgG_{2a+b}	α - $mIgG_{2a}$-Alexa647[d]
CD4	74-12-4	$mIgG_{2b}$	IgG_{2a+b}	α - $mIgG_{2b}$-Alexa488[d]
TcR-δ	PGBL22A[a]	$mIgG_1$	IgG_1	α - $mIgG_1$-PE[c]
CD3	PPT 3	$mIgG_1$	IgG_1	α- $mIgG_1$-PE[c]

[a]antibody from VMRD, Pullman, WA, USA; [b]MACS® magnetic particles from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; labelling antibodies from [c]SBA, Birmingham, AL, USA and [d]Invitrogen, Carlsbad, CA, USA; all other antibodies were produced at the Institute of Immunology, University of Veterinary Medicine Vienna, Austria.

^{3}H-thymidine incorporation assay

The proliferation of lymphocytes from blood, spleen and MLN was measured in ^{3}H-thymidine incorporation assays. Cells ($4x10^5$/well) were stimulated with three dilutions of antigen and mock control, corresponding to $150x10^{-5}$, $37.5x10^{-5}$, and $9.4x10^{-5}$ sporulated oocysts per lymphocyte, respectively. Cell culture medium was used to dilute antigen and controls. Cells were incubated for 6 days at 37°C, 5 % CO_2. For the last 17 h of culture 1 µCi methyl-^{3}H-thymidine (MP Biomedicals, Morgan, CA, USA) was added per well. Cells were harvested with a Packard Filtermate® (PerkinElmer, Boston, MA, USA) and finally the incorporation of ^{3}H-thymidine was measured in a TopCount (PerkinElmer).

CFSE-Assay

For staining with CFSE (Invitrogen, Carlsbad, CA, USA) approximately $1x10^7$ cells were washed with PBS and resuspended in 2 ml PBS with 5 % FCS. Two ml of 5 µM CFSE-solution were added and the cell suspension was vortexed immediately. After an incubation of 5 min at room temperature with occasional vortexing, 4 ml FCS (PAA Laboratories, Pasching, Austria) were added and the suspension was centrifuged for 8 min, 4°C at 350x*g*. Cells were subsequently washed twice in 10 ml cell culture medium and finally resuspended in cell culture medium at a concentration of $4x10^6$ cells/ml. CFSE-loaded cells were stimulated with antigen and controls as de-

scribed above and incubated for 7 days at 37°C, 7 % CO_2. Cells were then labelled with anti-CD3 mAb, anti-CD8β mAb, anti-CD4 mAb, or anti-TcR-δ followed by the corresponding secondary antibodies conjugated to a fluorochrome (Table 2) as described in *Publication 1*. In parallel, cells were labelled with isotype controls for the used isotypes instead of specific primary antibodies (Table 2). For FCM analysis, labelled cells were suspended in PBS containing 2 % (v/v) FCS. FCM analyses were performed on a FACSAria™ with FACSDiva™ Software Version 5.0.2 (BD Biosciences, San Jose, CA, USA) collecting at least 2×10^4 cells per sample.

Table 2: Antibodies used for FCM analysis of CFSE-assay.

Specifity	Clone Name	Isotype	Labelling
Isotype control	NCG2B.01[a]	$mIgG_{2b}$	α- $mIgG_{2b}$-Alexa647[c]
	NCG2A.01[a]	$mIgG_{2a}$	α - $mIgG_{2a}$-Alexa647[c]
	NCG01[a]	$mIgG_1$	α - $mIgG_1$-PE[d]
CD8β	PG164A[b]	$mIgG_{2a}$	α - $mIgG_{2a}$-Alexa647[c]
CD4	74-12-4	$mIgG_{2b}$	α - $mIgG_{2b}$-Alexa647[c]
TcR-δ	PGBL22A[b]	$mIgG_1$	α - $mIgG_1$-PE[d]
CD3	PPT 3	$mIgG_1$	α - $mIgG_1$-PE[d]

[a]Isotype controls from Dianova, Hamburg, Germany; [b]antibody from VMRD, Pullman, WA, USA; labelling antibodies from [c]Invitrogen, Carlsbad, CA, USA and [d]SBA, Birmingham, AL, USA; all other antibodies were produced at the Institute of Immunology, University of Veterinary Medicine Vienna, Austria.

3.3.2. Results

Lymphocytes from the spleen revealed an antigen-specific and dose-dependent production of IFN-γ (Fig. 4 A) compared to the mock control. Each animal showed a different frequency of reactive cells, ranging from 83 to 208 IFN-γ producing cells per $1x10^6$ lymphocytes after stimulation with a homogenate corresponding to $150x10^{-5}$ oocysts per lymphocyte. The stimulation of PBMC also revealed a specific production of IFN-γ, although a dose-dependency could only be detected for two animals (Fig. 4 B). The stimulation of MLN did not lead to antigen-specific production of IFN-γ (Fig. 4 C).

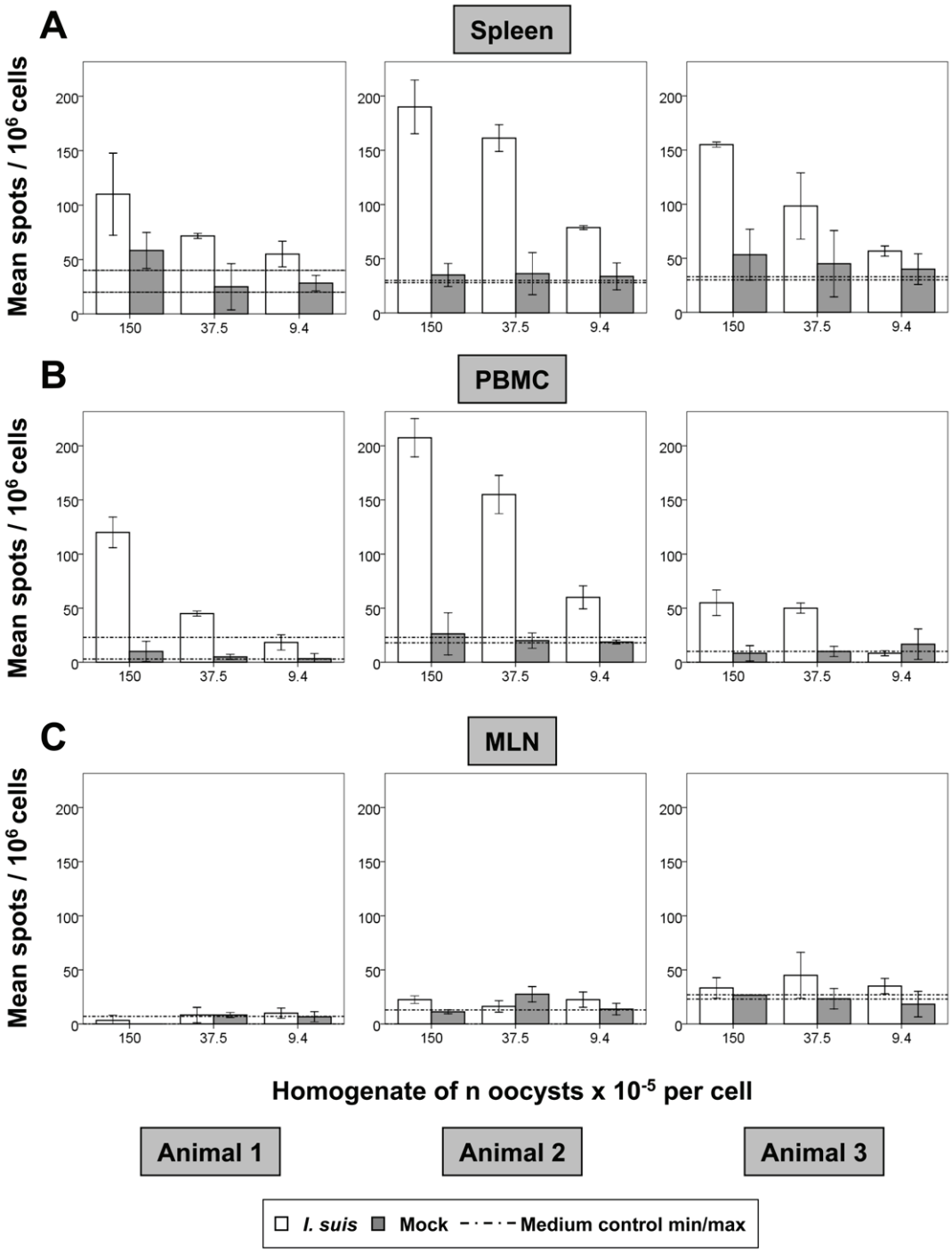

Fig. 4: Production of IFN-γ by (A) splenocytes, (B) PBMC and (C) lymphocytes from the MLN. Results were obtained by IFN-γ ELISPOT assays. One spot represents one cell producing IFN-γ. Error bars extend to ± 1 standard deviation.

All depleted cell populations showed a purity of higher than 98 % in the FCM-reanalysis. The depletion of CTL led to an increase in IFN-γ production (Fig. 5 B). The depletion of $CD4^+$ lymphocytes led to a clear reduction in one animal (Fig. 5 C). This is supported by results of two animals investigated in a pilot study where the depletion of $CD4^+$ cells also led to a reduction in IFN-γ production (data not shown). The removal of TcR-γδ T cells revealed a reduction in IFN-γ-response in one animal (Fig. 5 D). The most important finding from this experiment was a complete abrogation of IFN-γ production after the depletion of $CD3^+$ T cells (Fig. 5 E).

In contrast to the production of IFN-γ, no clear AG-specific proliferation could be observed among lymphocytes from the spleen (Fig. 6 A) or among PBMC (Fig. 6 B). Especially PBMC showed a very high level of spontaneous proliferation. Only lymphocytes from the MLN showed a clear and also dose-dependent proliferation after the *in vitro* restimulation with *I. suis* antigen (Fig. 6 C).

CFSE is a fluorescent dye for living cells. With every cell division each daughter cell receives only half of the fluorochrome load. Therefore, the proliferation of labelled cells can be measured by flow cytometry showing the dilution of CFSE with proceeding proliferation. Additionally, the phenotype of proliferating cells can easily be characterised with labelling of the cells under investigation against specific molecules. The CFSE-assay of MLN-lymphocytes of one animal showed that the majority of proliferating cells belonged to the T cells and that this reaction was antigen-specific when compared to the percentage of proliferating cells with the phenotype $CD3^+$ in the mock control (Fig. 7 A). The majority of the reacting cells seemed to belong to the subset of T_H cells (Fig. 7 B); but also CTL (Fig. 7 C) and TcR-γδ T cells (Fig. 7 D) showed a distinct *I. suis* specific proliferation.

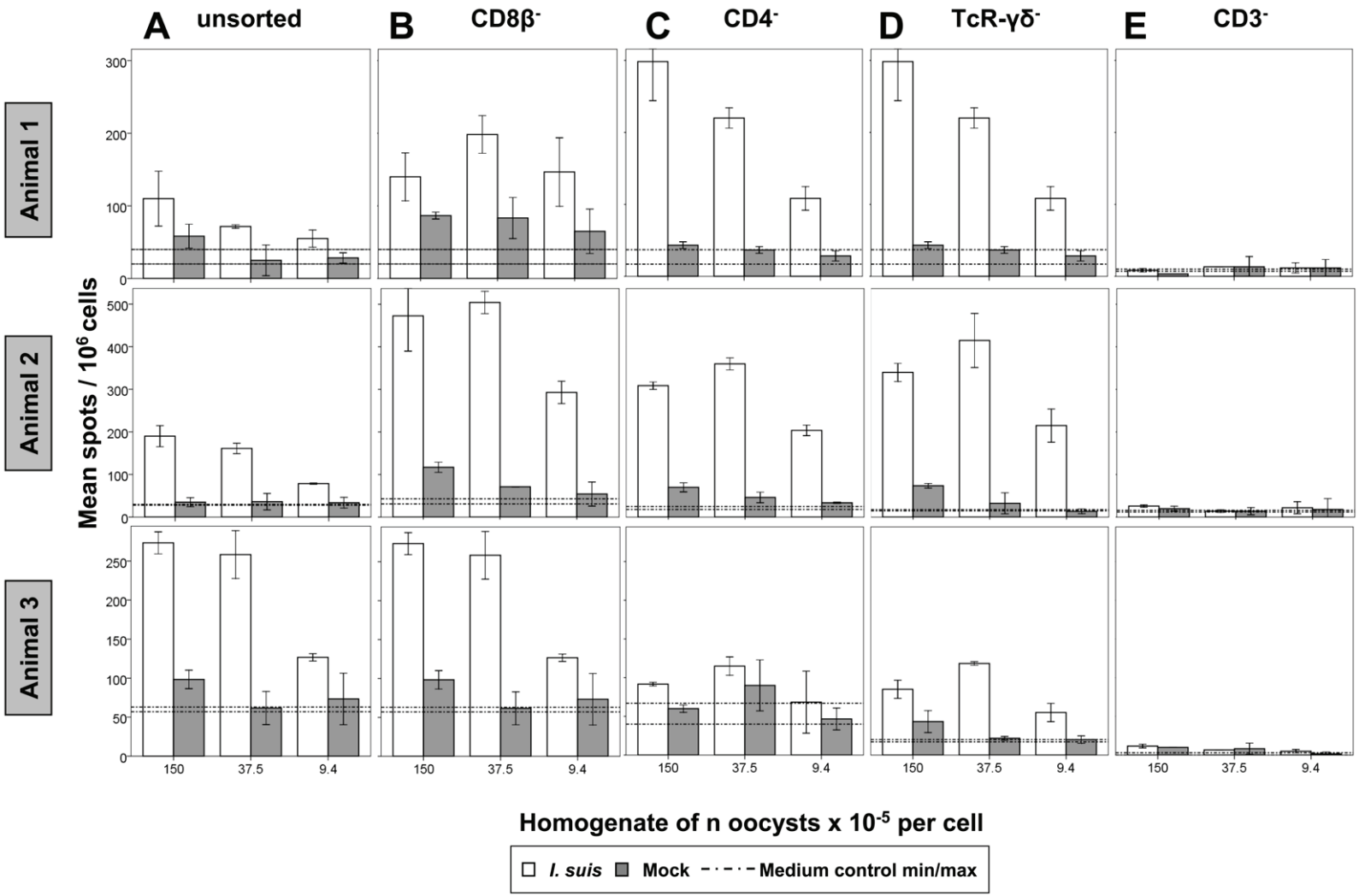

Fig. 5: Production of IFN-γ by (A) unsorted splenocytes, (B) CD8β$^-$, (C) CD4$^-$, (D) TcR-γδ$^-$ and (E) CD3$^-$ splenocytes. Lymphocyte subsets were depleted by MACS® separation of splenocytes. Results were obtained by IFN-γ ELISPOT assays. One spot represents one cell producing IFN-γ. Error bars extend to ± 1 standard deviation.

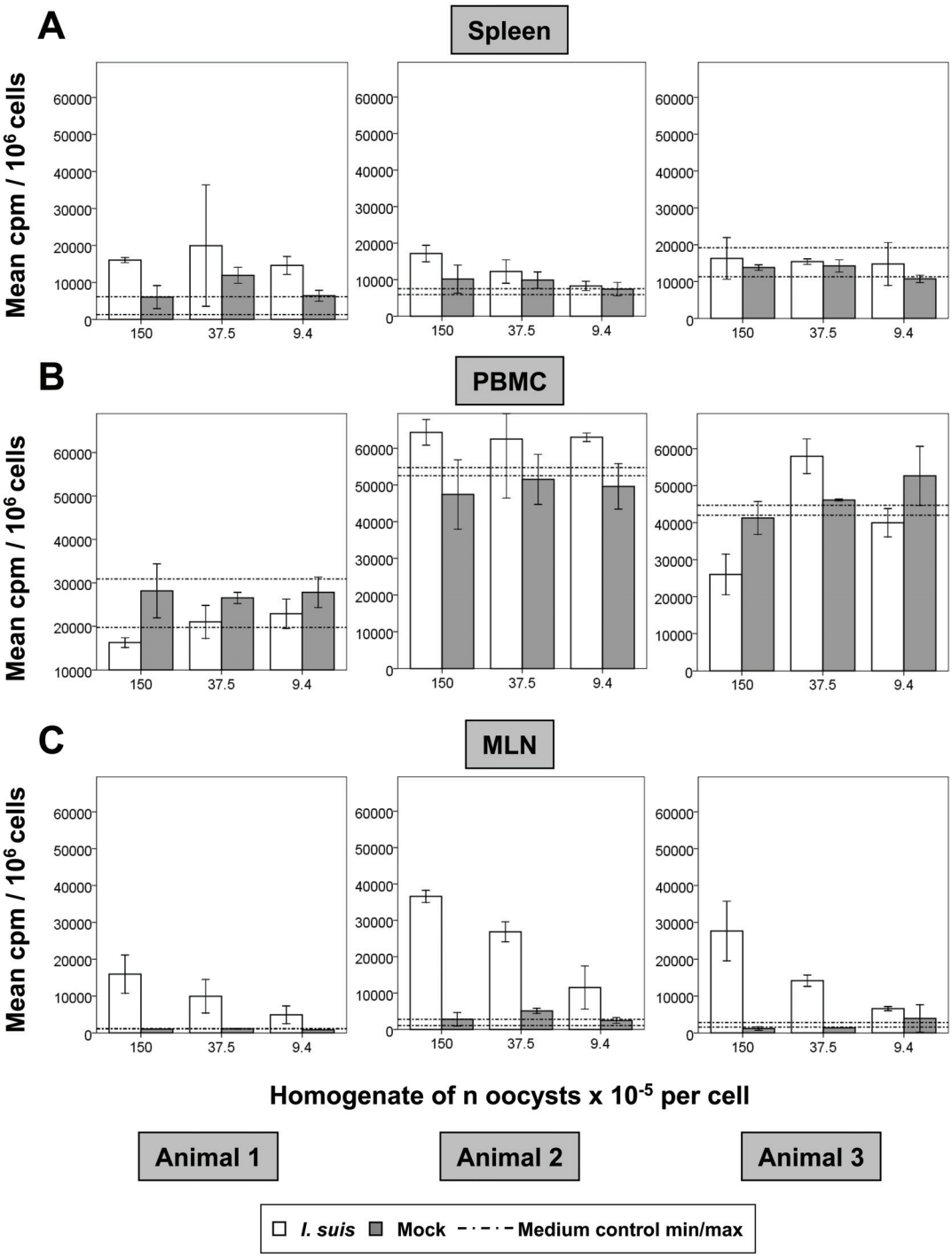

Fig. 6: Proliferation of lymphocytes isolated from (A) spleen, (B) blood and (C) MLN after *in vitro* restimulation with *I. suis* antigen. Results were obtained by ^{3}H-thymidine incorporation assays. Results are given in counts per minute (cpm) per 1x10^{6} cells. Error bars extend to ± 1 standard deviation.

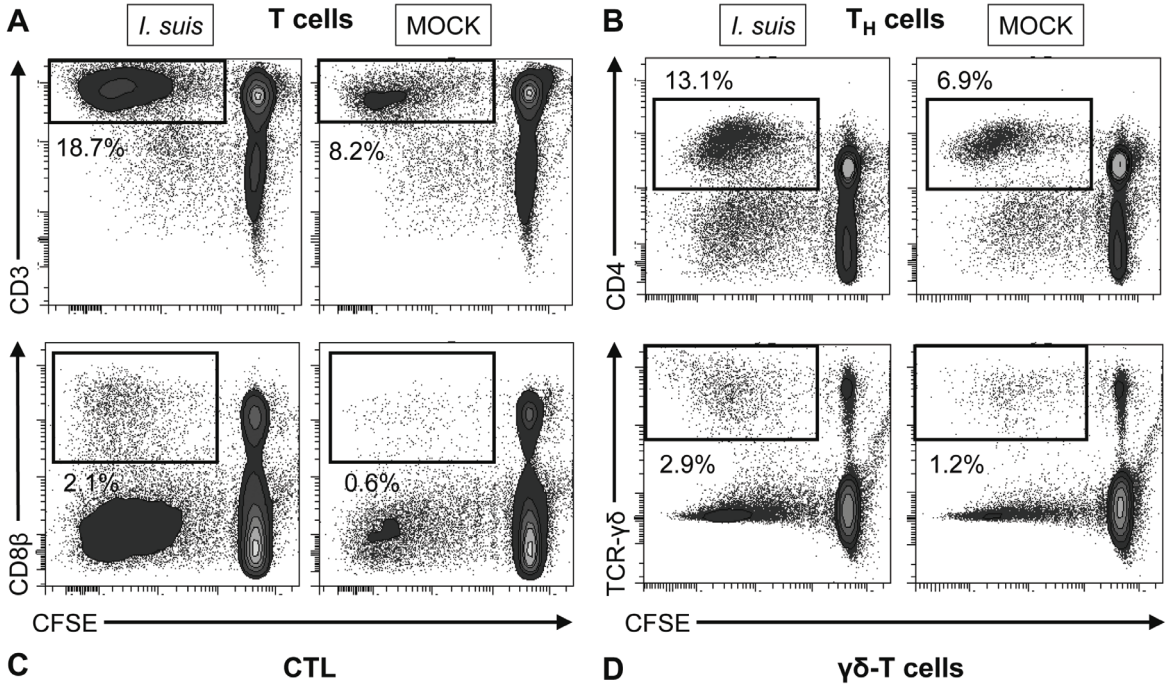

Fig. 7: CFSE assay of lymphocytes isolated from MLN of Animal 2. CFSE fluorescent intensity is plotted against expression of (A) CD3, (B) CD4, (C) CD8β and (D) TcR-γδ. Labelling with specific antibodies and FCM analyses were performed after seven days of incubation with *I. suis* antigen and respective mock controls. The given percentages refer to the proportion of the total lymphocytes with the marked phenotype.

4. CONCLUDING DISCUSSION

4.1. Phenotypic characterisation of immune response to *I. suis*

Functional investigations on lymphocytes might give the most detailed information about cells responsible for the immune response during a disease. Nevertheless, the phenotypical characterisation is the first step to get an idea about the involvement of different lymphocyte populations. In the case of neonatal porcine coccidiosis, nothing was known regarding involved cell populations. The only sources of information were suggestions of a possible involvement of lymphocytes and therefore of cellular immune response mechanisms in this disease (Taylor 1984) and studies about other coccidian parasite in other hosts (chicken, calves, rabbits, mice, rats). Those studies are not always comparable to the situation in newborn piglets since the immune system of pigs shows considerable peculiarities in comparison with other mammals and with chicken. Moreover, the postnatal development of the immune system has to be taken into account when investigating neonatal infections.

The study presented in *Publication 1* identified TcR-γδ$^+$ cells as the most promising target for further research of the local immune response in the gut. A strong increase of this cell population was detected in the jejunal lamina epithelialis (from 0.5 to 15 %) and the lamina propria (from 5 to 19 %) of infected piglets (age seven to 16 days). On the other hand, TcR-γδ$^+$ cells showed a severely reduced frequency in the peripheral blood and the MLN. This leads to the hypothesis of a migration of lymphocytes from blood and secondary lymphoid organs to the site of infection, besides a possible specific local proliferation. Especially in the lamina propria other T cells have to be involved since the increase in T cells in this compartment was not only caused by TcR-γδ$^+$ cells. These T cells might have been drained from blood, spleen or the MLN where CD3$^+$ cells have been reduced in infected piglets. In this study TcR-γδ$^+$ T cells have been the only subset of T cells investigated in the gut mucosa but in future studies especially jejunal T-helper cells but also NK cells as a part of the innate immune system should be investigated. This will be addressed in subsequent studies.

The status of TcR-γδ T cells as a linkage between adaptive and innate immune response and an important line of defence in young animals is in good agreement with the presented results. The ability of lymphocytes to respond to mitogens is limited in piglets in the investigated age group (Becker and Misfeldt 1993; Schwager and Schulze 1997),

reflecting the immature status of the neonatal porcine immune system. Therefore, a defence strategy which is at least partly independent of MHC-restricted antigen presentation seems likely.

NK cells ($CD3^{-}CD8\alpha^{high}CD4^{-}$) showed a higher frequency in the spleens of infected piglets which lead to the assumption that they are involved in the early immune response to *I. suis*, as shown for other infections with coccidia such as *Toxoplasma* or *E. papillata* (Schito and Barta 1997; Korbel *et al.* 2004). Additionally, also $CD21^{+}$ cells (a subpopulation of B cells) showed an increase in infected animals, in this case in the MLN. B cells are known to produce specific antibodies during coccidial infections but do not seem to provide protective immunity (Lillehoj and Trout 1996; Yun *et al.* 2000).

All other detected differences revealed a decrease of the distinct lymphocyte subpopulations. In the blood only lymphocytes showed this decrease, other subsets of leukocytes did not. As there is no blood loss due to haemorrhagic diarrhoea as seen in other coccidial infections, the reduction of lymphocytes in the blood is presumably caused by draining of the lymphocytes to the gut mucosa.

T-helper cells (including resting T_H and activated and memory T_H) were also significantly reduced in infected animals and are candidates for an involvement in the primary immune response to *I. suis*, as it was already shown for *Eimeria* infections in other hosts (reviewed in Worliczek *et al.* 2007).

To date, no data are available about the involvement of regulatory T cells in coccidiosis. Only TcR-γδ T cells were assumed to have a regulatory function in mice infected with *E. vermiformis* (Smith and Hayday 2000a). Since the demanded marker for regulatory T cells, the transcription factor Foxp3, is found in the majority of $CD4^{+}CD25^{high}$ lymphocytes but only in a small number of $CD4^{+}CD25^{dim}$ cells (Käser *et al.* 2008a) it can be assumed that the investigated $CD4^{+}CD25^{high}$ cells represent regulatory T cells in this study, and that regulatory T cells are involved in the immune response to *I. suis*, most likely in the control of inflammation and cellular immune response. However, referring to the immature status of the adaptive immune system in piglets and the strong increase in TcR-γδ T cells in the gut mucosa also those cells are candidates for a regulatory subpopulation of T cells.

The involvement of CTL ($CD8^{+}$) in the immune response to coccidiosis and especially their requirement for the development of a protective immunity is reported for infections with *E. tenella* and *E. acervulina* in chicken (Lillehoj and Trout 1996; Swinkels *et al.*

2006), but also for infections with *E. intestinalis* in rabbits (Pakandl *et al.* 2008) and for *E. bovis* in calves (Hermosilla *et al.* 1999). In the present study a significant age influence on the distribution of this cell population but not of the infection itself was found in the spleen. This indicates that the distribution of CTL in this organ depends on the age of the piglets and their involvement in the primary immune response to *I. suis* seems unlikely.

4.2. Functional characterisation of immune response to *I. suis*

To date, most functional studies on porcine lymphocytes have been performed with cells from animals much older than piglets susceptible for infections with *I. suis*. A pilot study showed that functional studies with lymphocytes from piglets up to three weeks of age would require extensive adaptation of the used investigation methods and the amount of cells that can be isolated from one piglet is quite limited. Therefore, the recall response to *I. suis* of lymphocytes from previously infected and challenged six month old pigs was investigated. With this setup already established readout systems for cellular immune response could be used with a sufficient number of isolated lymphocytes. The primary immune response of suckling piglets will be investigated functionally in consecutive studies.

The isolation of parasite material from faeces and a satisfactorily purification procedure to get antigen preparations suitable for restimulation experiments with lymphocytes was the major obstacle in this study. The only source of *I. suis* at the moment is faecal material with a high content of fat, bacteria and debris. Standard purification methods such as flotation or filtration did not lead to applicable parasite material. Therefore, a method using a density gradient centrifugation customised to sporulated *I. suis* oocysts and FACS was established (*Publication 2*). The described method provides oocysts of high grade purity. Nevertheless, bacteria and debris could not be removed completely. Hence, the cell sort was also used to produce a representative mock control containing all particles except the oocysts themselves for use in immunological assays.

It was shown for the first time that lymphocytes from blood and spleen but not from MLN produce specifically IFN-γ after *in vitro* restimulation in a dose-dependent pattern. In contrast, only lymphocytes isolated from the MLN showed specific proliferation, mainly caused by T cells including T_H cells, CTL and TcR-γδ T cells. Lympho-

cytes from the blood reacted with a high grade of spontaneous unspecific proliferation to the stimulus. This discrepancy between lymphocytes from different organs might be a result of the distribution of *I. suis* specific lymphocytes. Effector cells reacting with IFN-γ might be located predominantly in the blood and the spleen, whereas memory cells reacting with specific proliferation might be located in the MLN, although TcR-γδ T cells are not described as memory T cells.

The role of T cells, T_H cells, CTL and TcR-γδ T cells in the observed IFN-γ response was investigated in more detail. The reduction of IFN-γ response in one animal after the depletion of TcR-γδ T cells supports the suggestion of an active role of these cells at least in this animal. In two other individuals the response was increased after the depletion. This might have been caused by the accumulation of the other subsets or by a regulatory function of the depleted cells as shown for other coccidial infections (Roberts *et al.* 1996; Smith and Hayday 2000a; Smith and Hayday 2000b). The depletion of T_H cells led to a reduction of IFN-γ in one animal supporting the hypothesis that T_H cells are involved in the immune response to *I. suis*. After depletion of CTL, TcR-γδ T cells and T_H cells all investigated animals showed at least minor differences in the pattern of IFN-γ production regarding the dose dependency. This finding leads to the conclusion that the specific immune response to *I. suis* shows distinct individual differences. This phenomenon might explain the finding that piglets show individual differences in the severity of the pathology after experimental infections with *I. suis* under standardised conditions. Some piglets develop heavy diarrhoea, some show almost normal faecal consistency. The intensity of oocyst shedding might vary between individuals as well, possibly caused by individual differences in the reaction of the immune system.

A clear and reproducible reaction was detected after the depletion of T cells ($CD3^+$). The production of IFN-γ was completely inhibited. Thus, NK cells ($CD3^-$) do not seem to react in this setup and the *in vitro* IFN-γ response to antigen from sporulated *I. suis* oocysts seems to be completely dependent on T cells.

Further steps for functional research in the immune response to *I. suis* are going to be (a) the identification of the IFN-γ producing cells by directly intracellular labelling allowing also the investigation of the small number of lymphocytes that can be isolated from suckling piglets; (b) the retrieval of merozoites and sexual developmental stages as antigen to investigate their immunogenic potential which is assumed to be

higher than the immunogenicity of sporozoites due to their longer residence in the pig; (c) the investigation of lymphocyte trafficking in the piglet following an infection with *I. suis* trying to explain the decreasing proportion of T-cell subsets in blood and secondary lymphoid organs; (d) the adaptation of other immunological readout systems for the presented infection model such as ELISPOTs and intracellular labelling for other cytokines, realtime-PCR to detect mRNA of immunomodulatory molecules and e.g. killing assays for the detailed functional analysis of involved lymphocyte subsets.

The presented data provide new hypotheses and a fundamental basis for future immunological research in neonatal porcine coccidiosis verifying the feasibility of the infection model with *I. suis* for immunological research of a economically important neonatal infection in its natural host, the pig.

5. ABSTRACT

Isospora suis (Apicomplexa: Coccidea) is the causative agent of neonatal porcine coccidiosis, a common parasitic infection of suckling piglets worldwide. *I. suis* replicates inside the epithelium of the small intestine and destroys the host cell during this process, leading to a sloughing of the epithelial lining. This results in heavy non-haemorrhagic diarrhoea and a reduced capacity of nutrient absorption by the mucosa, and therefore to reduced and uneven weaning weights of the piglets. Thus, neonatal porcine coccidiosis is of considerable economic importance for pig production. Nevertheless, only very few data are available on the immune response to *I. suis*. The aim of this study was the characterisation of lymphocyte populations (T-cells and their subsets, natural killer cells, and B-cells) in blood, spleen, mesenteric lymph nodes (MLN) and the jejunal mucosa of infected piglets in comparison to healthy animals. First fundamental data about the involved cells of the immune system were obtained using a model of experimental infection of suckling piglets, multicolour flow cytometry of lymphocytes and immunohistochemistry of the gut mucosa. A significantly reduced frequency of T-cells, T-cell receptor-$\gamma\delta^+$ (TcR-$\gamma\delta^+$) cells, regulatory T cells and T-helper cells could be detected in blood, spleen and MLN of infected animals. B-cells showed an increase in the MLN, natural killer cells in the spleen. For cytotoxic T-lymphocytes (CTL) no difference could be detected between infected animals and controls. In the gut of infected piglets a strong and significant increase of T-cells, mainly caused by TcR- $\gamma\delta^+$ cells, was observed.

Furthermore, an experimental system for the functional characterisation of the immune response was established to identify antigen specific lymphocyte populations by *in vitro* restimulation. For this purpose ELISPOT-assays for the detection of IFN-γ production and proliferation assays were used as readout systems. Lymphocytes from blood and spleen showed an antigen-specific production of IFN-γ. This reaction was T cell dependent; $CD4^+$ T-helper cells and TcR-$\gamma\delta^+$ cells but not CTL and natural killer cells were involved in this response. An antigen-specific proliferation was exclusively detected in lymphocytes from the MLN. The majority of reactive cells were T cells, namely T-helper cells, TcR- $\gamma\delta^+$ cells and CTL.

These results indicate an involvement of innate and adaptive mechanisms in the primary immune response to *I. suis* and the development of an immunological memory to the parasite. Moreover it was shown for the first time that T cells react specifically to *I. suis* in terms of an *in vitro* recall response.

6. ZUSAMMENFASSUNG

Isospora suis (Apicomplexa: Coccidea) ist der Erreger der Saugferkelkokzidiose, einer in der Schweineproduktion weltweit verbreiteten parasitären Erkrankung mit hoher Morbidität und entsprechender wirtschaftlicher Bedeutung. *I. suis* vermehrt sich obligat intrazellulär in Epithelzellen des Dünndarms, die beim Austritt des Erregers zerstört werden. Diese Schädigung der Darmschleimhaut kann starken katarrhalischen Durchfall und in Folge dessen eine stark verminderte Nährstoffaufnahme im Darm bedingen, was zu einem Auseinanderwachsen der Würfe und zu reduzierten Absetzgewichten führt. Die Immunantwort gegen *I. suis* wurde bisher trotz der wirtschaftlichen Bedeutung der Saugferkelkokzidiose kaum untersucht. Das Ziel dieser Studie war es, die Veränderungen von Lymphozytenpopulationen (T-Zellen, natürliche Killerzellen, B-Zellen) in Blut, Milz, Mesenteriallymphknoten (MLN) und Dünndarmschleimhaut infizierter Tiere im Vergleich zu gesunden Ferkeln zu untersuchen um damit erste grundlegende Erkenntnisse über beteiligte Zellen des Immunsystems zu gewinnen. Diese Fragestellung wurde an experimentell infizierten Saugferkeln mittels Mehrfarben-Durchflusszytometrie und Immunhistochemie untersucht. In Blut, Milz und MLN infizierter Tiere konnte eine signifikant verminderte Frequenz von T-Zellen, T-Zellrezeptor-γδ$^+$ (TcR-γδ$^+$) Zellen, regulatorischen T-Zellen und T-Helferzellen beobachtet werden. B-Zellen zeigten eine Zunahme in den MLN, natürliche Killerzellen traten in der Milz vermehrt auf. In der Frequenz zytotoxischer T-Lymphozyten (CTL) konnte kein Unterschied zu gesunden Tieren festgestellt werden. In Lamina epithelialis und Lamina propria des Jejunums zeigte sich eine signifikante Zunahme von T-Zellen und in der Subpopulation der TcR-γδ T-Zellen.

Weiters sollten ein System zur funktionellen Charakterisierung der Immunantwort etabliert und damit antigenspezifische Lymphozytenpopulationen durch *in vitro* Restimulierung identifiziert werden. Die Reaktion von Lymphozyten reinfizierter Tiere auf Parasitenantigen wurde in ELISPOT-Assays (Produktion von IFN-γ) und Proliferationsassays untersucht. Eine *Isospora*-spezifische Produktion von IFN-γ konnte in Lymphozyten aus dem Blut und der Milz nachgewiesen werden. In der Milz wurden die reaktiven Zellen genauer charakterisiert. Für die Produktion von IFN-γ waren ausschließlich T-Zellen, vor allem CD4$^+$ T-Helferzellen und TcR-γδ$^+$ Zellen verantwortlich, nicht aber CTL und natürliche Killerzellen. Eine antigenspezifische Proliferation konnte nur in den MLN nachgewiesen werden. Ein Großteil der reaktiven Zellen

waren T-Zellen, wobei hier sowohl T-Helferzellen, TcR-$\gamma\delta^+$ Zellen als auch CTL eine deutliche antigenspezifische Proliferation zeigten.

Diese Ergebnisse lassen darauf schließen, dass sowohl angeborene als auch adaptive Mechanismen an der primären Immunantwort gegen *I. suis* beteiligt sind und nach einer Erstinfektion ein immunologisches Gedächtnis ausgebildet wird. Erstmals konnte gezeigt werden, dass eine spezifische zelluläre Immunantwort (Recall response) gegen *I. suis* stattfindet, in die unterschiedliche T-Zellsubpopulationen involviert sind.

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9. PUBLICATIONS

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Junior Award of the Austrian Society for Tropical Medicine and Parasitology (ÖGTP) in November 2008.

Memberships

Austrian Society for Tropical Medicine and Parasitology (ÖGTP)

World Association for the Advancement of Veterinary Parasitology (WAAVP)

Conference contributions with oral presentations

22nd Annual meeting of the German Society for Parasitology (DGP), February 22-25, 2006, Vienna, Austria: Interactions of *Isospora suis* with the porcine immune system.

40th Annual meeting of the ÖGTP, November 02-04, 2006, Linz, Austria.: Interaktionen von *Isospora suis* mit dem Immunsystem des Schweins: 1 - Charakterisierung beteiligter Mechanismen *ex vivo* und *in vitro*

Conference of the German Society for Veterinary Medicine (DVG), Section Parasitology and Parasitic Diseases, June 04-06, 2007, Celle, Germany: Zelluläre Immunantwort auf *Isospora suis* Infektionen beim Ferkel.

21st International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP) 2007 "From EPG to Genes", August 19-23, 2007, Gent, Belgium: Cellular immune response to *Isospora suis* infections in piglets

Conference of the German Society for Veterinary Medicine (DVG), Section Parasitology and Parasitic Diseases; July 9-11, 2008; Celle, Germany: Interaktionen von *Isospora suis* mit dem Immunsystem des Schweins: Auswirkungen der Infektion auf die Lymphozytenpopulationen in Blut, Milz und Mesenteriallymphknoten.

42nd Annual meeting of the Austrian Society for Tropical Medicine and Parasitology (ÖGTP); November 20-22, 2008; Innsbruck, Austria: Immunologie der Saugferkelkokzidiose - angeborene und adaptive Immunantwort gegen *Isospora suis*.

28th Scientific meeting of the German Society for Protistology; February 25-28, 2009, Naumburg (Saale), Germany: Porcine coccidiosis – immune response to *Isospora suis* in piglets

Conference of the German Society for Veterinary Medicine (DVG), Section Parasitology and Parasitic Diseases; June17-19, 2009; Leipzig, Germany: Antigenspezifische Immunantwort gegen *Isosora suis* - *in vitro* Untersuchungen zur Immunologie der Saugferkelkokzidiose.

22nd Conference of the World Association for the Advancement of Veterinary Parasitology WAAVP 2009 "One World. One Health. Parasites in a Changing Landscape"; August 9-13, 2009; Calgary, Alberta, Canada: Porcine coccidiosis - Investigations on the cellular immune response against *Isospora suis* & Age is more important than infection dose in determining the outcome of *Isospora suis* infections in suckling piglets.

43rd Annual meeting of the Austrian Society for Tropical Medicine and Parasitology (ÖGTP); November 19-21, 2009; Vienna, Austria: Ein *in vitro* Modell der Saugferkelkokzidiose - *Isospora suis* in der Zellkultur & The role of T cells in *Isospora suis* reinfected pigs.

11. LEBENSLAUF

Name	Hanna Lucia Worliczek
Geburtsdatum	02. April 1980
Geburtsort	Wien, Österreich
Nationalität	Österreich

Ausbildung

Juni 1998 Matura mit Auszeichnung bestanden

1998-2005 Diplomstudium Biologie – Mikrobiologie, Universität Wien.

März 2005 Diplom (Mag. rer. nat.) in Mikrobiologie, Universität Wien.

Diplomarbeit: Klassifizierung von Mykoplasmen auf der Basis von chemotaxonomischen und molekularbiologischen Untersuchungen. Durchgeführt am Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität Wien.
Betreuer: Dr. Hans-Jürgen Busse

März 2005- Doktoratsstudium der Naturwissenschaften, Genetik – Mikrobiologie,

Universität Wien.

Doktorarbeit: Immunologie von *Isospora suis*-Infektionen bei Saugferkeln. Durchführung am Institut für Parasitologie und Zoologie & Institut für Immunologie, Veterinärmedizinische Universität Wien.
Betreuerin: Univ. Prof. Dr. Anja Joachim

Auszeichnungen

Junior Award der Österreichischen Gesellschaft für Tropenmedizin und Parasitologie (ÖGTP) im November 2008.

Mitgliedschaften

Österreichische Gesellschaft für Tropenmedizin und Parasitologie (ÖGTP)

World Association for the Advancement of Veterinary Parasitology (WAAVP)

Vorträge auf wissenschaftlichen Tagungen

22. Jahrestagung der Deutschen Gesellschaft für Parasitologie (DGP), 22.-25.02.2006, Wien, Österreich: Interactions of *Isospora suis* with the porcine immune system.

40. Jahrestagung der ÖGTP, 02.-04.11.2006, Linz, Österreich: Interaktionen von Isospora suis mit dem Immunsystem des Schweins: 1 - Charakterisierung beteiligter Mechanismen ex vivo und in vitro.

Tagung der Deutschen Veterinärmedizinischen Gesellschaft (DVG), Fachgruppe Parasitologie und parasitäre Krankheiten "Diagnostik, Epidemiologie und Bekämpfung von Parasitosen bei Nutz-, Haus- und Heimtieren", 04.-06.06.2007, Celle, Deutschland: Zelluläre Immunantwort auf *Isospora suis* Infektionen beim Ferkel

21st International Conference of the World Association for the Advancement of Veterinary Parasitology WAAVP 2007 "From EPG to Genes", 19.-23.08.2007, Gent, Belgien: Cellular immune response to *Isospora suis* infections in piglets

Tagung der DVG, Fachgruppe Parasitologie und parasitäre Krankheiten "Diagnostik, Epidemiologie und Bekämpfung von Parasitosen bei Nutz-, Haus- und Heimtieren"; 9.-11.07.2008, Celle, Deutschland: Interaktionen von *Isospora suis* mit dem Immunsystem des Schweins: Auswirkungen der Infektion auf die Lymphozytenpopulationen in Blut, Milz und Mesenteriallymphknoten.

42. Jahrestagung der ÖGTP; 20.-22.11.2008, Innsbruck, Österreich: Immunologie der Saugferkelkokzidiose - angeborene und adaptive Immunantwort gegen *Isospora suis*.

28. wissenschaftliche Tagung der Deutschen Gesellschaft für Protozoologie, 25.-28.02.2009, Naumburg (Saale), Deutschland: Porcine Coccidiosis – Immune response to *Isospora suis* in piglets

Tagung der DVG, Fachgruppe Parasitologie und parasitäre Krankheiten "Diagnostik, Epidemiologie und Bekämpfung von Parasitosen bei Nutz-, Haus- und Heimtieren"; 17.-19.06.2009, 2009; Leipzig, Deutschland: Antigenspezifische Immunantwort gegen *Isosora suis* - in vitro Untersuchungen zur Immunologie der Saugferkelkokzidiose.

22nd Conference of the World Association for the Advancement of Veterinary Parasitology WAAVP 2009 "One World. One Health. Parasites in a Changing Landscape"; August 9-13, 2009; Calgary, Alberta, Canada: Porcine coccidiosis - Investigations on the cellular immune response against *Isospora suis* & Age is more important than infection dose in determining the outcome of *Isospora suis* infections in suckling piglets.

42. Jahrestagung der ÖGTP; 19.-21.11.2009, Wien, Österreich: Ein *in vitro* Modell der Saugferkelkokzidiose - *Isospora suis* in der Zellkultur & The role of T cells in *Isospora suis* reinfected pigs.