Host response to *Eimeria* infections

Gastheer respons op *Eimeria* infecties

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(met een samenvatting in het Nederlands)

Proefschrift

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Problems caused by *Eimeria*

Eimeria are obligate intracellular protozoan parasites belonging to the phylum Apicomplexa. The parasite *Eimeria* is responsible for the disease coccidiosis with a worldwide distribution. Clinical coccidiosis in poultry is characterized by diarrhea, ranging from mucoid and watery to hemorrhagic, mal-absortion, reduction in weight or weight gain and morbidity (Williams, R.B., 2002). Intestinal *Eimeria* infections are the dominating class of diseases in poultry. The short, homoxenous life-cycle, combined with the potential for massive reproduction, makes this group of parasites a serious problem for chickens that are reared under intensive farming conditions (Morris, G.M. and Gasser, R.B., 2006). This makes *Eimeria* infections economically important, as annual worldwide costs due to reduced food conversion, reduced growth, increased mortality and the necessary use of drugs are estimated at £500 million (Shirley, M.W. *et al.*, 2007). In poultry seven species of the genus *Eimeria* occur which are not only host, but also localization specific. Their characteristic niche in the gut facilitates coexistence of several species in a given flock. In chickens raised for meat (broilers) the three most diagnosed infections are *E.acervulina*, *E.maxima* and *E. tenella* (Shirley, M.W. *et al.*, 2005). *Eimeria acervulina* is known to infect the duodenum, *E.maxima* infects the jejunum and *E.tenella* infects the caecum (Johnson, J. and Reid, W.M., 1970).

Lifecycle

The protozoan *Eimeria* parasites have a complex life cycle with one part outside the host and another part in the intestinal tract, mainly within the epithelial cells. A schematic lifecycle of *Eimeria* is shown in Figure 1. We discuss the cycle starting from the unsporulated oocysts shed into the environment in the faeces of infected animals. Oocysts are thick walled structures, adjusted to survival outside the host that serve to transfer the parasite to new hosts. Given the correct environmental conditions (warmth, oxygen and moisture), the oocyst sporulates and becomes infective (Allen, P.C. and Fetterer, R.H., 2002; Graat, E.A.M. *et al.*, 1997; Williams, R.B., 1998). Sporulated oocysts can remain infectious for relatively long periods and can persist in the environment. After ingestion by a new host, the oocyst wall is cracked in the crop of the host under the influence of bile salts and gravel that is also ingested to facilitate digestion. The sporocysts that excystate during this process each form two sporozoites and subsequently these are able to infect the intestine (McDougald, L.R., 1998). In the intestinal tissue schizonts develop, which undergo at least two generations of asexual reproduction. These replicative phases, particularly the later schizont stages which are formed

in the lamina propria, lead to most damage to the intestinal tissues, causing varying degrees of digestive disturbances, fluid and blood loss, and increased susceptibility to other diseases (McDougald, L.R., 1998). After the asexual reproduction, micro- and macrogametes are formed. These gametes may fuse producing zygotes. The zygote matures into an oocyst and is subsequently excreted in the faeces after which a new lifecycle is ready to begin. The life cycles of the different poultry *Eimeria* species are largely similar, though differences occur in length of prepatent period, number of schizogony cycles, localization within the gut and in the amount and duration of oocyst shedding (Rose, M.E. *et al.*, 1996).

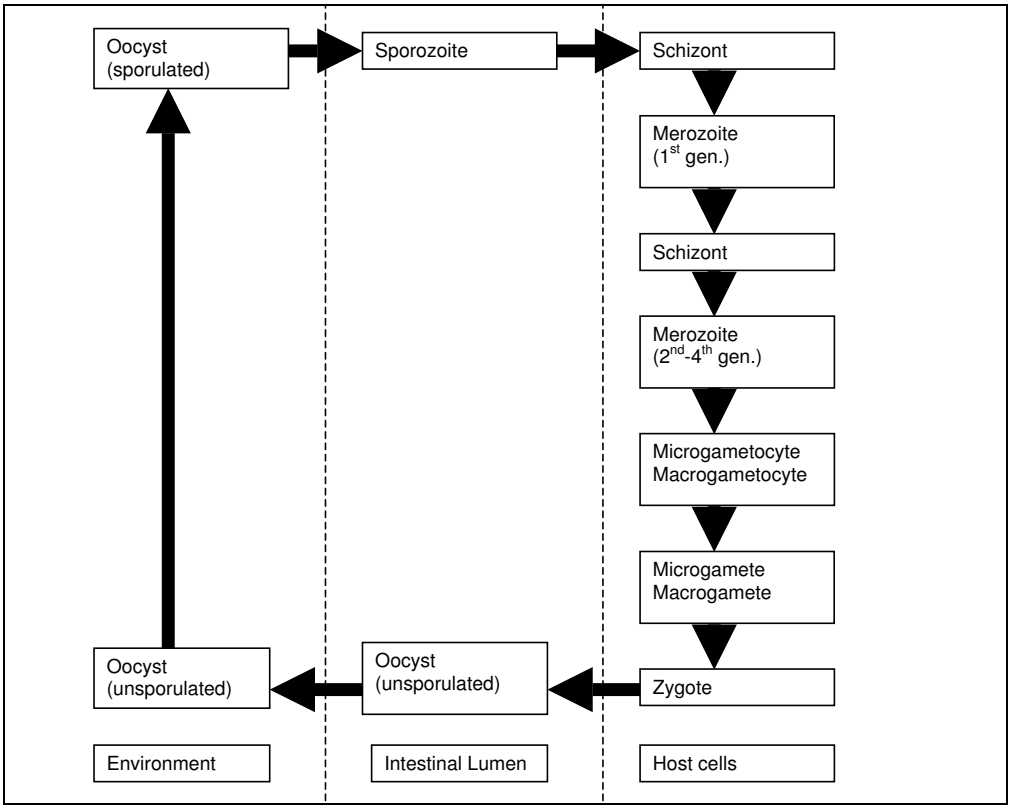

Figure 1: Schematic lifecycle of *Eimeria* parasites

Current methods in diagnosis

Due to the abundant shedding most diagnosis with respect to *Eimeria* infections are performed using oocyst enumeration . Then severity of an infection is assessed by counting the output of oocysts in the faeces (Chapman, H.D. and Rayavarapu, S., 2007). *Eimeria* species may be distinguished on basis of their size and shape. Unfortunately, these approaches can be unreliable, particularly because there can be an "overlap" in the sizes of oocysts of different species and multiple species of *Eimeria* can simultaneously infect the host (Long, P.L. and Joyner, L.P., 1984). Recently a new manner of identification is added to the range of possible diagnostic tools. By means of PCR it is possible to identify isolated *Eimeria* oocysts by means of their genetic fingerprint (Fernandez, S. *et al.*, 2003; Hnida, J.A. and Duszynski, D.W., 1999). The single drawback of this PCR method is the necessity of pre-purification of oocysts from the faeces. Until now it was not possible to perform a PCR on DNA directly extracted from faeces. This restricts the application of PCR to oocyst DNA obtained from faeces to a qualification of the species, but not for quantification thereof. The reason DNA purification results in losses of material cannot be accounted for. Though a quantitative PCR is presently not possible on faecal parasite stages, it proved possible on DNA directly extracted from tissue stages (This thesis).

Since the different species of *Eimeria* infect different niches in the intestine and characteristic lesions can appear at the site of infection, it is possible to use the macroscopic lesions as an indirect manner of identification of species and quantification of the severity of infection (Johnson, J. and Reid, W.M., 1970; Long, P.L. and Joyner, L.P., 1984). Some overlap exists between niches, but dissociation between species by means of lesions is in general an acceptable method for identification of species. Quantification of severity of the infection by means of lesion scores is less accurate, because there is a lack of direct correlation between microscopic and macroscopic lesion scores and the parasite frequency (Idris, A.B. *et al.*, 1997).

Host- pathogen interaction: Factors that may influence the nature of responses to an *Eimeria* infection

Responses to coccidia are extremely complex (Lillehoj, H.S. *et al.*, 2007). Upon infection chickens generate immune responses and can even develop protective immunity to homologous re-infections. In this thesis we cope with a selection of factors with an important impact on the host-pathogen interaction, which means that outcome of the infection is influenced by both pathogen and host. To start with, seven species of *Eimeria* are able to infect chickens. These seven species all have their specific niche in the chicken intestine and differ in immunogenic properties. This results in differences in development of host immunity to

re-infections (Stiff, M. and Bafundo, K., 1993). The protective immune response to re-infection with avian *Eimeria* is species specific and sometimes even strain specific (Basak, S.C. *et al.*, 2006; Blake, D.P. *et al.*, 2005; Blake, D.P. *et al.*, 2006; Dalloul, R.A. *et al.*, 2007). Thus infection history of the host is an important factor in resistance to a concurrent infection. Within the host, the *Eimeria* parasite has many intracellular developmental stages (Figure 1). All of these stages are characterized by specific antigens which add more complexity to the development of an appropriate protecting immune response (Tomley, F., 1994). The amount of oocysts infecting a host is also of importance on the host response *(Blake, D.P. et al., 2005)*. Different doses of *Eimeria* evoke host reactions that differ in qualitative/quantitative aspects such as balance in T-helper (Th) 1 and Th2 responses and sensitivity of the host to challenge infection (Blake, D.P. *et al.*, 2005). This difference could be due to the crowding effect, which states that a maximum infective dose exists above which the reproductive potential decreases (Williams, R.B., 2001). The genetic background is of importance for host resistance to *Eimeria* infections *(Li, G. et al., 2002)*. Lillehoj et al (1986) showed that different lines of chickens displayed a different susceptibility to the same *Eimeria* infections as shown by oocyst production, lesion score and clinical signs. (Lillehoj, H.S., 1986). It is well established that the intestinal immune system in newly hatched animals is still immature (Bar-Shira, E. *et al.*, 2003). Though chickens are also experimentally infected at a young age, differences in host response to *Eimeria* due to age of the host were not yet reported *(Chapman, H.D. et al., 2005; Ramsburg, E. et al., 2003)*.

Current methods in control of *Eimeria* infections

Coccidiosis is particularly problematic in the chicken industry, to a large extent due to the crowded rearing conditions (Wallach, 1997). Like most infections *Eimeria* infections can be limited by hygienic measures. Until recently, next to management, the most widely applied strategy to prevent and control *Eimeria* infections was by means of anti-coccidial drugs in feed. This strategy has two major drawbacks. First of all there is an emerging resistance of *Eimeria* to all drugs described so far (Peek, H.W. and Landman, W.J., 2006). Secondly, the public is becoming more and more aware of their consuming habits and of the possible detrimental effects of drug residues in poultry products (Peek, H.W. and Landman, W.J., 2003). New products that are being tested to control *Eimeria* infections include therefore natural medicinal products (Guo, F.C. *et al.*, 2004) and probiotics (Dalloul, R.A. *et al.*, 2004).

An alternative to therapeutics and preventive medication is vaccination. *Eimeria* infections induce a complex protective immunity that develops fast and is extremely strong (Allen, P.C. and Fetterer, R.H.,

2002; Vermeulen, A.N. *et al.*, 2001). Different types of vaccines are currently available: live vaccines, live attenuated vaccines, non-infective parasite derivates and genetically engineered subunit vaccines. Most effective are live vaccines. Live vaccines consist of infective parasites, that can induce the development of protective immunity. Risk of this method is that live oocysts not only can cause great damage in the host's intestine, but they are multiplied in the host intestine and are excreted in high amounts in the litter. As vaccinated chickens endure an infection, they can lose weight, have a higher food conversion and transmission of the disease to other members of the flock remains possible. This gives rise to the possibility of a clinical outbreak of coccidiosis. Most live vaccines therefore consist of attenuated strains of *Eimeria*. Attenuated strains differ from normal strains in the duration of their life cycle, multiplication factor and the level of damage inflicted to the host. A major problem with both of these live vaccines is that they are expensive. It is very labour intensive to produce live vaccines, since it is only possible to generate oocysts in live chickens (Allen, P.C. and Fetterer, R.H., 2002; Chapman, H.D. *et al.*, 2002; Hong, Y.H. *et al.*, 2006; Vermeulen, A.N., Schaap, D.C. and Schetters, T.P., 2001; Williams, R.B., 1998). Non infective parasite derivates have the same drawback, they need to be produced in chickens. Genetically engineered subunit vaccines, and especially the delivery of these antigens using live vectors may offer a solution to the problem but these types of vaccines have high development costs. Next to this, viral or bacterial expression vectors are able to stimulate cell mediated immune responses necessary for the build up of protective immunity. Much work has been done in order to find a suitable candidate useable for vaccination, thus far not resulting in an effective recombinant vaccine considered effective enough for control of coccidiosis in the field (Ding, X. *et al.*, 2004; Innes, E.A. and Vermeulen, A.N., 2006). Identification of new vaccine candidates might in future lead to successful approaches. Together with an appropriate adjuvans, for instance cytokines, these vaccines might provide methods to cope with the problem of coccidiosis (Asif, M. *et al.*, 2004).

Though vaccines are often used, the build-up of protective immunity both within individual animals and in a flock, and its interaction with control measures is still very poorly understood (Chapman, H.D., Cherry, T.E., Danforth, H.D., Richards, G., Shirley, M.W. and Williams, R.B., 2002; Graat, E.A. *et al.*, 1998). Knowledge of these processes is necessary in order to develop good control methods.

➢ The Chicken Immune System

General part: adaptive/ innate

Eimeria are protozoa that are intestinal parasites. Many non-specific barriers are present to protect the host from pathogens that enter. Factors playing a role in the non-specific defence (first line of defence) of the gastro-intestinal (GI) tract are, for instance, gastric secretion, bile salts, covering of the intestine with protective mucus and the expression of antimicrobial proteins. Host defence against intruders that crack the nonspecific defence barriers is regulated by the specific immune system. (McDonald, V., 1999).

The gut-associated lymphoid tissue (GALT) is the second line of defence against *Eimeria*. The three major compartments of the GI immune system are the organized lymphoid tissue, the lamina propria (LP) and the intraepithelial lymphocytes (IELs) present in the single layer of epithelium that lines the luminal surface of mucosal tissue (Figure 2). Localization of the *Eimeria* in the gut is dependent on the developmental stages either extracellular or intracellular as a result of invasion or phagocytosis. An entering pathogen, in the case of an intracellular protozoa, causes tissue damage and induces an immune response. After presentation of antigens, an (inflammatory) cytokine response is induced and this response results in expansion of helper and effector cells which induce an adaptive and thereby a protective response. The immune responses can be split into two types, innate and adaptive immunity. Innate immunity is a less specific immunity of which the cellular part is mediated by macrophages, natural killer (NK) cells and granulocytes. The innate immune system recognizes invading organisms by detecting a limited number of molecular structures present in pathogens, but absent in self-tissue. An important group of receptors recognizing pathogen associated molecular patterns (PAMPs) are Toll-like receptors (TLRs), as reviewed by Medzhitov (Medzhitov, R., 2001). A renewed encounter with the same pathogen in general results in a repeated innate immune response that is accompanied by a rapid adaptive immune response which is antigen specific. The development of protective immunity in individuals that have been infected repeatedly is of major importance for protozoan infections. This so-called acquired immunity probably plays a dominant role in the dynamics of coccidial infection (Lillehoj, H.S. and Lillehoj, E.P., 2000). The acquired immunity includes both cellular and humoral immune mechanisms.

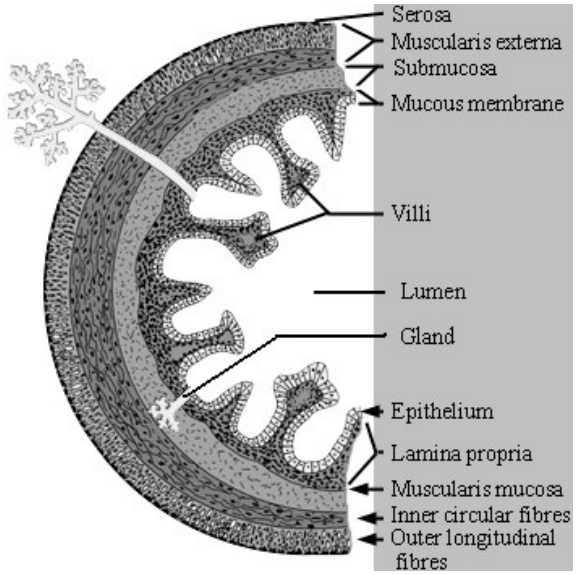

Figure 2: Schematic representation of the gastro-intestinal tract, adapted from http://www.uoguelph.ca/zoology/devobio/210labs/endo1.html

Cellular immune response

Cell-mediated immunity is directed primarily at pathogens that survive in cells as is the case for viruses and other intracellular pathogens. But it also plays a role in defending against fungi, cells expressing a modified self,cancer cells and, most important for this work: protozoa.

T-cells: The intra epithelial lymphocytes (IEL) population of the gut comprises mainly cells that do possess a T-cell receptor (TCR): T-cells (McDonald, V., 1999). The main type of effector cells in the chicken express the TCR$\alpha\beta$ (Chen, C.H. *et al.*, 1994). TCR$\alpha\beta$ expression on T-cells is found mostly in the lamina propria; another type of TCR, TCR$\gamma\delta$ expression is mostly found in the epithelium (Bucy, R.P. *et al.*, 1988). In the intestine, the TCR$\gamma\delta^+$ cells play a major role in immunity as compared to other parts of the host (Schild, H. *et al.*, 1994). TCR$\gamma\delta^+$ cells are able to mediate specific cellular immune functions without the requirement for antigen degradation and presentation and thereby are able to directly recognize invading pathogens or damaged cells (Schild, H. *et al.*, 1994). Most T-cells carry the accessory receptor molecules $CD4^+$ or $CD8^+$. The $CD8^+$ T-cells recognize antigens presented by major histocompatibility complex I (MHC I) molecules. MHC I is presented by all cells in an organism and presents peptides derived from proteins made within the cell which can be both self and non-self. This enables the cytotoxic $CD8^+$ T-cells to recognize and kill cells which are producing and presenting non-self or pathogen derived proteins. The $CD4^+$ T-cells recognize the MHC II

complex. This complex is able to present fragments of proteins which are not produced within the cell itself but are -part of- phagocytised foreign elements and may originate from pathogens. Cells expressing the MHC class II molecule are antigen presenting cells (APC) and include macrophages, dendritic cells and B-cells. $CD4^+$ T helper (Th) cells can generate Th1 (cell-mediated) or Th2 (antibody-mediated) immune responses by means of cytokines (McDonald, V., 1999). Part of the $CD4^+$ T-cells are T-helper cells that mainly activate macrophages and B-cells (Janeway, C.A. *et al.*, 1999).

Non-T-cells: The cell-mediated elimination of infections is accomplished with the help of different cell types. Macrophages phagocytose pathogens and cellular debris and stimulate lymphocytes and other immune cells to respond to the hall marks of the (intracellular) pathogen. In chickens interdigitating cells (IDC) are found in all T-cell regions of lymphoid tissues whereas follicular dendritic cells are found in the B-cell regions (Igyarto, B.Z. *et al.*, 2007). They are characterized by dendritic extensions and are probably the main APC during primary immune responses (Jeurissen, V., Janse, 1994). Natural killer (NK) cells are defined as large granular lymphocytes that do not express a TCR. They form a subpopulation of lymphocytes in the peripheral blood and spleen that mediate nonspecific cytotoxicity against tumor cells, microbes and microbially infected cells. NK cells are part of the innate immunity (Janeway, C.A., *et al.*, 1999). Mast cells are effector cells that are the cause of the acute phase response and promote local inflammatory responses, resulting in a recruitment of macrophages and lymphocytes. Though they play a role in the immune system, there is limited evidence for the role of mast cells in the pathofysiology of avian infectious diseases (Caldwell, D.J. *et al.*, 2004; Morris, B.C. *et al.*, 2004).

Though the immune system is relatively conserved in vertebrates, differences between animals occur. The adaptive immune system is most clearly described in mammalian species. Compared to mammals, differences are found in the cellular immunity of chickens. Chickens lack certain cellular components, for example chickens have fewer mast cells and basophils than mammals, chickens have few classical eosinophils and certain components of the Th2 response have not been observed so far. Birds have no neutrophils but instead they have heterophils (Petrone, V.M. *et al.*, 2002). The polymorphonuclear leukocytes (PMNL) that do resemble eosinophils in the chicken do not respond to inflammatory signals in a manner analogous to mammals (Kaiser, P. *et al.*, 2004). For evaluation of various functions in recent years for the chicken lymphocyte proliferation assays, NK-assays and assays to determinate the activity of phagocytes such as phagocytosis and killing have been developed. These assays have been adapted to the work with various important *Eimeria* strains used in this thesis. At this moment, in vitro techniques to measure antigen-

specific cytotoxic lysis of infected cells by T lymphocytes are not available. Nevertheless, an impression of the number of antigen-specific T lymphocytes can be obtained with an in vitro proliferation assay using (*Eimeria*) antigens (Breed, D.G.J. *et al.*, 1996). Since Vervelde et al. (1996) have shown that during a primary infection mainly macrophages are involved in the elimination of parasites, the Griess assay to measure macrophage activity in the form of NO_2^- has recently been adopted for our chicken lines (Ding, A.H. and Nathan, C.F., 1988; Vervelde, L. *et al.*, 1996).

Humoral immune response

B-cells: The humoral immune response is the part of the adapted immunity that is mediated by secreted antibodies, produced by B lymphocytes (B-cells), which in the chicken are generated in the Bursa. Unlike in mammals, there is no IgG sub-class switching in chickens, furthermore there is only a single chicken IgG equivalent, called IgY and in addition and chickens also lack IgE (Lillehoj, H.S. *et al.*, 2004). Secreted antibodies bind to antigens on the surfaces of invading microbes, which flags them for destruction through antibody-dependent cell-mediated cytotoxicity (ADCC) (Dalloul, R.A. and Lillehoj, H.S., 2006). The humoral immune response is a useful response for extracellular (stages of) pathogens. Since some of the *Eimeria* stages are extracellular this part of the immune response might play a role in immunity to *Eimeria* infections.

Cytokines

Initiation and regulation of innate and acquired responses is induced by cytokines. Interleukins are cytokines normally produced by macrophages and leukocytes, mainly $CD4^+$ T-cells (Janeway, C.A. *et al.*, 1999). Under the influence of cytokines, resting T helper cells (Th0) can differentiate in either the Th1 or the Th2 phenotype. The Th1 immune pathways play a dominant role in resolving intracellular pathogens, and in addition the Th2 immune pathways play a dominant role in resolving extracellular pathogens. Th1 cells typically produce IFN-γ. A high amount of IFN-γ is thought to be involved in parasite killing. The Th2-type cytokines could reduce the level of Th1 cytokines in the gut, performing a regulatory role to reduce immune-mediated damage (Inagaki-Ohara, K. *et al.*, 2006). Th2 type of responses include IL4, IL5 and IL13 (Degen, W.G. *et al.*, 2005). Finally some proinflammatory cytokines are described as being chemokines which are important mediators of cell migration during inflammation, amongst others IL-8 (Dalloul, R.A. and Lillehoj, H.S., 2006).

Immune responses of chickens during coccidiosis

Eimeria infections have been shown to induce a complex, multi-factorial immune response in the host. *Eimeria* infections evoke non-specific and specific immune responses involving many aspects of the cellular and humoral immune response. The complexity of the immune reaction to *Eimeria* differs for the individual species.

Increased NK activity was observed in the early stages after infection suggesting a role for NK cells in the control of parasite proliferation (Lillehoj, H.S., 1998). In addition, it was shown that during a primary infection mainly macrophages are involved in the elimination of parasites. Their role may be essential in antigen presentation during subsequent infections where cytotoxic ($CD8^+$) T-lymphocytes exert immunity (Vervelde, L., *et al.*, 1996). Depletion of $CD8^+$ cells with a monoclonal antibodies against $CD8^+$ led to an increased oocyst shedding after a primary infection with *E.acervulina* and *E.tenella* (Trout, J.M. and Lillehoj, H.S., 1996). The $CD4^+$ T-cells of the $TCR\alpha\beta^+$ subset most probably have a function in resistance to primary infection of most, but not all *Eimeria* species (Shirley, M.W. *et al.*, 2007). This suggests that, like already observed in mice, chicken $CD4^+$ T-cells play a role in the induction of immunity whereas $CD8^+$ T-cells play a role in the effector, parasite elimination function following challenge (Lillehoj, H.S., 1998; Rose, M.E. *et al.*, 1992). Lymphocytes, macrophages, dendritic cells and tissue cells are all able to secrete cytokines and pro-inflammatory molecules. These cytokines and pro-inflammatory molecules direct the type of cell mediated immunity in order to destruct the pathogen and develop a rapid protective response (Dalloul, R.A. and Lillehoj, H.S., 2006). The Th1-type cytokines are likely to play an important role in limiting parasite multiplication in the early stages of infection (Choi, K.D. *et al.*, 1999). The most studied Th1 cytokine is IFN-γ. Primary *Eimeria* infections lead to induction of IFN-γ at the place of infection as has been observed by many researchers during primary infections with *E. acervulina*, *E. maxima* and *E. tenella* (Byrnes, S. *et al.*, 1993; Lowenthal, J.W. *et al.*, 1997; Shirley, M.W., *et al.*, 2007), and though *E.acervulina* infects the duodenum an increase of IFN-γ was only observed in the cecal tonsils and the spleen (Choi, K.D. *et al.*, 1999). The putative role for IFN-γ was confirmed by treatment of chickens with IFN-γ that lead to inhibition of *E.tenella* growth (Lillehoj, H.S. and Choi, K.D., 1998). Interleukin-2 is a potent growth factor for a variety of cell types and is found to be increased after primary infections with E.acervulina (Choi, K.D. and Lillehoj, H.S., 2000). Immune responses to an infection of chickens with *Eimeria* do not only result in reduction of the number of parasites, but also result in sometimes severe tissue damage. The tissue damage may be the result of parasite penetration, damage inflicted by T-cells and cytokines on infected cells or from collateral damage from immune responses on

neighboring cells. Regulatory anti-inflammatory cytokines may play a vital role in limiting the immunopathology that is associated with pro-inflammatory cytokines that are induced by the infection. Down-regulation of the Th1 response is accomplished by IL-10. This cytokine alters the Th1-Th2 balance of the immune response (Rothwell, L. *et al.*, 2004). IL-10 is known to be produced during coccidiosis, but its role in disease pathogenesis has not been clarified (Rothwell, L. *et al.*, 2004). Interestingly, TCR$\gamma\delta^+$ cells form a large proportion of the intraepithelial lymphocyte population (Lillehoj, H.S. and Trout, J.M., 1994; Vervelde, L. *et al.*, 1996). After primary infections with *E.acervulina*, an increased percentage of TCR$\gamma\delta^+$ cells was observed in the duodenum (Choi, K.D. and Lillehoj, H.S., 2000). In mice infected with *E.veriformis* such TCR$\gamma\delta^+$ cells have been shown to play an immuno-regulatory role in helping to dampen the immunopathology caused by TCR$\alpha\beta^+$ cells and pro-inflammatory cytokines (Roberts, S.J. *et al.*, 1996). It is thought that the TCR$\gamma\delta^+$ also have this immuno-regulatory role during *Eimeria* infections. In addition it cannot be excluded, that the recently discovered Th17 immune cells, known to resolve tissue pathology in human autoimmune models, play a role in the balance between immunity paired with tissue damage on the one hand and damage reduction on the other hand (Schmidt-Weber, C.B. *et al.*, 2007).

About two weeks after an infection the first antibodies are formed against the parasite. Maternal transfer of such antibodies results in reduced parasite excretion of infected offspring (Smith, N.C. *et al.*, 1994; Wallach, M. *et al.*, 1992). This means that antibodies play a role in protection. However, antibody titers in both serum and intestine do not correlate with the level of protection after oral infection with coccidia (Dalloul, R.A. *et al.*, 2003). This might be explained by antibodies playing a role in *Eimeria* reduction by e.g. ADCC. The minor role of antibodies is further demonstrated after removal of the bursa. Bursectomy did not affect the ability of the animal to generate a protective secondary immune response, confirming the dominance of the cellular response to an *Eimeria* infection (Lillehoj, H.S. and Ruff, M.D., 1987; Rose, M.E., 1970). Though specific antibodies against *Eimeria* parasites are found after infection, the protective immune response seems still predominantly T-cell mediated (Allen, P.C. and Fetterer, R.H., 2002; Lillehoj, H.S. and Lillehoj, E.P., 2000; Yun, C.H. *et al.*, 2000).

Protection to a subsequent challenge is only possible to a homologous infection (Dalloul, R.A. and Lillehoj, H.S., 2006). Less or no protection occurs when the host is infected with an *Eimeria* species different from the primary infection. After an infection with E.maxima reduced or even no protection is observed after secondary infection with a different strain (Allen, P.C. *et al.*, 2005). Contrary to observations with other *Eimeria* species, hosts that were primed to *Eimeria maxima*

displayed a low responsiveness to a secondary infection (Rothwell, L. *et al.*, 1995). In *Eimeria* immune hosts the precise process of inhibition of parasite growth and development is not known (Dalloul, R.A. and Lillehoj, H.S., 2006; Shirley, M.W., Smith, A.L. and Blake, D.P., 2007). Like in primary infections, characterisation of the mechanisms responsible for immunity against and secondary infection with most *Eimeria* species also has revealed a critical role for T-cells (Byrnes, S. *et al.*, 1993; Lowenthal, J.W. *et al.*, 1997). Great variations have been observed in putative protective immune mechanisms against secondary infection of the different *Eimeria* strains. Roles therein for both $CD4^+$ and $CD8^+$ T cells have been suggested especially for the $TCR\alpha\beta^+$ cell subsets. Lillehoj and Bacon found an increase in $CD8^+$ $TCR\alpha\beta^+$ cells after a secondary infection with E.acervulina, which might be an indication that T-cells expressing $CD8^+$ and $TCR\alpha\beta^+$ in the duodenum are induced and may be important for protection against coccidiosis (Yun, C.H., *et al.*, 2000). Like in primary infections, an increased percentage of $TCR\gamma\delta^+$ cells and an increased amount of Il-2 were observed in the duodenum after secondary infections with *E.acervulina* (Choi, K.D. and Lillehoj, H.S., 2000).

➢ This Thesis

The broader context

The work presented in this thesis is part of a joint research program of the Expertise Centre Animal Sciences and Health (WUR, UU and ASG) called Immuno-epidemiology, ecology and control of coccidial infections in poultry. The goal of this program is to obtain insight into the within-animal dynamics and between-animal transmission of *Eimeria* parasites of poultry and specifically into the way in which these two processes are connected and how they interact to determine the infection dynamics within flocks and between flocks. A central question is how the natural resistance to the parasite interacts with control measures and how this natural resistance can be used in intervention strategies. Therefore it is necessary to provide insight into several of the key parts that determine transmission of *Eimeria* spp. in a chicken flock. The overall focus is the interaction between the parasite and the gut ecosystem, the immune system and the external environment. The program's approach on the way to these insights consists of characterising "natural immunity" of hosts (being the effect of the immune response of the host on the parasite, the host pathogen interactions dynamics), developing protocols and methods for transmission experiments with *Eimeria*, and developing a mathematical model that connects non-trivial descriptions of within and between-animal dynamics. The first is the topic of this PhD thesis, the second is the topic of the future PhD thesis of F.C. Velkers, the modelling was a postdoc

project performed by D. Klinkenberg. All projects interacted within this project and one joint experiment and analysis was carried out. This latter work is reported in both PhD theses (see below). Since it appears that antibody-mediated responses play a minor role in the protective immunity against *Eimeria* infections, this thesis will focus on the cellular immune responses.

The traditional measure of reduction in oocyst output after challenge to characterize immune status of birds is not sufficiently precise and more accurate measures need to be developed. Next to the classical output measurements, immunological measures have recently been developed in order to assess chicken immune responses. For the most important chicken cytokines and T-cell subsets RT PCR quantification has been developed. For evaluation of cellular functions various functional assays have been developed. This will allow carefully evaluation of the immune response mechanism at the level of the GI-tract. Recent advances have made it possible to monitor the expression of thousands of genes on a micro-array. For the chicken an array is available containing 20.673 oligo probes providing almost a total evaluation of the chicken immune response. Transmission experiments can provide insight into the relation between the development of protective immunity, initial contamination and control. There are indications from earlier work that this relationship may well be non-linear (i.e. leaving an intermediate level of infestation between flocks might be preferable to not cleaning and to trying to clean very well). Both gut response and transmission to the environment are dynamic processes that, on the one hand influence each other and that are, on the other hand, influenced by control measures. The new and previous experimental results and insights can form a basis for a population dynamical model to further explore the population consequences of individual variation. This model will combine within-host immune response with between-host transmission in a flock of interacting animals. Mathematical modelling is ideally suited to address this complex problem and will serve the purpose of integrating the two experimental projects. The experimental projects are linked through the monitoring of immune status. Analysis of the model in different settings can be used to study the effects of different control strategies extending the ideas in Roberts & Heesterbeek (Roberts, M.G. and Heesterbeek, J.A., 1998) to include a more realistic description of immune response tailored to existing knowledge of the *Eimeria*-poultry system. Initial progress has been made in the work of Klinkenberg (Klinkenberg, D. and Heesterbeek, J.A., 2005; Klinkenberg, D. and Heesterbeek, J.A., 2007) and Severins (Severins, M. *et al.*, 2007).

Outline of this thesis

The aim is to obtain insight in host response to *Eimeria* infection, quantifying the dynamics of immune status and infectivity. We do this in relation to various factors of influence during a natural infection; infection dose, parasite species, broiler line, age of the host and individual exposure history.

In chapter 2 the effect of a primary *E.acervulina* infection on the (T-cell) immune responses of young broilers, in relation to the number of parasites used for infection is described. In our experiment we infected one-day-old broilers with a low ($5 * 10^2$ oocysts) and a high ($5 * 10^4$ oocysts) dose of *E.acervulina.* This resulted in the conclusion that, based on the kinetics of observed T-cell and cytokine responses, a primary infection with a high dose of *E.acervulina* in one-day-old broilers seems to generate an immune response that shows a peak at the time of oocyst excretion, whereas the immune response to a low dose is less explicit.

In chapter 3 the effect of *Eimeria* strains on the immune response are investigated by means of a single and multi-species infections. In field studies in general the presence of various *Eimeria* species simultaneously is reported. It has been suggested that infection with one species may enhance reactions to infections with other species. Our results indicate that different *Eimeria* species give different cell mediated responses and a multiple species infection does not induce additional T-cell and cytokine responses. The response to a multiple-species infection is formed by an accumulation of the species specific responses to all different administered *Eimeria* species.

In chapter 4 a study on differences in immune reactions to an *E.acervulina* infection in two different broiler lines is described. The lines used were a commercial fast-growing broiler line and a slow-growing type of broiler as used in organic farming. In conclusion, based on weight gain both lines have different growth rates and control and infected conditions. Based on the T-cell kinetics a primary infection with *E.acervulina* in 7-day-old broilers seems to generate an early $CD8\alpha^+$ and $TCR\gamma\delta^+$ response in fast-growing broilers compared to the slow-growing broilers accompanied by IL-8 and IL-18 cytokine responses . Fast growing broilers also showed a lower amount of *E.acervulina* DNA in their duodenum as compared to slow growing broilers. The difference in immune reaction after an *E.acervulina* infection could account for the different *Eimeria* load in the duodenum.

In chapter 5 the effect of host age and development at the time of primary infection with *E.maxima* is investigated. Broilers were infected at seven fourteen and twenty-one days of age. Only broilers infected at day 1 of age showed a significant weight loss 7 after infection. Strength and kinetics of the T-cell and cytokine Th1 type of response increased as the age of the host increased.

In chapter 6 the effect of previous infection is described. Differences in infection history were created by re-infecting broilers with an homologue or heterologue species of *Eimeria*. When re-infected with a homologue strain a complete reduction of oocyst exretion was found. T-cells and cytokines showed a low reactivity the re-infection with a homologue species. A micro-array showed many comparable results between the primary and secondary infection. Specific responses to the re-infection are also observed and these might play a role in protection.

In chapter 7 the knowledge of the within-host aspects of the infection was linked to the knowledge of transmission aspects. If we take an epidemiological, i.e. population, view of immunology, we have to deal with both the immunological dynamics in individual chickens, the dynamics of the infection acting in a population of chickens, and the complex way in which these processes influence each other. A transmission experiment was performed in which immune (T-cells) and transmission parameters (Oocyst excretion) were determined in pairs of broilers that differed in infection history. It was found that transmission in pairs of broilers was lowered most when seeders, and not receivers, had endured a previous infection. When broilers were able to infect each other repeatedly for a prolonged time, transmission decreased to zero, and complete immunity developed. No T-cells were found playing a role in protective immunity.

In the general discussion (chapter 8) the findings and observations are summarized and discussed in a broader context.

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Chapter 2

Immune responses in *Eimeria acervulina* infected one-day-old broilers compared to amount of *Eimeria* in the duodenum, measured by real-time PCR

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Abstract

T-cell responses are supposed to be the major immune reactions in broilers infected with *Eimeria.* The nature of such T-cell responses is influenced by the species of *Eimeria* involved*,* age of the host, amount of parasites and the preceding infection history.
In young chicks the intestine is still developing in length while the lymphocyte populations in the gut develop and differentiate. In chicks infected at young age the immune response may be different in quality as compared to responses in adults.
We investigated the (T-cell) immune responses of young broilers to a primary *E.acervulina* infection in relation to the number of parasites used for infection. In our experiment we infected one-day-old broilers with a low (5 x 10^2 oocysts) and a high (5 x 10^4 oocysts) dose of *E.acervulina*. We used a newly developed species specific real-time PCR to quantify total amount of parasites in the duodenum as the number of oocysts in faeces may not be representative for the exposure of the gut immune system. We characterized T-cell subsets in the duodenum by means of FACS-analyses, lymphocyte proliferation assays with spleen lymphocytes and the mRNA profiles of different cytokines (TGF-β2, -4, IFN-γ, IL-2, -6, -8 and -18) in the duodenum by means of real-time PCR.
From day 5 p.i. broilers with a high dose of *E.acervulina* had a significantly lower body weight than the control group. No increase in $CD4^+$ cells, but a strong increase in $CD8^+$ cells was observed at day 7 and 9 p.i. in the duodenum of broilers infected with a high dose *E.acervulina*. IL-8 mRNA responses were observed after infection with low and with high infection doses, but no IFN-γ and TGF-β mRNA responses were found in the duodenum. The specific proliferative T-cell responses to a low infectious dose were not significantly different as compared to the control group.
In conclusion, based on the kinetics of observed responses a primary infection with a high dose of *E.acervulina* in one-day-old broilers seems to generate an immune response that shows a peak at the time of oőcyst excretion, whereas the immune response to a low dose is less explicit.

Key words: *Eimeria acervulina*; Immunology; Real-time PCR; Cytokines; T-cells

Introduction

Coccidiosis is an intestinal disease that is caused by protozoan parasites of the genus *Eimeria* (Constantinoiu, C.C. *et al.*, 2004). *Eimeria* are obligate intracellular parasites with a complicated life cycle, which consists of sexual and asexual stages (Augustine, P.C., 2001). An *Eimeria* life cycle can be characterized by three stages: 1) sporogony, 2) schizogony and 3) gametogeny (Allen, P.C. and Fetterer, R.H., 2002). Coccidiosis is spread worldwide and *Eimeria* species have been recognized to infect different types of animals like rodents, rabbits, horses, pigs, sheep, cows and poultry (Augustine, P.C., 2001; Hermosilla, C. *et al.*, 1999; Hnida, J.A. and Duszynski, D.W., 1999; Lyons, E.T. and Tolliver, S.C., 2004; Reeg, K.J. *et al.*, 2005; Renaux, S. *et al.*, 2003; Yunus, M. *et al.*, 2005). *Eimeria* species are host specific, the species infecting chicken include *Eimeria acervulina*, *brunetti*, *maxima*, *mitis*, *necatrix*, *praecox* and *tenella* (Lillehoj, H.S. and Lillehoj, E.P., 2000). The most prevalent *Eimeria* species in broilers in the Netherlands is *E. acervulina* (Graat, E.A. *et al.*, 1998; Peek, H.W. and Landman, W.J., 2003). Each *Eimeria* species has its specific place within the intestine of this host. *E. acervulina* infects the duodenum, whereas *E. tenella* infects the caeca. Clinical coccidiosis is characterized by diarrhoea ranging from mucoid and watery to haemorrhagic, reduction in weight or weight gain and morbidity. Sometimes acute death is seen in young animals (Williams, R.B., 2002; Yunus, M., Horii, Y., Makimura, S. and Smith, A.L., 2005). Severity of an *Eimeria* infection is mostly assessed by means of lesion scores of the intestine, alternatively weight loss of the animal, excretion of oöcysts, depigmentation, coloration of the serum and even mortality are also used (Idris, A.B. *et al.*, 1997). For quantification of *Eimeria* counting oocysts of chicken coccidia is the mostly used method. Unfortunately this technique only allows counting oocysts in faeces. Currently no technique is available to assess the amount of parasites present in the intestine of the infected bird. To assess the severity of an infection, *Eimeria* presence in the intestine might be more representative than oocyst counts in the faeces; therefore a real-time PCR was developed to measure the amount of *Eimeria* in the intestine.

Numerous studies have demonstrated a dominant role for T-cells in the immune response to *Eimeria* (Dalloul, R.A. and Lillehoj, H.S., 2005). Each *Eimeria* species generates a specific immune response. Host immune responses are triggered during the sporozoite migration as the parasite cycle progresses and a strong protective immunity generally takes place after a primary infection. Infection triggers a local lymphocyte stimulation and proliferation but there is also a significant participation of other intestinal areas and systemic components (Bessay, M. *et al.*, 1996) *Eimeria* infection seems to rapidly induce, locally at the site of parasite

development, a dramatic modification of the proportions of T-cell subsets in intraepithelial leucocytes (IEL), accompanied by systemic variations that are generally opposing, in the lymphocyte populations (Bessay, M. *et al.*, 1996)..

After a primary *Eimeria maxima* infection $CD4^+$ cells were found almost exclusively in the lamina propria while $CD8^+$ cells were found in both the intestinal epithelium and lamina propria (Rothwell, L. *et al.*, 1995; Yun, C.H. *et al.*, 2000). Th1 responses seem to be dominant during coccidiosis, as best manifested by proven involvement of IFN-γ (Dalloul, R.A. and Lillehoj, H.S., 2005). $CD8^+$ T cells are also thought to play a role in sporozoite transport (Lillehoj, H.S., 1998; Lillehoj, H.S. and Trout, J.M., 1996). Intraepithelial lymphocytes (IEL) are sparse in newly hatched chickens but increase as the chickens are exposed to environmental antigens. IEL in contrast to other lymphocytes include a high percentage of $TCR\gamma\delta^+$ cells. These $TCR\gamma\delta^+$ can mediate specific cellular immune responses without the requirement for antigen processing and can recognize invading pathogens or damaged cells directly (Schild, H. *et al.*, 1994; Yun, C.H., Lillehoj, H.S. and Lillehoj, E.P., 2000).

The composition of different T cell subsets will depend upon the age of the host, region of the gut examined and the genetic background of the host (Lillehoj, H.S. and Chung, K.S., 1992; Lillehoj, H.S. and Trout, J.M., 1996). Many experiments on broilers focus on infections in animals of about 7 days of age. However, *Eimeria* infections can occur directly after hatch. At that time the composition of the gut immune system and microflora is quite different from the colonized gut. Here we investigated the developing immune system of one-day-old broilers and its reaction after an *Eimeria acervulina* infection. We inoculated one-day-old-broilers with a either a relatively low or high single dose *Eimeria acervulina*. In order to find the relation between the immune responses in the duodenum of broilers infected at day 1 of age and the presence of the parasite in the duodenum we measured both the amount of the parasite with the developed real time PCR and the immune responses in the duodenum. It was found that the majority of the immunological changes occurred after the parasite had disappeared from the duodenum.

Materials and methods

Chickens

A total of 170 one-day-old Ross 308 broilers were obtained from a commercial breeder (Pronk's broederij, Meppel, the Netherlands). The chickens were divided into three groups of 55 animals. The one-day-old broilers were orally inoculated. All animals in group 1 were orally inoculated with 0,5 ml phosphate buffered saline (PBS pH 7.4), animals in group two were orally inoculated with 0,5 ml PBS containing 5×10^2 sporulated oocysts of *Eimeria acervulina* and animals in group three were orally

inoculated with 0,5 ml PBS containing 5 x 10^4 sporulated oocysts of *Eimeria acervulina*. Sporulated *E.acervulina* oocysts were provided by the Animal Health Service in Beekbergen. All chickens were immunised against infectious bronchitis (IB) and Newcastle disease virus (NDV) with a spray vaccine (Intervet®, Boxmeer, The Netherlands). Chicks were reared on wire cages to prevent contact with faeces. Feed and water were provided ad libitum. The study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

Experimental design and inocula

On day 0 a control group of 5 chickens was killed by cervical dislocation before infection. Chickens were killed by cervical dislocation at 1, 2, 3, 4, 5, 7, 9 and 11 days post inoculation (p.i.) The number (n) of chickens per group for post mortem analysis was 5, except for days 4,7 and 9 where n was 10. From these chickens individual body weight was measured, droppings were collected and blood samples were obtained by cardiac puncture before section. The duodenal loop was collected, snap-frozen in liquid nitrogen and stored at -70°C, while the adjacent part of the duodenum was collected in cold PBS. On days 4, 7 and 9 spleens were collected in PBS containing Penicillin and Streptomycin (pen/ strep) (Gibco, UK) at a concentration of 100 IU/ L.

Spleen lymphocyte isolation

The spleens that were kept in PBS pen/ strep were squeezed through a 100µm nylon-mesh (Becton Dickinson, Franklin Lakes, NJ, USA). The mesh was flushed with 10 ml RPMI 1640 (Gibco, UK). Cells were loaded on a 10 ml Ficoll® gradient (Amersham Pharmacia Biotech Buckinghamshire, UK) and centrifuged at 550 *g* for 20 minutes without a brake to separate leukocytes from other cells. The leukocytes were washed twice with RPMI 1640. The cells were suspended in RPMI 1640 to a cell concentration of 5 x 10^6 cells/ ml.

Intraepithelial lymphocyte isolation

5 cm Duodenum was opened longitudinally, washed with PBS and cut into pieces of 1 cm. These pieces were incubated at 37° for 45 minutes in Medium I (PBS containing 1mM EDTA and 5mM DTT). The suspension thus obtained held the intraepithelial lymphocytes (IEL) and was kept at 4°C until use. The remaining pieces of intestine were further incubated at 37°C for 90 minutes in medium II (RPMI + 5% fetal calf serum + 400 FALGPA units Collagenase per liter (Sigma, St Louis, MO, USA) + 60000 Kunitz units DNase I per liter (Sigma, St Louis, MO, USA)) while shaking. After incubation the suspension contained the lamina propria lymphocytes (LPL). The IEL and LPL fractions were mixed and after centrifugation the pellet was resuspended in 10 ml Medium III (RPMI + 1% Fetal calf serum + 60000 Kunitz units DNase I per liter). The suspension was purified on a 25% percoll (Sigma, St Louis, MO, USA) gradient centrifuging for 15 minutes at 2000 rpm. The pellet was washed twice with PBS and cells were resuspended in 20 x 10^6 cells per ml.

Lymphocyte proliferation assay (LPA)

The spleen cells were used at 5 x 10^6 viable cells per well in 200 µl RPMI 1640 (Gibco, Life Technologies) containing 1% normal chicken serum (Gibco, Life Technologies) and 100 IU/ L Penicillin and Streptomycin in flat bottom 96-well plates (Costar, Corning, Inc., Corning, NY). The cells are incubated with ConA (5 µg/ml)(Sigma, St Louis, MO, USA) for 68 h in a humidified incubator at 41 °C with 5% CO_2 in order to measure (re)stimulation in vitro. After the incubation, 0.4 µCi/well [^{3}H] thymidine

(Amersham Pharmacia Biotech, UK) was added and 4 h later the plates were harvested onto fiberglass filters and counted by liquid scintillation spectroscopy (Betaplate, Wallac Oy, Turku, Finland).

Flow cytometry and antibodies

The total leukocyte isolates and proportions of lymphocyte subpopulations were estimated by flow cytometry. For the flow cytometric analysis the concentration of the isolated lymphocytes was brought to 20 x 10^6 cells/ml, and 50 µl was transferred into a 96 well plate on ice. Cells were washed with PBS supplemented with 1% FCS. A normal mouse serum (1%) was applied to block non-specific bindings, followed by either of the monoclonal combinations CD4-PE / CD3-FITC; TCR$\gamma\delta$-PE / CD8α-FITC; CD45-PE / Ia-FITC (southern Biotechnology Associates, Birmingham). Ia will further be referred to as MHC II^+.

After 15 min incubation at 4°C, the cells were washed twice with PBS/FCS and re-suspended in 200 µl ice-cold PBS/FCS. A total of 10^4 cells per sample were analysed by flow cytometry (FACS Calibur™)(Beckton Dickinson, Leiden, The Netherlands). The data were analysed using a flow cytometry computer programme. The lymphocyte population was gated and counted.

RNA extraction and cDNA preparation

The duodenum samples were homogenised with liquid nitrogen using a mortar and pestle. Total RNA was extracted from 50-100 mg tissue samples of duodenum with TRizol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's recommendations with minor modifications. The homogenised tissue samples were dissolved in 1 ml of TRizol reagent per 50-100mg of tissue using a syringe and 21-G needle passing the lysate for 10 times. After centrifugation the supernatant was transferred to a fresh tube. Subsequently a phase separation with chloroform was performed as described by the manufacturer. The RNA was precipitated. 200ng RNA was incubated with random hexamers (0.5µg, Promega Benelux BV, Leiden, The Netherlands) at 70°C for 10 min, then reverse transcribed in a final reaction volume of 20 µl containing Superscript RNase H $^-$ reverse transcriptase (200 Units, Invitrogen, Breda, The Netherlands), RNAsin (40 U, Promega Benelux BV, Leiden, The Netherlands), dNTP (2 mM Promega Benelux BV, Leiden, The Netherlands), 5xFirst Strand Buffer (Invitrogen, Breda, The Netherlands) and 0.1M DTT (Invitrogen, Breda, The Netherlands) for 50 min at 37°C. The reaction was stopped by heating at 70 °C for 10 min and the cDNA product was stored at -20°C until use. The cDNA product was 10 times diluted before use.

DNA preparation from tissue

About 0.02 to 0.04 gram of tissue was homogenised with liquid nitrogen using a mortar and pestle. For the isolation of DNA from the intestinal tissues the *chemagic DNA tissue kit* (Baseclear, Leiden; the Netherlands) was used according to the manufacturers manual. After cell lysis, DNA was bound to magnetic beads which were separated from the supernatant using the chemagic stand. DNA was eluted from the beads using 100 µl of the elution buffer as provided by the manufacturer.

Real- time PCR

Real-time PCR was employed with on line detection of the PCR reaction based on fluorescence monitoring (LightCycler, Roche Diagnostics, Mannheim, Germany). Primer and probe sequence were selected with the software from TIB MolBiol (Berlin, Germany). Hybridisation probes (TIB MolBiol, Berlin, Germany) were used to monitor the amount of specific target sequence product.

Table 1: Primer and probe sequences as used in the real-time PCR.

Target			Probe / primer sequence (5'-3')	Annealing temp.
IL-2	Probe	IL-2-FL IL-2-LC	CTTGTTGTTAGCTTCACAGATCTTGCATTCACT X CGGTGTGATTTAGACCCGTAAGACTCTTGAG p	58 °C
	Primer	IL-2-FOR IL-2-REV	CAGTGTTACCTGGGAGAAGTG GCAGATATCTCACAAAGTTGGTC	
IL-6	Probe	IL-6-FL IL-6-LC	CGAACAGGCCGCTGGAGAGC X CGTCAGGCATTTCTCCTCGTCGAAG p	59 °C
	Primer	IL-6-For IL-6-Rev	AGGACGAGATGTGCAAGAAG TGCTGTAGCACAGAGACTCG	
IL-8	Probe	IL-8-FL IL-8-LC	CATCCGAAGAAGGCATCATGAAGC X TTCCATCTTCCACCTTCCACATCG p	56 °C
	Primer	IL-8-For IL-8-Rev	ATTCAAGATGTGAAGCTGAC AGGATCTGCAATTAACATGAGG	
IL-18	Probe	IL-18-FL IL-18-LC	CTCCTTCCCTAAATCGAACAACCAT X TCCCATGCTCTTTCTCACAACACAT p	56 °C
	Primer	IL-18-For IL-18-Rev	CGTCCAGGTAGAAGATAAGAG AGGAGTCTTCTTCCTCAAAG	
IFN-γ	Probe	IFN-γ-FL IFN-γ-LC	GAAAGATATCATGGACCTGGCCAAGC X CCCGATGAACGACTTGAGAATCCAG p	55 °C
	Primer	IFN-γ-For IFN-γ--Rev	TTCGATGTACTTGGAAATGC TTGCATCTCCTCTGAGACTG	
TGF-β-2	Probe	TGF-β-2-FL TGF-β-2-LC	TGTCTGCGTCCACTTTATATTGACTTC GAGGGATCTTGGCTGGAAATGGAT	58°C
	Primer	TGF-β-2-For TGF-β-2-Rev	TACAGACTTGAGTCCAAC AATCATGTTGGACAGCTG	
TGF-β-4	Probe	TGF-β-4-FL TGF-β-4-LC	ACCTTTGGGTTCGTGGATCCAC CCACTGCAGATCCTTGCGGA	56°C
	Primer	TGF-β-4-For TGF-β-4-Rev	GATGAGAAGAACTGCTGC CATATTGGAGAGCTGCTC	
28S	Probe	28S –FL 28S–LC	GCGGGTGGTAAACTCCATCT X GGCTAAATACCGGCACGAGACCG p	56 °C
	Primer	28S-For 28S-Rev	CAAGTCCTTCTGATCGAG TCAACTTTCCCTTACGGTAC	
Eimeria acervulina	Probe	Eacer-FL Eacer-LC	gATgACCgCTCgCCTAgTTgT-FL gTCCCCAATgCTCTgTTTgTCgT p	62°C
	Primer	Eacer-F Eacer-A	CCTCCgAgTTTCTCACCC CCCCCAAATCACTCgAA	

For is forward; Rev is reverse; FL is Fluorescein; LC is light cycler red 640.

Quantitative results were determined as described by Loeffler *et al* (Loeffler, J. *et al.*, 2000). For the quantification a standard curve of the plasmid with the insert of interest was used, constructed in PGEM-T easy (Promega). PCR reactions were performed according to the LightCycler kit instructions (FastStart DNA Master Hybridization Probes, Roche Diagnostics, Mannheim, Germany). Real-time PCR was done as described by Rebel et al (Rebel, J.M.J. *et al.*, 2005). Primer and probe combinations and PCR conditions are depicted in Table 1. To avoid contamination, filter pipet tips were used and reagents were mixed in rooms separate from rooms where DNA was present. A negative control containing reagents only and a standard dilution series (cytokine and 28S) was included in each run. For all cytokine and 28S PCR reactions the same cDNA sample was used in order to standardise and normalise the data.

Normalisation of mRNA concentration

A standard curve was generated from the threshold cycles (C_t) of the standard dilution series by the LightCycler software version 3.5. The standard curve had a slope around -2.97, meaning that the overall reaction efficiency for the standard curve was between 1.0 and 1.3.
The amplified PCR product performed with the LightCycler can be described as $N_n=N_o*E^n$ (Mygind, T. *et al.*, 2001), where N the is concentration of a sample which is identified by a given cycle number, N_o is the starting concentration of the sample, E is the overall reaction efficiency and n is the crossing point in number of cycles. From the cytokine specific standard curve, the concentrations of the samples were calculated. To normalise the concentration of cytokine cDNA, the sample concentration of each individual chicken was divided by the sample concentration of 28S of the same chicken. The mean was then used of the sample concentrations of five chickens at each time point.

Statistics

On all data a student-t test was performed. Each infected group was compared to the control group and both infected groups were compared to each other. Data were called significant when $p<0.05$.
For the interleukins a different approach was needed because of the presence of samples with amounts below detection limits.
For the cytokine analysis the original observations were replaced by rank numbers within each time point. The rank numbers were analysed with an analysis of variance model, comprising main effects for times and treatment groups. The F-test for groups was used as a significance test for overall differences between groups over time. To increase the power of the test, the factor for treatment groups was replaced by an explanatory variable with values 1 (control), 2 (=infection with 5×10^2 oocysts) and 3 (=infection with 5×10^4 oocysts). A one-sided test was performed (P-values equal to half of the P-values of the two-sided F-test).

Results

Body weight

An oral inoculation in one-day-old broilers with sporulated *Eimeria acervulina* induced a weight gain depression (figure 1). In the group infected with 5×10^4 sporulated oocysts of *Eimeria acervulina* the weight depression was significant at days 7, 9 and 11 as compared to the control group. The inoculation with 5×10^2 sporulated oocysts of *Eimeria*

acervulina only generated a significant weight gain depression at day 5 as compared to the control group.

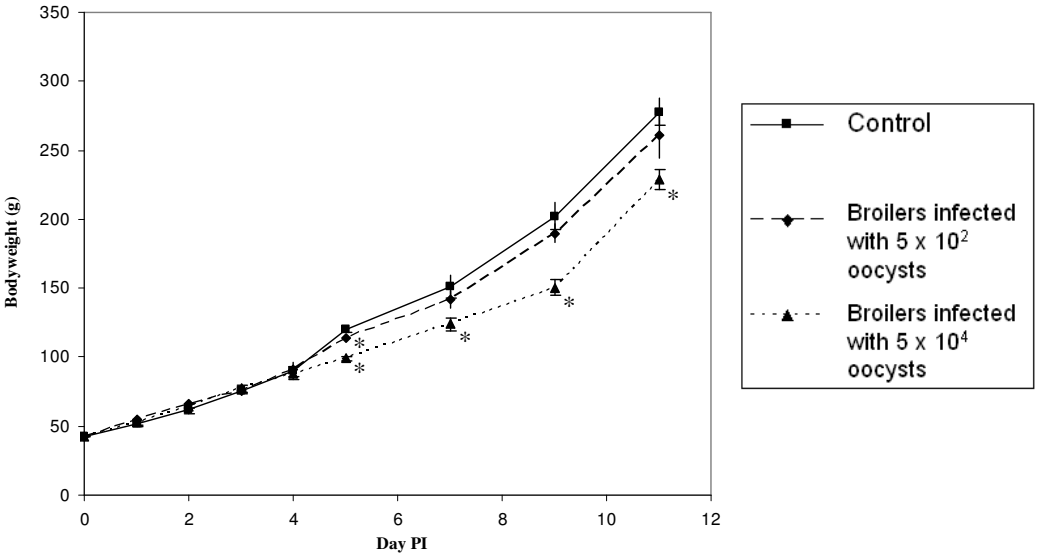

Figure 1: Body weight of three groups of broilers .
One-day-old broilers were mock infected, infected with 5 x 10^2 sporulated oocysts of *Eimeria acervulina* or infected with 5 x 10^4 sporulated oocysts of *Eimeria acervulina*. Body weight as depicted is the mean weight of 5 animals ± SEM. *= p≤ 0.05

Presence of Eimeria Acervulina in the duodenum

Infection with 5 x 10^2 and 5 x 10^4 sporulated oocysts of *Eimeria acervulina* resulted in a transient presence of the parasite in the intestine with a load between days 3 and 5 post infection as determined by real-time PCR (figure 2.) The duodenal parasital load in the group infected with 5 x 10^2 oocysts was highest at day 3 post infection whereas in the group infected with 5 x 10^4 oocysts the duodenal load reached a peak at day 4 post infection. At days 7 and 9 post infection no parasital DNA could be detected in the duodenum with the real-time PCR for both infectious doses.

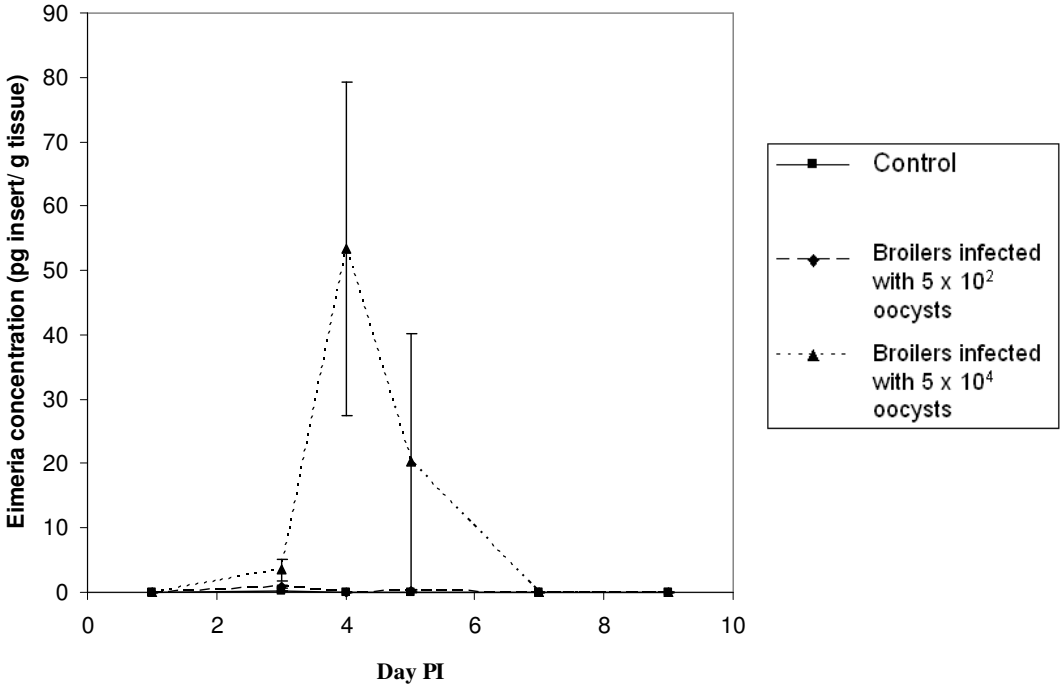

Figure 2: Amount of *Eimeria acervulina* ±SEM present in the duodenum after inoculation with 5 x 10^2 sporulated oocysts or 5 x 10^4 sporulated oocysts. Amounts are the mean of 5 samples.

Flow cytometric analysis of intestinal cells

The cell population was analysed as a percentage of gated intestinal lymphocytes from the duodenum. Figure 3 shows the percentage of $CD4^+$, $CD8\alpha^+$, $TCR\gamma\delta^+$, MHC II^+ and $CD45^+$ cells from day 0 to day 11. Significant differences between infected and control groups are depicted.

The percentage of $CD45^+$ cells in the duodenum increased with time. At day 0 the percentage of $CD45^+$ cells in the duodenum in the control group is about 10% of the gated cells and increased to almost 45% at day 11.

The population of $TCR\gamma\delta^+$ cells in broilers infected with a high dose of *Eimeria acervulina* was elevated from day 5 p.i. onwards as compared to the control group. In the group inoculated with 5 x 10^2 oocysts the $TCR\gamma\delta^+$ percentage elevation was observed but the difference was not significant. At days 5 and 9 a significantly higher amount of $TCR\gamma\delta^+$ cells was observed in the group infected with 5 x 10^4 oocysts when compared to the group infected with 5 x 10^2 oocysts.

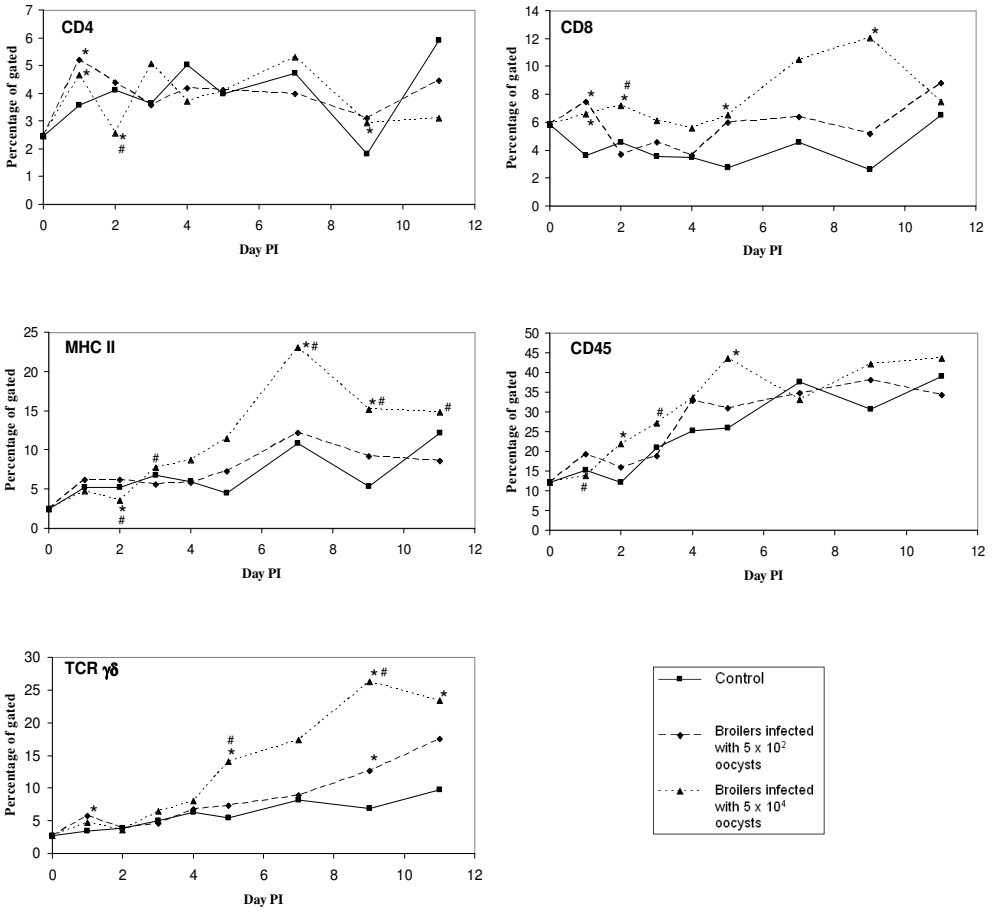

Figure 3: FACS analysis of cell populations isolated from duodenum of broilers infected with PBS (control), 5 x 10^2 sporulated *Eimeria acervulina* oocysts or 5 x 10^4 sporulated *Eimeria acervulina* oocysts. The percentage of $CD3^+$, $CD4^+$, $CD8^+$, $TCR\gamma\delta^+$, MHC II^+ and $CD45^+$ cells are the percentages of gated lymphocytes. Values are means of 5 chickens. *= Significant difference between control and infected group (P≤0.05). #= Significant difference between the groups infected with 5 x 10^2 or with 5 x 10^4 sporulated *Eimeria acervulina* oocysts (P≤0.05).

The percentage of the $CD4^+$ cells fluctuates with time. At days 1, 2 and 8 p.i. significantly increased values were observed for the groups infected with sporulated oocysts of *Eimeria acervulina* as compared to the control group. $CD8^+$ cell percentages were significantly elevated at days 1,2,5 and 9 p.i. in the group inoculated with a high dose when compared to the control group. In the group inoculated with 5 x 10^2 oocysts of *Eimeria acervulina* the percentage of $CD8^+$ gated cells was significantly elevated when compared to the control group only at day 1, while at days 5 and 11 p.i. the elevation was not significant (p=0.099 and p=0,085). At day 2 p.i. the $CD8^+$ percentages in the group inoculated with 5 x 10^4 oocysts were significantly higher than in both other groups.
The percentage of MHC II^+ cells was significantly higher in the broilers infected with 5 x 10^4 sporulated oocysts of *Eimeria acervulina* at days 7 and 9 post infection when compared to the control animals.

Cytokine mRNA expression

Except for IL-8, none of the tested cytokines showed a significant increase or decrease in time as compared to the control group (figure 4). Both inoculations with 5 x 10^2 and 5 x 10^4 sporulated oocysts of *Eimeria acervulina* induced an elevation of IL-8. In the broilers infected with 5 x 10^2 sporulated oocysts of *Eimeria acervulina* this elevation was of shorter duration than in the broilers inoculated with 5 x 10^4 sporulated oocysts of *Eimeria acervulina*. Though not significant, elevation of IL-6 mRNA amount can be found in animals infected with 5 x 10^4 sporulated oocysts of *Eimeria acervulina* at day 9 p.i.. At day 7 post infection in both inoculated groups the level of IL-18 mRNA was elevated compared to the control group. The IL-2 levels fluctuated but infection did not influence the levels of this cytokine. TGF-β2, TGF-β4 and IFN-γ mRNA levels could not be detected in both control and infected animals of this age.

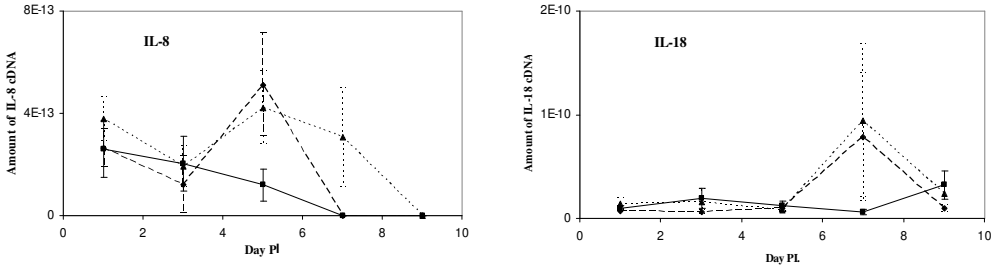

Figure 4: Mean relative amounts of cytokine mRNA ±SEM of 5 control chickens, 5 chickens infected with 5 x 10^2 oocysts, 5 chickens infected with 5 x 10^4 oocysts. Note the difference in the scales.

Lymphocyte proliferation

The *in vitro* proliferation of spleen lymphocytes after stimulation with Con-A showed no difference in reactivity at day 4 p.i. between control and infected groups (figure 5). At day 7 p.i. asignificant increase in reactivity of spleen lymphocytes in both inoculated groups was observed when compared to the control group. No difference could be found between the control groups at day 4 and 7 p.i.. At 9 p.i. a significant reduced reactivity was observed in spleen lymphocytes of broilers infected with 5 x 10^4 sporulated oocysts when activated with Con-A as compared to both control group and the group infected with 5 x 10^2 sporulated oocysts. At day 9 p.i. no difference in reactivity between control group and the group infected with 5 x 10^2 sporulated oocysts could be observed.

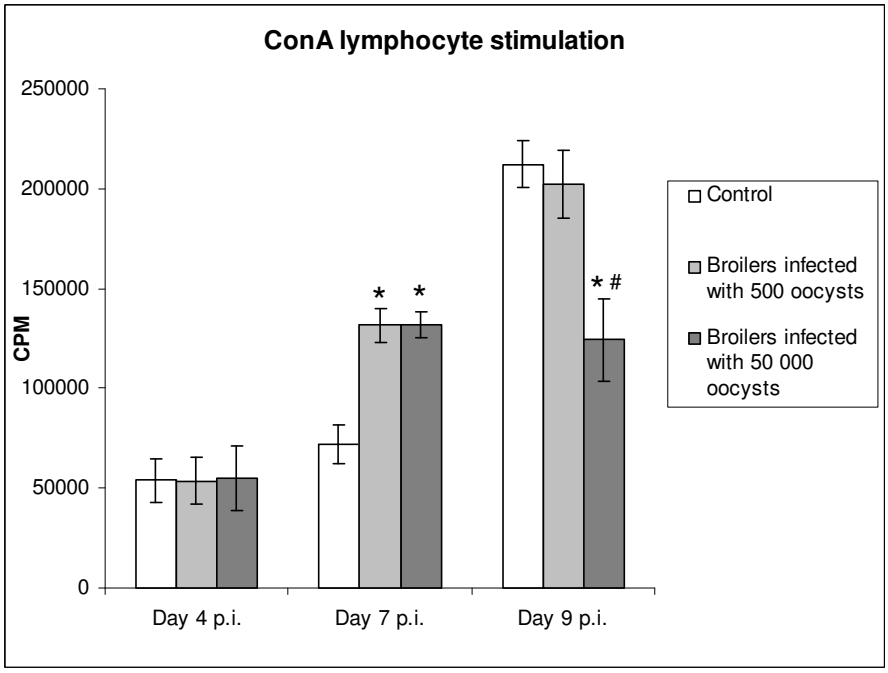

Figure 5: Lymphocyte stimulation assay with Con-A ±SEM.
*= Significant difference between control and infected group (P≤0.05).
#= Significant difference between the groups infected with 5 x 10^2 or with 5 x 10^4 sporulated *Eimeria acervulina* oocysts (P≤0.05).

Discussion

One-day-old broilers infected with *Eimeria* were monitored for their immune responses and the amount of *E.Acervulina* DNA present in the duodenum. All infected animals showed clinical signs as was illustrated by the weight gain depression (figure 1) and intestinal lesions of infected animals (data not shown). After hatch the intestine rapidly develops from relatively immature and unexposed tissue into a fully mature gut ecosystem. The kinetics of the immune parameters that were observed in the control group in part will be the result of this normal intestinal development.

The amount of *Eimeria* DNA in time found in the duodenum follows the life cycle of *E.acervulina* (Allen, P.C. and Fetterer, R.H., 2002). Therefore this will be a representative technique for the measurement of *E.acervulina* DNA in the duodenum. The duration of the transient presence of the parasite in the intestine as shown by real-time PCR was longer in the animals infected with 5 x 10^4 sporulated oocysts as compared to animals infected with only 5 x 10^2 oocysts. The amount of *Eimeria* DNA reached its peak in the duodenum 1 day later in the animals infected with 5 x 10^4 oocysts than in the animals infected with 5 x 10^2 oocysts. Thus with this real-time PCR differences in amount of *Eimeria* DNA in the duodenum will be recognised. Comparing the immune responses to both different doses, a striking result of this study was the relatively low level of T-cell infiltration in the duodenum after an inoculation with 5 x 10^2 sporulated oocysts of *Eimeria acervulina*. The kinetics of most measured parameters in broilers infected with 5 x 10^2 oocysts resembled the control situation. The reason for this relative unresponsiveness may reside in the low level of intestinal damage that was inflicted. Alternatively the broiler immune system may be too immaturely developed at this time or the amount of 10^2 oocysts might be below the threshold dose required for responsiveness in one-day-old broilers.

In contrast, at day 1 a significant influx of both $CD4^+$ and $CD8^+$ could be observed in the gut of chickens inoculated with 5 x 10^4 sporulated *Eimeria acervulina* oocysts as compared to the control group. This response to an inoculation with 5 x 10^4 oocysts in terms of both $CD4^+$ and $CD8^+$ cell percentages at day 1 p.i., was also observed in the group infected with only 5 x 10^2 oocysts. During the experiment the $CD4^+$ levels in both infected groups after day 2 p.i. remained the same as the control situation. In contrast, the proportion of $CD8^+$, MHC^+ and TCR^+ cells was higher in the group infected with 5 x 10^4 oocysts than in the control group throughout the experiment. This indicates that there indeed may exist a threshold value for the response to the infection in one-day-old broilers. As *Eimeria* parasites develop intracellularly and are not accessible to

antibodies it is assumed that the parasites to a large extent are eliminated by destruction of the infected cells. The elevation of $CD8^+$ and TCR^+ cells might account to this process, because the frequencies of these cell types elevate at the same time. In the broilers infected with the high dose of *Eimeria*, the MHC II^+ level rises from day 7-11 post infection. Uptake of oocysts by macrophages may result in activation and up regulation of MHC II expression. Most likely these T-cells are recruited from the spleen, where the reactivity of T-cells was increased at day 7 p.i.. At the moment the $CD8^+$ and $TCR\gamma\delta^+$ cells reach maximum percentages in the intestine of animals infected with 5 x 10^4 oocysts, the reactivity in the spleen is decreased, what might be due to the high recruitment by the affected intestine.

Over time, IL-8 mRNA was significantly higher in the infected groups than in the control group. The height of the IL-8 mRNA responses were similar, independent on the infection dose, but the time during which the mRNA remained elevated was longer in the infection with the 5 x 10^4 sporulated oocysts. As the chemotactic factor IL-8 can be released after epithelial necrosis (Petrone, V.M. *et al.*, 2002); this might account for the high amounts of IL-8 mRNA present at days 5 and 7. At these time points the oocysts have completed their intracellular cycle and are excreted. The damage that is inflicted to the gut may therefore result in such a response. However, we did not find *Eimeria* DNA in the duodenum from day 7 onwards. This would implicate that at the time of relatively high IL-8 levels the infection is already declining in severity. The group infected with 5 x 10^4 oocysts had more parasites in the duodenum; this might explain the more severe intestinal damage and might also account for longer lasting IL-8 mRNA signal.

It is known, that an *Eimeria* infection may evoke high IFN-γ levels in chickens that are at least three weeks of age when infected. However, even than gene expression of IFN-γ was only found in the ceacal tonsil (Choi, K.D. *et al.*, 1999). In contrast, in this experiment no IFN-γ mRNA response in the duodenum was observed at all. The lack of IFN-γ reaction might be due the age of the animals and the chicken lines that were used for the present experiments. However at day 7 post infection IL-18 (IFN-γ inducing factor) mRNA increased, although not significant in both infectious doses.

In general a low dose of *Eimeria* induced a minimal response in the broilers. A higher dose of parasites generated a stronger immune response early after infection which resulted in delay of parasite development but could not prevent coccidiosis. The immune responses at days 7 and 9 as found in this experiment could not be related to presence of the parasite in the duodenum as measured with our new developed real-time PCR. We assume that the onset of immunological reactions is due to intestinal damage as a result of excretion of parasites.

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Chapter 3

Host response to multi-species infection with *Eimeria acervulina*, *maxima* and *tenella*: A cumulation of species specific responses.

W.J.C. Swinkels, J.B.W.J. Cornelissen , J.M.J. Rebel

Abstract

It is well known that broilers may be infected by different *Eimeria* strains at the same time and that different species infect specific parts of the gut. Co-infection with different types of pathogens may evoke simultaneously different pathogen specific T-helper types of response. Depending on the compatibility of these responses, a T-helper conflict or synergy can rise. Here we analyzed the cell-mediated response to a mono-species and multi-species infection with *E.acervulina*, *E.maxima* or *E.tenella* in the duodenum, jejunum and caecum of all animals. As immune parameters we used T-cell population analysis in the intestine, and cytokine expression levels in the intestine. These parameters were related to the level of *Eimeria* infection that was measured in the intestine with an *Eimeria* strain specific quantitative PCR. The results showed that the strongest immune response was induced in the specific part of the intestine that was affected by each *Eimeria* strain. An *E.acervulina* infection mainly induced a $CD8^+$ T-cell response and a IL-18 cytokine response whereas an *E.maxima* infection mainly induced a $CD4^+$ T-cell response and IFN-γ and IL-4 cytokine responses and *E.tenella* induced a $CD4^+$ T-cell response and IL-2 and IL-4 cytokine responses. The different *Eimeria* species gave different cell-mediated responses and a multiple-species infection did not induce any additional or stronger responses. Thus a multi-species infection with *Eimeria* does not lead to a response synergy or a T-helper conflict, but gives rise to a third possibility, namely an accumulation of the results of the mono-species infections.

Key words: chicken, *Eimeria*, multiple-species infection, cytokine, T-cell

Introduction

An infected host can immunologically respond to a pathogen in different manners. The immune response can be classically divided into two different pathways. The T-helper 1 (Th1) immune pathway resolves intracellular pathogens, and the T-helper 2 (Th2) immune pathway which resolves extracellular pathogens. Most often, animals are not solely infected with one pathogen, but with multiple pathogen species at the same time. When the different types of pathogen in a co-infected animal evoke different T-helper types of response, a T-helper conflict can rise. But when the host reacts with the same T-helper types of response to the different pathogens, the co-infection might lead to a response synergy which can enhance the host response (Graham, A.L., 2002). In mice for example a T-helper conflict is described for a co-infection of *Borrelia burgdorferi* and *Babesia microti,* leading to an increase in arthritis severity (Moro, M.H. *et al.*, 2002). Mice co-infected with *Trichuris muris* and *Schistosoma mansoni*, however, acquire the capacity to resolve *T.muris* infection (Curry, A.J. *et al.*, 1995).

In the field broilers may be simultaneously infected by different *Eimeria* species. Eimeria infections are the cause of coccidiosis, which is an intestinal disease. *Eimeria* are host specific, the seven members that infect chickens are *E.acervulina*; *E.brunetti*; *E.maxima*; *E.mitis*; *E.necatrix*; *E.praecox* and *E.tenella*. In intensively reared poultry, like meat producing chickens (broilers), infections with *E.acervulina*; *E.maxima* or *E.tenella* are the most frequently diagnosed. All these *Eimeria* species have site specificity and lesions are found in the intestines at a specific place for each *Eimeria* species. In chickens, *E.acervulina* infects the duodenum, whereas *E.tenella* infects the caeca and *E.maxima* infects the jejunum (Williams, R.B., 2005; Yunus, M. *et al.*, 2005).

In a mono-species infection, the different species of *Eimeria* induce specific host responses at their locations in the GI tract. The T-cell mediated immunity plays a major role in all *Eimeria* infections (Dalloul, R.A. and Lillehoj, H.S., 2005; Lillehoj, H.S., 1998). At the site of *Eimeria* infection a change is rapidly induced in the proportions of T-cell subsets of intraepithelial leucocytes (IEL). The effects of T-cells in protection against *Eimeria* are partially mediated by cytokines. Such cytokine responses can be distinguished into Th1 or Th2 type of responses (Hong, Y.H. *et al.*, 2006a). The Th1 response seems to be dominant during coccidiosis. Th1 cells typically produce IFN-γ , which is found to be involved in *E.acervulina*, *E.maxima* and *E.tenella* infections in birds older than 7 days. A high amount of IFN-γ is involved in parasite killing (Choi, K.D. *et al.*, 1999; Laurent, F. *et al.*, 2001; Yun Cheol, H. *et al.*, 2000). Th2-type cytokines could reduce the level of Th1 cytokines in the gut, performing a regulatory role to reduce immune-mediated damage (Inagaki-Ohara, K. *et*

al., 2006). One of the cytokines to counteract the protective effect of IFN-γ is IL-10 (Rothwell, L. *et al.*, 2004).
The effectors in the response to a mono-species infection are roughly known, but it is not known what the influence of a multi-species *Eimeria* infection is on the immune response in broilers. The aim of this study is to investigate whether the immune response to a multi-species infection with three different types of *Eimeria* leads to a response synergy which can enhance the clearance of the parasite or to a T-helper conflict which exacerbates the disease in the host. To gain insight in the mechanisms of host responses, the cell-mediated response in the duodenum, jejunum and caecum to a mono-species infection with *E. acervulina*, *E. maxima* or *E.tenella* and to a multi-species infection with these three species were analysed. This was done by investigating changes in cell subpopulations and cytokine expression in relation to the local presence of *Eimeria* DNA in the intestine.

Materials and methods

Chickens

A total of 200 one-day-old fast-growing (Ross 308) male broilers were obtained from commercial breeders (Pronk's Broederij, Meppel, the Netherlands). All chickens were immunised against infectious bronchitis (IB) and Newcastle disease virus (NDV) with a spray vaccine (Intervet®, Boxmeer, The Netherlands). At day 7 of age the chickens were randomly divided into five groups. The animals in the control group were orally inoculated with 1 ml phosphate buffered saline (PBS pH 7.4). The animals in the four infected groups were orally inoculated with 1 ml PBS containing 5 x 10^4 sporulated oocysts of *E.acervulina* (Weybridge strain); 1 x 10^4 sporulated oocysts of *Eimeria maxima* (Weybridge strain); 1 x 10^4 sporulated oocysts of *E.tenella* (Houghton strain) or a mix containing 5 x 10^4 sporulated oocysts of *E.acervulina*, 1 x 10^4 sporulated oocysts of *E.maxima* and 1 x 10^4 sporulated oocysts of *E.tenella*. The *Eimeria* doses chosen will lead to pathology but not to mortality. Sporulated *Eimeria* oocysts were provided by the Animal Health Service in Deventer, The Netherlands. Chicks were reared in wire cages to prevent intensive contact with feces. Feed and water were provided ad libitum. The study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

Experimental design and inocula

Five chickens from each group were killed by cervical dislocation at 1, 4, 6, 7, 10, 13, 17 and 20 days p.i.. From these chickens individual body weight was measured and for DNA and RNA extraction one cm of the duodenum, caecum and the jejunum was collected, snap-frozen in liquid nitrogen and stored at -70°C until use. For the immunohistochemistry an adjacent cm of each intestinal part was collected in liquid nitrogen and stored at -70°C until use.

DNA preparation from tissue

In order to quantify parasital DNA about 20 to 40 mg of tissue was homogenized with liquid nitrogen using a mortar and pestle. For the isolation of DNA from the intestinal

tissues the *Chemagic DNA Tissue Kit* (Baseclear, Leiden; The Netherlands) was used according to the manufacturer's manual. After cell lysis, DNA was bound to magnetic beads which were separated from the supernatant using the Chemagic Stand. DNA was eluted from the beads using 100 µl of the elution buffer as provided by the manufacturer. DNA was 10 times diluted for use in a real-time PCR.

RNA extraction and cDNA preparation

The frozen jejunum samples were homogenized with liquid nitrogen using a mortar and pestle. Total RNA was extracted from 50-100 mg of this homogenized tissue with TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's recommendations with minor modifications. The homogenized tissue samples were dissolved in 1 ml of TRIzol reagent per 50-100 mg of tissue using a syringe and 21-G needle passing the lysate for 10 times. After centrifugation the supernatant was transferred to a fresh tube. Subsequently a phase separation with chloroform was performed as described by the manufacturer. The RNA was precipitated using 500 µl 2-propanol. A total of 200 ng RNA was incubated with random hexamers (0.5µg, Promega Benelux BV, Leiden, The Netherlands) at 70°C for 10 min, then reverse transcribed in a final reaction volume of 20 µl containing Superscript RNase H $^-$ reverse transcriptase (200 Units, Invitrogen, Breda, The Netherlands), RNAsin (40 U, Promega Benelux BV, Leiden, The Netherlands), dNTP (2 mM Promega Benelux BV, Leiden, The Netherlands), 5xFirst Strand Buffer (Invitrogen, Breda, The Netherlands) and 0.1M DTT (Invitrogen, Breda, The Netherlands) for 50 min at 37°C. The reaction was stopped by heating at 70 °C for 10 min and the cDNA product was stored at -20°C until use. The cDNA product was 10 times diluted before use.

Real- time PCR

Real-time PCR was employed with on-line detection of the PCR reaction based on fluorescence monitoring (LightCycler, Roche Diagnostics, Mannheim, Germany). Real-time PCR was done as described by Rebel et al. (Rebel, J.M.J. *et al.*, 2005). Primer and probe combinations and PCR conditions are depicted in Table 1. For all cytokine and 28S PCR reactions the same cDNA sample was used in order to standardise and normalise the data.

Normalisation of mRNA concentration

A standard curve was generated from the threshold cycles (C_t) of the standard dilution series by the LightCycler software version 3.5. The standard curve had a slope around -2.97, meaning that the overall reaction efficiency for the standard curve was between 1.0 and 1.3.

The amplified PCR product performed with the LightCycler can be described as $N_n=N_o*E^n$ (Mygind, T. *et al.*, 2001), where N the is concentration of a sample which is identified by a given cycle number, N_o is the starting concentration of the sample, E is the overall reaction efficiency and n is the crossing point in number of cycles. From the cytokine-specific standard curve, the concentrations of the samples were calculated. To normalize the concentration of cytokine cDNA, the sample concentration of each individual chicken was divided by the sample concentration of 28S of the same chicken. The mean was then used of the sample concentrations of five chickens at each time point.

Immunohistochemistry

Immunohistological staining by an indirect immunoperoxidase method was performed on frozen tissue sections (10 µm thick) collected at day 6 p.i.. This date was based on previous results (Swinkels, W.J. *et al.*, 2007). The sections were loaded on glass slides, air-dried, and fixed in acetone for 10 min. After drying, endogenous peroxidase was

Table 1: LC pcr table

Target			Probe/primer sequence (5'-3')	Annealing temp.
IL-2	Probe	IL-2-FL IL-2-LC	CTTGTTGTTAGCTTCACAGATCTTGCATTCACT X CGGTGTGATTTAGACCCGTAAGACTCTTGAG p	58 °C
	Primer	IL2-FOR IL-2-REV	CAGTGTTACCTGGGAGAAGTG GCAGATATCTCACAAAGTTGGTC	
IL-4	Probe	IL-4 FL IL-4-LC	AAACTgAgCTCTTATgCAAAgCCTCC- FL LC640-CAATTgTTTgggAgAgCCAgCACT-p	60°C
	Primer	IL-4 For IL-4Rev	gTgCCCACgCTgTgCTTAC gACgCATgTTgAggAAgAgAC	
IL-8	Probe	IL-8-FL IL-8-LC	CATCCGAAGAAGGCATCATGAAGC- FL TTCCATCTTCCACCTTCCACATCG-p	56 °C
	Primer	IL-8-For IL-8-Rev	ATTCAAGATGTGAAGCTGAC AGGATCTGCAATTAACATGAGG	
IL-18	Probe	IL-18-FL IL-18-LC	CTCCTTCCCTAAATCGAACAACCAT X TCCCATGCTCTTTCTCACAACACAT p	56 °C
	Primer	IL-18-For IL-18-Rev	CGTCCAGGTAGAAGATAAGAG AGGAGTCTTCTTCCTCAAAG	
IFN-γ	Probe	IFN-γ-FL IFN-γ -LC	GAAAGATATCATGGACCTGGCCAAGC-FL CCCGATGAACGACTTGAGAATCCAG-p	55 °C
	Primer	IFN-γ-For IFN-γ--Rev	TTCGATGTACTTGGAAATGC TTGCATCTCCTCTGAGACTG	
28S	Probe	28S –FL 28S–LC	GCGGGTGGTAAACTCCATCT-FL GGCTAAATACCGGCACGAGACCG-p	56 °C
	Primer	28S-For 28S-Rev	CAAGTCCTTCTGATCGAG TCAACTTTCCCTTACGGTAC	
Eimeria acervulina	Primer	Eacer F EacerA	CCTCCgAgTTTCTCACCC CCCCCAAATCACTCgAA	62°C
	probe	Eacer-FL Eacer-LC	gATgACCgCTCgCCTAgTTgT-FL 640-gTCCCCAATgCTCTgTTTgTCgT	
Eimeria maxima	primer	Emax S Emax A	CACCATTggATATgTggAAAAgTTAA CCgAgTCTAgCTTAgCCATAgg	58°C
	probe	Emax FL Emax LC	TgATCCAAgAATgAACgAACTTAgTgCCC-FL 640-TCAATgTTCAgCACATggCAgCAACCC	
Eimeria tenella	primer	Eten F Eten R	ATTTTCTgTgACTggCgTCC GCTCACATACTgCAACAATggA	57°C
	probe	Eten FL Eten LC	gCgTAgATCTgCACACAgggCC-FL 640-gCCAAATACgTCATgAAATgCTTCAAAgAC	

inhibited for 5 minutes at room temperature (2 % NaN_3 in TRIS-HCl 0.05M pH 7.5 + 0.06% H_2O_2). The slides were washed with PBS with 0.1% BSA and were subsequently incubated for 1 h with monoclonal antibodies against monocytes and macrophages (1:568.1; CVI-ChNL-68.1 (Jeurissen, S.H. *et al.*, 1988)), $CD4^+$ T cells (1:200; CT-4, Southern Biotech) or $CD8^+$ T cells (1:200; CT-8, Southern Biotech), followed by peroxidase-conjugated rabbit anti-mouse Ig (1:80; P161, Dakopatts, Denmark). Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M Tris-HCl solution (pH 7.5) containing 0.03% H_2O_2. The sections were further colored with 1% $CoCl_2$ for 5 minutes. After washing the nuclei were counter-stained with hematoxylin. The sections were dehydrated and mounted in Eukitt. The images were acquired and analyzed with Image-Pro Plus (version 5.1, media cybernetics).

Statistics

Data were analyzed for statistical significance using a Student T-test. Data are expressed as the mean ± standard error of the mean (SEM). A P value < 0.05 was taken as the level of significance.

Results

Weight and lesions

All infected animals showed intestinal lesions in the part of the intestine specifically infected by each *Eimeria* species (data not shown). Chickens that received a multi-species infection showed lesions in the duodenum, jejunum and caecum.

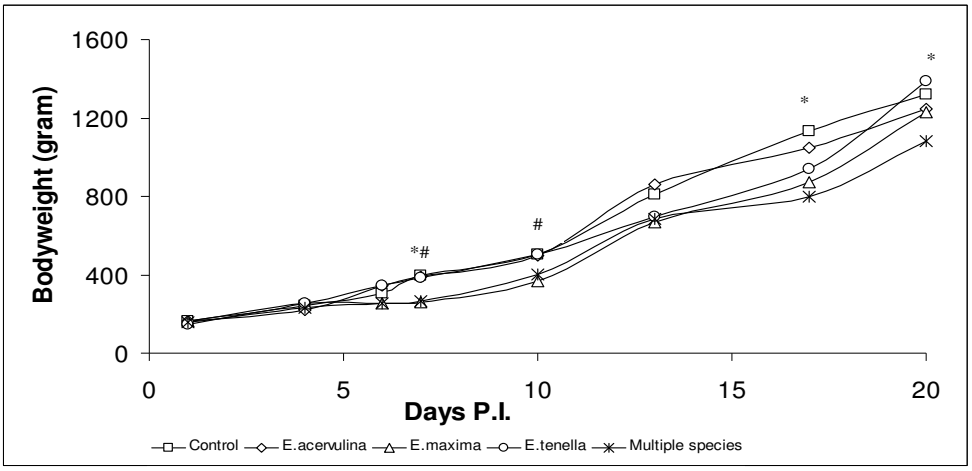

Figure 1: Weight of chickens infected with *E.acervulina*, *E.tenella*, *E. maxima* or a multi-species infection with all three. Mean weight of 5 chickens per group. * $p<0.05$ when the multiple species infection group was compared to the control group. # $p<0.05$ when the *E. maxima* group was compared to the control group.

Weight gain depression was observed from day 7 onwards in the animals that were infected with a multi-species infection or with *E. maxima* only. The weight of the *E. maxima* infected chickens recovered at day 20 while the chickens which received a multi-species infection did not recover their weight over the measured period. There was a small reduction in weight in the *E.tenella* infected animals. No weight gain depression was found in the animals infected with *E. acervulina* (Figure 1).

Presence of Eimeria DNA

The amount of parasitic DNA in the intestine at day 6 p.i. was quantified using a quantitative species-specific PCR. In the multi-species infected chickens *Eimeria* was detected in all parts of the gut, though all species were only found at their specific sites. The amount of species-specific *Eimeria* DNA that was locally present in specific parts of the intestine in chickens with a mono-species infection was similar to the amounts locally observed for the same species in the same specific intestinal segment after a multi-species infection (Figure 2). No *Eimeria* DNA was detected in birds not infected with *Eimeria*. Also only the DNA was detected of the *Eimeria* species that was used for inoculation in the mono-species infected chickens .

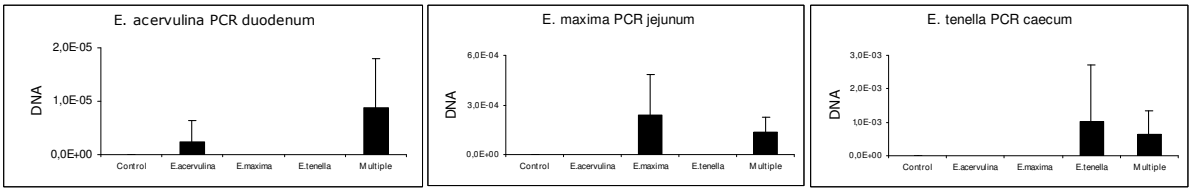

Figure 2: Amount of *E. acervulina*, *E. maxima* or *E. tenella* DNA present in duodenum; jejunum or in ceacum. Amount of DNA is the mean of 5 chickens ± SD at 6 days p.i.

Immunohistochemistry

With immunohistochemistry the amount of $CD8^+$, $CD4^+$ T-cells and macrophages at 6 days p.i. was quantified (Figure 3).

In response to an *E.acervulina* infection $CD4^+$ T-cells were found to increase in the jejunum and a slight increase was also found in the duodenum when compared to control birds (P= 0.06). In the duodenum also the $CD8^+$ T-cells increased after an *E.acervulina* infection.

An *E.maxima* infection caused an increase of $CD4^+$ T-cells in the jejunum, no $CD8^+$ T-cell response was observed.

Due to the *E.tenella* infection the proportion of both $CD4^+$ T-cells and macrophages increased in the caecum.

A multi-species infection caused an increase of $CD4^+$ and $CD8^+$ T-cells in the duodenum and in the caecum. In animals with a multi-species

infection the proportion of macrophages increased in all segments, when compared to the control group.

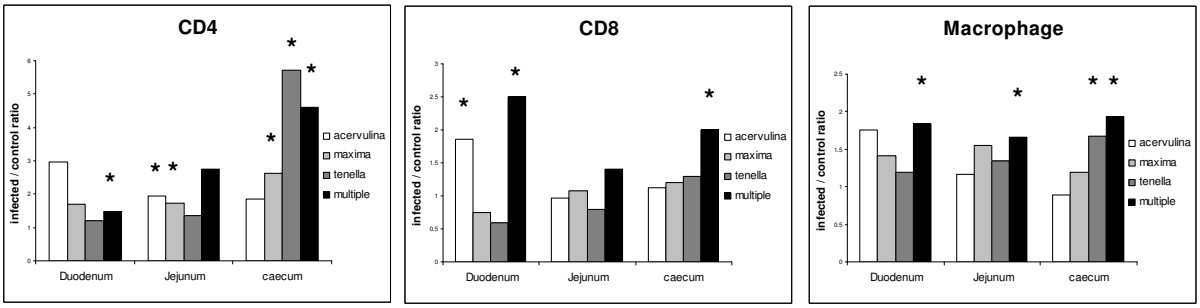

Figure 3: Frequency of $CD4^+$, $CD8^+$ T-cells and macrophages in the intestine.
$CD4^+$, $CD8^+$ or macrophages are immunohistochemically stained and the frequency of positive cells in the intestinal segment is counted. Mean of 25 observations is shown.

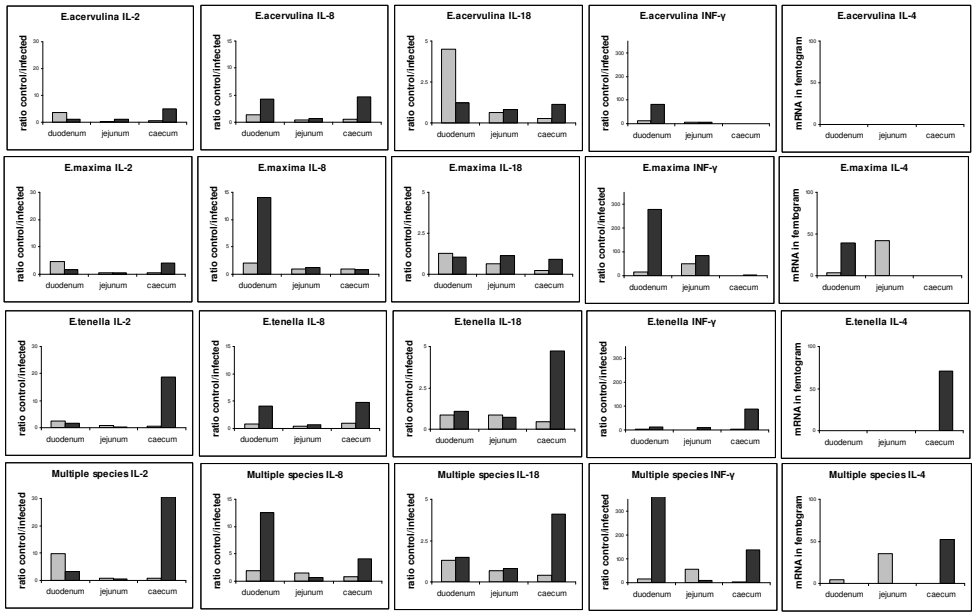

Figure 4: Ratio of cytokine mRNA induction in *E.acervulina*, *E.tenella*, *E.maxima* and multi-species infection at days 4 and 6 post infection. For IL-2, IL-8, IL-18 and IFN-γ ratios are given. Note the difference in scale on the y-axis. For IL-4 the amount of amplified product is given in femtogram. Light columns represent ratio or amount of cytokines at day 4 p.i., dark columns represent day 6 p.i. ratio or level. Mean of 5 chickens is shown.

Cytokine mRNA expression

The induction of cytokine mRNA in the duodenum, jejunum and caecum was analyzed (Figure 4). For IL-2, Il-4, IL-8, IL-18 and INF-γ significant differences were detected between infected groups and control groups. For IL-4 no expression could be detected in the non-infected animals, therefore no ratio of mRNA induction was calculated.

An *E.acervulina* infection caused an increase of the IL-18 mRNA level in the duodenum at day 4 p.i.

No increase of IL-18 mRNA was observed in *E.maxima* infected chickens, while IL-4 induction was observed at days 4 and 6 pi. The *E. maxima* infected group showed the highest up-regulation of IL-8 when compared to the other infected groups. Interferon gamma was regulated by *E.maxima* in both duodenum and jejunum at days 4 and day 6 p.i..

After an *E.tenella* infection a significant increase in IL-2 and IL-4 mRNA induction was observed in the caecum at 6 day p.i.. At day 6 p.i. after an *E.tenella* infection an increase of IFN-γ and IL-18 was also observed in the caecum.

After a multi-species infection a significant increase in IL-2 mRNA induction was observed in the caecum 6 day p.i.. In the multi-species infected group IL-4 induction was found in duodenum, jejunum and caecum. Interferon gamma was regulated by the multi-species infection in both duodenum and jejunum at days 4 and day 6 p.i..

Summary of the results

A summary of the results is given in table 2. An *E.acervulina* infection causes the least amount of changes in the measured parameters when compared to the non-infected group, *E.tenella* causes the strongest reaction. In the multi-species infected animals the reactions are an accumulation of the reactions to a mono-species infection.

Table 2: Overview of reactions on the different infections.

	Acervulina	***Maxima***	***Tenella***	**Multiple-species**
Weight		- d7 pi		- d7 pi
CD4+	+	+	++	++
CD8+	++			++
Macrophage			+	+
IL-2			+ d6 pi	+ d6 pi
IL-4		+ d4 pi	+ d6 pi	+ d4 pi
IL-8	+ d6 pi	++ d6 pi	+ d6 pi	++ d6 pi
IL-18	+ d4 pi		+ d6 pi	+ d6 pi
IFN- γ		++ d6 pi	+ d6 pi	+ d6 pi

Discussion

After a multi-species infection the DNA of the different *Eimeria* species was only found at their specific localization sites in intestinal segments of infection (Williams, R.B., 2002; Yunus, M. *et al.*, 2005). This experiment therefore suggests that there is no change in the site specificity of each *Eimeria* species even when multiple parasite species with different localization patterns were simultaneously administered. Next to that, the amount of *Eimeria* parasite DNA determined in their specific segment per species did not differ between the mono-species infections and in the multi-species infections. It appears that due to the site confinement there is a lack of competition for intestinal space. This agrees with earlier findings by Williams (Williams, R.B., 2001), that no interspecies crowding effect takes place and each species seems able to grow freely, unhindered by other species of *Eimeria* infecting the chicken. A weight loss due to infection was observed only in the mono-species *E.maxima* and the multi-species infection at day 7 p.i.. It is therefore assumed that the weight loss in the multi-species infection may be ascribed to the *E.maxima* infection.

In our experiment, host immune reactions to each *Eimeria* species were found to occur mostly at the site of infection. We observed different induction patterns of $CD8^+$, $CD4^+$ T-cells and macrophages by the different *Eimeria* species. The different *Eimeria* species do not only induce different cellular responses, but related to this are differences in induction of cytokine responses. A mono-species infection with the species *E.acervulina* caused an intermediate $CD4^+$ response at the site of infection and a high increase of $CD8^+$ cells, a mono-species infection with *E.maxima* caused an intermediate increase of $CD4^+$ cells in the jejunum and the caecum and a mono-species infection with *E.tenella* caused a strong increase of $CD4^+$ cells at the site of infection. Though *E.maxima* is not present in the caecum, after an *E.maxima* infection an increase of $CD4^+$ cells was found in the caecum. This increase could be due to T-cell recruitment to the caecal tonsils, the lymphoid structures located at the ileo-colonic junction. Weight loss was found after an *E.maxima* infection, and is probably caused by a damage to the jejunal tissue. Tissue lesions cause T-cells to be unable to gain access to the cells causing the infection, which could be an explanation for the T-cells residing in the caecum after an *E.maxima* infection. The multi-species infection with three species of *Eimeria* led to significant changes in $CD4^+$, $CD8^+$ T-cells in the caecum and to an increased frequency of macrophages in all segments. In the mono-species infections only *E.acervulina* seemed to be able to generate a strong local $CD8^+$ T-cell response, whereas after a multi-species infection the frequency of $CD8^+$ cells is increased both in the lymphoid structure in

the caecal tonsil as well as in the duodenum. These cellular responses seem to reflect an accumulation of the host immune reactions to all three species. Mono-species infections gave a slight non-significant increase of specific cell subpopulations, but in the multiple species infection these effects combined into a significant effect on the level of cell subpopulations.
After an *E.acervulina* infection the clinical effect was minimal. This might suggest that the most prominent cellular response, in the case of *E.acervulina* the $CD8^+$ response, contributed to protection to clinical effects of a primary infection. However, from the current results it cannot be concluded whether the $CD8^+$ T-cell responses that were observed indeed protected against further clinical damage.

Differences in host immune reactions were not only observed in the cellular responses, but also in the cytokine responses. Though most cytokine responses are locally expressed, reactivity was also observed in adjacent parts of the intestine. An increase of IL-8 mRNA was observed in all infections. IL-8 expression is induced by tissue damage and wound healing processes and therefore its presence after *Eimeria* infections which lead to lesions in the gut is expected. The recruiting activity of IL-8 might explain some of the changes in cell distributions.

E.acervulina particularly induced an early IL-18 response in the duodenum. The IL-18 response might be part of an effective protective response, as the *E.acervulina* infection showed the least lesions and effect on weight as compared to the other *Eimeria* strains. Though IL-18 is mainly known as a Th1 mediator and an inducer of IFN-γ , no IFN-γ was induced after the *E.acervulina* infection. The measured cytokine response could therefore also be responsible for other, not measured, reactions. Next to the Th1 activity, IL-18 is also known to take part in a Th2 pathway. It may play a protective role as an inducer of IL-4 and IL-13 in the acute phase of infection (Reuter, B.K. and Pizarro, T.T., 2004).

E.maxima infections led to a local induction of both IFN-γ and IL-4, which did not change the frequencies of $CD4^+$ or $CD8^+$ cells in the intestine. The relatively late IFN-γ production could come from the $CD4^+$ cell population and is a Th1-type of response that was described earlier for *E.maxima* (Hong, Y.H. *et al.*, 2006b). The Th2-type cytokine IL-4, however, might reduce the level of Th1 cytokines in the gut, performing a regulatory role to reduce immune-mediated damage (Inagaki-Ohara, K. *et al.*, 2006). But following *E.maxima* infections the intestinal lesions were severe and resulted in weight depression.

E.tenella particularly induced growth and modulating factors IL-2 (Th1) and IL-4 (Th2). IL-2 is a cytokine that among other things induces the proliferation of T-cells and is critical for the generation of both humoral and cell mediated immunity. In this role IL-2 might be responsible for the enhanced $CD4^+$ frequencies that were found.

The *Eimeria* species that we studied in the present experiments evoked different levels of clinical effects and different immune response characteristics in a mono-species *Eimeria* infection, but in a multi-species infection the responses per intestinal segment displayed a cumulative response as compared to the mono-species primary infections. No additional responses due to a multi-species infection were found. Moro et al. found that after a co-infection the level of IFN-γ decreased, probably regulated by the increased level of IL-4 (Moro, M.H. *et al.*, 2002). In our experiment no such regulation was found. Responses were confined to their specific segment and did not have any regulatory effect on each other. Contrary to the findings in mice by Curry et al., no increased clearance of parasites was observed due to a co-infection (Curry, A.J. *et al.*, 1995). Thus a multi-species infection with *Eimeria* does not lead to a response synergy or a T-helper conflict, but gives rise to a third possibility, namely an accumulation of the results of the mono-species infections. To our knowledge no other co-infections lead to such an accumulation. Most studies on multiple infections however, are not on co-infection, but are on different infections which are not administered at the same time.

In conclusion, host reactions to an *Eimeria* infection were limited to the infection site for each species. Although all species gave a different cell-mediated reaction pattern of the host, no additional major changes in the cell-mediated response were detected in the intestinal segments after a multi-species infection as compared to mono-species infections. Broilers that were infected with a multi-species infection respond per intestinal segment with an accumulation of the responses to the mono-species infections.

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Chapter 4

Immune responses to an *Eimeria acervulina* infection in different broilers lines

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Abstract

The (T-cell) immune responses of two different broiler lines to a primary *E. acervulina* infection were investigated. The lines used are a commercial fast-growing broiler line and a slow-growing type of broiler as used in organic farming.

We infected seven day-old broilers of both lines with 5 x 10^4 oocysts of *E. acervulina*. The animals were weighed and a species-specific real-time PCR was used to quantify total amount of parasites in the duodenum. In the fast-growing line lower parasite load was seen from day 4 onward compared to the slow-growing line. In both lines the intestinal peak of *Eimeria* DNA was observed at day 5 post infection. In the duodenum no increase in $CD4^+$ T-cells was found in both infected lines, but a fast increase in $CD8^+$ T-cells was observed in the fast-growing line. At day 3 p.i. in the slow-growing broilers an IL-18 mRNA response was observed. At day 4 p.i. strong IFN-γ and IL-8 mRNA responses were found in both lines. No IL-4 mRNA responses were found in the duodenum.

In conclusion, it was clear that both lines have different growth rates and control and infected conditions. Based on the kinetics of observed phenomena a primary infection with *E. acervulina* in 7 day old broilers seems to generate an early $CD8\alpha^+$ response in fast-growing broilers compared to the slow-growing broilers. This difference in immune reaction after an *E. acervulina* infection could result in a different *Eimeria* load in the duodenum.

Key words: Eimeria acervulina; Immunology; Real-time PCR; Cytokines; T-cells; Broilers

Introduction

Coccidiosis is an intestinal disease caused by protozoan parasites of the genus *Eimeria* that occurs world wide. *Eimeria* species are known to infect different types of animals

(Augustine, P.C., 2001; Hermosilla, C. *et al.*, 1999; Hnida, J.A. and Duszynski, D.W., 1999; Lyons, E.T. and Tolliver, S.C., 2004; Reeg, K.J. *et al.*, 2005; Renaux, S. *et al.*, 2003; Yunus, M. *et al.*, 2005). Seven types of *Eimeria* are able to infect chickens. The most prevalent *Eimeria* species in broilers in the Netherlands is *E. acervulina* (Graat, E.A. *et al.*, 1998; Peek, H.W. and Landman, W.J., 2003). *Eimeria* species are not only host specific, but also site specific, *E. acervulina* is known to infect the duodenum (Lillehoj, H.S. and Lillehoj, E.P., 2000).

Eimeria infections can be controlled by anticoccidial drugs, however this has resulted in drug resistance (Peek, H.W. and Landman, W.J., 2003; Williams, R.B., 2006). The emergence of drug resistance and the societal pressure against the use of antibiotics is driving coccidiosis research towards vaccine development and breeding of more resistant types of animals (Williams, R.B., 2006). Because broiler chickens are bred mainly for economically important traits such as growth rate and feed conversion, almost no specific selection on disease resistance has occurred (Leshchinsky, T.V. and Klasing, K.C., 2001). This might have resulted in the indirect selection of several unfavorable traits. These indirect selections may result in a decrease in general resistance leading to disease susceptibility or a bad adaptation capacity against enteric disorders. It is beneficial both for welfare and economics to breed broilers with a better resistance against infectious diseases and a better adaptation capacity against for example enteric disorders.

Nowadays consumers become more demanding with aspect to animal husbandry, which has resulted in organic animal husbandry (free-range). Organic husbandry of broilers in Europe requires that broilers have a growing period of at least 81 days before reaching slaughter weight. Thus free-range animals kept in this type of system need to have a slower growth rate. Instead of restricting the food of the animals and thereby slowing the growth, there is a need for genetically different and slow-growing broilers (Fanatico, A.C. *et al.*, 2005). Originating either from present rapid growing birds or from traditional slow-growing birds, these slow-growing breeds might be immunological different from the fast-growing type of broilers (Pinard-Van Der Laan, M.H. *et al.*, 1998).

Host immune reactions to parasites are complex and cell-mediated immunity plays a major role in disease resistance to coccidial infection (Dalloul, R.A. and Lillehoj, H.S., 2005; Lillehoj, H.S., 1998). The composition of different T-cell subsets will depend upon the age of the host, region of the gut and the genetic background of the host (Lillehoj,

H.S. and Chung, K.S., 1992; Lillehoj, H.S. and Trout, J.M., 1996; Swinkels, W.J. *et al.*, 2006). Genetic selection for superior growth affects the cell-mediated immune responses and the ratio of $CD4^+$ to $CD8^+$ T-cells in turkeys (Bayyari, G.R. *et al.*, 1997). In broiler chickens it is also known that selection for high weight gain results in alterations in the immune response to phytohemagglutinin-P (PHA-P) when compared to slower growing lines (Cheema, M.A. *et al.*, 2003).

As *Eimeria* infections present a major problem in the production of chickens it is relevant to investigate how the resistance against this infection is influenced by the selection on slower growing breeds.

Materials and methods

Chickens

A total of 120 one-day-old fast-growing (Ross 308) and 120 one-day-old slow-growing (Hubbard JA 957) male broilers were obtained from commercial breeders (Pronk's Broederij, Meppel, The Netherlands; Kuikenbroederij Morren, Lunteren, The Netherlands). All chickens were immunized against infectious bronchitis (IB) and Newcastle disease virus (NDV) with a spray vaccine (Intervet®, Boxmeer, The Netherlands), and injected against Marek's disease (Fort Dodge, Weesp, The Netherlands). Chicks were reared in wire cages to prevent intensive contact with faeces. At 7 days of age (day 0 of the experiment) chickens of each line were randomly divided into two groups, one control group (n=60) and one experimentally infected group (n=60). The same day the broilers were inoculated. All animals in the control groups were inoculated with 0,5 ml phosphate buffered saline (PBS pH 7.4), the animals in the infected groups were orally inoculated with 0,5 ml PBS containing 5×10^4 sporulated oocysts of *Eimeria acervulina*. Sporulated *E. acervulina* oocysts were provided by the Animal Health Service in Deventer, The Netherlands. Feed and water were provided ad libitum. Both lines were kept under the same conditions, and were used at the same age. Since this setup was an experimental one and did not fit the organic housing requirements, we cannot use the terms organic, biological, or free range and we will further specifically use the term slow-growing line. The study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

Experimental design

On day 0 a group of five chickens from each control group was killed by cervical dislocation before infection. Chickens from all four groups were killed by cervical dislocation at 1, 2, 3, 4, 5, 7, 9, 11, 14 and 17 days post inoculation (p.i.) The number (n) of chickens per group for post mortem analysis was five. From these chickens individual body weight was measured and the duodenal loop was collected, snap-frozen in liquid nitrogen and stored at -70°C, while the adjacent part of the duodenum was collected in cold PBS.

Intraepithelial lymphocyte isolation

About five cm duodenum adjacent to the duodenal loop was opened longitudinally, washed with PBS and cut into pieces of 1 cm. These pieces were incubated at 37° for 45 minutes in Medium I (PBS containing 1mM EDTA and 5mM DTT). The suspension thus obtained held mainly intraepithelial lymphocytes (IEL) and was kept at 4°C until use. The

remaining pieces of intestine were further incubated at 37°C for 90 minutes in medium II (RPMI + 5% fetal calf serum + 400 FALGPA units Collagenase per liter (Sigma, St Louis, MO, USA) + 60000 Kunitz units DNase I per liter (Sigma, St Louis, MO, USA)) while shaking. After incubation the suspension mainly contained lamina propria lymphocytes (LPL). Because the isolations did not yield pure IEL or LPL fractions, both isolates were mixed. After centrifugation the pellet was resuspended in 10 ml Medium III (RPMI + 1% Fetal calf serum + 60000 Kunitz units DNase I per liter). The suspension was purified on a 25% percoll (Sigma, St Louis, MO, USA) gradient centrifuging for 15 minutes at 2000 rpm. The pellet was washed twice with PBS and cells were resuspended in 20 x 10^6 cells per ml.

Flow cytometry and antibodies

In the total leukocyte isolates the proportions of lymphocyte subpopulations were estimated by flow cytometry. For the flow cytometric analysis 50 µl of cell suspension was transferred into a 96 well plate on ice. Cells were washed with PBS supplemented with 1% FCS. Normal mouse serum (1%) was applied to block non-specific binding. Cells were stained by one of the monoclonal combinations CD3-PE / CD4-FITC; TCR$\gamma\delta$-PE / CD8α-FITC; CD45-PE / Ia-FITC (Southern Biotechnology Associates, Birmingham). Ia will further be referred to as MHC II^+.
After 15 min incubation at 4°C, the cells were washed twice with PBS/FCS and re-suspended in 200 µl ice-cold PBS/FCS. A total of 2 x 10^4 cells per sample was analyzed by flow cytometry (FACS Calibur™, Beckton Dickinson, Leiden, The Netherlands, and a life gate was set for the lymphocyte population in the forward and side scatter plot. The data was analyzed using a flow cytometry computer program (CellQuest™ version 3.3).

RNA extraction and cDNA preparation

The duodenum samples were homogenized with liquid nitrogen using a mortar and pestle. Total RNA was extracted from 50-100 mg of this homogenized tissue with TRizol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's recommendations with minor modifications. The homogenized tissue samples were dissolved in 1 ml of TRizol reagent per 50-100mg of tissue using a syringe and 21-G needle passing the lysate for 10 times. After centrifugation the supernatant was transferred to a fresh tube. Subsequently a phase separation with chloroform was performed as described by the manufacturer. The RNA was precipitated using 250 µl high salt buffer (0,8 M Sodium Citrate and 1,2 M NaCl in Diethyl Pyrocarbonate (DEPC) treated water) and 250 µl 2-propanol. 200ng RNA was incubated with random hexamers (0.5µg, Promega Benelux BV, Leiden, The Netherlands) at 70°C for 10 min, then reverse transcribed in a final reaction volume of 20 µl containing Superscript RNase H^- reverse transcriptase (200 Units, Invitrogen, Breda, The Netherlands), RNAsin (40 U, Promega Benelux BV, Leiden, The Netherlands), dNTP (2 mM Promega Benelux BV, Leiden, The Netherlands), 5xFirst Strand Buffer (Invitrogen, Breda, The Netherlands) and 0.1M DTT (Invitrogen, Breda, The Netherlands) for 50 min at 37°C. The reaction was stopped by heating at 70 °C for 10 min and the cDNA product was stored at -20°C until use. The cDNA product was 10 times diluted before use.

DNA preparation from tissue

About 20 to 40 mg of tissue was homogenized with liquid nitrogen using a mortar and pestle. For the isolation of DNA from the intestinal tissues the *Chemagic DNA Tissue Kit* (Baseclear, Leiden; The Netherlands) was used according to the manufacturer's manual. After cell lysis, DNA was bound to magnetic beads which were separated from the supernatant using the Chemagic Stand. DNA was eluted from the beads using 100 µl of the elution buffer as provided by the manufacturer.

Real- time PCR

Real-time PCR was employed with on-line detection of the PCR reaction based on fluorescence monitoring (LightCycler, Roche Diagnostics, Mannheim, Germany). Primer and probe sequences were selected with the software from TIB MolBiol (Berlin, Germany).
Hybridization probes (TIB MolBiol, Berlin, Germany) were used to monitor the amount of specific target sequence product.

Table 1: Primer and probe sequences as used in the real-time PCR.

Target			**Probe/primer sequence (5'-3')**	**Annealing temp.**
IL-4	Probe	IL-4 FL	AAACTgAgCTCTTATgCAAAgCCTCC- FL	60°C
		IL-4-LC	LC640-CAATTgTTTgggAgAgCCAgCACT-p	
	Primer	IL-4 For	gTgCCCACgCTgTgCTTAC	
		IL-4Rev	gACgCATgTTgAggAAgAgAC	
IL-8	Probe	IL-8-FL	CATCCGAAGAAGGCATCATGAAGC- FL	56 °C
		IL-8-LC	TTCCATCTTCCACCTTCCACATCG-p	
	Primer	IL-8-For	ATTCAAGATGTGAAGCTGAC	
		IL-8-Rev	AGGATCTGCAATTAACATGAGG	
IL-18	Probe	IL-18-FL	CTCCTTCCCTAAATCGAACAACCAT- FL	56 °C
		IL-18-LC	TCCCATGCTCTTTCTCACAACACAT-p	
	Primer	IL-18-For	CGTCCAGGTAGAAGATAAGAG	
		IL-18-Rev	AGGAGTCTTCTTCCTCAAAG	
IFN-γ	Probe	IFN-γ-FL	GAAAGATATCATGGACCTGGCCAAGC-FL	55 °C
		IFN-γ-LC	CCCGATGAACGACTTGAGAATCCAG-p	
	Primer	IFN-γ-For	TTCGATGTACTTGGAAATGC	
		IFN-γ--Rev	TTGCATCTCCTCTGAGACTG	
28S	Probe	28S –FL	GCGGGTGGTAAACTCCATCT-FL	56 °C
		28S–LC	GGCTAAATACCGGCACGAGACCG-p	
	Primer	28S-For	CAAGTCCTTCTGATCGAG	
		28S-Rev	TCAACTTTCCCTTACGGTAC	
Eimeria acervulina	Probe	Eacer-FL	gATgACCgCTCgCCTAgTTgT-FL	62°C
		Eacer-LC	gTCCCCAATgCTCTgTTTgTCgT-p	
	primer	Eacer-For	CCTCCgAgTTTCTCACCC	
		Eacer-Rev	CCCCCAAATCACTCgAA	

For is forward; Rev is reverse; FL is Fluorescein; LC is light cycler red 640.

Quantitative results were determined as described by Loeffler *et al* (Loeffler, J. *et al.*, 2000). For the quantification a standard curve of the plasmid with the insert of interest was used, constructed in PGEM-T easy (Promega). PCR reactions were performed according to the LightCycler kit instructions (FastStart DNA Master Hybridization Probes, Roche Diagnostics, Mannheim, Germany). Real-time PCR was done as described by Rebel et al. (Rebel, J.M.J. *et al.*, 2005). Primer and probe combinations and PCR conditions are depicted in Table 1. To avoid contamination, filter pipette tips were used and reagents were mixed in rooms separate from rooms where DNA was present. A negative control that contained reagents only and a standard dilution series (cytokine and 28S) was

included in each run. For all cytokine and 28S PCR reactions the same cDNA sample was used in order to standardize and normalize the data.

Normalization of mRNA concentration

A standard curve was generated from the threshold cycles (C_t) of the standard dilution series by the LightCycler software version 3.5. The standard curve had a slope around -2.97, meaning that the overall reaction efficiency for the standard curve was between 1.0 and 1.3.

The amplified PCR product performed with the LightCycler can be described as $N_n=N_o*E^n$ (Mygind, T. *et al.*, 2001), where N the is concentration of a sample which is identified by a given cycle number, N_o is the starting concentration of the sample, E is the overall reaction efficiency and n is the crossing point in number of cycles. From the cytokine specific standard curve, the concentrations of the samples were calculated. To normalize the concentration of cytokine cDNA, the sample concentration of each individual chicken was divided by the sample concentration of 28S of the same chicken. The mean was then used of the sample concentrations of five chickens at each time point.

Statistics

Weights, interleukin data and *Eimeria* load were analyzed with an analysis of variance model. The model comprised main effects for factors day (e.g. 11 levels for days 0, 1…5, 7, 9, 11 and 17 for weight), treatment (control or infected) and line (fast-growing or slow-growing). Residual plots for weight indicated that the variance increased with the mean. Therefore, log transformed weights were analyzed. Consequently, effects of the aforementioned experimental factors were multiplicative for weight on the original scale.

For weight the final analysis comprised linear and quadratic curves in time (days), replacing the initial factor with 11 levels for days. Profiles for interleukin and *Eimeria* load included less time points, they were more irregular and could not be described by simple curves.

Log transformation was applied for a number of variables. Prior to log transformation, any observation equal to 0 was replaced by half of the lowest positive observation among the data.

Overall tests were performed with the F-test. In addition, means were compared pairwise by Fisher's LSD method (Rao, 1965).

Percentages in the FACS data often approached 0 or 100%. Therefore, most of the variables for the FACS data were analyzed with a generalized linear model (GLM), comprising a logit link function and the binomial variance function with a multiplicative dispersion parameter. Effects for days, treatments and lines were introduced on the logit scale and estimated by maximum quasi-likelihood. The dispersion parameter was estimated from Pearson's chi-square statistic. For details we refer to (McCullagh, N., 1989) (Sections 4.3 and 4.5 and Ch. 9).

All calculations were performed with GenStat (2000).

Results

Body weight

The weight differed between the lines from day 1 p.i. onward (Figure 1). The average weight gain of the fast-growing broilers between day 0 and 17 p.i. was 917,2 gram, whereas the average weight gain of the slow-growing broilers was only 638 gram. This is an average weight gain per

day of 53,95 gram in the fast-growing line and 37,53 gram in the slow-growing line respectively.
The bodyweight of slow-growing broilers was not affected by an E. acervulina infection, whereas at day 3 p.i. a significant lower bodyweight was seen in infected fast-growing broilers.

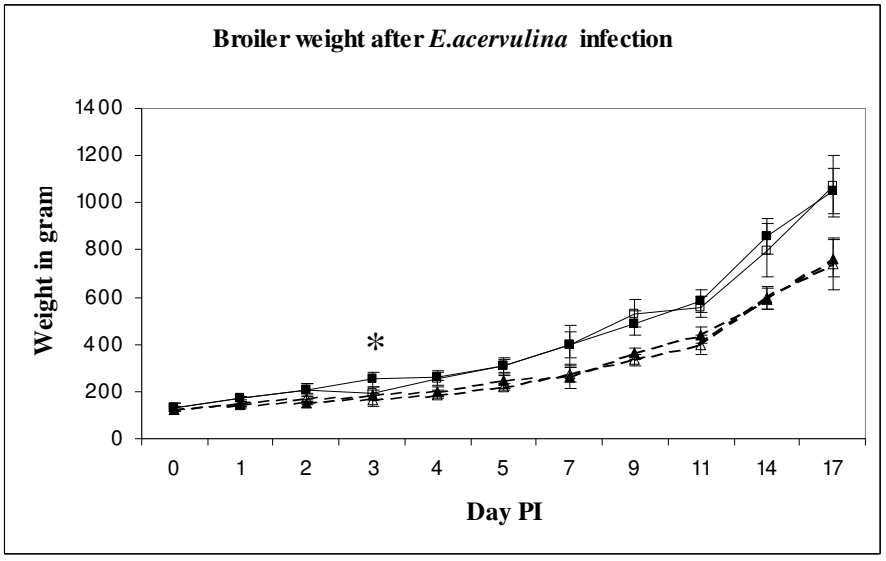

Figure 1: Body weight of four groups of broilers.
Seven day old fast- or slow-growing broilers were mock infected or infected with infected with 5 x 10^4 sporulated oocysts of *Eimeria acervulina*. Body weight as depicted is the mean weight of 5 animals ± SEM. *= Significant difference between control and infected group (P≤0.05).

Presence of Eimeria acervulina in the duodenum

An *E. acervulina* infection in fast- and slow-growing lines resulted in the presence of *Eimeria* DNA in the duodenum as determined by real-time PCR (Figure 2). At day 3 p.i. the infected groups of both lines contained an similar amounts of *E. acervulina* DNA in a sample of the duodenum. At day 4 p.i. the amount of *E. acervulina* DNA has increased in the duodenum of the slow-growing broilers. Over time the slow-growing broilers have a significantly higher load (P=0.04) of *E. acervulina* as compared to the fast-growing broilers. From day 4 to 7 however, both lines displayed the same kinetics of parasite growth. In the control animals no *E. acervulina* DNA was detected in the duodenum (data not shown).

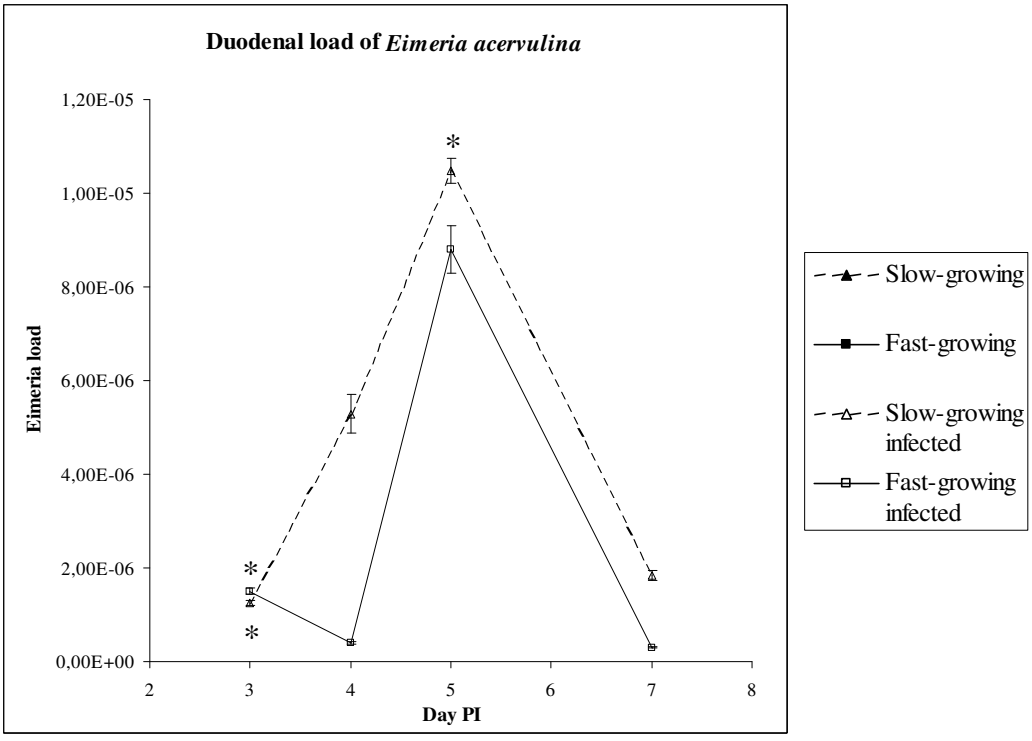

Figure 2: Amount of *Eimeria acervulina* present in the duodenum after inoculation with 5 x 10^4 sporulated oocysts. Amounts are the mean of 5 samples. *= Significant difference between control and infected group (P≤0.05).

Flow cytometric analysis of intestinal cells

Cell populations were analyzed as a percentage of gated intestinal lymphocytes from the duodenum. Figure 3 shows from both lines the infected/ control ratio of $CD3^+$, $CD4^+$, $CD8\alpha^+$, $CD45^+$, $MHCII^+$, $TCR\gamma\delta^+$ and $CD8\alpha^+TCR\gamma\delta^+$ cells from day 0 to day 17 p.i.. Significant differences between infected and control group are depicted. The percentage of $CD45^+$ cells in the duodenum is comparable in both lines and does not change throughout the experiment. With time the infected groups develop a higher percentage of $CD45^+$ cells than their controls. During the experiment the percentage of $CD3^+$ cells in both lines, both in control and infected animals increased. In the fast-growing infected animals however, this increase is significantly higher, compared to the fast-growing control group. The $CD8\alpha^+$ percentage showed similar increase during the course of the experiment in both infected groups as compared to their controls. A fast increase of the $CD8\alpha^+$ percentage was found on days 2 and 4 in the fast-growing infected group when compared to the fast-growing control group. Though the increase in the slow-growing infected broilers is not significant when compared to their controls, their levels show the same kinetics as the $CD8\alpha^+$ percentage in the fast-growing broilers. The ratio (infected / control birds) of $CD8\alpha^+$levels is similar for both lines. Contrary to the $CD8\alpha^+$ percentage, the $CD4^+$ percentage does not show changes between lines and treatments during the course of the experiment. The percentage of $MHCII^+$ cells increased during the course of the experiment. No differences were observed between lines, the infected fast-growing broilers have a significantly higher percentage of $MHCII^+$ cells as compared to their fast-growing controls. The percentage of the $TCR\gamma\delta^+$ cells increased from day 7 to 14 p.i. due to infection in both lines. The percentage of $TCR\gamma\delta^+CD8\alpha^+$ cells is the highest in the infected fast-growing broilers .

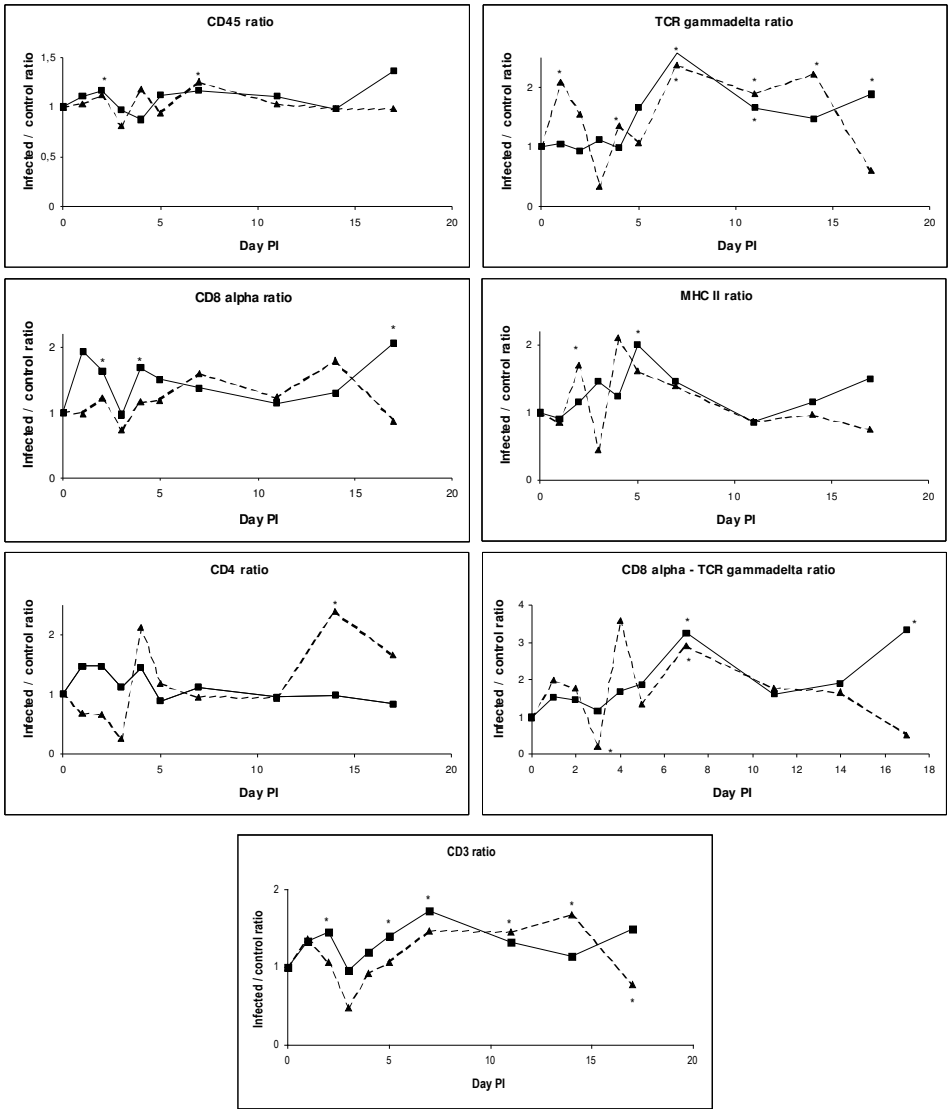

Figure 3: FACS analysis of cell populations isolated from duodenum of broilers infected with PBS (control) or 5 x 10^4 sporulated *Eimeria acervulina* oocysts. The percentage of $CD3^+$, $CD4^+$, $CD8^+$, $TCR\gamma\delta^+$, MHC II^+, $CD45^+$ and $CD8^+TCR\gamma\delta^+$ cells are the percentages of gated lymphocytes. Data is displayed as a ratio of infected / control. Triangles represent the ratio of Slow-growing infected divided by the Slow-growing control animals. Squares represent the ratio of Fast-growing infected divided by the Fast-growing control animals. Values are the mean of 5 chickens. *= Significant difference between control and infected group ($P \leq 0.05$). Note the difference in the scales.

Cytokine mRNA expression

After an *E. acervulina* infection a significant increase in the amount of IL-18 mRNA was found at day 3 p.i. in the slow-growing broilers (Figure 4). At day 4 p.i. an IFN-γ mRNA increase was found in both lines. Both infected lines also showed an increase of IL-8 mRNA at day 4 post infection as compared to controls. At 7 p.i., slow-growing broilers had a higher IL-8 level than infected fast-growing broilers. No IL-4 responses was detected (data not shown).

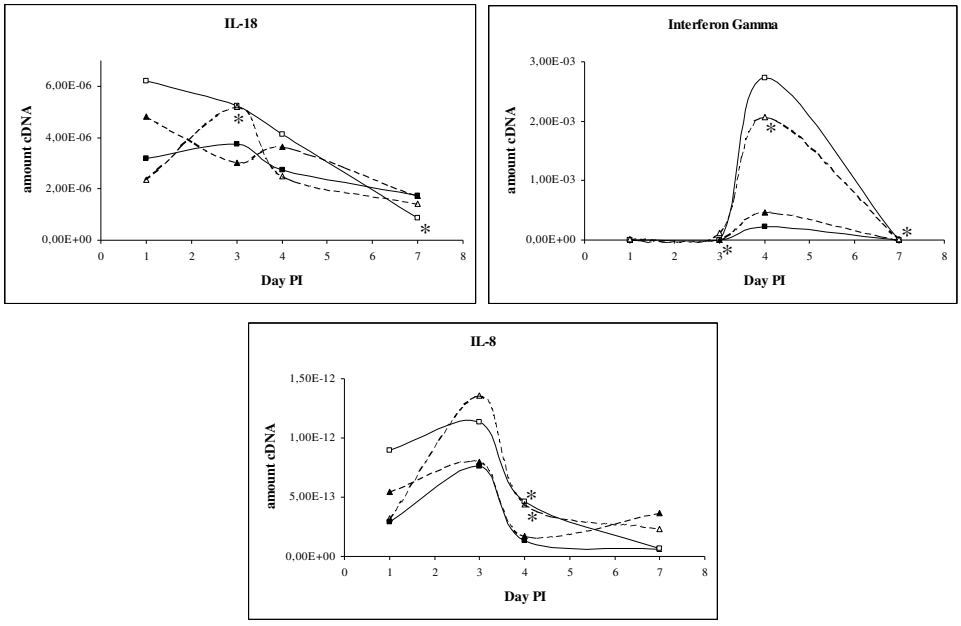

Figure 4: Mean relative amounts of cytokine mRNA ±SEM of 5 control chickens or 5 chickens infected with 5 x 10^4 oocysts from both broiler lines. Note the difference in the scales. Values are means of 5 chickens. *= Significant difference between control and infected group (P≤0.05).

Discussion

A clear difference in growth characteristics was observed between fast- and slow-growing broilers. After an *Eimeria acervulina* infection, no difference in weight gain was observed within the lines between infected chickens and control animals.

The dynamics of the frequencies of the T-cell subpopulations after an *E. acervulina* infection were comparable in both broiler lines. Contrary to experiments by Choi et al. (Choi, K.D. *et al.*, 1999) no increase in the $CD4^+$ T-cells was observed after infection. However, the T-cells expressing CD3, CD8α or TCR$\gamma\delta$ antigens were upregulated. The upregulated cell types showed the same biphasic increase in frequency as described in earlier experiments with *E.maxima* (Rothwell, L. *et al.*, 1995). Though part of the changes in the T-cell populations may be related to the development of the immune system in young animals, even at day 14 p.i. the elevation of both $CD8\alpha^+$ and $TCR\gamma\delta^+$ T-cells was still detected in the infected animals when compared to control animals. But at this time no *E. acervulina* DNA could be detected in the duodenum by means of real-time PCR. This indicated that a single infection was able to induce a change in the intestinal immune system of both lines till at least 14 days post infection. Whether these changed T-cell compositions have a function in protection to re-infections remains to be investigated.

$TCR\gamma\delta^+$ cells mediate specific cellular immune functions without the requirement for antigen degradation and presentation and thereby are able to directly recognize invading pathogens or damaged cells (Schild, H. *et al.*, 1994). Therefore $TCR\gamma\delta^+$ cells are considered part of an innate response. However, in this experiment their up-regulation is a relatively late response as compared to the kinetics of the parasite presence in the gut. $CD8\alpha^+$ cytotoxic T-cells play an important role during the recovery phase of infections. Therefore the increase in $CD8\alpha^+$ T-cells may reflect enhanced acquired immune status (Lillehoj, H.S., 1994). In this experiment infection in both lines resulted in an increase of $CD8\alpha^+$ T-cells. The level of $CD8\alpha^+$ T-cells like the $TCR\gamma\delta^+$ level remained elevated until the end of the experiment. The increase of $CD8\alpha^+$ T-cells occurred simultaneously to the increase of the $TCR\gamma\delta^+$ T-cells. This is in agreement with earlier studies described by Bessay et al. and Swinkels et al. (Bessay, M. *et al.*, 1996; Swinkels, W.J. *et al.*, 2006). They observed simultaneous increases of both $CD8\alpha^+$ and $TCR\gamma\delta^+$ T-cells in the duodenum after an *E. acervulina* infection. A double labeling experiment was performed which proved that the $TCR\gamma\delta^+$ and $CD8\alpha^+$ markers do not only display a parallel increase, but are found on the same cells.

The cytokines that were elevated after an *E. acervulina* infection indicated that the reaction showed a tendency toward a cell-mediated profile. IL-8 is a pro-inflammatory cytokine that is able to attract chicken T-cells (Lam, K.M., 1999). In this experiment elevated levels of IL-8 mRNA were observed in infected fast-growing broilers at day 1 p.i. and in both infected lines at day 3 post infection. This could have recruited the $CD8\alpha^+$ T-cells. The fluctuations in IL-8 level in the non infected chickens probably resulted from a normal development of the intestine (Uni, Z. *et al.*, 2000). IL-18 is known to stimulate the development of intestinal intra-

epithelial lymphocyte populations and can be induced in macrophages 8h after *E. acervulina* exposure (Dalloul, R.A. *et al.*, 2007). Here the elevation of IL-18 was found at day 3 p.i.. in the infected slow-growing broilers. It is not known whether macrophages are the source of this IL18. IFN-γ is considered an important component of host cell mediated immunity (CMI) during coccidiosis and regulates acquired immunity by activating lymphocytes (Kaspers, B. *et al.*, 1994). IFN-γ is known to be differentially expressed following infection with different *Eimeria* species. After an infection with *E. acervulina*, Choi et al. found an increase of IFN-γ in the caecum, but almost none in the duodenum (Choi, K.D. *et al.*, 1999). In this experiment we provide evidence that the increase of IFN-γ also occurs in the duodenum in seven day old fast-growing broilers infected with *E. acervulina*. In an earlier experiment we found that the IFN-γ response is absent in animals infected at one day of age (Swinkels, W.J. *et al.*, 2006), but the same strain of broilers infected at 7 days of age respond as shown here. Therefore vulnerability to *Eimeria* infections may be dependent on age and developmental stage of the immune system of the animal investigated.

Both broiler lines displayed similar late responses after an *E. acervulina* infection. The immunological differences therefore are observed during the early phases of the response. In contrast to the slow-growing broilers, the fast-growing broilers have early IL-8, IL-18, TCR$\gamma\delta^+$, CD3$^+$ and CD8α^+ responses. Altogether these responses could be the cause of the of the lower amount of *E. acervulina* DNA found in the duodenum of the infected fast-growing broilers as shown by real-time PCR. The differences in part might be ascribed to differences in kinetics of development of the immune system in the broiler lines that were compared.

In conclusion we now have evidence that fast- and slow-growing broilers lines have different early responses to an *E. acervulina* infection, which coincide with a different *Eimeria* load in the duodenum. Late immune responses are similar in both lines, last at least 14 days p.i. and are of cellular character. Finally, it seems that apart from the genetic background, the developmental stage of the immune system influences the host response to an *E. acervulina* infection in young chickens.

Acknowledgments: We thank our animal care takers for their technical support.

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Chapter 5

Immune reactions in one-day-old, seven-day-old and twenty-one-day-old broilers after primary infection with *Eimeria maxima*

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W.J.A. Boersma, J.M.J. Rebel

Abstract

Young animals have an immature immune and gastrointestinal (GI) tract system. It is therefore likely that the nature of immune responses generated in the GI –tract at young age are different from those in older animals. Here the (T-cell) immune responses of broilers of different ages, following a primary *E.maxima* infection were investigated. Broilers of one day, seven days or twenty-one days of age were infected with 2 x 10^4 sporulated oocysts of *Eimeria maxima*. The presence of parasites in the jejunum was quantified by a species-specific real-time PCR. In addition the number of *Eimeria maxima* parasites in the feces was determined by microscopy. Frequencies of T-cell subsets in the jejunum and expression of mRNA of cytokines (IFN-γ, IL-4, IL-10) were determined.

In general, it was concluded that the strength and kinetics of immune responses to a primary *E.maxima* infection increased with the age of the broilers. With increasing age the broilers were able to respond earlier after infection with a stronger influx of T-cells and a higher production of cytokines measured as specific mRNAs. This was particularly demonstrated by the frequencies of $CD8^+$ cells and accompanying cytokine profiles. One-day-old birds tended to respond in a Th2 mode, but as the age of the birds increased the responses to an *Eimeria* infection became more Th1 biased. Interestingly, these age-related differences in immune reactivity were not associated with a difference in protection from a secondary infection. All birds infected at day one or day seven of age were fully protected to a secondary infection with *E.maxima*.

Key words: Chicken, *Eimeria*, ageing, immunology, infection, T-helper immunity

Introduction

Age plays an important role in the development of immunity. Neonates are not fully immune deficient, but certain features of the immune system and the GI-tract are immature (Adkins, B. *et al.*, 2004). In order to be able to improve the effect of vaccination and of anti-microbial therapies it is of importance to understand the basic cellular immune mechanisms in young and older animals. For infections such as *Mycoplasma gallisepticum* and the tick *Argas persicus* it was described that the age of birds is of influence on the clearance of an infection (Dusbabek, F. *et al.*, 1994; Gaunson, J.E. *et al.*, 2006; Lillehoj, H.S. and Chung, K.S., 1992). However, age-related differences towards important parasitic infections such as *Eimeria* spp. are not well documented.

Coccidiosis is among the most commonly reported diseases in chickens. Coccidiosis is a disease with a worldwide distribution, that is caused by protozoan parasites of the genus *Eimeria*. *Eimeria* infections occur in a wide range of animals including chickens (Williams, R.B., 2002). In various studies the immune responses were described for *Eimeria* infections in chickens, but age was not used as a variable parameter. The immature small intestines of a newly hatched chick go through various changes during the first days after hatch, such as increase of gut length and surface and development and differentiation of the Gut Associated Lymphoid Tissue (GALT) (Uni, Z. *et al.*, 2000). Villus length and surface area are smaller in young birds as compared to villus-size mature animals (Geyra, A. *et al.*, 2001). The maturation of the GALT occurs in two stages or waves. The primary wave arises during the first week post-hatch and a second wave occurs during the second week (Bar-Shira, E. *et al.*, 2003). Therefore in chicks infected with *Eimeria* at young age the mechanisms of the immune response induced in the gut may differ dependent on the stage of development of the gut and GALT until these functions are fully developed.

In general, homologous protective immunity is established after an *Eimeria* infection. However, the character of the cellular mechanisms and the relationship with age-related development of the chicken immune system until now remained unclear. It was suggested that the immune response to an *Eimeria* infection is mostly cell mediated (Dalloul, R.A. and Lillehoj, H.S., 2005). T-cells carrying the alpha-beta T-cell receptor seemed to be involved in protection of the host against primary infection, whereas gamma-delta T-cells, which are mostly found within epithelia of the GALT, might play a role in repair of lesions and in response regulation (Roberts, S.J. *et al.*, 1996). Gamma-delta cells occur more frequently in the gut of healthy non-infected young mice than in healthy adult mice (Hayday, A.C. *et al.*, 2000). This could also be the case in chickens.

It was assumed that the effects of T-cells in protection against *Eimeria* are partially mediated by cytokines. Such cytokine responses can be distinguished into Th1- or Th2 type of responses (Hong, Y.H. *et al.*, 2006a). The Th1 immune pathways resolve infections with intracellular pathogens and the Th2 immune pathways resolve infections with extracellular pathogens. Th1 cells typically produce IFN-γ. IFN-γ is involved in parasite killing. The Th2-type cytokines modulate the level of Th1 cytokines in the gut and perform a regulatory role to reduce damage resulting from Th1 immune responses (Inagaki-Ohara, K. *et al.*, 2006). Though it recently was shown that three-week old chickens are able to mount both Th1- and Th2-biased cytokine responses (Degen, W.G. *et al.*, 2005) it is not known whether such responses are balanced as in the matured situation. Neonatal mice express a Th2 bias during all phases of the immune response. Whether this is also the case in chickens remains to be investigated (Adkins, B. *et al.*, 2001).

To address the effect of age-related maturation of the immune system on the chicken response to parasites we compared the immune responses of young broilers of different ages and with different stages of GALT maturation. The broilers were one-day-old, seven-day-old or twenty-one-day-old, respectively, and were exposed to a primary *E.maxima* infection. Two features of the immune response were studied. First, differences in mRNA transcripts Th1 cytokine interferon gamma (IFN-γ), Th2 cytokines interleukin-4 (IL-4) and IL-10 were studied. In addition, the changes in the T-cell subpopulations that expressed $CD4^+$, $CD8^+$, TCRαβ and TCRγδ were studied. Using real-time PCR the relative amounts of parasitic DNA at the site of infection were determined.

Materials and methods

Chickens

A total of 155 one-day-old Ross 308 male broilers were obtained from a commercial breeder (Pronk's Broederij, Meppel, The Netherlands). All chickens were immunised against infectious bronchitis (IB) and Newcastle disease virus (NDV) with a spray vaccine (Intervet®, Boxmeer, The Netherlands), and vaccinated against Marek's disease (Fort Dodge, Weesp, The Netherlands).

At day of hatch (day 1), the chickens were randomly divided into six groups. One group was inoculated with PBS (0,5 ml phosphate buffered saline, pH 7.4) at day 1 of age (control day 1; n=30) and one group was orally inoculated with 0,5 ml PBS containing 2×10^4 sporulated oocysts of *Eimeria maxima* (Weybridge strain) at day 1 of age (infected day 1; (n=25). A third group was inoculated with PBS at day 7 of age (control day 7; n=30) and group 4 was inoculated with 2×10^4 sporulated oocysts of *Eimeria maxima* at day 7 of age (infected day 7; n=25). Group 5 was inoculated with PBS at day 21 of age (control day 21; n=25) and group 6 was inoculated with 2×10^4 sporulated oocysts of *Eimeria maxima* at day 21 of age (infected day 21; n=20). All groups were housed in separate cages to prevent cross-infection.

Five broilers of the groups that were inoculated at day 1 or day 7 of age were reinfected with 2 x 10^4 sporulated oocysts of *Eimeria maxima* at 14 days post primary infection.

Sporulated *E. maxima* oocysts were provided by the Animal Health Service in Deventer, The Netherlands. Chicks were reared in wire cages to prevent intensive contact with feces. Feed and water were provided ad libitum. The study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

Experimental design and inocula

From each control group five chickens were killed by cervical dislocation right before inoculation. Five chickens from each of the six groups (control and infected at different ages) were killed by cervical dislocation at 1, 2, 4, and 7 days post infection (p.i.). From the chickens killed individual body weight was measured and one cm of the jejunum next to the Meckel's diverticulum was collected, snap-frozen in liquid nitrogen and stored at -70°C until use. From this frozen section of jejunum DNA was extracted in order to quantify the amount of parasitic DNA by means of real-time PCR, whereas RNA was extracted in order to quantify the mRNA expression of cytokines with real-time PCR.
For the immunohistochemistry an adjacent part of the jejunum was collected in liquid nitrogen and stored at -70°C until use.
The groups challenged at 14 days post primary infection were kept for collection of fecal samples at day 7 post secondary infection.

Fecal sample collection, processing and oocyst counts

Fecal samples were either collected after spontaneous defecation during weighing, or by emptying the intestinal tract during the postmortem examination. To determine the number of oocysts per gram of droppings (OPG), the McMaster counting chamber technique according to Long and Rowell (Long, P.L., and J. G. Rowell., 1958) was used. Oocysts were counted in the McMaster counting chamber using a light microscope under 10 x 10 magnification.

DNA preparation from tissue

About 20 to 40 mg of tissue was homogenised in liquid nitrogen using a mortar and pestle. For the isolation of DNA from the intestinal tissues the *Chemagic DNA Tissue Kit* (Baseclear, Leiden; The Netherlands) was used according to the manufacturer's manual. After cell lysis, DNA was bound to magnetic beads which were separated from the supernatant using the Chemagic Stand. DNA was eluted from the beads using 100 µl of the elution buffer as provided by the manufacturer. Quality and quantity of the DNA was measured with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies).

RNA extraction and cDNA preparation

The frozen jejunum samples were homogenised in liquid nitrogen using a mortar and pestle. Total RNA was extracted from 50-100 mg of this homogenised tissue with TRizol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's recommendations with minor modifications. The homogenised tissue samples were dissolved in 1 ml of TRizol reagent per 50-100mg of tissue using a syringe and 21-G needle passing the lysate for 10 times. After centrifugation the supernatant was transferred to a fresh tube. Subsequently a phase separation with chloroform was performed as described by the manufacturer. The RNA was precipitated using 500 µl 2-propanol. A total of 200ng RNA was incubated with random hexamers (0.5µg, Promega Benelux BV, Leiden, The Netherlands) at 70°C for 10 min, then reverse transcribed in a

final reaction volume of 20 µl containing Superscript RNase H ⁻ reverse transcriptase (200 Units, Invitrogen, Breda, The Netherlands), RNAsin (40 U, Promega Benelux BV, Leiden, The Netherlands), dNTP (2 mM Promega Benelux BV, Leiden, The Netherlands), 5xFirst Strand Buffer (Invitrogen, Breda, The Netherlands) and 0.1M DTT (Invitrogen, Breda, The Netherlands) for 50 min at 37°C. The reaction was stopped by heating at 70 °C for 10 min and the cDNA product was stored at -20°C until use. The cDNA product was 10 times diluted before use.

Table 1: Primer and probe sequences as used in the real-time PCR

Target			**Probe/primer sequence (5'-3')**	**Locus**	**Annealing temp.**
IL-2	Probe	IL-2-FL IL-2-LC	CTTGTTGTTAGCTTCACAGATCTTGCATTCACT CGGTGTGATTTAGACCCGTAAGACTCTTGAG	AJ224516	58 °C
	Primer	IL-2-FOR IL-2-REV	CAGTGTTACCTGGGAGAAGTG GCAGATATCTCACAAAGTTGGTC		
IL-4	Probe	IL-4 FL IL-4-LC	AAACTgAgCTCTTATgCAAAgCCTCC CAATTgTTTgggAgAgCCAgCACT	AJ621249	60°C
	Primer	IL-4 For IL-4Rev	gTgCCCACgCTgTgCTTAC gACgCATgTTgAggAAgAgAC		
IL-6	Probe	IL-6-FL IL-6-LC	CGAACAGGCCGCTGGAGAGC CGTCAGGCATTTCTCCTCGTCGAAG	AJ309540	59 °C
	Primer	IL-6-For IL-6-Rev	AGGACGAGATGTGCAAGAAG TGCTGTAGCACAGAGACTCG		
IL-8	Probe	IL-8-FL IL-8-LC	CATCCGAAGAAGGCATCATGAAGC TTCCATCTTCCACCTTCCACATCG	AJ009800	56 °C
	Primer	IL-8-For IL-8-Rev	ATTCAAGATGTGAAGCTGAC AGGATCTGCAATTAACATGAGG		
IL-10	Probe	IL-10-FL IL-10-LC	TCACCTgCgAgAAgAggAgCAA gCCATCAAgCAgATCAAggAgACg	AJ621254	58°C
	Primer	IL-10-For IL-10-Rev	gCTgTCACCgCTTCTTCA CCgTTCTCATCCATCTTCTCgAA		
IFN-γ	Probe	IFN-γ-FL IFN-γ-LC	GAAAGATATCATGGACCTGGCCAAGC CCCGATGAACGACTTGAGAATCCAG	Y07922	55 °C
	Primer	IFN-γ-For IFN-γ--Rev	TTCGATGTACTTGGAAATGC TTGCATCTCCTCTGAGACTG		
28S	Probe	28S –FL 28S–LC	GCGGGTGGTAAACTCCATCT GGCTAAATACCGGCACGAGACCG	DQ018756	56 °C
	Primer	28S-For 28S-Rev	CAAGTCCTTCTGATCGAG TCAACTTTCCCTTACGGTAC		
Eimeria maxima	Primer	Emax S Emax A	CACCATTggATATgTggAAAAgTTAA CCgAgTCTAgCTTAgCCATAgg	AY571588	58°C
	Probe	Emax FL Emax LC	TgATCCAAgAATgAACgAACTTAgTgCCC TCAATgTTCAgCACATggCAgCAACCC		

For is forward; Rev is reverse; FL is Fluorescein; LC is light cycler red 640.

Real- time PCR

Real-time PCR was employed with on-line detection of the PCR reaction based on fluorescence monitoring (LightCycler, Roche Diagnostics, Mannheim, Germany).
Quantitative results were determined as described by Loeffler *et al* (Loeffler, J. *et al.*, 2000). For the quantification a standard curve of the plasmid with the inserts of interest were used, constructed in PGEM-T easy (Promega). PCR reactions were performed according to the LightCycler kit instructions (FastStart DNA Master Hybridization Probes, Roche Diagnostics, Mannheim, Germany), with modifications as described by Rebel et al. (Rebel, J.M.J. *et al.*, 2005). Primer and probe combinations and PCR conditions are depicted in Table 1. To avoid contamination, filter pipet tips were used and reagents were mixed in rooms separate from rooms where DNA was present. A negative control that contained reagents only and a standard dilution series (a plasmid containing the cytokine of interest or 28S) was included in each run. For all cytokine and 28S PCR reactions the same cDNA sample was used in order to standardise and normalise the data.

Normalisation of mRNA concentration and DNA concentration

A standard curve for each reaction was generated from the threshold cycles (C_t) of the standard dilution series by the LightCycler software version 3.5. The standard curves of all cytokines and 28S had slopes around -2.97, meaning that the overall reaction efficiency for each standard curve was between 1.0 and 1.3.
The amplified PCR product performed with the LightCycler can be described as $N_n=N_o*E^n$ (Mygind, T. *et al.*, 2001), where N the is concentration of a sample which is identified by a given cycle number, N_o is the starting concentration of the sample, E is the overall reaction efficiency and n is the crossing point in number of cycles. From the cytokine specific standard curve, the concentrations of the samples were calculated. To normalise the concentration of cytokine cDNA, the sample concentration of each individual chicken was divided by the sample concentration of 28S of the same chicken. After normalisation of each individual sample the mean was taken of 5 chickens within one group.
The amount of *Eimeria* DNA was normalised with the weight of the tissue used.

Immunohistochemistry

From each animal, jejunum sections (10 μm thick), collected at day 2, 4 and 7 pi were immunohistologically stained by an indirect immunoperoxidase method. The sections were loaded on glass slides, air-dried, and fixed in acetone for 10 min. After being dried, the slides were immersed in PBS with 0.1% BSA and were subsequently incubated for 1 h with monoclonal antibodies against $CD4^+$ T cells (1:200; CT-4, Southern Biotech), $CD8^+$ T cells (1:200; CT-8, Southern Biotech), TCR alphabeta (1:50; TCR2, Southern Biotech) or TCR gammadelta (1:400; TCR1, Southern Biotech) followed by peroxidase-conjugated rabbit anti-mouse Ig (1:80; Dakopatts, Denmark). Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M Tris-HCl solution (pH 7.5) containing 0.03% H_2O_2. The slides were further coloured with 1% $CoCl_2$ for 5 minutes. After washing the nuclei were counter-stained with hematoxylin. The sections were dehydrated and mounted in distyrene-tricresyl phosphate-xylene (DPX). Images were acquired and analysed with Image-Pro Plus (version 5.1, media cybernetics). Per animal and staining 5 images were acquired and analysed, giving a total of 25 datapoints for each group and each staining.

Statistics

Eimeria DNA load was analysed using an ANOVA and significant data were further analysed using a Student T-test. All other data were analysed for statistical significance using a Student T-test. Data are expressed as the mean ± standard error of the mean (SEM). A P value < 0.05 was taken as the level of significance.

Results

Body weight

An oral inoculation in one-day-old broilers with sporulated *E. maxima* induced a significant weight gain depression of approximately 20 percent only at day 7 p.i. when broilers were infected at day 1 of age when compared to control animals of the same age (Figure 1). In the groups infected at day seven or day twenty-one of age, no significant weight depression was detected compared to the control age matched groups.

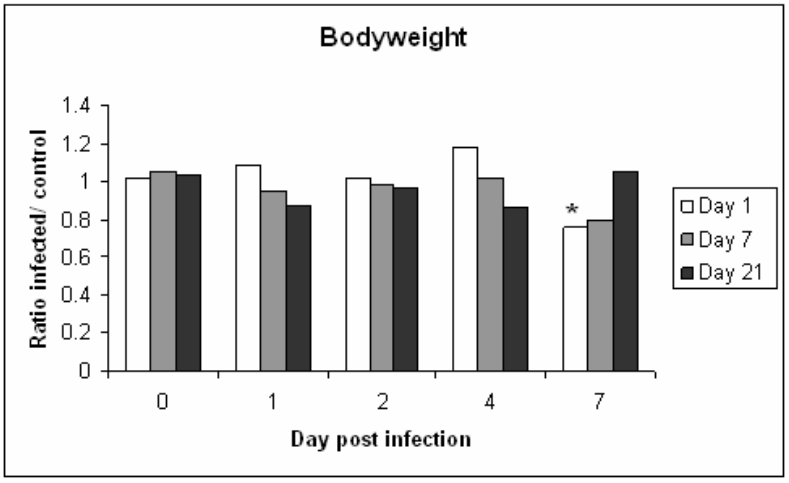

Figure 1: Body weight of three age groups of broilers. The data are displayed as ratios of infected / control. Body weights as displayed are the mean weight of five animals. *= Significant difference between control group and the group infected at day 1 of age at 7 days p.i. ($P \leq 0.05$).

Parasite dynamics

Log-transformed data of *E.maxima* DNA presence in the jejunum are shown in Figure 2a. Infection with 2 x 10^4 sporulated oocysts of *E.maxima* resulted in presence of the parasite DNA in the intestine, at days 2, 4 and 7 p.i., as determined by real-time PCR. In all infected groups the *Eimeria* DNA concentration increased rapidly after infection. No parasitic DNA or oocysts in droppings were found in any of the control groups (data not shown).

In animals infected on day 7, the amount of *Eimeria* DNA in the jejunum was higher four days p.i. when compared to animals infected on day 1. This was the only significant difference in DNA load found between the infected groups.

In all groups at day 7 p.i. oocysts in faeces were counted. Data were log transformed and no significant differences were observed between the primary infected groups (Figure 2b). In the droppings of the groups re-infected at 14 days post primary infection no oocysts were found (data not shown).

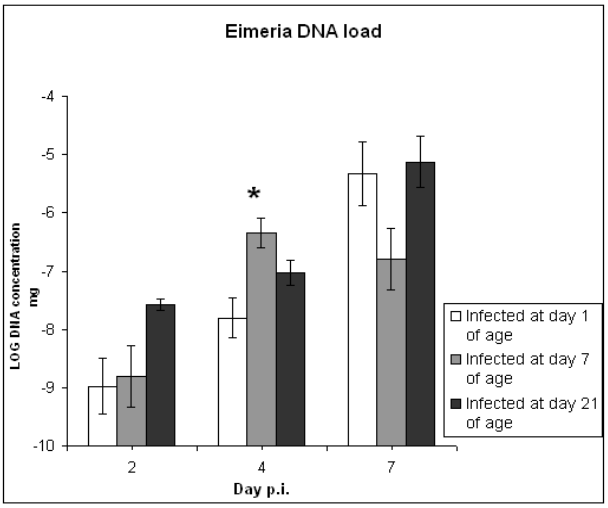

Figure 2a: The concentration of *Eimeria maxima* DNA in the jejunum. DNA concentration was measured by means of a Real-time PCR, LOG transformed and are the mean of 5 samples ±S.E.M. *= Significant difference between groups infected at day 1 and 7 of age at 4 days p.i. (P≤0.05). All infected groups at all time points were significantly increased compared to their age control counterparts.

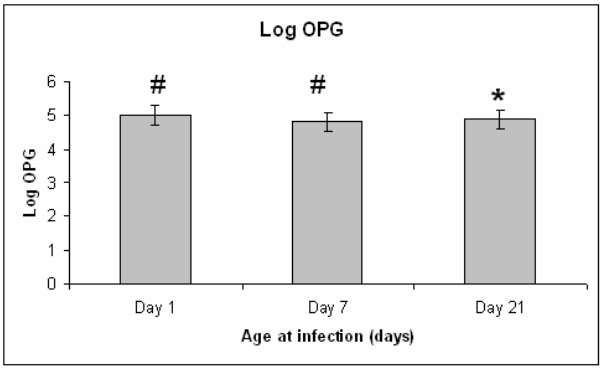

Figure 2b: The number of *Eimeria maxima* oocysts present in the feces day 7 postinoculation.The frequencies of oocysts were measured by means of oocyst counting according to the McMaster counting chamber technique, LOG transformed and represent the mean of 5 samples ±S.E.M. *= Significant difference between control and infected group (P≤0.05). #= difference between control and infected group (0.05 ≤ P ≤ 0.06).

Histochemistry

In immunohistochemical stainings the positive cells were counted. Figure 3 (A-D) shows the ratio of frequencies of infected/control of $CD4^+$, $CD8\alpha^+$, $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells from chickens at days 4 and 7 after infection. Significant differences between infected and corresponding control groups are depicted.

In all groups of infected animals the amount of $CD4^+$ cells reached levels about twice the frequency of the control situation. The amount of $CD4^+$ cells increased significantly at day 4 p.i. in the groups infected at day one and twenty-one of age. No significant increase of $CD4^+$ cells was found at day 7 p.i. in the animals infected at day one of age due to a high variation between the chickens.

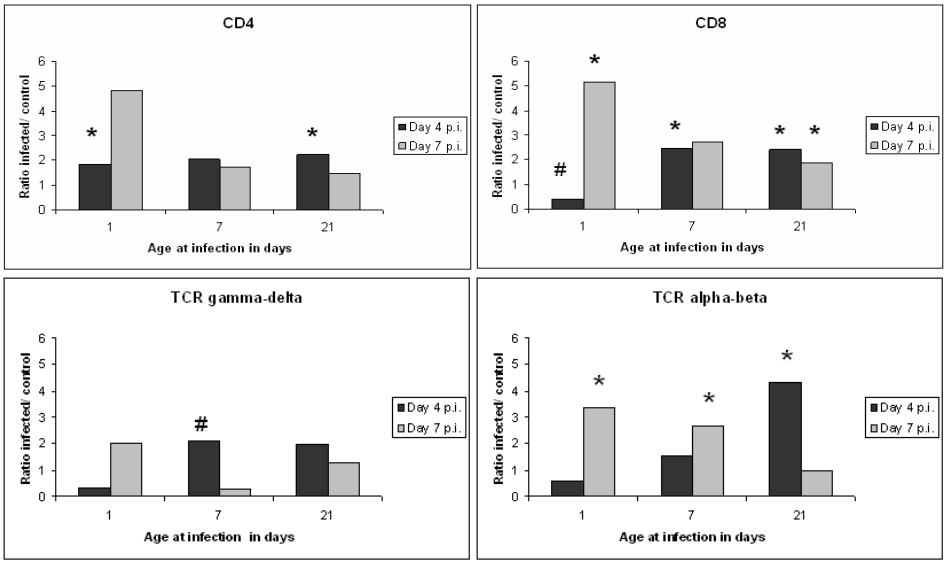

Figure 3: Histochemistry analysis of cell populations present in the jejunum.
The ratio of $CD4^+$, $CD8^+$, $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells are shown. The data are displayed as a ratio of cell frequencies in infected / control animals. Values are the mean of 5 chickens. *= Significant difference between control and infected group ($P \leq 0.05$) at day post infection as indicated. #= difference between control and infected group ($0.05 \leq P \leq 0.06$) at day post infection as indicated.

The level of $CD8^+$ cells was increased at day 7 p.i. in the animals infected at day 1 of age, in the other infected groups the increase was already found at day 4 p.i. In the 1-day-old animals the induction of $CD8^+$ cells was slower, but also stronger. The $TCR\alpha\beta^+$ cells were increased as compared to the control group at day 7 p.i. in the animals infected at day one and seven of age, and already at day 4 p.i. in the animals infected at day twenty-one of age. For the $TCR\gamma\delta^+$ cells no significant changes were

observed, but a slight increase was found at day 4 p.i. in the animals infected at day seven of age.

Cytokine mRNA expression

At day 4 p.i. the two groups infected at one and seven days of age have a significant increase in IFN-γ mRNA response as compared to the controls at the same time, while the 21-day old infected group showed no significant increase of IFN-γ mRNA at day 4 p.i. At day 7 p.i., however, broilers of all ages show a significant IFN-γ response. For IL-2 mRNA induction no significant changes between control and infected animals were observed in any of the groups. In the majority of the animals no levels of IL-4 and IL-6 cDNA were detected. Only in the group infected at day twenty-one of age a significant increase of the amount of IL-8 cDNA was found at days 4 and 7 p.i. (data not shown). Significant increased levels of IL-10 cDNA were found at day 7 p.i. in broilers infected at day seven and day twenty-one of age. Broilers infected at day 1 of age also showed an increased IL-10 mRNA expression which is not significant compared to the control group of the same age (p value 0,09).

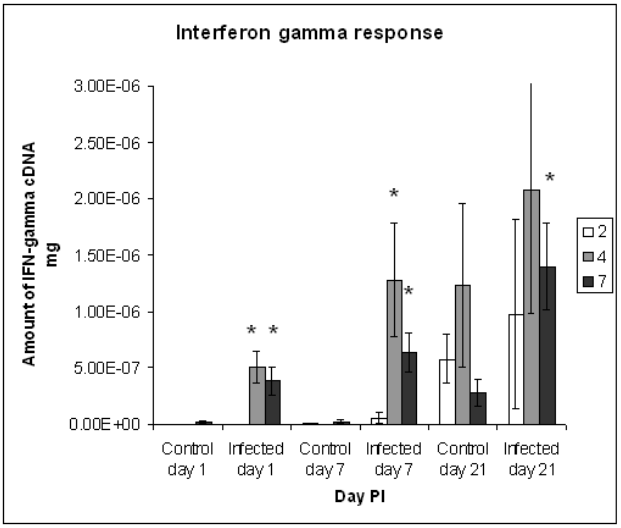

Figure 4a: Cytokine amounts ±SEM from infected and control broilers of three different ages.

Figure 4a1: Mean relative amounts of IFN-γ cytokine cDNA. *=Significant difference between control and infected group at day 4 p.i. in the groups infected at days 1 and 7 of age, and at day 7 p.i. in the groups infected at all ages (P≤0.05). The data represent results of 5 control chickens or 5 chickens of each age group infected with 2 x 10^4 sporulated *Eimeria maxima* oocysts Note the difference in the scales for the different cytokines.

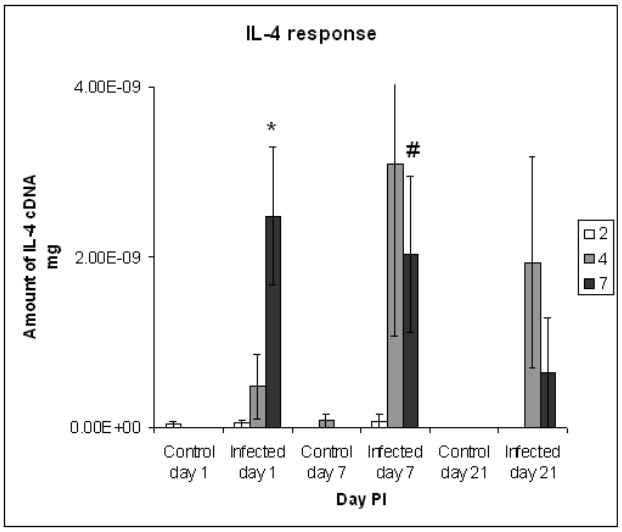

Figure 4a2: Mean relative amounts of IL-4 cytokine cDNA. *=Significant difference between control and infected group infected at day 1 of age (P≤0.05). #= difference between control and infected group infected at day 7 of age (0.05 ≤ P ≤ 0.06). The data represent results of 5 control chickens or 5 chickens of each age group infected with 2 x 10^4 sporulated *Eimeria maxima* oocysts Note the difference in the scales for the different cytokines.

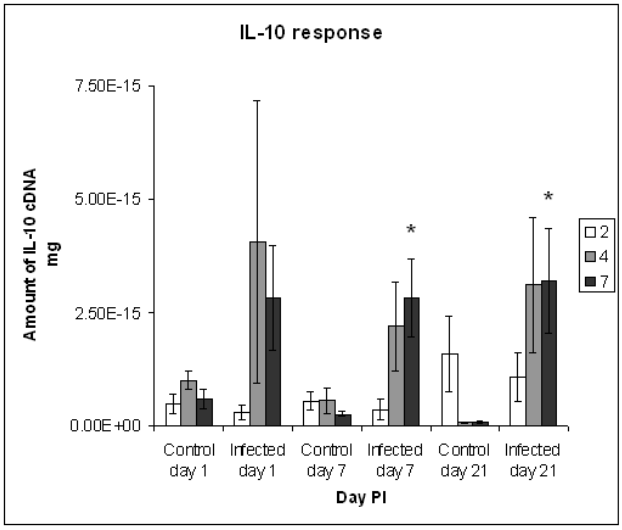

Figure 4a3: Mean relative amounts of IL-10 cytokine cDNA. *=Significant difference between control and infected groups infected at 7 and 21 days of age (P≤0.05).
The data represent results of 5 control chickens or 5 chickens of each age group infected with 2 x 10^4 sporulated *Eimeria maxima* oocysts Note the difference in the scales for the different cytokines.

In Figure 4b the IFN-γ / IL-10 ratio for the infected animals is shown as a representation of the Th1 - Th2 cytokine response. No ratio of INF-γ/IL-4 was shown due to the lack of detectable IL-4 responses in most of the animals. The early Th1-type response of IFN-γ gets dominant as the age of the broiler increases. In addition the dominant IFN-γ response appeared earlier after infection with increasing age of the chickens.

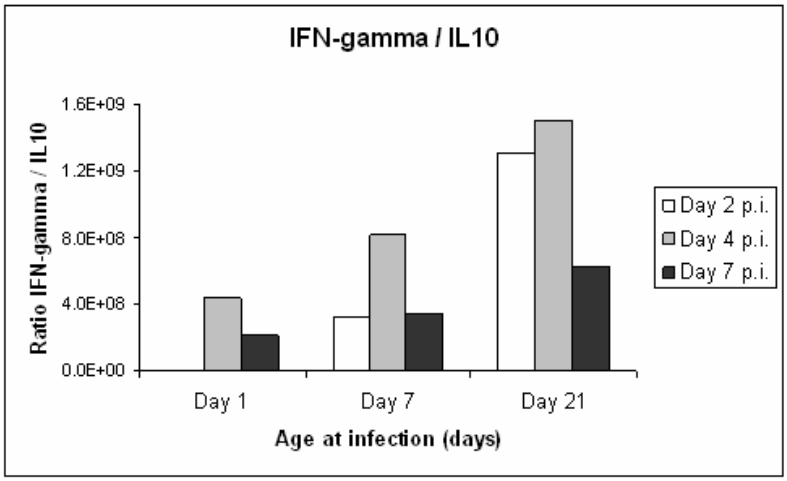

Figure 4b: The ratio of IFN-γ / IL-10. IFN-γ and IL-10 are two cytokines representing the Th1 and Th2 type of reactions. Amounts of IFN-γ and IL-10 are as shown in figure 4a.

Discussion

Broilers of the three age groups were infected with *E.maxima* as demonstrated by oocyst output in faeces and specific DNA in the GI-tract. With increasing age the cellular immune response to the infection became more pronounced. In addition to the increase in the relative frequency of $CD8\alpha^+$ and $TCR\alpha\beta^+$ cells in the GALT with time p.i. also expression was observed of an increased IFN-γ / IL-10 ratio.

In broilers infected with *E.maxima* at day one of age, the frequency of $CD8\alpha^+$ was decreased as compared to controls at day 4 p.i., but at day 7 p.i. the frequency of $CD8\alpha^+$ cells was much higher than in controls in these broilers. The broilers infected at day seven and day twenty-one of age were able to mount an $CD8\alpha^+$ response more rapid than was observed in the broilers infected at day one of age but a further increase at day 7 p.i. was not observed. Chickens infected at days 7 and 21 did not show

any difference, indicating that the age-related induction of the response at the CD8α^+ level was stabilised at least from day 7 on. Such increased levels of CD8α^+ cells may reflect enhanced acquired immune status (Bumstead, J.M. *et al.*, 1995; Lillehoj, H.S., 1994; Lillehoj, H.S. and Lillehoj, E.P., 2000).

In general, the development of the CD4$^+$ cell population was highly similar as observed for the CD8α^+ cells. However, as the CD4$^+$ cell frequencies at day 4 p.i. in chickens infected at day one are similar to those observed in chicken infected at day 7 or at day 21 it is concluded that the induction of CD4$^+$ responses may stabilise slightly earlier than observed for CD8α^+ cells. No significant increase in amount of CD4$^+$ cells was detected at day 7 p.i in animals infected at day one while the mean of the CD4$^+$ cells increased. This increase was not significant; some of the animals responded with an increased frequency of CD4$^+$ cells while other animals responded with CD4$^+$ cell frequencies similar to the control values. Early after infection an age-related increase in responsiveness was found for TCR$\alpha\beta^+$ cells. The cell frequencies of TCR$\alpha\beta^+$ infected/ control ratio at day 4 p.i. increased with the age of the host at the time of infection. Mature response levels were not observed in chickens infected at days one and seven. The ratio of TCR$\alpha\beta^+$ at day 7 p.i. decreased with the age of the host at the time of infection. This might represent a faster response regulation that leads to a less severe reaction and limitation of the damage caused by the host immune reactivity. Though no significant differences between control and infected groups were observed for TCR$\gamma\delta^+$ cells, the same tendency was observed for the response kinetics of these cells. The T-cell responses are an indication for age related kinetics of the development of the cellular response to an *E.maxima* infection. We hypothesize that the developmental stage of the immune system is responsible for the kinetics of this response. The immaturity of the GALT may account for a reduced support of healing of gut lesions. As a result the absorbance of nutrients is decreased, which could explain the observed weight-gain decrease of almost 20% after infection at young age.

Although the T-cells found in the GALT of young broilers were phenotypically mature, they still may be functionally immature (Bar-Shira, E. *et al.*, 2003). This was investigated by measuring the cytokine responses in control and infected animals. Different kinetics in host response for broilers infected at a different ages were not only found for host T-cell frequencies, but also for the host cytokine responses of the Th1 and Th2 signature. Thus the immaturity of the GALT was also expressed by the inability of young animals to mount a full cytokine response. IL-10 responses in chickens infected at days 7 and 21 were highly similar. However, the response of one day old chickens showed slightly different kinetics. In one day old chickens the maximum response was observed at

day 4 p.i. while in older animals the response increased gradually until day 7 p.i. This might reflect a Th2-type down-regulation of Th1 responses in young birds. The IL-10 response at day 7 p.i. was independent on the age of the animals at the time of infection, indicating that the IL-10 response induction is similar in young birds. In animals infected at day one of age, the IL-10 response at day 7 p.i. was relatively weak (p=0,09), which suggests that the response may be present but was not fully induced in all the tested animals.

The response kinetics of IFN-γ was similar for infections in all age groups: a rapid response is still low at day 2 p.i. but reaches a maximum at day 4 p.i. and is reduced rapidly thereafter (day 7 p.i.). In control animals the capacity to generate IFN-γ increased with age. This confirmed earlier observations that IFN-γ mRNA levels in chickens increase as chickens age (Rebel, J.M. *et al.*, 2005). Indeed, a clear increase in relative response level was observed from day one of age to day 21 of age (see Figure 4a). For the Th1 cytokine IFN-γ in day one and day seven old chicks the increase in mRNA levels on day 4 p.i. was significant compared to their control counterparts. The control levels of IFN-γ in animals infected at 21 days of age were relatively high compared to chicks of age one and seven days.

The ratio of IFN-γ / IL-10 for the infected animals was determined as an indication for Th1 or Th2-type of response induction at the different ages. A high IFN-γ / IL-10 ratio reflects a Th1-type of response, whereas a relatively low ratio is indicative for a Th2-type of response (de Groot, J. *et al.*, 2005; Degen, W.G. *et al.*, 2005; Rothwell, L. *et al.*, 2004). In other experiments with poultry or mice the inability of neonates to mount IFN-γ responses has already been described (Adkins, B., Bu, Y. and Guevara, P., 2001; Swinkels, W.J. *et al.*, 2006). In our present experiments the IFN-γ / IL-10 ratio at all time points measured day 2, 4, and 7 p.i. increased with age. From Figure 4b it can be deduced, that the Th1 response character is overruling in a response to this *E.maxima* infection (Hong, Y.H. *et al.*, 2006b). The resulting Th1 response is stronger with increasing age of the broiler. Relative down-regulation at day 7 p.i. is most effective in young birds where the Th1 response is still low and decreases with age. This is similar to the findings of Adkins et al. who found that neonatal mice were able to mount Th1 responses, but appeared to be biased to Th2 lineage function (Adkins, B. *et al.*, 2001).

Though the quality of the GALT response to *Eimeria* was age dependent, we did not observe any effect on the amount of parasites in the gut and in excreta. During the period that was investigated (2-7 days p.i.) *Eimeria* kept replicating in the gut and the oocyst numbers remained constant.

An alternative method to estimate the local presence in the gut of *Eimeria* parasites is to determine the amount of species-specific DNA.

Using this method it was clear that the absolute levels of *Eimeria* in the gut increased with time. At day 7 p.i. in both intestine (DNA) and faeces (oocysts) no differences were observed between the infected groups of the three ages. It is well established that primary *Eimeria* infections generate protective responses to secondary infections, but our observations in both intestine and in faeces demonstrated no influence of the detected immune responses on parasite proliferation.

It therefore cannot be excluded that the immune reactions observed in the intestine are not responsible for termination of the ongoing infection, but for protection against secondary homologous infections. In the groups that were primary infected at day one or day seven of age, no parasites were found in the faeces after secondary infection, suggesting a full protection against homologous parasites. However, the full protection to secondary infections as observed in our experiment is in part in contradiction with previous findings by Chapman et al (2005) who found only a partial immunity following infection of newly hatched chicks (Chapman, H.D. *et al.*, 2005). The different doses used for infection in these experiments might account for these dissimilar findings, as a threshold exists for a single dose immunization (Danforth, H.D. *et al.*, 1997).

The different immune reactions found in infected broilers of different ages and the shift to a Th1-type of reaction was not associated with differences in the course of a primary infection, as well as with differences in protection to a secondary-infection. This might indicate that while different immune responses were induced in different age groups, these responses all were associated with effective protection. One reason might be that other cell types than the ones analysed in our experiment may be responsible for the clearing of parasites. $CD8^+$ cells have been implicated to play a role in the recovery phase of gut damage and wound healing, but not in innate resistance to the parasite as previously stated for *E.acervulina* infections (Lillehoj, H.S., 1994). However, this is in contradiction with findings that $CD8^+$ T cells, cytotoxic T-lymphocytes (CTLs), are often observed around infected epithelial cells and proliferate upon the encounter of antigen in vitro, and that CTLs can act against the intracellular stages of *Eimeria* by lysis of infected cells, and thus reduce the production of merozoites or gametes (Allen, P.C. and Fetterer, R.H., 2002; Klinkenberg, D. and Heesterbeek, J.A., 2005; Lillehoj, H.S. and Lillehoj, E.P., 2000).

In conclusion, the age of broilers when first infected is of influence on the strength and kinetics of the immune responses to an *E.maxima* infection. Young animals tend to have a less pronounced Th1 reaction, and as age increases the reaction to a primary *Eimeria* infection becomes more Th1 biased. Although different in timing and strength, the quality of the primary immune responses detected does not seem to influence the level

of excretion of parasites or the protection to ongoing primary and secondary infection. Infection at a young age resulted in a fully protected animal. This implies that vaccination is possible at an extremely young age.

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Chapter 6

Immune reactions after a homologous or heterologous challenge of broilers primed with *Eimeria maxima*

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Abstract

Eimeria are obligate intracellular protozoan parasites which can affect chickens. After exposure to *Eimeria* chickens establish (partial) protective immunity to the homologous strain. In this paper we investigate the process responsible for *Eimeria* protection. In order to find host reactions specificly involved in protection to homologous re-infection we investigated the host reactions after primary infection and a homologous or heterologous secondary infection.

Broilers were mock infected or infected with *E.maxima* (Max) at one week of age. Two weeks later broilers were mock infected, infected with *E.maxima* or *E.acervulina.* Oocyst output, T-cell population and cytokine mRNA expression profiles and *Eimeria* DNA profiles were measured 2, 4 and 7 days pi. Specific regulation of gene expression profiles was monitored by a whole genome oligo-array containing 20.673 oligo´s at 8 and 24 hours pi.

No *E.maxima* DNA or oocysts were found after a homologous re-infection, while *E.acervulina* DNA and oocysts were observed after a the heterologue re-infection in chickens primed with *E.maxima*. Frequencies of T-cell populations in the jejunum and Th1 type of cytokine profiles were induced after primary infection by either *E.acervulina* or *E.maxima*. Only $CD8^+$ T-cells were increased at day 4 p.i. in a homologous infection compared to a heterologous infection. No specific induction of $CD4^+$ T-cells, $TCR\alpha\beta^+$ T-cell or $TCR\gamma\delta^+$ T-cells, pro-inflammatory, Th1 or Th-2 cytokines were found after a homologous secondary infection in comparison to a heterologous infection. With micro-array analysis we found common genes that were induced in al infected groups. Specific genes that were only differentially expressed within the Max-Max group and not regulated in all other groups were observed and these could be responsible to induce protection. These genes are mainly involved in specific T-cell signalling and in translation. In conclusion there are T-cell based responses that induce *Eimeria* species specific protection.

Introduction

Many drugs are used to treat and control infections in livestock and poultry. For *Eimeria*, the parasite that causes coccidiosis in poultry, the frequent use of anti-helmintics results in drug-resistance (Peek, H.W. and Landman, W.J., 2003; Williams, R.B., 2006). New preventive treatments for animals need to be developed to make the use of anti-helmintics redundant. Vaccination is the preventive method of choice. Different types of vaccines are currently available: live vaccines; live attenuated vaccines, non-infective parasite derivates and genetically engineered subunit vaccines (Hong, Y.H. *et al.*, 2006a). Most effective are live vaccines. But live parasite vaccines are labor-intensive and expensive to manufacture, which is why alternative technologies are required. In addition, live vaccines produce most of the unwanted side effects a natural infection brings. Infected chickens lose weight, have a higher food conversion and can transmit the infection to other members of the flock. In order to design new vaccines for *Eimeria*, it is pivotal to first understand the nature of the host-pathogen interaction i.e. the nature of the protective response of cells and cytokines.

In chickens raised for meat (broilers), the three most diagnosed *Eimeria* infections are *E.acervulina*, *E.maxima* and *E.tenella* (Shirley, M.W. *et al.*, 2005). Broilers under continuous exposure of these three strains are capable to establish protective immunity to *E.acervulina*, *E.maxima* and *E.tenella* (Stiff, M.I. and Bafundo K.W., 1993). For *E.maxima* the ingestion of a small number of *Eimeria* oocysts is already enough to induce full protection to a subsequent homologous challenge (Blake, D.P. *et al.*, 2005). Protection however is in general restricted to homologous or species specific infections and sometimes even to strain specific immunity (Bessay, M. *et al.*, 1996). Primary infections have been studied in great detail and the resulting protection to a secondary homologous infection is well known, but the process responsible for this protection is less well understood. The knowledge of these processes is crucial in the development of new vaccines, adjuvantia to existing vaccines and strategies to combat *Eimeria* infections.

In this paper we describe the host reactions occurring after a primary infection and the comparison with the response to a challenge with either a homologous or heterologous strain of *Eimeria*. By comparing the host reactions after a primary infection to those after homologous and heterologous challenge, the factors responsible for protection in principle might be identified. The host reactions were monitored for T-cell populations, cytokine profiles and and for gene expression profiles with the use of an oligonucleotide array

Materials and methods

Chickens

A total of 125 one-day-old Ross 308 male broilers were obtained from a commercial breeder (Pronk's Broederij, Meppel, The Netherlands). All chickens were immunised against infectious bronchitis (IB) and Newcastle disease virus (NDV) with a spray vaccine (Intervet®, Boxmeer, The Netherlands), and vaccinated against Marek's disease (Fort Dodge, Weesp, The Netherlands).

The chickens were randomly divided in five groups according to the groups as described in table 1. Priming occurred at day 7 of age. Three groups were inoculated with 0,5 ml PBS, the remaining two groups were orally inoculated with 0,5 ml PBS containing 2 x 10^4 sporulated oocysts of *Eimeria maxima*. A challenge followed at day 21 of age. This challenge administered orally and included 0,5 ml PBS containing 2 x 10^4 sporulated oocysts of *Eimeria Maxima* or 0,5 ml PBS containing 5 x 10^4 sporulated oocysts of *Eimeria Acervulina,* thus forming the five groups as shown in table 1.

Fresh sporulated *E.acervulina* and *E.maxima* oocysts were provided by the Animal Health Service in Deventer, The Netherlands. Chicks were reared in wire cages to prevent contact with faeces. Control and infected animals were housed in separate battery cages in order to prevent transmission between groups. Feed and water were provided ad libitum. The study was approved by the institutional Animal Experiment Commission in accordance with the Dutch regulations on animal experimentation.

Table 1: Group names and corresponding names and treatments.

Group	Priming	Challenge	Name in figures
Control	PBS	PBS	PBS-PBS
Primary *E.maxima*	PBS	*E.maxima*	PBS-Max
Primary *E.acervulina*	PBS	*E.acervulina*	PBS-Ac
Homologous challenge	*E.maxima*	*E.maxima*	Max-Max
Heterologous challenge	*E.maxima*	*E.acervulina*	Max-Ac

Experimental design

Five chickens from each group were killed by cervical dislocation at 8 and 24 hours, 2, 4 and 7 days post infection. From these chickens individual body weight was measured and, three samples of one cm of jejunum located at 2 cm of the Meckel's diverticulum were collected for histology and extraction of DNA or RNA and one sample of one cm of duodenum located the duodenal loop was collected for extraction of DNA. These samples were, snap-frozen in liquid nitrogen and stored at -70°C until use.

RNA extraction

The frozen jejunum samples were homogenized in liquid nitrogen using a mortar and pestle. Total RNA was extracted from 50-100 mg of this homogenized tissue with TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's recommendations with minor modifications. The homogenized tissue samples were dissolved in 1 ml of TRIzol reagent per 50-100 mg of tissue using a syringe and 21-G needle passing the lysate for 10 times. After centrifugation the supernatant was transferred to a fresh tube. Subsequently a phase separation with chloroform was

performed as described by the manufacturer. The RNA was precipitated using 500 µl 2-propanol and used for analysis by real-time PCR and micro-arrays. Quality of the RNA was verified by Nanodrop and the Bioanalyzer (Agilent, Santa Clara, U.S.A) before use.

cDNA preparation and Real-time PCR

A total of 200 ng RNA was incubated with random hexamers (0.5µg, Promega Benelux BV, Leiden, The Netherlands) at 70°C for 10 min, then reverse transcribed in a final reaction volume of 20 µl containing Superscript RNase H $^-$ reverse transcriptase (200 Units, Invitrogen, Breda, The Netherlands), RNAsin (40 U, Promega Benelux BV, Leiden, The Netherlands), dNTP (2 mM Promega Benelux BV, Leiden, The Netherlands), 5xFirst Strand Buffer (Invitrogen, Breda, The Netherlands) and 0.1M DTT (Invitrogen, Breda, The Netherlands) for 50 min at 37°C. The reaction was stopped by heating at 70 °C for 10 min and the cDNA product was stored at -20°C until use. The cDNA product was diluted 10 times before use.

Real-time PCR

Real-time PCR was employed with on-line detection of the PCR reaction with an ABI7500 fast real-time PCR system (Applied Biosystems, Foster City, U.S.A.). Real-time PCR was done with the SYBR® Green PCR Master Mix as described by the manual (Applied Biosystems, Foster City, U.S.A.). Primer combinations and PCR conditions are depicted in Table 2. A standard curve was generated from the threshold cycles (C_t) of a standard dilution series of a plasmid containing a cytokine specific sequence. The standard curve had a slope around -2.97, meaning that the overall reaction efficiency for the standard curve was between 1.0 and 1.3. From the cytokine specific standard curve, the concentrations of the samples were calculated. To normalize the concentration of cytokine cDNA, the sample concentration of each individual chicken was divided by the sample concentration of 28S of the same chicken. The mean value was then used of the sample concentrations of five chickens at each time point.

Quantification of parasite load

In order to quantify parasital DNA about 20 to 40 mg of tissue was homogenized with liquid nitrogen using a mortar and pestle. For the isolation of DNA from the intestinal tissues the Chemagic DNA Tissue Kit (Baseclear, Leiden; The Netherlands) was used according to the manufacturer's manual. After cell lysis, DNA was bound to magnetic beads which were separated from the supernatant using the Chemagic Stand. DNA was eluted from the beads using 100 µl of the elution buffer as provided by the manufacturer. DNA was 10 times diluted for use in a Real-time PCR. Real-time PCR on diluted DNA was employed with on-line detection of the PCR reaction based on fluorescence monitoring (LightCycler, Roche Diagnostics, Mannheim, Germany. Real-time PCR was done as described by Rebel et al. (Rebel, J.M. *et al.*, 2005). Primer and probe combinations and PCR conditions are depicted in Table 3.

Table 2: real-time PCR table

Target	Name	Primer sequence (5'-3')	Annealing temp.
IL-2	IL2-For IL-2-Rev	TTCAAAATATCGAAAAGAACCTCAAG CGGTGTGATTTAGACCCGTAAGAC	59 °C
IL-4	IL-4 -For IL-4-Rev	GTGCCCACGCTGTGCTTAC AGGAAACCTCTCCCTGGATGTC	59°C
IL-7	IL-7 -For IL-7-Rev	GTGTCAAATATGAGAACATACTCAG CTTTTCCTTTTTATGTCTTTCA	
IL-8	IL-8-For IL-8-Rev	ATTCAAGATGTGAAGCTGAC AGGATCTGCAATTAACATGAGG	59 °C
IL-10	IL-10-For IL-10-Rev	CGCTGTCACCGCTTCTTCA TCCCGTTCTCATCCATCTTCTC	
IFN-γ	IFN-γ-For IFN-γ-Rev	GTGAAGAAGGTGAAAGATATCATGGA GCTTTGCGCTGGATTCTCA	59 °C
28S	28S-For 28S-Rev	GGCGAAGCCAGAGGAAACT GACGACCGATTTGCACGTC	56 °C
Eimeria maxima	Emax-For Emax-Rev	CACCATTGGATATGTGGAAAAGTTAA CCGAGTCTAGCTTAGCCATAGG	59°C

Table 3: real-time PCR table for *Eimeria* DNA detection

Target			Probe/primer sequence (5'-3')	Annealing temp.
Eimeria acervulina	Primer	Eacer F EacerA	CCTCCgAgTTTCTCACCC CCCCCAAATCACTCgAA	62°C
	probe	Eacer-FL Eacer-LC	gATgACCgCTCgCCTAgTTgT-FL 640-gTCCCCAATgCTCTgTTTgTCgT	
Eimeria maxima	primer	Emax S Emax A	CACCATTggATATgTggAAAAgTTAA CCgAgTCTAgCTTAgCCATAgg	58°C
	probe	Emax FL Emax LC	TgATCCAAgAATgAACgAACTTAgTgCCC-FL 640-TCAATgTTCAgCACATggCAgCAACCC	

Immunohistochemistry

Immunohistological staining by an indirect immunoperoxidase method was performed on frozen tissue sections (10 μm thick) collected at day 4 and 7 post infection. The sections were loaded on glass slides, air-dried, and fixed in acetone for 10 min. After drying, endogenous peroxidase was inhibited for 5 minutes at room temperature (2 % NaN_3 in TRIS-HCl 0.05M pH 7.5 + 0.06% H_2O_2). The slides were washed with PBS with 0.1% BSA and were subsequently incubated for 1 h with monoclonal antibodies against monocytes and macrophages (1:500; CVI-ChNL-68.1 (Jeurissen, S.H. *et al.*, 1988). $CD4^+$ T cells (1:200; CT-4, Southern Biotech, Birmingham, U.S.A.), $CD8^+$ T cells (1:200; CT-8, Southern Biotech), TCR$\alpha\beta^+$ cells (1:50; TCR2, Southern Biotech) or TCR$\gamma\delta^+$ cells (1:400; TCR1, Southern Biotech) followed by peroxidase-conjugated rabbit anti-mouse Ig (1:80; P161, Dako, Glostrup, Denmark). Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M Tris-HCl solution (pH 7.5) containing 0.03% H_2O_2. The sections were further colored with 1% $CoCl_2$ for 5 minutes. After washing the nuclei were counter-stained with hematoxylin. The sections were dehydrated and mounted in Eukitt (Electron Microscopy Sciences, Hatfield, U.S.A). The images were acquired and analyzed with Image-Pro Plus version 5.1 (Media Cybernetics, Bethesda, U.S.A.). Per animal and staining 5 images were aquired and analyzed, giving a total of 25 datapoints for each group and each staining.

Statistics for immunohistochemistry, Real-time PCR

Immunohistochemistry and Real-time quantitative PCR data were analysed for statistical significance by an ANOVA followed by a Student T-test. Data are expressed as the mean ± standard error of the mean (SEM). A P value < 0.05 was taken as the level of significance. *Eimeria* PCR data were log transformed, in case of a negative sample, half the amount of the lowest positive sample was taken as a value. All groups were compared to the control group. When significant differences were found between an infected group and the control group, a comparison between groups was also done.

Micro-array

Oligonucleotide arrays were obtained from Operon (Operon, Cologne, Germany). These arrays contained 20.673 chicken oligo probes (*Gallus gallus* Roslin/ARK CoRe V1.0). Biotin- or fluorescein-labeled cDNA was generated from 5 µg of total RNA from chicken jejunum per reaction. The cDNA synthesis time was increased to 3 hours at 42°C. To hybridize the microarray, the MICROMAX TSA labeling and detection kit (PerkinElmer, Waltham, U.S.A.) was used. The TSA probe labeling and array hybridization were performed as described in the instruction manual with minor modifications. After a 65°C overnight incubation, the slides were left for 5 minutes at room temperature. Then the slides were washed with 2* SSC + 0.2% SDS at 42°C for 15 minutes. This was followed by 15 minutes incubation with 2*SSC and finally a 15 minutes incubation with 0.2*SSC. After signal amplification the microarrays were dried and scanned in a Genepix autoloader 4200AL (Molecular Devices Corporation, Sunnyvale, U.S.A.) The image was processed with Genepix software version 6.1 (Molecular Devices Corporation, Sunnyvale, U.S.A.) and spots were located and integrated with the spotting file. Reports were created of total spot information and spot intensity ratio for subsequent data analyses. Analysis of the Microarray Data After background correction the data were presented in an M/A plot were M=log2R/G and A=log2_(R×G). An intensity-dependent normalization was performed using the Lowess function in the statistical software package R (Yang, Y.H. *et al.*, 2002). The normalization was done with a fraction of 0.2 on all data points.

In the results file effects are given for all levels for each factor, compared to the reference which was the PBS-PBS group. The reference group itself is therefore omitted.

Missing values were possibly due to a bad signal to noise ratio. Differential expression was denoted as the M-value. A gene was considered to be differentially expressed when the mean value of the ratio was > 1.58 or < -1.58 and the cDNA was identified with significance analysis of microarrays (based on SAM (Yang, Y.H. *et al*., 2002) with a False discovery rate < 1%. Because a ratio is expressed in a log2 scale, a ratio of > 1.58 or < -1.58 corresponds to a more than threefold up- or down-regulation, which is the expression difference limit indicated by the manufacturer of the MICROMAX TSA labeling and detection kit.
When homologies or functions of observed genes were not already known a tBLASTx search was performed (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Next to common genes induced in all infected groups, we looked for genes specifically upregulated in the Max-Max group. Genes that were included for this further analysis had to meet the following criteria: their expression was altered more than threefold (log_2 value 1,58) in the homologous re-infected group (Max-Max) at 8 or 24 hours after infection, their expression was not three fold up altered in the other groups, the corresponding spots on the micro-array were of good quality in at least 4 chickens of one group and a name or similarity of the oligo had to be available. We further investigated the top ten up or down-regulated genes for both time points, thus yielding 40 genes.

Results

Infection characteristics
After priming or after challenge with the indicated dose of oocysts no differences in weight were observed between the experimental groups between 0 and 8 days p.i. Nevertheless lesions characteristic for *Eimeria infections* were present in all primary and secondary infected groups. Log transformed data of *E.maxima* and *E.acervulina* DNA presence are shown in Figure 1.

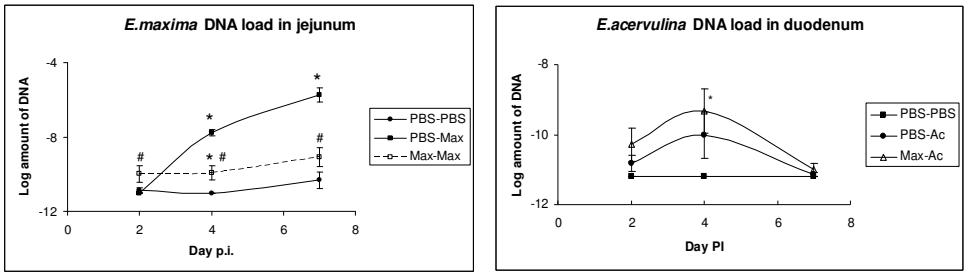

Figure 1: The concentration of *Eimeria maxima* DNA in the jejunum and *Eimeria acervulina* DNA in the duodenum. DNA concentration was measured by means of a Real-time PCR, LOG transformed and are the mean of 5 samples ±S.E.M. * Significant difference between PBS- PBS (control) and infected group ($P \leq 0.05$). # Significant difference between infected groups ($P \leq 0.05$).

Administration of *E.maxima* parasites both in a primary and secondary heterologous infection resulted in presence of *E.Maxima* DNA in the

chicken duodenum. In the primary infected animals the *Eimeria* DNA concentration increased rapidly after infection. A significantly lower amount of *E.Maxima* DNA was found in the jejunum of animals that had received a homologous re-infection as compared to the primary infected animals. At days 4 and 7 post infection in the droppings of the homologous infected group no *E.maxima* no oocysts were observed (data not shown). In the group of animals infected with *E.acervulina* a significant increase of DNA as compared to the control group was found at day 4 p.i. in the primary infected group. No differences in *Eimeria* DNA load were found between the groups primary or after secondary heterologous infection with *E.acervulina*.

Cytokine mRNA expression

No differences in mRNA expression of the cytokines observed in this experiment were found between the Max-Max group and the Max-Ac group.

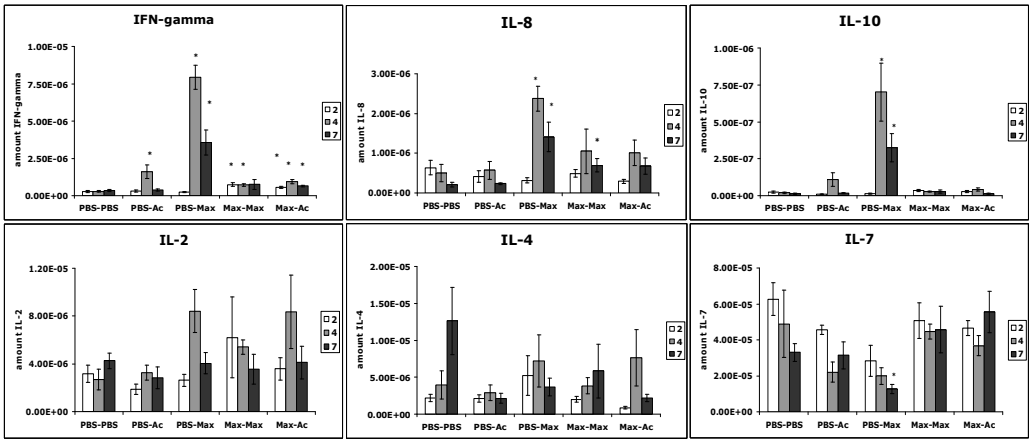

Figure 2: Mean amounts of cytokine mRNA. Cytokine amounts ± S.E.M. of five control chickens or five infected chickens at days 2, 4 and 7 post infection. Note the difference in the scales. Values are means of five chickens ± SEM. * Significant difference between PBS- PBS (control) and infected group ($P \leq 0.05$).

The group PBS-Max showed the most increase in cytokine cDNA levels (Figure 2). In this group the cytokines IFN-γ, IL-8 and IL-10 were up-regulated at days 4 and 7 post infection as compared to the control group. The cytokine IL-7 was down-regulated in the PBS-Max group at day 7 post infection as compared to all other groups.

In the Max-Max group at days 2 and 4 post infection the level of IFN-γ was increased, while the level of IL-8 was increased at day 7 post infection, as compared to the control group. The levels of IFN-γ and IL-8

mRNA were significantly higher in the PBS-Max group as compared to the Max-Max group. In the Max-Ac group the only upregulated cytokine was IFN-γ, this cytokine was up-regulated at day 7 post infection as compared to the control group. Also in the PBS-Ac group IFN-γ was found to be upregulated at day 4 p.i. as compared to the control group. No differences in the IFN-γ mRNA amount in both *E.acervulina* groups was observed at day 4 and 7 as compared to the Max-Max group .

No significant differences in IL-2 and IL-4 mRNA expression between infected and non infected groups were detected in our experiment.

Immunohistochemistry

The investigated cells showed the same increase in Max-Max infected animals as in Max-Ac infected animals as compared to the control group (Figure 3). The only difference between the Max-Max animals and the Max-Ac animals was an increased frequency of $CD8^+$ T-cells at day 4 post infection in the Max-Max group.

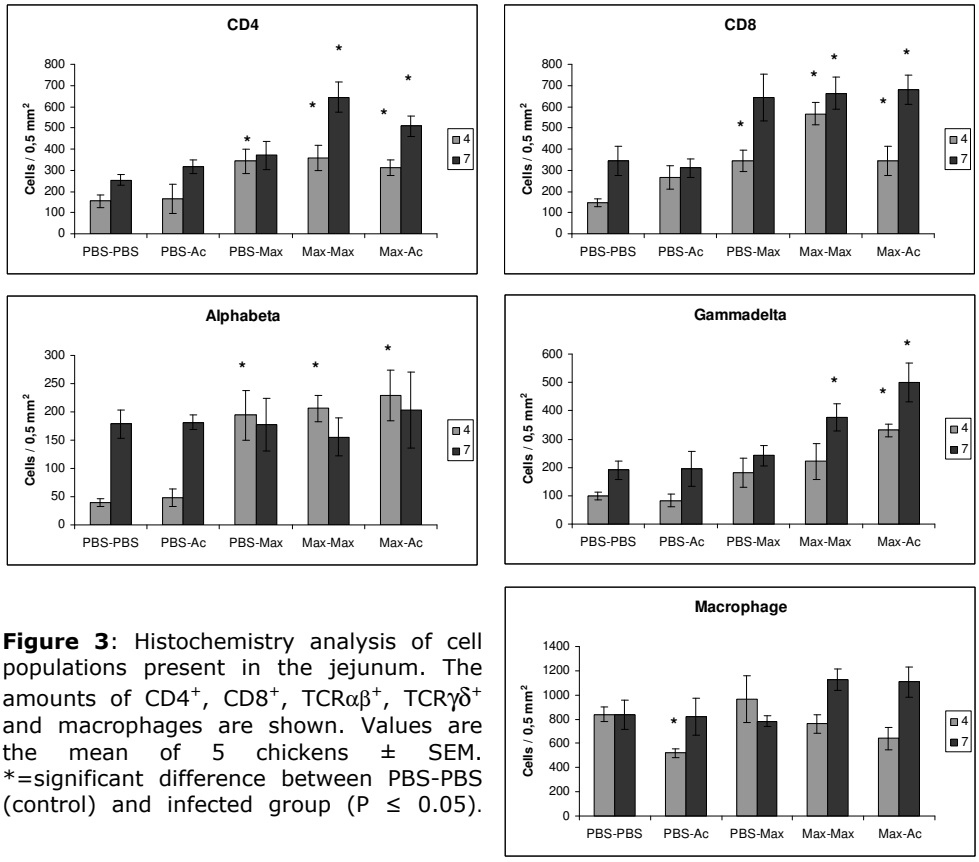

Figure 3: Histochemistry analysis of cell populations present in the jejunum. The amounts of $CD4^+$, $CD8^+$, $TCR\alpha\beta^+$, $TCR\gamma\delta^+$ and macrophages are shown. Values are the mean of 5 chickens ± SEM. *=significant difference between PBS-PBS (control) and infected group ($P \leq 0.05$).

The Max-Max and the Max-Ac group showed a significant increase of the frequency of $CD4^+$ and $CD8^+$ at days 4 and 7 post infection, $TCR\alpha\beta^+$ cells were increased at day 4 post infection and $TCR\gamma\delta^+$ cells were increased at day 7 post infection as compared to the control group. The Max-Ac group showed a significant increase of the frequency of $TCR\gamma\delta^+$ cells at 4 days post infection as compared to the control group but not when compared to the Max-Max group.

At day 4 post infection the group PBS-Max showed an increase of the frequency of $CD4^+$, $CD8^+$ and $TCR\alpha\beta^+$ cells. The group PBS–AC showed a decreased frequency of macrophages at day 4 post infection.

Table 4: Fold change (Log_2) of top 10 genes up-regulated 8 hours after a homologous *E.maxima* re-infection, combined with the fold changes (Log_2) of these genes in the rest of the experimental groups.

	Gene name	Max-Max 8pi	Max-Max 24pi	Max-Ac 8pi	Max-Ac 24pi	PBS-Max 8pi	PBS-Max 24pi	PBS-Ac 8pi	PBS-Ac 24pi	Unigene
1	Gallus gallus BAC clone CH261-29M9	5.23	6.58	6.96	6.63	4.85	5.49	5.70	6.35	ENSGALT00000018039
2	PREDICTED: Gallus gallus RAB4A, member RAS oncogene family (RAB4A)	3.72	5.23	4.47	4.82	4.58	4.73	3.71	4.25	Gga.16164
3	PREDICTED: Gallus gallus similar to JAZF zinc finger 1	3.17	4.26	3.89	4.55		3.95	4.10	3.77	Gga.11067
4	PREDICTED: Gallus gallus similar to Chromosome 1 open reading frame 96	3.12	5.28	4.97	6.48	5.54	4.95	4.96	5.00	Gga.30184
5	Gallus gallus phosphodiesterase 4B, cAMP-specific	3.02	2.13	3.77	3.49	3.73	3.44	3.36	3.86	Gga.19233
6	PREDICTED: Gallus gallus similar to PCAF associated factor 65 beta	2.96	3.29	3.98	3.53	2.33	3.25	2.89	3.74	Gga.22918
7	PREDICTED: Gallus gallus hypothetical protein LOC772470	2.85	2.34	2.53	2.88	3.39	2.67	2.80	2.58	ENSGALT00000018075
8	Gallus gallus BAC clone CH261-75C12	2.73	3.23	3.66	2.63	1.83		2.09		Gga.2909
9	PREDICTED: Gallus gallus similar to 40S ribosomal protein S29	2.70	3.21	2.67	2.99	2.03	2.60	2.48	2.27	ChEST468g22
10	Gallus gallus finished cDNA, clone ChEST130f3	2.61	2.31	2.52	1.94	2.92	2.24	2.48	2.92	Gga.13425

Differentially expression of mRNA

In each group the analysis of the micro-array data lead to about 600-1000 oligonucleotides of which expression was either significantly up or down-regulated compared to the PBS-PBS group, with a false discovery rate lower than 0,1%. The top 10 up-regulated genes in the Max-Max group 8 hpi were compared to the other groups and those results are depicted in table 4. In this table 4 the oligo's that were found had no homologue with described genes or proteins. The 'top-ten'-list of genes that were upregulated in the Max-Max group 8 hpi were found in all infections. In the PBS-Max group and in the PBS-Ac group genes were excluded due to the analysis restrictions.

The genes solely up-regulated in the homologous re-infection are depicted in table 5. Remarkable is that only 2 genes (Gallus gallus T-cell receptor beta chain and Lymphocyte antigen 6 complex) which were differentially expressed at 8hpi are also found at 24hpi. Of the ten genes solely up-regulated in the homologous infected group, one gene (MHC class II alpha chain) found at 24 hours p.i. was also significantly down-regulated at 8 hours after a homologous re-infection. Of the ten down regulated genes at 24 hours in a homologous re-infection only one gene (Gallus gallus transmembrane 9 superfamily member 3) was also found to be significantly down-regulated at 8 hours after a homologous re-infection (table 5).

Table 5: Fold change (Log_2) of top 10 genes up- or down-regulated after a homologous *E.maxima* re-infection, combined with the fold changes (Log_2) of these genes in the rest of the experimental groups. Genes are sorted for the Max-max groups 8 or 24 hours post infection (8pi and 24pi).

	Gene name	Max-Max 8pi	Max-Max 24pi	Max-Ac 8pi	Max-Ac 24pi	PBS-Max 8pi	PBS-Max 24pi	PBS-Ac 8pi	PBS-Ac 24pi	Unigene
1	Gallus gallus T-cell receptor beta-chain	2.53	1.65	1.21	1.13		1.27	1.14	1.01	Gga.19803
2	Lymphocyte antigen 6 complex, locus E	1.97	1.64	1.51	1.12	0.47	0.70	1.38	0.60	Gga.1171
3	Gallus gallus T-cell receptor beta chain constant region	1.94	0.42	1.30	1.03	0.39	1.08	0.95	0.47	Gga.1311
4	Gallus gallus similar to phospholipase A2 inhibitor gamma subunit B	1.91	1.49	1.37	1.07	-0.02	0.98	1.31	0.75	Gga.7788
5	Ribosomal protein L22	1.86	0.60	1.04	0.95	1.14	1.57	1.34	1.12	Gga.9333
6	Ribosomal protein S25	1.86	1.49	1.34	1.28	1.15	1.56	1.54	1.13	ChEST401o22
7	TM2 domain containing 2	1.84	1.20	0.76	0.94	1.41	1.13	1.31	1.15	Gga.14161
8	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit E	1.82	0.81	1.12	1.14	1.00	1.16	1.33	1.25	Gga.6091
9	Similar to ribosomal protein L30	1.80	0.86	0.96	1.11	0.61	1.31	1.43	1.11	Gga.15527
10	Gallus gallus ribosomal protein S15a	1.77	1.44	0.58	0.52	0.56	1.02	1.02	0.95	ENSGALT00000010956
	Gene name	**Max-Max 8pi**	**Max-Max 24pi**	**Max-Ac 8pi**	**Max-Ac 24pi**	**PBS-Max 8pi**	**PBS-Max 24pi**	**PBS-Ac 8pi**	**PBS-Ac 24pi**	**Unigene**
1	Similar to Maltase-glucoamylase (alpha-glucosidase)	0.22	2.28	1.09	1.49	1.35	1.41	0.41	1.06	Gga.28909
2	Taeniopygia guttata clone 0058P0009A05 RIKEN cDNA 9430023L20-like	0.33	1.96	0.66	0.57	0.67	0.60	0.52	0.48	ENSGALT00000028127
3	Gallus gallus heat shock 90kDa protein 1, beta	0.61	1.93	0.88	1.15	0.91	0.88	0.87	-0.06	Gga.2962
4	Gallus gallus pro-alpha 2(I) collagen)	-0.52	1.92	1.13	1.05	0.53	0.34	-0.14	0.54	Gga.33654
5	Gallus gallus platelet-derived growth factor receptor, alpha polypeptide	-0.66	1.90	0.86	0.73	1.40	0.82	-0.29	0.43	ENSGALT00000016193
6	Gallus gallus MHC class II alpha chain, complete	-1.81	1.88	1.10	0.66	0.16	-0.09	-1.32	-0.94	Gga.33711
7	Gallus gallus hydroxyacyl-Coenzyme A dehydrogenase	1.26	1.86	1.38	1.53	0.66	1.14	1.44	1.27	Gga.3036
8	Gallus gallus Gatinaise disrupted tyrosinase gene	0.08	1.82	0.90	0.13	0.33	0.71	0.25	0.34	Gga.10608
9	Gallus gallus phosducin-like	1.22	1.82	1.34	1.23	1.02	1.25	0.90	1.18	ENSGALG00000001275
10	Gallus gallus survival motor neuron domain containing 1	0.86	1.82	1.26	1.45	0.58	1.02	1.31	1.26	Gga.21189

	Gene name	Max-Max 8pi	Max-Max 24pi	Max-Ac 8pi	Max-Ac 24pi	PBS-Max 8pi	PBS-Max 24pi	PBS-Ac 8pi	PBS-Ac 24pi	Unigene
1	Transmembrane protein 53	-2.49	-0.94	-1.08	-0.71	-1.41	-1.51	-1.49	-0.98	Gga.11943
2	Similar to leukemia virus-b receptor	-2.38	-1.57	-0.98	-1.53	0.11	-1.24	-1.55	-1.23	Gga.3329
3	Similar to Leucine rich repeat containing 1	-2.35	0.01	0.25	-0.30	-1.30	-0.67	-0.93	-0.89	Gga.16766
4	Gallus gallus ATPase8, ATPase6 genes for F0-ATP synthase subunit	-2.20	-0.35	-0.78	-0.32	-0.71	-0.82	-1.48	-1.31	Gga.7069
5	Serum/glucocorticoid regulated kinase 2	-2.17	-0.40	-0.78	-1.34	-0.73	-0.96	-1.49	-1.52	Gga.9334
6	Gallus gallus heat shock cognate protein HSP 90-beta	-2.11	-0.12	-1.02	-0.71	-1.17	-0.96	-1.17	-1.19	Gga.4332
7	Gallus gallus c-ets-1 oncogene	-2.08	0.06	-0.33	-0.60	-0.17	-1.08	-1.10	-1.33	ChEST596k12
8	Gallus gallus platelet-derived growth factor receptor, alpha polypeptide	-2.06	-0.87	-1.00	-0.98	-1.23	-1.38	-1.33	-0.82	Gga.274
9	Similar to SLC26A1 anion exchanger Monodelphis domestica	-2.04	-0.85	-1.30	-0.32		-1.06	-1.07	-1.20	ENSGALT00000024816
10	Phosphatidylinositol-specific phospholipase C, X domain containing 2	-2.01	-0.88	-1.40	-0.91	-1.50	-1.01	-1.18	-1.43	Gga.10625
	Gene name	**Max-Max 8pi**	**Max-Max 24pi**	**Max-Ac 8pi**	**Max-Ac 24pi**	**PBS-Max 8pi**	**PBS-Max 24pi**	**PBS-Ac 8pi**	**PBS-Ac 24pi**	**Unigene**
1	Gallus gallus LanC lantibiotic synthetase component C-like 3	-1.05	-2.27	-1.34				-1.31		ENSGALG00000016265
2	Similar to RanBP17 Ornithorhynchus anatinus		-2.23		-1.33	0.89	-0.86	-1.07	-0.68	Gga.4167
3	Gallus gallus potassium inwardly-rectifying channel, subfamily J, member 12		-2.12	-1.41	-1.49				-1.08	Gga.254
4	Similar to Creb5 protein Mus musculus		-2.05	-0.89	-1.26		-0.67	-1.07		ENSGALT00000020678
5	Gallus gallus cysteine dioxygenase, type I	-1.57	-2.04	-1.04	-0.31	-1.27	-1.09	-0.77	-0.47	Gga.6921
6	Gallus gallus eukaryotic translation initiation factor 1A, X-linked	-0.53	-1.96	-1.57	-1.50	-0.99	-0.95	-0.91	-0.76	Gga.15791
7	Gallus gallus solute carrier family 25, member 29	-0.37	-1.90					-0.65		ENSGALG00000011220
8	Gallus gallus transmembrane 9 superfamily member 3	-1.62	-1.86	-1.41	-1.39		-1.55	-1.26		Gga.4101
9	Gallus gallus BTB and CNC homology 1, basic leucine zipper transcription factor 2		-1.83	-1.29			-1.49			Gga.11181
10	Gallus gallus POU class 2 associating factor 1	0.41	-1.82	-0.12	-0.57		-0.67			Gga.4907

Discussion

To investigate the differences between the protective and non-protective immune reactions following *Eimeria* infections we studied infected naïve or *E.maxima* primed chickens after infection with either *E.maxima* or *E.acervulina*. Chickens primed with *E.maxima* after a homologous re-infection were supposed to mount protective immunity (Blake, D.P. *et al*., 2005). The local gut host immune reactions to different *Eimeria* infections were studied by histological counting of specific T-cells and mRNA expression with a oligo micro-array and real-time quantitative PCR for specific cytokine levels.

The present infections can be considered as mild *Eimeria* infections. Though 2,0 – 5,0 x 10^4 oocysts were used for infection no significant differences in weight gain were found in this experiment. Nevertheless typical gut lesions were observed in all infected animals that were investigated.

After a homologous re-infection no oocysts were found in the faeces on days 2, 4 and 7 after infection. This indicated that the "memory" induced upon primary infection resulted in efficient and protective responses to secondary infection. This phenomenon was confirmed by a real-time PCR for *Eimeria* DNA on intestinal samples. A cleared primary infection led to control of *Eimeria* infections following homologous secondary exposure, but this was not observed in a heterologous challenge. Therefore we set out to determine which factors might contribute to this protective response.

Cellular responses in terms of changes in subpopulation frequencies, were present after primary and secondary-infection. An up-regulation of the T-cells expressing the $CD4^+$ and $CD8^+$ marker was found in both homoloqous and heterologous infected groups at the same moments. The cells expressing the $CD8^+$ marker were more up-regulated after homologous re-infection at day 4 post infection as compared to the heterologous infected group. This might be one of the major components of a protective response, because it is thought that the $CD8^+$ T-cells play a role in the recovery phase of infections and thus might reflect enhanced acquired immune status (Lillehoj, H.S., 1988; Swinkels, W.J. *et al.*, 2007). The kinetics of the $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells did not follow the profile of $CD4^+$ and $CD8^+$ contrary to results from other experiments (Swinkels, W.J. *et al.*, 2007). No specific cell subpopulation that was investigated was observed was increased in frequency only after a homologous re-infection. This might be an indication that the the cell subpopulations examined in our experiment might not include the key cell types playing a role in the protective response to a re-infection. It might however also be possible that the contribution of T-cells and macrophages occurs earlier after infection. than was investigated in the present experiments.

We observed the cytokine mRNA production at 2, 4 and 7 days post infection in all groups. This yielded an up-regulation of the cytokine IFN-γ for all infected groups, with the highest up-regulation found after the primary *E.maxima* infection. Up-regulation of the cytokine IFN-γ was described earlier (Min, W. *et al.*, 2003; Swinkels, W.J. *et al.*, 2007). After any infection with *E.acervulina* or *E.maxima* a conserved Th1 type of reaction was induced, IFN-γ is involved in Th1 processes, a type of reaction which is known to resolve intracellular infections. The profile of the IFN-γ up-regulation resembles the profiles found for the chemokine IL-8 and the regulatory interleukin IL-10. The chemokine IL-8 was found to be induced only in the jejunum of primary and secondary *E.maxima* infected animals. The only cytokine which was found to be down-regulated was IL-7 after a primary *E.maxima* infection. In mice IL-7 is an extrathymic regulator of TCRγδ$^+$ IEL differentiation and growth (Okazawa, A. *et al.*, 2004). and these cells are thought to play a role in repair of lesions and in response regulation (Roberts, S.J. *et al.*, 1996). The primary *E.maxima* infected group has the highest parasite load and as a result of parasites leaving host cells this group may have the most lesions. We hypothesized that in the max group the most recruitment of TCRγδ$^+$ cells, and therefore the highest level of IL-7 was expected. This however was not the case. The TCRγδ$^+$ T-cells could have differentiated and grown in the chicken spleen, followed by a recruitment of mature cells to the site of infection. The low reactivity of T-cells of the homologous re-infected animals is in line with the findings of Hong et al. and Min et al. who also found that coccidiosis induces a diverse and robust primary cytokine / chemokine response, but a more subdued secondary response (Hong, Y.H. *et al.*, 2006a; Hong, Y.H. *et al.*, 2006b; Min, W. *et al.*, 2003).

Since we did not find many factors which could be involved in protection against the homologous re-infection, we investigated the mRNA expression profiles in early host responses. Comparing the 'top ten' expressed genes of all groups at 8 and 24 hours post infection yielded more similarities than differences between a primary infection and a secondary infection. This indicates that early after infection the interaction of the host with *Eimeria* leads to induction of the same set of genes independent of the character of the infection i.e. primary vs secondary infection or even secondary infection with a homologous parasite. The 5 genes that showed the highest expression in the Max-Max infected group at 8 hours post infection were also found as highly up-regulated genes of all groups, except for the PBS-Max and Max-Max group. In this latter group at 24 hours post infection phosphodiesterase 4b was up-regulated 4,4 times, which is almost a factor 2 lower as the observed up-regulation in all other groups. The regulation of genes independent of the kind of eimeria infection is in line with the findings of Dalloul et al. who found a

set of core response elements comprising 25 genes in animals infected with *E.acervulina*, *E.tenella* and *E.maxima* (Dalloul, R.A. *et al.*, 2007).

We searched for the specific genes only present in the early host response of animals in the Max-Max group as compared to animals from all of the other groups. First we sorted for up-regulation solely in homologous re-infection, at 8 hours post infection. The genes in this comparison were T-cell receptor genes or proteins mostly expressed on lymphocytes. Four other genes of the top ten up-regulated genes in homologous re-infected chickens at 8 hours post infection were involved in ribosomal composition. When gene expression was sorted for up-regulation solely in homologous re-infected chickens, at 24 hours post infection, only one of the top ten genes was involved in antigen binding (MHCII alpha chain) and no ribosomal proteins were found. The few specific genes observed in this study which were only found after a homologous re-infection could possibly be related to specific protection, but this is not proven yet by functional assays. Further studies will be needed to understand the function and pathways of the genes described here. As early as 8 hours post infection after a homologous *E.maxima* re-infection, the host increases the expression of T-cell markers and production of proteins. A T-cell reaction may result in a recognition of intruders and as a result of this, elimination of these cells. Due to the up-regulation of ribosomal proteins the host cells may facilitate more translation of mRNA thus producing proteins involved in protection to homologous *E.maxima* re-infection. In vaccination programs up-regulation of T-cell and ribosomal proteins might be a useful method to restrict *Eimeria* infections.

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Chapter 7

Immunity against *Eimeria acervulina*: the influence of exposure history on transmission in broiler chickens

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Abstract

We experimentally study, for the first time, individual effects of previous exposure to *Eimeria acervulina* on immune response and oocyst output after a subsequent infection and, simultaneously, the resulting transmission at the population level. A pair-wise transmission experiment was carried out with different combinations of inoculated (I) and contact (C) birds, either previously exposed (primed, p) or not, i.e. pairs of I-C, I-C_p, I_p-C and I_p-C_p birds and naïve non-inoculated control chickens. Transmission rates (number of new infections due to one animal per unit of time) were calculated, oocyst output was quantified in feces of individual birds and $CD4^+$, $CD8^+$, $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T-cell subsets and macrophages were quantified in duodenum sections. No single cell type could characterize protection level. Naïve contacts, however, had an increase in the frequency of $CD4^+$ T-cells during the infection cycle and an increase of $TCR\gamma\delta^+$ T-cells starting at the end of the infectious cycle. Transmission rate was reduced with 98% in the I_p-C_p group, compared to I-C birds. Reduction in transmission rate was higher in the I-C_p group, compared to the I_p-C group. The study clarifies the importance of time in the development of immunity in a natural setting.

Introduction

Coccidiosis, caused by protozoan parasites of the genus *Eimeria*, is one of the most prevalent diseases in the poultry industry and is responsible for considerable economic losses (Williams, R.B., 1999). Upon ingestion of infectious *Eimeria* spp. oocysts, chickens develop (partial) immunity which can protect them against clinical signs during subsequent infections, later on during the production cycle. The immune response of the host depends on the infection exposure history and will affect the oocyst output (Lillehoj, H.S., 1988a; Stiff, M.I. and Bafundo, K.W., 1993; Williams, R.B., 1995b).

This 'circle of interaction' determines the overall infection dynamics, which can be very complex and even unpredictable in host-parasite systems in general (Roberts, M.G. and Heesterbeek, J.A., 1998; Roberts, M.G. and Heesterbeek, J.A.P., 1995) and for *Eimeria* spp. infections in particular (Klinkenberg, D. and Heesterbeek, J.A., 2007). As oocyst uptake is a stochastic process, heterogeneity in acquired immunity of individuals in a flock arises due to interactions between intracellular parasite stages and the immune system of the individual bird (within-host dynamics). This results in variability in oocysts excretion, heterogeneous distribution of oocysts in the environment, and variability in oocyst ingestion, which in turn influences the rate of transmission of the parasite in the flock (between-host dynamics). These processes cause a flock to become a collection of interacting individuals with different and evolving immune status, with associated differences in reaction to infection.

Mathematical models have shown that heterogeneity in immune status between individual birds can have large effects on the course and severity of *Eimeria* spp. infections on individual and flock level and on the effectiveness of control measures (Klinkenberg, D. and Heesterbeek, J.A., 2007; Severins, M. *et al.*, 2007). Therefore, both the population-dynamic characteristics of coccidiosis and within-host dynamics of *Eimeria* spp. infections, *i.e.* the full 'circle of interaction', should be taken into account when studying the dynamics and control of coccidiosis.

Various control measures have been developed and applied in the past. Most studies that have been carried out to evaluate these control measures, have focused either only on reduction of clinical signs and oocyst excretion (Chapman, H.D. *et al.*, 2002; Williams, R.B., 2002) or primarily on the immune response (Allen, P.C. and Fetterer, R.H., 2002; Dalloul, R.A. and Lillehoj, H.S., 2005). Up to now there are no experimental results that quantify the full infection cycle.

The aim of this study was not only to determine and quantify the effect of previous exposure to *Eimeria* spp. on immune response and oocyst output individually, but is also the first study to interpret and quantify

consequences for a population of birds regarding transmission dynamics of *Eimeria* spp. infections. A transmission experiment was carried out in which transmission rate (the number of new infections due to one inoculated animal per unit of time) and intestinal immune response were quantified between pairs of broilers with different exposure history. The pair-wise design enables interpretation of the relation between oocyst output and transmission rate and assessment of the relative importance of level of infectiousness of infected birds and level of susceptibility of contact-exposed birds within the population dynamics of an *Eimeria* spp. infection.

The methods described in this paper can be used for studying *Eimeria* spp. infections and might also be applicable for studying other host-parasite interactions where, analogous to *Eimeria* spp. infections, the response to infection is determined largely by the acquired immunity, which is built up gradually by the environmentally-driven heterogeneous exposure history to the pathogen.

Materials and Methods

Chickens and management

At day 0 of age (day 0 of the experiment), a total of 198 male broiler chickens were obtained from GD-Animal Health Service (Deventer, The Netherlands). The chicks originated from a specified pathogen free (SPF) parent flock, a crossbred of Cobb, Hybro and Ross, bred in an *Eimeria* spp. free environment. All chicks were reared in battery cages (Tecniplast®, Technilab-BMI, The Netherlands) with smooth, perforated Noryl® floors (height x width x depth = 95x65x65 cm) from day 0 to day 25. After this date, chicks were housed in pairs on sawdust litter in floor pens (height x width x depth = 80x60x50 cm), except for the control birds which were housed in battery cages with sawdust litter in groups of five birds. The room was cleaned with a 10% solution of ammonia and the wooden floor pens consisted of solid floors and high solid walls, that had not been in contact with chicken feces before the experiment started, to reduce the chance of between-pen transmission with *Eimeria* spp. parasites.

Room temperature was 32 °C at day 0 and was gradually decreased to 18 °C at day 50. The chicks were subjected to a lighting scheme of 23 hours of light per day. A broiler ration (12 MJ/kg metabolizable energy) without anticoccidial drugs, and drinking water were available *ad libitum*.

The birds were observed twice daily for signs of illness or welfare impairment. Birds were housed, handled and treated following approval by the Animal Experimental Committee of Utrecht University (Utrecht, The Netherlands), in accordance with the Dutch law on experimental animals.

Experimental design

A transmission experiment was carried out with pairs of birds that were naïve or had previously been exposed to *E. acervulina*. The controlled exposure history to *E. acervulina* was established by a single oral exposure to 5 x 10^4 sporulated oocysts suspended in 1 ml water at day 6 of age (referred to as "priming"), based on the results from previous experiments by Swinkels et al. (Swinkels, W.J. *et al.*, 2006; Swinkels,

W.J. *et al.*, 2007) who demonstrated a temporary change in number of intestinal $CD8^+$ and $TCR\gamma\delta^+$ cells as the result of inoculation with a single dose of 5 x 10^4 sporulated oocysts after this treatment.

At day 24 of age, 12 primed birds (p) and 62 non-primed birds were orally inoculated with 5 x 10^4 sporulated *E. acervulina* oocysts in 1 ml of water (referred to as "inoculated" birds). From day 25 onwards birds were housed pair-wise in a floor pen. Four different combinations of inoculated and contact birds were used in the experiment:

- 31 pairs of inoculated non primed birds and contact non primed birds (I-C)
- 31 pairs of inoculated birds and primed contact birds (I-C_p)
- 6 pairs of primed inoculated birds and contact birds (I_p-C)
- 6 pairs of primed inoculated birds and primed contact birds (I_p-C_p).

Transmission and the number of oocysts per gram of feces (OPG) was studied using data from 6 pairs for all combinations. From these birds single individual droppings were collected daily from day 10 to day 23 (only I_p and C_p birds) and daily from day 28 to day 50 (all birds).

The immunohistochemistry was studied in 25 pairs of the I-C and 25 pairs of the I-C_p combination. Twenty-five naïve non-inoculated chickens were kept in groups of five birds as negative controls for immune parameter analysis. Five pairs of I-C and I-C_p birds and five birds of the control group killed by cervical dislocation at day 28, day 31, day 33, day 36 and day 39 respectively. One cm of duodenal loop was snap-frozen in liquid nitrogen and stored at -70°C until further examination by immunohistochemistry.

Group composition, housing conditions and measured parameters are outlined in Table 1.

Table 1. Outline of the experimental design; composition of groups, housing conditions and measurements.

Description[1]	# of chicks[2]	Housing > D25	Measurements
Control group	5x5	Groups	T-cells on D28, D31, D33, D36, D39
I+C	6x2	Pairs	OPG from D28-50
	25x2	Pairs	T-cells on D28, D31, D33, D36, D39
I+C_P	6x2	Pairs	OPG daily from D28-50
	25x2	Pairs	T-cells on D28, D31, D33, D36, D39
I_P+C	6x2	Pairs	OPG daily from D28-50
I_P+C_P	6x2	Pairs	OPG daily from D28-50

[1] I = chicks inoculated at D24, C = contact chicks, not inoculated at day 24, Control group = not inoculated at day 24. When subscript $_p$ is present, birds received a previous infection at day 6.
[2] First number represents number of cages and the second number gives the number of birds per cage

Immunohistochemistry
Frozen duodenum sections (8 µm thick), collected from chickens of 28, 31, 33, 36 and 39 days of age were immunohistologically stained by an indirect immuno-peroxidase method. The sections were loaded on glass slides, air-dried, and fixed in acetone for 10 min. After drying, endogenous peroxidase was inhibited for five minutes at room temperature (2% NaN3 in TRIS-HCl 0.05M pH 7.5 + 0.06% H2O2). The slides were washed with PBS with 0.1% BSA and were subsequently incubated for 1 h with monoclonal antibodies against $CD4^+$ T-cells (1:200; CT-4, Southern Biotech), $CD8^+$ T-

cells (1:200; CT-8, Southern Biotech), TCR$\alpha\beta^+$ cells(1:50; TCR2, Southern Biotech), TCR$\gamma\delta^+$ cells(1:400; TCR1, Southern Biotech) or macrophages (1:50; KUL1, Southern Biotech) followed by peroxidase-conjugated rabbit anti-mouse Ig (1:80; P0161, Dakopatts, Denmark). Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M Tris-HCl solution (pH 7.5) containing 0.03% H2O2. The slides were further colored with 1% $CoCl_2$ for five minutes. After washing, the nuclei were counter-stained with hematoxylin. The sections were dehydrated and mounted in Eukitt™ Mounting Medium. The images were analyzed with Image-Pro Plus (version 5.1, media cybernetics) to quantify the number of stained T-cells per 0.5 mm^2. Per bird five images were acquired and analyzed and the mean of the five log_{10} transformed T-cell counts per bird was used for the statistical analysis.

A linear mixed model was applied using SAS software (SAS, 2004) with log_{10}-transformed mean cell counts as dependent variable and litter cage as random factor. Bird type (5 different bird types, i.e. inoculated and contact birds of the I-C and I-C_p group and control birds), day (day 28, 31, 33, 36 and 39) and the interaction group*day were entered into the model as fixed factors. The two-tailed partial F-test (type III) was used as the elimination criterion for the model building and the fit of the model was assessed by the Akaike Information Criterion. The best model for $CD4^+$ and $CD8^+$ T-cells consisted of day and the interaction between group*day. The final model for TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ cells contained day, group and group*day as fixed factors. Differences in T-cell counts were assessed per day between inoculated birds of both groups and control birds, between contact bird of both groups and control birds, between inoculated birds of the I-C group and inoculated birds of the I-C_p group and between contact birds of the I-C group and contact birds of the I-C_p group. Bonferonni corrected pair-wise multiple t-tests were carried out to test the difference between each above mentioned pair of means. Model assumptions were evaluated by examining normality and equality of variances of the residuals. The level of statistical significance was set at $P < 0.05$.

Droppings collection, processing and oocyst counts

Absence of excretion of oocysts was verified on days 6 and 9 by examining pooled samples of all birds with the sedimentation-flotation technique according to Long et al. (Long P.L. *et al*., 1976). Single individual droppings were collected daily from primed birds from day 10 to day 23 and from inoculated and contact birds daily from day 28 to day 50 or until oocysts were not detected for three consecutive days. The single droppings were collected according to Velkers et al. (submitted) by placing each chick in a cardboard box, with a clean paper sheet for one to two hours, The single dropping was weighed and the number of oocysts per gram of feces (OPG) was determined, according to a modification of a McMaster oocyst counting chamber technique described by Long and Rowell (Long P.L. and Rowell J.G., 1958). When OPG was zero the sedimentation flotation technique was carried out

Oocyst counts (OPG) were not Gaussian distributed and therefore counts were log_{10} transformed, after adding 1 to the OPG to allow computations. The average oocyst production was determined by calculating the mean of the log_{10} transformed OPG per day for $I_{(p)}$- and $C_{(p)}$-birds per group. In cases of missing values due to absence of single droppings, the mean was calculated using the available oocyst counts.

The average log_{10} transformed AUC (area under the curve; written as logAUC) was calculated for $I_{(p)}$- and $C_{(p)}$-birds per group using the daily log_{10} transformed OPG results. The period in which OPG was intermittently above zero (last day of positive OPG minus first day of positive OPG results = PERIOD) and the highest measured log_{10} (OPG) peak output (PEAK) were determined for $I_{(P)}$- and $C_{(P)}$- birds per group.

A linear mixed model was applied with logAUC, √PERIOD or PEAK as dependent variable, bird type (8 different bird types, i.e. $I_{(P)}$- and $C_{(p)}$-birds of each of the four

different groups) as fixed factor and litter cage as random factor using SAS software (SAS, 2004). The two-tailed partial F-test (type III) was used as the elimination criterion for the model building and the fit of the model was assessed by the Akaike Information Criterion. Bonferonni corrected pair-wise multiple t-tests were carried out to test the difference between each pair of means. Model assumptions were evaluated by examining normality and equality of variances of the residuals. The level of statistical significance was set at $P < 0.05$.

Transmission rate calculations

The transmission rate is defined as the number of new infections that occurs due to one infectious animal per unit of time in a large completely susceptible population (Diekman, O. and Heesterbeek, J.A.P., 2000). The epidemic model for a pair-wise transmission experiment with *Eimeria* spp. consists of only two population states, i.e. one state where one infectious inoculated bird and one contact bird are present and another state where the contact bird has become infected and both the newly infected contact bird and the inoculated bird are "infectious". It is assumed that transition from the "infectious" state to a "recovered state", which means that the bird is no longer able to infect contact birds, does not take place during the experiment, as excretion continues during most of the experiment.

To estimate the probability of infection and subsequently the transmission rate, it is necessary to determine for each contact bird the number of days without successful transmission, i.e. the number of days that has passed between the first day when it is considered possible for contact birds to start their infectious period and the day when the contact birds actually started to excrete oocysts (referred to as "infection lag time"). When contact birds first showed positive McMaster oocyst counts they were considered to have become infectious. The first day when contact birds could have started their infectious period was assumed to be from D32 onwards, based on the pre-patent period of 4 days (Edgar, S.A., 1955; Joyner, L.P. and Long, P.L., 1974) for both inoculated and contact bird. It was assumed that sporulation time was negligible, as it is known that small numbers of sporulated oocysts can be present already after a few hours following excretion (Graat, E.A.M. *et al.*, 1994) and as oocyst excretion was already observed in contact birds from D32 onwards.

The probability of infection, denoted by $\hat{p}$, was estimated using maximum likelihood, based on the total number of successful transmissions from an inoculated to a contact bird ($\underline{k}$), the number of days without successful transmission added for all contact birds ($\underline{y}$), the number of observed days in the experiment (from day 32 to day 50 = 19 days = n_x) and the number of pairs of inoculated and contact birds (n_k=6). With the maximum likelihood estimation for the probability of infection the transmission rate can be estimated applying: $\hat{\beta} = -2\ln(1-\hat{p})$

The 95% confidence interval around the probability of infection was calculated using a cumulative distribution function of the maximum likelihood estimator for $\hat{p}$. A negative binomial distribution was used that described the distribution the distribution of $\underline{y}$ failures before $\underline{k}$ successes and the number of pairs $\underline{k}$ out of n_k in which the contact bird did become infected during n_x days. Subsequently the 95% confidence interval for the transmission rate was calculated. The calculations and equations are given in detail by Velkers et al. (Velkers F.C. *et al.*, In preparation) and have been based on similar calculations by Velthuis et al. (Velthuis A.G. *et al.*, 2002).

Results

Immunohistochemistry

In Figure 1 T-cell profiles are given for the inoculated and contact birds of the I-C and I-C_p groups for days 28, 31, 33, 36 or 39. Significant differences in T-cell counts between contact birds of the I-C and I-C_p group were not found for any of the T-cell types at any of the observed days.

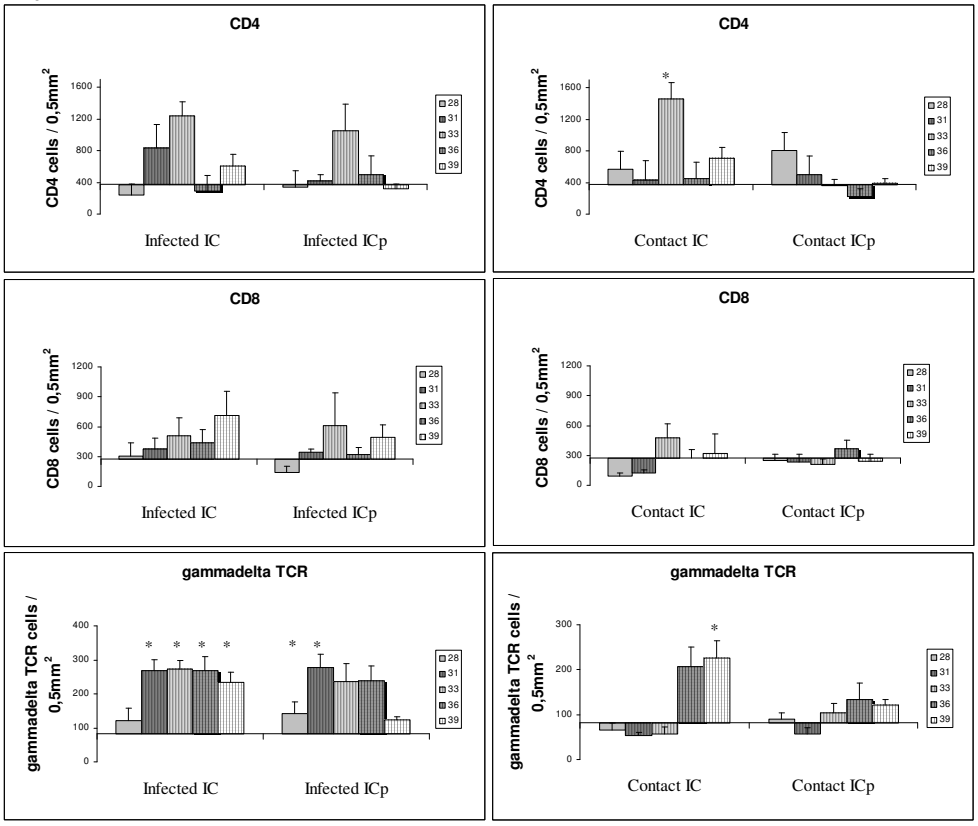

Figure 1: The number of $CD4^+$, $CD8^+$ and $TCR\gamma\delta^+$ cells in duodenum sections from inoculated and contact chickens of the I-C group and the I-Cp group compared to a baseline formed by chickens of the control group at 28, 31, 33, 36 and 39 days of age.$CD4^+$, $CD8^+$ and $TCR\gamma\delta^+$ T-cells were stained and the amount of positive cells in an intestinal segment was counted. Mean of 5 chickens is shown. Baseline of the graphs is not set at zero, but at the mean amount of the control values. (*) Significant differences in the numbers of the different T cell types between I or $C_{(p)}$ birds and the control group ($P \leq 0.05$). Note the difference in the scales. Plain columns represent measurements before transmission, striped columns represent data when infectious animals are able to infect their contact animals and checked columns represent data when both animals are able to infect each other.

The contact birds of the I-C group had significantly higher $CD4^+$ T- cell counts compared to the control birds on day 33. A non-significant increase was observed for $CD4^+$ T-cells in inoculated birds in both the I-C and the I-C_p groups. Contact chickens of the I-C_p group showed no increase in their intestinal $CD4^+$ T-cell amount. Non-significant $CD4^+$ T-cell increases were observed at day 39 in both infected and contact animals from the I-C group.

The frequency of TCR$\gamma\delta^+$ T-cells in the inoculated birds from the I-C group differed significantly from the frequency observed in control birds from day 31 onwards. For inoculated birds from the I-C_p group, the frequency of TCR$\gamma\delta^+$ T-cells differed significantly from the control birds at days 28 and 31. A significantly higher frequency of TCR$\gamma\delta^+$ T-cells was also found in the contact birds from the I-C group at day 39 of age when compared to the control birds.

Significant differences in $CD8^+$ T-cells and macrophages were not observed between inoculated and contact birds of the I-C and I-C_p groups when compared to control birds.

The frequency of the TCR$\alpha\beta^+$ T-cells in control birds showed high fluctuations for all observed days.

Oocyst excretion patterns

The priming inoculation at day 6 resulted in excretion from day 10 to day 23 with an average $\log_{10}$(AUC) of 6.44 (SD 0.26).

In Table 2 the number of excreting birds, average period in which OPG was intermittently positive, $\log_{10}$(AUC), and the $\log_{10}$(OPG) of the peak output are summarized for the oocyst excretion period after inoculation, from day 28 to day 50. All inoculated birds of the I-C and I-C_p group excreted oocysts, whereas only four inoculated birds of the I_p-C group and one inoculated bird of the I_p-C_p group excreted oocysts. All contact birds of the I-C and I_p-C groups excreted oocysts. In the I-C_p group five contact birds and in the I_p-C_p group, one contact bird showed positive OPG results.

Non-primed I and C birds had a significantly longer OPG positive period, higher $\log_{10}$(AUC) and higher peak outputs compared to the primed I_p and C_p birds. These oocyst excretion parameters were significantly higher for contact infected birds from the I_p-C group than for contact birds from the I-C_p group. For primed inoculated birds in the I_p-C group, these parameters were significantly higher than for primed inoculated birds in the I_p-C_p group.

In Figure 2 average oocyst excretion patterns are visualized for the four groups. In the I-C and I-C_p group most of the inoculated birds started oocyst excretion on day 28. Inoculated birds of the I_p-C and I_p-C_p group only showed output of oocysts from day 42 and 31, respectively. Contact birds of the I-C group and of the I-C_p and I_p-C group started shedding

oocysts on day 32 and 33 respectively. Contact birds of the I_p-C_p group only showed oocyst output on day 34.

Table 2: Duration of oocyst excretion period, total number of excreted oocysts during entire excretion period (AUC) and peak value of the oocyst output for $I_{(p)}$- and $C_{(p)}$- birds for all groups according to McMaster oocyst counts.

Group	Bird	Excreting birds	OPG Positive period[1] Mean (SD)	^{10}log(AUC)[2] Mean (SD)	Peak output[3] Mean (SD)
I-C	I	6	14.67 (3.93)^{a}	6.29 (0.30)^{a}	5.82 (0.26)^{a}
I-C	C	6	13.83 (0.98)^{a}	6.83 (0.51)^{a}	6.57 (0.53)^{a}
I-C_P	I	6	13.00 (3.10)^{a}	6.36 (0.39)^{a}	6.03 (0.51)^{a}
I-C_P	C_p	5	6.00 (5.76)^{b}	2.46 (1.42)^{b}	2.18 (1.21)^{b}
I_P-C	I_p	4	2.17 (3.43)^{b}	1.59 (1.40)^{b}	1.63 (1.44)^{bc}
I_P-C	C	6	14.50 (2.17)^{a}	5.52 (0.47)^{a}	5.23 (0.44)^{a}
I_P-C_P	I_p	1	0.17* (0.41)^{c}	0.49 (1.20)^{c}	0.48 (1.18)^{c}
I_P-C_P	C_p	1	0.17* (0.41)^{c}	0.24 (0.58)^{c}	0.23 (0.57)^{c}

[1]= period (in number of days) of (intermittent) oocyst excretion, which is the last day with OPG larger than 0 minus the first day with OPG larger dan 0
[2]= AUC which represents oocyst output during the entire oocyst excretion period
[3]= highest OPG, measured during the entire oocyst excretion period
[a-c] Values within the same column and with different superscripts differ significantly ($P<0.05$)

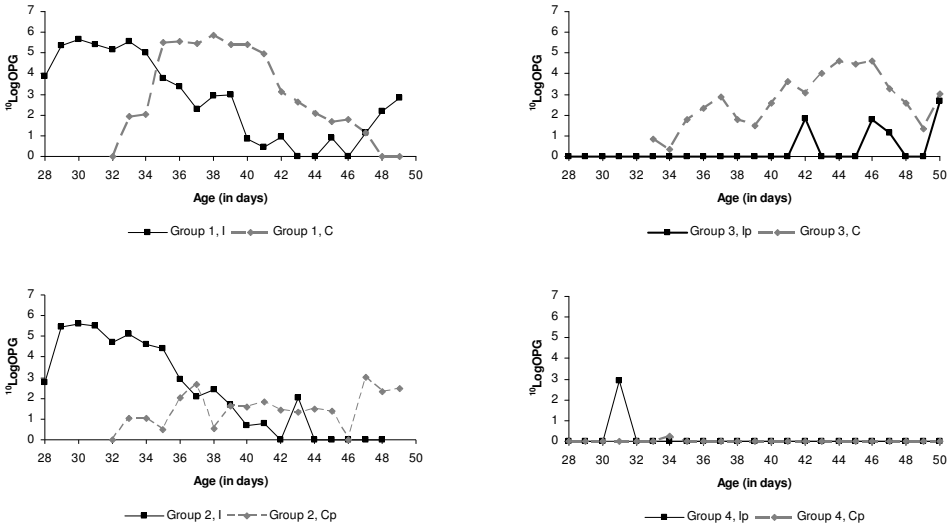

Figure 2: Average oocyst output of 6 pairs of chickens from groups 1 to 4.

Tables 3a to 3d show McMaster oocyst counts results and sedimentation-flotation results for each individual bird. For the I_p-C group, oocysts were not detected in two of the inoculated birds, whereas all contact birds from this group became positive for oocysts. In the I_p-C_p group inoculated birds were positive for only one to two days, which started for two birds on day 30, for one bird on day 33 and for two birds on day 35, whereas their contact birds did not become positive. In one pair the contact bird was positive on day 34 for one day without a single positive result of its inoculated pen mate.

Sedimentation-flotation results generally show positive results at the start of the excretion period before positive McMaster oocyst counts are found and remain positive for a longer period than McMaster oocyst counts at the end of the excretion period. In addition, the sum of the infection lag times for each contact bird (time between D32 and start of excretion of the contact birds) and the total number of infected contact birds per group are presented in Tables 3a-3d and were used to calculate the transmission rate.

Table 3a: Group 1: I + C

Bird[1] / Age[2]	827i	803c	764i	756c	311i	823c	741i	847c	310i	839c	795i	857c	Total infection lag time[4]	Total number of infected C birds
28	+*		-*		+*		+*		+*		+*			
29	+*		+*		+*		+*		+*		+*			
30	+*		+*		+*		+*		+*		+*			
31	+*		+*		+*		+*		+*		+*			
32	+*	*-	+*	*-	+*	--	+*	--	+*	--	+*	*-		
33	+*	+-	+*	*-	+*	++	+*	--	+*	--	+*	+-		
34	+*	--	+*	++	+*	++	+*	++		--	+*	-+		
35	+*	++	+*	++		++	+*	+*	-*	++	+*	++		
36	+*	+*	+*	+*	+*	+*	+*	+*	--	+*	+*	+*		
37	+*	+*	+*	+*	-*	+*	+*	+*	+*	+*	+*	+*		
38	++	+*	+-	+*	++	+*	+-	+*	+-	+*	+-	+*		
39	++	+*	++	+*	+*	+*	++	+*	+*	+*	+*	+*		
40	-*	+*	-*	+*	-*	+*	-*	+*	+*	+*	+*	+*		
41	-*	+*	--	+*	-+	+*	-+	+*	-*	+*	+*	+*		
42	++	+*	-+	+*	-+	+*	++	+*	--	-*		+*		
43	--	+*	*-	+*	*-	+*	--	-*	*-	+*	-+	-*		
44	*-	+*	-+	+*	*-	+*	-+	-*	*-	+*	--	+*		
45	*-	+*	*-	+*	*-	+*	-+	--	*-	-*	++	-+		
46		+*		+*		-*	--	++		-+		++		
47	-+	+*	*-	-*	*-	+*	*-	--		+-	++	--		
48	*-	--		--		-*	*-	--		--	++	-+		
49	*-	--		-+		--		*-	*-		++	-+		
50														
Infection lag time C bird[3]		1		2		1		2		3		1	10	6

Table 3b: Group 2: I + C_p

Bird[1] / Age[2]	760i	771cp	838i	814cp	853i	858cp	789i	849cp	870i	749cp	796i	779cp	Total infection lag time[4]	Total number of infected C birds
28	+*		+*		+*		-*		+*		+*			
29	+*		+*		+*		+*		+*		+*			
30	+*		+*		+*		+*		+*		+*			
31	+*		+*		+*		+*		+*		+*			
32	+*	*-	+*	-+	+*	-+	+*	-+	+*		+*	*-		
33	+*		+*	--	+*	--	+*	++	+*	--	+*	+-		
34	+*		+*	-+	+*	++	+*	+*	+*	--	+*	--		
35	+*	--	+*	-+	+*	-+	+*	++	+*	-+	+*	-+		
36	+*	--	-*	++	+*	*-	+*	++	+*	*-	+*	*-		
37	+*	*-	++	++	+*	*-	+*	*-	+*	*-	-*	*-		
38	+-	-+	++	*+	+-	++	+-	-+	-+	*-	*-	*-		
39	+*	*-	++	++	++	+-	-*	*-	-+	--	*-	*-		
40	+*	*-	-*	+*	-*	-*	++	*-	*-	*-	-+	*-		
41	+*	++	++	+*	-+	-*	--	+-	--	*-	--	*-		
42	*+	+-	*-	+*	-+	*-	--	-*	*-	*-	*-	*-		
43	++	-*	+-	+*	*-	++	++	*-	*-	*-	*-	*-		
44	*-	*-	--	++	*-	-*	*-	*-	*-	*-	*-	*-		
45	-+	--	-+	++										
46	--	-*	--											
47	--	*-	--	++										
48	-+		*-	+*										
49		*-		+*										
50														
Infection lag time C bird[3]		9 (6)		4 (0)		2 (0)		1 (0)		3		1	17 (10)	5 (6)

Table 3c: Group 3: Ip en C

Bird[1] / Age[2]	797ip	301c	318ip	778c	821ip	319c	320ip	866c	787ip	788c	892ip	862c	Total infection lag time[4]	Total number of infected C birds
28	*-		*-		*-		*-		*-		--			
29	*-		*-		*-		*-		*-		--			
30	*-		*-		*-		*-		*-		*-			
31	*-		*-		*-		*-							
32	*-	*-	*-		*-	*-	*-		*-	*-	*-	*-		
33	*-	+-	*-	--	-+	+-	*-	--	*-	--	*-	--		
34	--	--	--	--	--	+-	--	--	--	--	--	--		
35	*-	+-	*-	--	*-	--	*-	++	*-	--	*-	++		
36	*-	-*	*-	--	*-	++	*-	+*	*-	++	*-	+*		
37		--		++		+*		+*		+*		+*		
38	*-	--	*-	-*	*-	-*	*-	+*	*-	+*	*-	+*		
39		*-		--		-+		+*		+*		-*		
40	*-	*-	*-	++	*-	++	*-	+*	*-	+*	*-	++		
41		++		+*		+*	-*	+*	-*	+*		+*		
42	*-	-*	*-	+*	*-	+*	++	+*	++		*-	+*		
43		*-		-*		+*	*-	+*	*-	+*		+*		
44	*-	++	--	+*	*-	+*	*-	+*	-+	+*	*-	+*		
45	*-	++	*-	+*	*-	+*	*-	+*	*-	+*	*-	+*		
46				+*	++	+*		+*			-+	+*		
47	*-	++	*-	+*	++	+*	*-	+*	--	+*	++	+*		
48		+*		+*	*-	+*		+*		-+	*-	+*		
49		+*		+*	-+	-+		--			-*	--		
50		+*							+*					
Infection lag time C bird[3]		1		5		1		3		4		3	17	6

Table 3d: Group 4: I_p + C_p

Bird[1] / Age[2]	805ip	763cp	777ip	900cp	840ip	743cp	861ip	898cp	831ip	331cp	865ip	781cp	Total infection lag time[4]	Total number of infected C birds
28	*-		*-		*-		*-		--		*-			
29	*-		--		*-		*-		--		*-			
30	*-		*-		-+		*-		*-		-+			
31	*-		*-		++		*-		*-		*-			
32		*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-		
33	*-	--	*-	--	*-	--	-+	--	*-	--	*-	--		
34	--	--	--	--	--	--	--	--	--	+-	--	--		
35	-+		-+	--	*-	--	*-	--	*-	--	*-	--		
36	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-		
37		*-		*-		*-		*-		*-		*-		
38	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-		
39		*-		*-		*-		*-		*-		*-		
40	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-		
41		*-		*-		*-		*-		*-		*-		
42		*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-		
43		*-		*-		*-		*-		*-		*-		
44	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-	*-		
45														
46														
47														
48														
49														
50														
Infection lag time C bird[3]										2			2	1

[1] = Individual tag number followed by i for I and c for C birds.
[2] = Age of the birds is given in days.
[3] = The observed time in days between day 32 (surrounded by borders in the table) and actually becoming infectious according to McMaster oocyst counts or, between brackets, based on sedimentation flotation test when positive results occurred earlier in time than according to the McMaster oocyst counts.
[4] = The sum of the infection lag times for all contact birds together.
Oocyst output data following each I and C bird: ab = a represents the results of McMaster oocyst counts, b shows the result of sedimentation flotation slides.
+: oocysts were detected, *: not done, -: no oocysts were detected.
Dark grey marked sections represent OPG values, obtained from McMaster oocyst counts, larger than 0.
Light grey marked sections represent samples that had positive results with the sedimentation flotation technique but had missing or negative values for OPG.

Transmission rate

In Table 4 transmission rates are given per group. In I-C_p birds, transmission was not reduced compared to the non-primed I-C group when the sedimentation-flotation results were used to determine time of infection. When calculations were based on McMaster oocyst counts for the I-C_p group, transmission rate was reduced by 72% compared to the naïve I-C group, For the I_p-C birds transmission rate was reduced by 36% and for the I_p-C_p group by 98% compared to the I-C group.

Table 4: Results of the transmission rate calculations, including 95% confidence interval and the percentage of transmission for each group in comparison with the naïve I-C group.

Group	Transmission rate	95% confidence interval	Reduction in transmission rate compared to I-C group
I-C	0.94	0.35-1.77	-
I-C_P	0.26	0.15-0.50	72 %
I_P-C	0.60	0.23-1.21	36 %
I_P-C_P	0.02	0.00045-0.0932	98 %

Discussion

The aim of this study was to experimentally quantify the effects of previous exposure to *E. acervulina* on immune response and on oocyst output in individual birds after secondary infection, simultaneously with assessing transmission of *E. acervulina* infections within a population. As explained in the introduction, the natural 'circle of interaction' between within-host dynamics and environmental feedback through transmission, causes flocks to become heterogeneous collections of individuals with respect to their level of protection (immune status). Differences in exposure history lead to individual differences in reaction to infection, for example becoming apparent in different excretion patterns (infectivity) and the effect the parasite has on the individual host. To our knowledge this is the first time that an experiment has been carried out where the full natural cycle of infection and feedback has been explicitly taken into account.

A good measure for increased immune status of individuals, e.g. an experimentally quantifiable measure that correlates well with infectivity or susceptibility of birds, would be helpful to characterise differences between individual hosts, and would be very useful to track, interpret and

predict the population dynamics of infection in a flock. In this study immune parameters that describe T-cell and macrophage response were examined for this purpose.

In contact animals from the I-C group a significant increase in the amount of $CD4^+$ T-cells was observed after contact infection compared to naïve control birds. Contact chickens of the I-C_p group, however, showed no increase in their intestinal $CD4^+$ T-cell amount. This suggests that $CD4^+$ T-cells are only recruited to the intestine by replicating *Eimeria* and do not take part in resistance to infection of *E. acervulina* in primed chickens. It confirms the conclusion of Trout & Lillehoj (Trout, J. M. and Lillehoj, H. S., 1996) that $CD4^+$ T-cells are unlikely to play a role in the resistance to re-infection. An intestinal $CD4^+$ T-cell increase therefore seems a good marker only for the presence of a primary infection of *E. acervulina* in animals without acquired immunity.

As oocysts in the litter can remain viable for weeks (Williams, R.B., 1995a) inoculated and contact birds can re-infect themselves throughout the duration of the experiment. These repeated infections affect immunity and the response to infection (Blake, D.P. *et al.*, 2005; Klinkenberg, D. and Heesterbeek, J.A., 2005). This phenomenon should be considered when extrapolating to different populations, i.e. small populations where birds are continuously exposed to excreted oocysts versus populations where birds do not stay in the same local environment, due to movements in a larger area or because they are moved to another poultry house. This process of re-infection has most likely also occurred in this study, as indicated by the prolonged oocyst excretion period of the inoculated birds. Also, non-significant $CD4^+$ T-cell increases were observed at day 39 in both infected and contact animals from the I-C group, which might be the result of re-infection. Furthermore, this second increase of $CD4^+$ T-cells in non-primed birds, suggests that the development of immunity takes longer than the ten days observed in this experiment (day 28-39 for the inoculated birds).

The $CD8^+$ T-cells are known to be up-regulated after *E. acervulina* infections (Bessay, M. *et al.*, 1996; Swinkels, W.J. *et al.*, 2006). It is thought that the $CD8^+$ T-cells play a role in the recovery phase of infections and thus might reflect enhanced acquired immune status (Lillehoj, H.S., 1988b; Swinkels, W.J. *et al*., 2007). The birds in our experiment, however, showed no clear difference in their pattern of $CD8^+$ T-cell kinetics as compared to control birds. This might be due to the gradual acquisition of parasites, or because the amount of parasites picked up by means of a contact infection might be too low to induce enough intestinal damage to recruit a significant amount of $CD8^+$ T-cells.

In mice, intestinal $TCR\gamma\delta^+$ T-cells influence the growth and differentiation of epithelial cells, shown by the observation that mice lacking $TCR\gamma\delta^+$ T-cells had an impaired development of the intestinal

epithelia (Boismenu, R. and Havran, W.L., 1994). In all chickens except for the C_p chickens the TCR$\gamma\delta^+$ T-cell reactions to an *E. acervulina* infection seem to take place after the infection cycle has been completed. Thus only in the non-protected animals the increase of the TCR$\gamma\delta^+$ T-cells was observed. Therefore in chickens, recruitment of the TCR$\gamma\delta^+$ T-cells can be a reaction to intestinal damage or to the final *Eimeria* stages present in the intestine. Schild *et al.* (Schild H. *et al*., 1994) already suggested that TCR$\gamma\delta^+$ T-cells can mediate cellular immune functions without a requirement for antigen processing. This enables TCR$\gamma\delta^+$ T-cells to directly recognize pathogens, damaged tissues, or even T cells (Schild, H., *et al*., 1994). If less shedding of oocysts takes place, there is less intestinal damage and less need for a recovery phase mediated by the TCR$\gamma\delta^+$ T-cells. An intestinal TCR$\gamma\delta^+$ T-cells increase therefore seems a good marker only for intestinal damage after *E. acervulina* infections in animals without acquired immunity.

The high fluctuation of TCR$\alpha\beta^+$ T-cells in control animals over the five days on which this variable was measured did not show a clear pattern and could not be explained; therefore no conclusions can be drawn from the TCR$\alpha\beta^+$ kinetics of inoculated or contact birds. Next to this, we did not find any differences in macrophage recruitment between all compared groups as compared to the control group (data not shown).

Momentarily we might have T-cell markers for either a current *E. acervulina* infection (CD4$^+$ T-cells) or intestinal damage due to an *E. acervulina* infection (TCR$\gamma\delta^+$ T-cells). In this experiment, however, no single cell type was found which showed a sustained increase in frequency after infection in primed contact broilers and not in non-primed contact broilers. Thus no cell type or types were identified that could indicate "protection level" and predict oocyst output, infectivity, susceptibility or transmission of primed birds. Though increase of certain specific cell populations after infection was observed, this does not indicate what their role may be in the outcome of the *Eimeria* infection. It is unknown whether protection is dependent on a single factor or if it is a multi-factorial process, but these results point to a multi-factorial nature. Possibly, protection is not related to a major increase in some cell type, but to the possibility of recruiting specific groups of T-cells when needed for the elimination of the parasite. These antigen specific cells however could not be visualized with the current available techniques.

Apart from the effects on immune parameters, effects of previous exposure to *Eimeria* on oocyst output and transmission were also observed. The transmission rate was reduced in groups with an *Eimeri*a exposure history. The highest reduction in transmission rate, compared to the non-primed I-C group, was observed in the I_p-C_p group (98%). In the I-C_p group reduction in transmission rate was two times higher (72%) compared to the I_p-C group (36%). This might suggest that the reduced

susceptibility of contact birds after secondary infection with *E. acervulina* was mainly responsible for the observed reduction in oocyst output and transmission. Aiming control measures towards influencing the susceptibility of birds, if possible, might be most effective in reducing transmission of *Eimeria* spp. in a population.

The discrepancy between expected reduction in transmission rate for the I_p-S_p group when multiplying transmission rates of the I-C_p and I_p-C groups (84%), compared to the observed reduction in transmission rate (98%) suggest that the within-host and between-host dynamics might be more complex than simply combining the effects of reduced susceptibility and infectivity. Other factors, such as stochastic, environmental or behavioural processes regarding the uptake of (sporulated) oocysts might play an additional role. Furthermore, when oocyst output is low, detection of oocysts might also be delayed due to lack of sensitivity of the sedimentation-flotation technique as well as the McMaster oocyst counts. This is also demonstrated by the two contact birds from the I_p-C and one of the I_p-C_p groups that excreted oocysts, where their inoculated pen mates did not show oocyst output for the entire duration of the experiment. This suggest that small numbers of oocysts, that remain undetectable for the McMaster and sedimentation flotation technique, may still result in a sufficient ingestible infectious dose to cause effective transmission to contact birds. Furthermore, the low oocyst output in I_p birds might have resulted in a low uptake of oocysts by the contact birds, resulting in delayed labeling of these birds as "infectious". If so, the transmission rate in the I_p-C and I_{p-} C_p birds might be a slight underestimation, or alternatively the rate calculated may not be linearly correlated to the doses of oocysts that act in the natural infection process.

Very low numbers of oocysts were detected by the sedimentation-flotation test in contact birds of the I-C_p group days before McMaster counts were positive. This suggests that transmission rate might be higher when sedimentation-flotation data were used to determine infection lag time for the transmission rate calculations, in stead of McMaster oocyst count data. The low numbers of oocysts, however, most likely indicate that these oocysts were not actively excreted following multiplication in the intestinal tract, but might be unsporulated oocysts passing through, after uptake from the environment as described by Williams (Williams, R.B., 1995c).

Although the immune parameters that were studied in this experiment are not applicable to make a thorough prediction about infection dynamics in a flock, a first step has been made towards linking the effects of both within-host dynamics and between-host dynamics in a single experiment. The unique combination of studying within-host dynamics and between-host dynamics in the same setting has taught us the importance of time in developing immunity in a natural setting. A key finding is that primed

contact birds in the I-C_p group were better protected against contact infection than inoculated birds in both I-C and I-C_p pairs were against immediate re-infection. This follows from (1) a lack of $CD4^+$ T-cell influx in the primed contact birds compared to a second $CD4^+$ T-cell rise in the inoculated birds (as described above) and (2) from lower excretion of *E. acervulina* oocysts on days 36-40 in the contact birds compared to the inoculated birds, which is the time of peak excretion from secondary infection. Because the only difference between the groups is the time of their primary infection, the conclusion can be drawn that there is a delay between infection and build-up of immunity. This delayed immunity may have considerable impact on dynamics in a larger population, which is not yet covered by our present models (Klinkenberg, D. and Heesterbeek, J.A., 2007; Severins, M. et al., 2007); this again stresses the importance of combining experiments and models.

The oocyst excretion pattern and transmission rate provided by transmission experiments might be used in mathematical models to predict or choose the best methods to minimize clinical signs in the flock. Furthermore, this study can contribute to a better understanding of host-parasite interactions, for *Eimeria* and might provide useful estimates for future studies, i.e. for evaluating control measures. Finally this study can contribute to a better understanding of other host-parasite interactions of hosts infected by parasites with an environmental stage and a gradual build up of immunity upon (re)infection.

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Chapter 8

Discussion

General findings

For protozoa like Eimeria preventive measures for infections in chicken include the use of antibiotics and the use of attenuated live vaccines. Both remedies cannot be regarded as sustainable solutions for such infections. The goal of the project of which the work has been described in this thesis was to link the immunology and epidemiology of Eimeria infections in chickens. It was expected that better understanding of the mechanisms of host pathogen interactions and spreading of Eimeria infections might provide clues to new safe vaccine designs and application strategies.

The parasite Eimeria causes coccidiosis, which is a major intestinal infection in chickens. Parasite proliferation is followed by destruction of the intestinal epithelium (Fernando, M.A., 1990). The intestines become swollen, bloody diarrhea may occur and the host may mount an inflammatory reaction. Chicken immune reactions are the result of host-pathogen interactions, that are dependent on many features of both Eimeria and chicken. Mathematical modeling of the infection process and epidemiology is of use in designing new strategies to restrict both spreading of and damage caused by Eimeria. In order to generate valid mathematical models it is necessary to have knowledge of mechanisms and parameters that are representative for the infection characteristics either at the level of the individual that is infected as well as on the level of a flock or farm. The response of the chicken influences the outcome of the Eimeria infections and transmission. The link between the immunology and epidemiology of coccidiosis could serve as a basis to develop mathematical models for parasitic infections and transmission with biologically sensible within-host dynamics.

This thesis is focused on the host aspects of the host-pathogen interaction that determines the outcome of Eimeria infections. We started with the investigation of the factors that exert external influences on the host immune status. In hosts with relatively high similarity the parasite characteristics, amount and type of parasites were varied. The Eimeria species used in this thesis are E.acervulina, infecting in the duodenal tissue, E.maxima, infecting the jejunum and E.tenella infecting the caecum. E.acervulina was used in most studies. Together with E.maxima and E.tenella it is one of the three Eimeria species that form the major problem in intensive poultry breeding in the Netherlands (Peek, H.W. and Landman, W.J., 2003). An experimental advantage of the use of E.acervulina infections above infections with other strains are the characteristic lesions formed by the parasite (Johnson, J. and Reid, W.M., 1970).

Host responses can be measured systemically in reactivity in various tissues or at the local site of infection in the gut. After infection with

E.acervulina Choi et al. found an increase of IFN-γ mRNA in the caecal tonsils and splenic lymphocytes (Choi, K.D. *et al*., 1999). In our experiments we investigated the local gut immune responses that seemed to be the major responses to an *Eimeria* infection. Most of the cellular responses that were observed to the single E.acervulina, E.maxima and E.tenella infections were localized in their corresponding intestinal segments. The local T-cell influxes found after a multiple species infection were similar to the responses to a single infection, and thus seemed to be mounted solely against the local intruders. The local responses seem to be the most important responses and detailed measurements may lead to elucidation of the protective responses to an Eimeria infection.

Each pathogen species gives a different host reaction with respect to immune reactivity and localization of the infection related pathogenesis and next to the pathogen-dependent variables, variables that are host dependent were evaluated in this thesis. Summarizing all the experiments described in this thesis, the conclusion can be drawn that all variables either at the pathogen or host side that were investigated influenced the host immune reaction.

Many Eimeria animal infection models have been described in the literature. Every group working on Eimeria infections uses it's own standard chicken line to test their hypothesis (Leshchinsky, T.V. and Klasing, K.C., 2001; Lillehoj, H.S., 1986; Lillehoj, H.S., 1994; Lillehoj, H.S. and Li, G., 2004; Lillehoj, H.S. and Ruff, M.D., 1987). Each research group uses its own standard number of parasites and even strains of Eimeria to infect chickens (Allen, P.C. and Danforth, H.D., 1984; Trout, J.M. and Lillehoj, H.S., 1996; Williams, R.B., 2003). Therefore it is difficult to compare our experiments to all of the experiments described in the literature. Though many contradicting host cytokine and T-cells reactions to Eimeria infections have been described in experimental settings, it was possible to extract two general features of reactions to Eimeria infections described in the literature which were also found in our experiments. Next to that also two specific findings will be discussed. The overall findings within all experiments that were observed during our studies, which will be discussed below more extensively, are:

1. A typical biphasic response consisting of an early and a late response. The frequencies of observed T-cells were increased in two phases. First T-cell increases were observed during the parasite replication cycle (see introduction), the second wave of reactions was observed in the phase where no parasites were found in the gut or excretions of the host.
2. A Th1 reaction profile. An increase in the frequency of T-cells was observed after a primary infection. The cytokine profiles described, mainly due to the relatively high expression of IFN-γ, pointed into the direction of a Th1 profile.

The specific findings within our experiments, which will be discussed below more extensively, are:

3. Correlates of protection. Although in our studies young chickens reacted with a Th2 type of reaction and older chickens with a bias toward Th1 type of reactions this did not influence the secondary homologous reaction where in both cases no parasites could replicate. Reaction type to a primary infection does not correlate with protection to a re-infection.
4. A low responsiveness to re-infection. In our studies animals that were subjected to homologous re-infected with E.maxima showed no excretion of parasites and thus had a full protection. The immunological parameters that we measured in response to re-infection were either low or absent. Increase in $CD4^+$ and $CD8^+$ T-cell amounts was found when chickens were infected and re-infected with E.maxima, though the cytokine mRNA amounts did not show significant increase as compared to non-infected controls. In our transmission experiment with E.acervulina no T-cell reactions were observed to a homologous secondary infection.

1. Biphasic T-cell response

After E.acervulina infection in animals of one or of seven days of age the $CD4^+$ and $CD8^+$ T-cell influxes were found to come in a biphasic manner. This confirms the biphasic response for T-cells that was described earlier by Rothwell et al. (1995) and Hong et al. (2006b) in analyses of the responses to an E.maxima infection (Hong, Y.H. *et al*., 2006b; Rothwell, L. *et al*., 1995). In our experiments, a first wave of T-cell influx in the gut was found between days 0 and 3 post infection. The cells taking part in the influx in this phase could play a role in the response to the Eimeria parasites that were locally present. This T-cell influx occurs during the period that the parasites are present in the intestine of the animal. The pre-patent time of Eimeria maxima is about 121 hrs and the peak production of oocysts occurs between 168 and 192 hrs after infection (Joyner, L.P., 1978). The second wave of T-cell influx appeared from day 5 onwards and lasted at least until day 14 p.i.. This period coincides with the time period in which the Eimeria parasites are excreted by the host. The second T-cell influx seems to be no direct reaction to parasites since in that phase no replicating parasites were present to evoke a response. This reaction was most likely mounted against parasite remains or pathological damage caused by the Eimeria oocysts leaving their host cells. A schematic representation of the T-cell frequencies over time is shown in Figure 1.

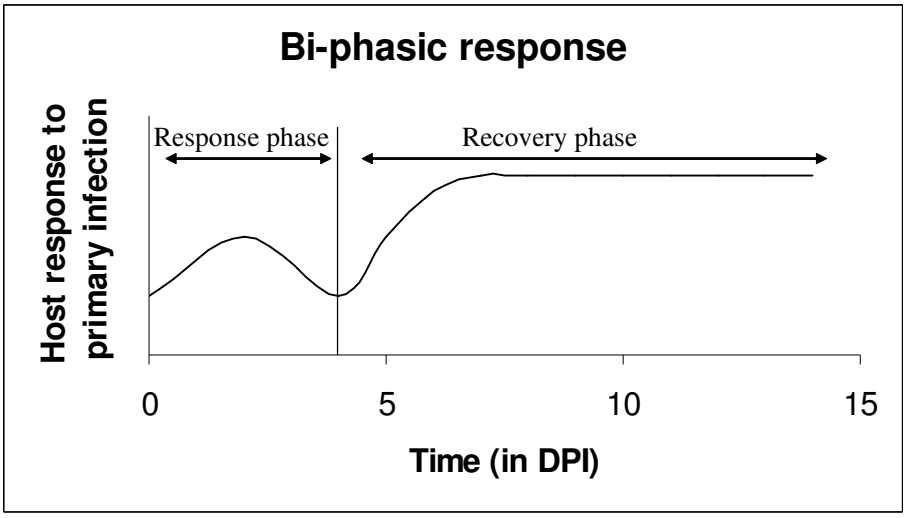

Figure 1: Schematic representation of the bi-phasic T-cell influx after Eimeria infections.

Our hypothesis is that the early response lasting from day 0 to day 5 p.i. is the phase in which the protective response develops. During this phase the parasite is present and replicates in the chicken intestines and from there tries to invade the chicken. The parasites enter the gut and attack the enterocytes in order to enter the host. This causes damage to the intestine to which the host will react with restoration where possible. In our paper describing immune reactions of fast and slow growing broiler lines infected with Eimeria the fast growing line showed the lowest output of oocysts after infection. The major early response in these birds was an increase in proportions of $CD8^+$ T-cells. In our experiment describing the effects of a dose of 500 or $5x10^4$ sporulated oocysts on the immune system of one-day-old-broilers, next to the increase in $CD8^+$ T-cells, an increase in $CD4^+$ T-cells was found. Since both $CD4^+$ and $CD8^+$ T-cells were found to be increased in frequency after infection, both cell types could function as effector cells against the intruding Eimeria parasites. In the dose response experiment, a proliferation assay was performed with spleen lymphocytes stimulated with E.acervulina antigens or ConA. An increased proliferation of spleen cells was observed in animals that had endured an infection. The total lymphocyte population was observed and the $CD4^+$ and $CD8^+$ cell types were not investigated separately in the functional test, thus it was not elucidated which of these cell types was effective in reducing parasite growth. It might even be possible that, next to the two cell types observed in our experiments, cell types that were not investigated separately in our experiment cooperate in host immunity to Eimeria infection.

Early IL-8 responses were found in our experiments. One day post infection with $5x10^4$ E.acervulina oocysts in day-old broilers an increase of IL-8 mRNA was found. When fast growing broilers were infected at day seven of age with $5x10^4$ E.acervulina oocysts the increase of IL-8 mRNA was observed at days one to four post infection, whereas in slow growing broilers only an increase was observed of IL-8 mRNA at day 4 post infection. At days four and seven after a primary E.maxima infection of animals of twenty-one days of age the increase of IL-8 mRNA was also found. The third parasite used in our experiments, E.tenella, also induced locally in the caecum an increase of IL-8 mRNA six days after an infection at seven days of age. IL-8 is a chemokine, a chemotactic factor attracting cells, oa heterophils, to the site of infection. It can be released due to epithelial necrosis caused by penetration of the parasite in the mucosa (Petrone, V.M. *et al*., 2002). This might explain the observation of early IL-8 responses in infected broilers.

The late response, mainly consisted of an increase in the frequency of TCR$\gamma\delta^+$ T-cells, started at the moment the first oocyst excretion was found in faeces and lasted until at least day 14 p.i.. The increase in TCR$\gamma\delta^+$ cells which was found towards the termination phase of the infection was also described by Rothwell et al. (Hong, Y.H. *et al*., 2006b; Rothwell, L. *et al*., 2004). The influx of TCR$\gamma\delta^+$ T-cells is thought to be a reaction to the damage to epithelial cells caused by the parasite. But most likely no replicating parasites are present in the intestine at the time that these responses are found. At the end of their cycle, parasites burst out of their host cells and leave the host in the form of oocysts. At this time the characteristic lesions appear at the site of infection. These lesions need to recover and in the phase this occurs, the TCR$\gamma\delta^+$ T-cell influx at the site of infection was observed which suggests a role for these cells in wound healing. This role for TCR$\gamma\delta^+$ T-cells in growth and differentiation of intestinal cells was already described for mice (Boismenu, R. and Havran, W.L., 1994).

2. T-cell profile

The focus of this thesis is on gut immunity, in which T-cells play an important role. In the classic model of the immune system response, naïve T-cells differentiate into two types of cells. Th1 cells are involved mainly in cell mediated inflammatory reactions and Th2 cells play a role in humoral immunity (Mosmann, T.R. and Sad, S., 1996). Since Mosmann described the Th1-Th2 dichotomy, many researchers have provided additional information (Mosmann, T.R. and Coffman, R.L., 1989). Most of the cytokines have been characterized as promoting either Th1 or Th2 type of reactivity, although not for all cytokines their role is clear. Also relatively recent characterized cell types are found that do not fit the Th1-Th2 dichotomy. These include adaptive regulatory T-cells and the most

recently discovered Th17 cells. T helper cell differentiation, and a part of the signaling molecules involved are shown in figure 2 (Reiner, S.L., 2007).

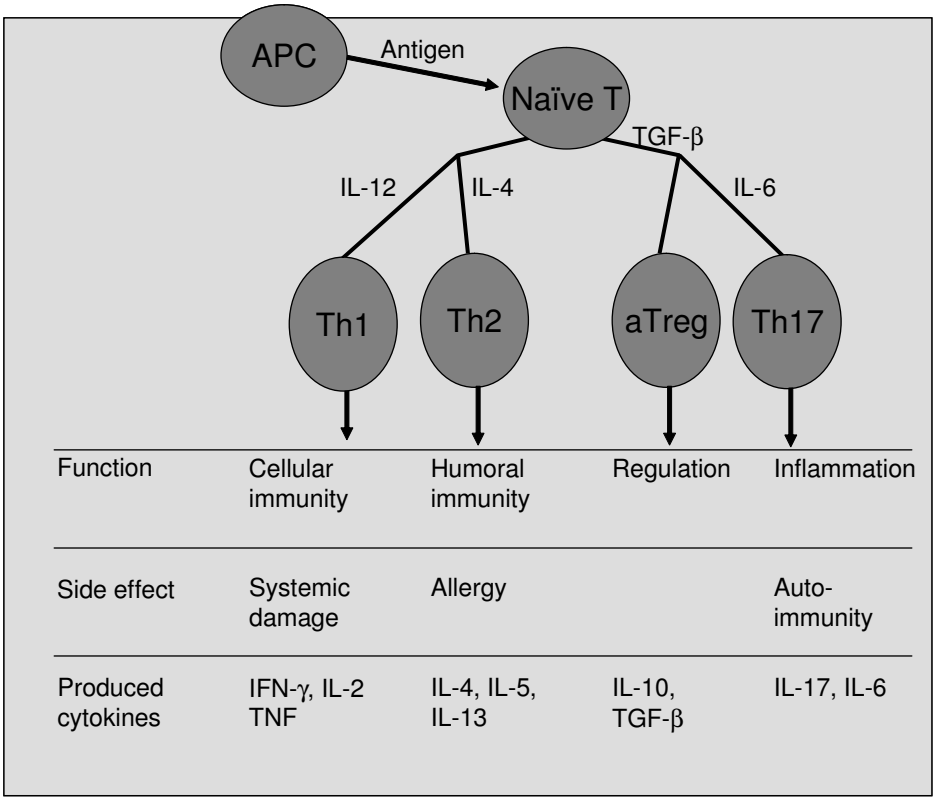

Figure 2: Types of Helper T Cells
Development from a naïve T-cell into the different T-cell subsets is shown. Cytokines play a critical role in the induction or repression of the lineages. The different helper T-cell subsets have distinct protective functions (listed below). Host defense is orchestrated by the three major cell types, Th1, Th2, and Th17. Adaptive regulatory T (aTreg) cells can regulate immune responses.

The Th1 cells driven by early IL-12 and IL-18 responses typically produce IFN-γ (Gobel, T.W. *et al*., 2003; Mosmann, T.R. and Sad, S., 1996). In our experiments in chickens of one day, one week, and three weeks old an IFN-γ mRNA increase was found in birds that were infected with E.maxima (chapter 5). No IFN-γ was produced in one day-old birds infected with E.acervulina though local production of the IFN-γ inducer IL-18 was demonstrated (chapter 2). In chapter 5 and 6 IL-10 mRNA expression was found. IL-10 is not only expressed by, but also induces the

Th-1 cells. The cytokine IL-10 has a regulatory function on the Th1-Th2 balance (Hong, Y.H. *et al*., 2006b; Rothwell, L. *et al*., 2004). In chapter 5 the calculated ratio of IFN-γ / IL-10 was taken as a ratio for Th1 / Th2, this ratio was in favor of the Th1 cytokines. Th1 cells may be the cause of observed pathological lesions. It is thought that IL-10 expression dampens the Th1 response in order to reduce the possible damage caused by the inflammatory and protective responses. Adaptive regulatory T (aTreg) cells can down regulate immune responses, although details of their physiological role in vivo is yet uncertain (figure 2).

The findings in our studies are in line with the findings of other authors and research groups that intracellular parasites induce Th1 dominated responses (Degen, W.G. *et al*., 2005; Jankovic, D. *et al*., 2001), and the current thought that the immunity to Eimeria infections is cell mediated (Hong, Y.H. *et al*., 2006b). Th1 cytokines such as IFN-γ can activate macrophages well beyond their innate capacities (Erf, G.F., 2004; Wigley, P. and Kaiser, P., 2003). In our experiments the number of macrophages in the intestine were not increased after infection, but an additional activation of the existing population might play an important role in parasite removal.

The typical Th2 cytokine, IL-4, was not detected with PCR in all of our experiments. Due to the lack of IL-4 expression in our experiments, the quality of the Th2 profile could not be determined. The recently discovered Th17 form a relative new group of cells complicating the current thoughts on protective immune responses. Their complete signaling pathway is still unclear, but it is suggested that successful signaling of Th17 requires a cooperation between TGF-β and IL-6 while suppressing both the Th1 and Th2 pathways (Bettelli, E. *et al*., 2006). Next to the lack of expression of TGF-β, no IL-6 expression was observed in our experiments. Therefore also Th17 does not appear to play a major role. The lack of IL-6 mRNA expression as found in our experiments is contradicted by other articles (Hong, Y.H. *et al*., 2006a; Lynagh, G.R. *et al*., 2000). Since seemingly minor differences in experimental design may result in a differences at the level of immune parameters that were measured these results may not be a contradiction. Especially since Lynagh et al. explicitly state in their article that their results concerning chicken IL-6 must be treated with caution (Lynagh, G.R., Bailey, M. and Kaiser, P., 2000). In this thesis the group of adaptive regulatory T-cells consisting of Th3 cells, Tr1 cells and $CD4^+CD25^-$ cells that can become $CD4^+CD25^+$, of which the latter is still under debate, will not be further discussed (Romagnani, S., 2006).

3. Correlates of protection

Many different host responses are observed after an Eimeria infection. Due to the many contradictory results between all types of experiments as described in the literature it is hard to interpret the many changes observed in infected chickens in a comprehensive picture of immunological pathways and to link T-cell and cytokine reactions to reduction of parasite load. We tried to link the processes as observed in our experiments to a decrease of parasites in the intestine of the host. The increased capacity of animals of twenty-one days of age to mount Th1 type of reactions did not result in a reduced parasite load at the site of infection in the animals when compared to animals infected at day one of age. When E.acervulina, E.maxima and E.tenella were simultaneously present in the chicken gut, the host handled parasites independently, no influence on parasite growth or on host T-cell influxes and cytokine mRNA levels was observed with the methodology we applied.

As shown in chapter 5, the primary immune responses as observed in our experiments had no influence on concurrent infections. In one-day-old chickens, that have a less pronounced Th1 reaction profile, the same amount of intestinal parasites per gram intestinal tissue was observed as in animals which were infected at day twenty-one of age. The possibility existed that priming of animals at a very young age when the Th2 character was dominant resulted in a reduced protection to re-infection. This was evaluated in our experiment infecting chickens at day one or day seven of age with $2x10^4$ E.maxima oocysts, followed by a challenge 14 days after priming with $2x10^4$ E.maxima oocysts. Both groups, with and without a pronounced Th1 profile, were similarly effective protected against re-infection and showed no excretion of parasites. The minimum oocyst dose required to stimulate complete protective immunity against a homologous challenge is dependent on the host genotype (Blake, D.P. *et al*., 2005). With this threshold dose where a full protective immunity develops, it might be possible to elucidate the reaction profile playing a role in chicken protective immunity to Eimeria infections.

4. Low responsiveness to re-infection

The responses to a primary infection consisted of many factors. Most conditions lead to a Th1 profile and the early $CD8^+$ influx seemed to play a role in the generation of protection to this primary infection. Acquired immunity developed following exposure to Eimeria parasites. In general a secondary infection results in a decreased or even a lack of excretion of parasites. This was observed in many experiments and is the basis of Eimeria vaccination programs. The decreased excretion of parasites is an indication for the development of a protective immune response. The components of this protective immune response are until now unknown. In our experiment with homologous and heterologous E.maxima re-infections, no cytokine inductions were found after a secondary homologous E.maxima infection, and the cytokines investigated can not be related to protection. Influx of T-cells was observed after both a homologous and a heterologous re-infection. Since the same profiles were observed in a protected and a non-protected situation, these T-cell influxes again could not be related to protection to re-infection. Though very counter intuitive, this is in line with the findings by Hong et al. (2006b), who also stated that coccidiosis induces a diverse and robust primary cytokine / chemokine response, but a more subdued secondary response (Hong, Y.H. *et al*, 2006a; Hong, Y.H. *et al*., 2006b). Since it is proven by means of the reduced oocyst excretion that the protective responses to secondary infections are present, it must be possible to identify the cells or signals responsible for this response in the future (see future research).

The immuno-epidemiology of Eimeria infections

In order to understand the infection biology of Eimeria and to construct mathematical models for vaccination or for therapeutic studies, it is necessary to have a thorough knowledge of processes that are of influence on the infection characteristics. Recent mathematical models such as the one that was delineated from experimental data in the literature by Klinkenberg and Heesterbeek (Klinkenberg, D. and Heesterbeek, J.A., 2005) form important attempts to explain typical experimental results such as the relation between quantitative and kinetic aspects of parasite uptake and the excretion and the gradually increasing immunity during trickle infections. In these mathematical models parameters need to be estimated that quantify the host immunity. To do better than estimates a better understanding is needed of the immunological response processes. In our experiments we showed that host response is dependent on age, genetic background and infection history of the host, and amount of the Eimeria parasites and niche in the GI-tract.

The central question in our project was to know what the impact is of immune status in flocks that show divergence at the level of Eimeria specific resistance on the epidemiology of the infection. To investigate this question, we have performed the experiments described in order to find reactions displaying enhanced immunity in animals. At the start of our project it was clear from the literature that an Eimeria infection resulted in resistance to re-infection with a homologous species. Each species displayed different immunogenic properties with E.maxima being the most immunogenic Eimeria species to infect chickens. In our experiment, we carefully chose the background of our hosts. The experiment was set up with pair-wise housed animals which had received a mock infection or a priming with E.acervulina in order to create groups that had clearly distinct infection histories and thus differed in immunological reaction to Eimeria. The experiment addresses the central question of our project. In the natural 'circle of interaction', the within-host dynamics and environmental feedback lead to individual differences in reaction to infection. An individual protective immune status results in a lowered transmission.

Eimeria quantification

Resistance to infection is mostly expressed in decreased oocyst output. It is convenient to use the environmental stage to quantify parasite load, in order to quantify the intestinal load it is necessary to kill the chicken, which dramatically increases the amount of animals needed in an experiment. In order to have a link between the Eimeria epidemiology and immunology, a new sensitive way in which the counting of oocysts could be improved had to be developed. The currently used method to count oocysts in faeces is a sedimentation flotation method followed by a McMaster counting under a microscope (Hodgson, J.N., 1970), a method which is not only very labour intensive, but also has to be performed on fairly fresh faeces. Many research groups are trying to find a way in simplifying this protocol (Haug, A. *et al*., 2005). We tried to replace the oocyst counting method could by a quantitative PCR on parasite DNA. Having this PCR at our disposal would save some work during the course of an experiment. Next to this, microscopic identification of oocyst samples is very difficult, the possession of a species specific quantitative PCR would make it possible to have a better idea of the species composition in a field sample containing a mixed population.

Many protocols existed to extract DNA from oocysts (Zhao, X.M. *et al*., 2001). All of these protocols however used purified Eimeria species. We tried to extract DNA directly from faeces, using adaptations of the present protocols. After testing the glass bead grinding of the rigid oocyst wall on purified oocysts in order to release the DNA, we performed a PCR on dilutions of the extracted DNA. In this experiment it was possible to

have a positive signal when the DNA of at least 10 oocysts was present per reaction. In this way, we showed the possibility to extract DNA from purified oocysts. Next, we tried to extract oocyst DNA directly from faeces. DNA isolated from faeces spiked with oocysts showed an extremely reduced amount of specific PCR product as compared to DNA isolated from the same amount of purified oocysts. Testing several stool kits for extracting DNA from human stool samples led to the same results. This resulted in the hypothesis that either DNA isolation from chicken faeces or the following PCR reaction is disturbed by unknown factors present in this faeces. This limited the possibilities of the PCR. We tried to purify the oocysts from the faeces before DNA extraction. Unfortunately this resulted in the loss of an unpredictable amount of oocysts, making this method only useful for species identification of Eimeria present in large amounts. Besides, the method was more labour intensive than the McMaster method it was supposed to replace. It was however possible to use the PCR on DNA obtained from infected intestinal segments (used in this thesis). In the intestinal segments the parasite is present in another developmental stadium which is not surrounded by the rigid oocyst wall (Belli, S.I. *et al*., 2006), furthermore and most important, in tissue the disturbing factors of faeces are not present. To establish a reliable quantitative PCR for faeces samples therefore remains a major challenge.

Future research

The scope of this thesis was to get insight into the mechanisms that determine the immune status of the host in relation to Eimeria infections and to link the pre-existing immune status of the host to infection epidemiology. Therefore we investigated factors that relate both to the host and to the pathogen influence on response to Eimeria infections. Most likely all major factors that determine the outcome of an Eimeria infection are not identified. An additional factor that could be included in an infection model is the fundamental difference between sexes in responses to Eimeria infection (Zhu, J.J. *et al*., 2000). It is expected that male and female broilers do not have the same type of reaction to an Eimeria infection. Differences may for instance exist in Th1 / Th2 cytokine profile. In pigs such sex differences already were observed for cytokine production levels. Gilts were more prone to express IL-2, whereas boars produced higher levels of IL-4 (de Groot, J. *et al*., 2005).

Main regulators in the immune response are cytokines. Due to the lack of antibodies against chicken cytokines and the fast degradation of cytokines we measured the mRNA of cytokines as a representative of cytokine expression. In our experiments the mRNA extraction was done on whole tissue. Tissues are composed of immune cells, epithelial cells, connective tissue cells, muscle cells and nerve cells which all contain their specific mRNA's. Due to the different origins of mRNA, the signals

originating from the immune cells might be underestimated. Thus we could have missed responses. This problem can be solved by isolation of specific cell populations or even single cells. By means of laser-capture micro-dissection (LCM) it is possible to cut specific regions out of a tissue slice (Espina, V. *et al*., 2006). This regions might even be as small as a single cell. By cutting out the specific immune regions or cells it is possible to get a more focused mRNA expression profile of infected animals. It even is possible to extract mRNA from single dissected cells and perform a quantitative real-time PCR (Okuducu, A.F. *et al*., 2005). Specific cell types which could be isolated to investigate gene expression are the TCR$\gamma\delta^+$ cells. These cells were found to be present mostly in the epithelial regions. We cut out the tip of the villus, containing mostly TCR$\gamma\delta^+$ cells, the middle of the villus and the crypt from a section of paraffin embedded jejunal tissue isolated from animals that were re-infected with E.maxima in order to find out region specific differences in mRNA expression. From these small pieces it was still possible to extract RNA and determine expression profiles of ribosomal RNA. This method provided enough mRNA to detect immune related genes.

It is expected that the response to complex organisms such as a protozoa is dependent on a multi-factorial process. It still remains to be established which of the parameters that demonstrate changes during Eimeria infections are pivotal in determining the outcome of primary and of secondary infections. The experiments with birds of one, seven or twenty-one days of age, fast growing or slow growing birds and birds with pre-existing expositions to homologous or heterologous Eimeria infections, indicated that responses of dissimilar hosts resulted in different types of immune responses upon exposure to Eimeria parasites. From our results it may cautiously be concluded that the parameters described in this thesis may not be the main parameters responsible for the regulation of host protection to Eimeria infections. This raises the question which cell types or cytokine responses or other responses are responsible for protection.

To start with, the specific candidate populations are the cell populations that change of frequency after infection. The function of these cells should be investigated in further detail to determine their role in host immune reactions to Eimeria infections. It is therefore logical to investigate the role of single labeled macrophage, $CD4^+$ or $CD8^+$ T-cell populations in a primary infection. Next to that it is also necessary to look at double labeled cells. In mice for instance, the $CD4^+$ and $CD8^+$ cells are divided into "naïve" and "memory" phenotype cells on the basis of CD44 expression. Naïve T-cells had a low expression of CD44 and memory T-cells had a high expression of this marker (Tough, D.F. and Sprent, J., 1998). Memory cells might play a role in the protection to re-infection. For chickens, antibodies to identify the CD44 marker are now available (Southern Biotech). Though in chickens the memory function of the T-cells

expressing the $CD44^+$ T-cell marker is not yet demonstrated, studies with this marker might also reveal a role of memory T-cells in chicken acquired immunity.

The findings described in this thesis suggest many roles for the T-cell types that were found to be increased in number after infection with E.acervulina, E.maxima or E.tenella strains. Though increase of certain specific cell populations after infection was observed, this does not indicate what their role may be in the outcome of the Eimeria infection. Since the gut derived lymphocytes contain bacterial flora originating from the gut, in vitro assays with intestinal T-cell populations are not easily developed. If it is possible to perform in vitro functional tests on gut lymphocytes, Flow-cytometric cell sorting with specific antibodies might be a manner to get enriched sterile populations of specific T-cells derived from the gut. The function of for instance isolated $CD4^+$, $CD8^+$ and $TCR\gamma\delta^+$ T-cells might be tested in an in vitro assay or by administering to a naïve host followed by a challenge. This could reveal the cell type responsible for protection to homologue re-infection. Though it seems a laborious protocol, administering sorted cell populations to naïve chickens originating from inbred lines might transfer cell type responsible for the protective response that leads to immunity to Eimeria infections. This type of research has already been done by Rose and Heskett who intravenously injected spleen cells or peripheral blood lymphocytes, and were thus able to transfer immunity to naïve chickens (Rose, M.E. and Hesketh, P., 1982). But such experiments can only be performed using inbred chickens. The currently available methods could provide a way to extend this type of research.

It is still possible that the currently investigated cell types are not responsible for the removal of parasites. This illustrates the necessity of the development of new cell type markers for many types of chicken cells. With new markers it might be possible to reveal which cell types are increased in protected animals. With liquid chromatography-mass spectrometry (LC-MS) or matrix-assisted laser desorption/ionization (MALDI) it is possible to identify expressed proteins. A protein expressed on immune cells of animals with acquired immunity may be the marker specific for protection against Eimeria infections. Another way to identify new markers is by the expression of specific mRNA. We determined mRNA expression profiles in order to identify bio-markers for altered Eimeria specific immune status by means of a micro array. This method provides an unbiased approach to analyze expression of many genes at the same moment, where no genes or proteins are chosen beforehand. In this way we were able to compare the changes in mRNA profiles in primary and secondary infected animals. Genes were called up- or down-regulated only when the expression differences were higher than a beforehand defined threshold, like 3 or 4 times. The advantage of this approach is that the

percentage of false positive signals is low, the disadvantage is that by setting our specific cut-off value we could have missed genes with small but significant changes in up- or down-regulation. We chose to focus on genes which were significant lower or higher expressed in animals with a secondary homologue infection as compared to the control group and showed no differential expression in animals which were primary infected or animals that had received a secondary infection with a heterologous Eimeria species. This comparison revealed a huge amount of genes which might play a role in protective responses. Further comparing between groups might reveal even more genes differentially expressed between primary infections and homologue or heterologous re-infections. Comparing between groups however remains difficult due to limitations of the currently available bio-informatical models. Furthermore statistical analyses can be improved in order to gain better insight in the differences between naïve, infected and protected chickens. Since the annotation of the full genome and cDNA's is not complete, completion of this process might provide new information to our work. Fitting micro-array data into pathway analysis will perhaps also shed a new light on the data. Comparison with known pathways in other animal species will be possible. Currently data analysis and interpretation tools need to be further developed to facilitate data interpretation. Due to time limits, we were not able to fit the significantly up or down regulated genes to biological pathways, but plans exist to perform a pathway analysis on the data generated in the experiments described in this thesis**.**

Chapter 9

Summary

The protozoan parasite *Eimeria* is responsible for the disease coccidiosis and has a worldwide distribution. Intestinal *Eimeria* infections are the dominating class of diseases in poultry causing great economical damage and considerably affecting animal welfare. In the Netherlands in chickens raised for meat (broilers) the three most diagnosed coccidiosis infections are *E.acervulina*, *E.maxima* and *E. tenella. Eimeria acervulina* is known to infect the duodenum, *E.maxima* infects the jejunum and *E.tenella* infects the caecum. After recovery of primary infections, chickens generate protective immune responses and can even develop a full protective immunity to homologous re-infections.The parasite passes through several different developmental stages within the host. All of these stages display different antigens. Due to the antigen switching between developmental stages of the parasite, the host reaction to *Eimeria* infections is a complex process.

The work presented in this thesis is part of a joint research program. The goal of this program was to link insight into the within-animal dynamics with insight into the between-animal transmission of *Eimeria* parasites of poultry. We want to investigate how these two processes interact to determine the infection dynamics within flocks and between subsequent flocks, thus being able to develop intervention methods. Before being able to perform a transmission experiment where within-host dynamics is monitored, it was necessary, for the within-host part, to find out which factors influence the host immune responses. In this thesis we investigated a selection of factors that may influence the development of the host-pathogen interactions and therefore modulate the response of the host to the *Eimeria* infection; the factors that we investigated were: number of parasites in a single primary infection, different *Eimeria* species, genetic background of the broilers, age of the host and the effect of a preceding infection. The transmission dynamics between hosts are studied in a companion PhD project of F.C. Velkers.

In our experiments, we analysed the immune response of the host. As immune parameters we used T-cell population analysis in the intestine, and cytokine mRNA expression levels in the intestine. The cytokine expression profile displays the type of response which is generated to an infection. T helper (Th) 1 cells can generate cell-mediated immune responses by means of cytokines, and mostly react on intracellular

infections. Th2 cells are mainly associated with antibody-mediated responses and are a response to extracellular (stages of) pathogens. A species-specific real-time PCR was used to quantify total amount of Eimeria DNA in the intestine.

In **chapter 2** we investigated the effect of the number of parasites that was used to infect broilers. In an experiment we infected one-day-old broilers with a low ($5 * 10^2$ oocysts) or a high ($5 * 10^4$ oocysts) dose of *E.acervulina.* The presence of *Eimeria* in the gut was determined by measuring the amount of *Eimeria* specific DNA in the gut. In order to quantify the amount of *Eimeria* DNA present in the intestine of the host we have developed a quantitative PCR. The kinetics of the $CD8\alpha^+$ T-cell population in the gut and the local IL-8 cytokine responses to an infection of one-day-old broilers with a high dose of *E.acervulina* indicated that an immune response was induced that increased with time after the first oocyst excretion that was expected around day four post infection. In contrast, the immune response to a low dose of *E.acervulina* was relatively weak, for no increase of $CD8\alpha^+$ T-cells in the gut was observed after the expected moment of first oocyst excretion.

The second variable factor that was investigated on the pathogen side was the comparison of the effect on the host response to infections with different strains of *Eimeria.* In chickens seven species of *Eimeria* exist, each with a specific localization in the gut following infection. This study was initiated as most studies focus on infections with one species of *Eimeria* whereas in the field hosts may be infected with multiple species simultaneously. In **chapter 3** we compared the effects on the immune response to an infection with a single species to responses to an infection with multiple species. The three *Eimeria* species used in this experiment (*E.acervulina*, *E.maxima* and *E.tenella*) gave rise to distinct response profiles. An *E.acervulina* infection mainly induced a $CD8^+$ T-cell response and a IL-18 cytokine response whereas an *E.maxima* infection mainly induced a $CD4^+$ T-cell response and IFN-γ and IL-4 cytokine responses and *E.tenella* induced a $CD4^+$ T-cell response and IL-2 and IL-4 cytokine responses. A multi-species infection with three Eimeria *Eimeria* species did not lead to a response synergy or a T-helper conflict, but gave rise to a third possibility, namely an accumulation of the results of the mono-species infections.

Factors which could influence the host immune reaction to an *Eimeria* infection also may be of host origin. Therefore we investigated three host-dependent factors with a possible influence on the immunity to *Eimeria* infections: genetic background of the broilers, age of the host and the effect of a preceding infection.

The first host-dependent factor was the choice of the broiler lines that we used in the experiment. Broilers are grown in genetic lines. This breeding process based on genetic selection may influence both the innate and specific immune response potential of the host. In **chapter 4** we described an experiment in which two different broiler lines were infected with *E.acervulina*. In this experiment we used a conventional rapidly growing broiler line with a life span of about 7 weeks and a slow-growing broiler line with a time to slaughter of about 12 weeks. This latter type of broiler is normally used in organic farming. In these chickens we observed line-dependent reaction profiles to the *Eimeria* infection. Fast-growing broilers seem to generate a relatively early $CD8\alpha^+$ and $TCR\gamma\delta^+$ response accompanied by IL-8 and IL-18 cytokine responses as compared to slow-growing broilers. In addition, the fast growing broilers showed a reduced amount of *E.acervulina* DNA in the duodenum as compared to the amounts of DNA determined in the gut of the slow growing line.

The animals as used in the experiments that are described in chapters 2 and 4 differed in age at primary infection. Age as a host-dependent parameter also seemed to influence the reactivity to an *Eimeria* infection. Especially the lack of IFN-γ mRNA expression in broilers infected at day one of age as compared to broilers infected at day seven of age was a difference observed between these experiments. However, a direct comparison of the experiments in chapters 2 and 4 is dangerous as the two different age groups were not investigated within the same experiment. In **chapter 5** a subsequent experiment is described, where we explicitly investigated the influence of host age on a primary infection with *E.maxima*. Broilers were infected at seven, fourteen and twenty-one days of age. Though *Eimeria* infections are known to cause weight loss in infected animals, only in the animals that were infected at day one of age a weight loss due to the infection was observed. T-helper (Th) cells can generate Th1 (cell-mediated) or Th2 (antibody-mediated) immune responses by means of cytokines. The ratio of IFN-γ / IL-10 for the

infected animals was determined as an indication for Th1 or Th2-type of response induction at the different ages. Though the Th1 type of response to a primary infection was less pronounced in animals infected at day one of age than in animals infected at twenty-one days of age, the development of protective immunity was not immature in the animals infected at day one of age as compared to the responses that were observed in the birds infected at seven days of age.

Chickens are known to develop a protective immunity to *Eimeria* infections after a primary infection. This protection is known to be *Eimeria*-species specific and therefore was expected also to be localization specific. In **chapter 6** the effect of preceding *E.maxima* infection both by a homologous (*E.maxima*) and by a heterologous (*E.acervulina*) challenge is described. After the homologous challenge a complete protection was observed, no oocysts were excreted. A heterologous challenge did not result in a decreased output of oocysts as compared to a primary *E.maxima* infection. In contrast, after a homologous re-infection, T-cells and cytokines showed a low response. With micro-array analysis we found common genes that were induced in all infected groups. Specific genes were observed that were only differentially expressed within the homologously re-infected group, and not regulated in all other groups; these genes could be responsible to induce protection. These genes are mainly involved in specific T-cell signalling and in translation, and might play a role in protection, but this remains to be further investigated.

As described in this thesis, both host and pathogen-dependent factors influence the immune responses to an *Eimeria* infection. Host responses modulate the excretion of oocysts and thus may influence the transmission of *Eimeria*. The knowledge gained from our experiments, together with the knowledge gained in the companion PhD project of F.C. Velkers concerning the between-host transmission, finally allowed us to jointly investigate the relationship between differences in immune responses due to preceding exposure to *Eimeria* and transmission of *Eimeria*. This transmission experiment is described in **chapter 7.** The host responses were linked to the transmission of *Eimeria* infections. A pair-wise transmission experiment was carried out with different combinations of inoculated (I) and contact (C) birds, either previously exposed (primed, p) or not. The experiment thus consisted of pairs of I-C, I-C_p, I_p-C and I_p-

C_p birds and naïve non-inoculated control chickens. In these pairs, transmission rates (number of new infections due to one animal per unit of time), were calculated. Reduction in transmission rate was higher in the group consisting of pairs of I-C_p birds, as compared to the group consisting of pairs of I_p-C birds, showing that a difference in in exposure history influences transmission dynamics and protection level. No single observed T-cell cell type could characterize this protection level.

In **chapter 8** the the main findings of the preceding chapters and some of the final conclusions and suggestions for further research are given. Summarizing all the experiments described in this thesis, the conclusion may be drawn that all tested variables: amount of parasites, *Eimeria* species, genetic background of the broilers, age of the host and the effect of a preceding infection, had an influence on the host immune reaction. Though all factors investigated influenced the host response to *Eimeria* infections, some general principles could be identified. An *Eimeria* infection induces a typical biphasic response consisting of an early and a late response. The early phase of the response reflects a reaction to the proliferating parasite and the late phase of the response seems a reaction to cellular damage. Mainly due to the high IFN-γ mRNA levels observed after *Eimeria* infection, the bi-phasic responses to the *Eimeria* infections have a Th1 reaction profile. Though host responses to a primary infection became more Th1 biased as hosts increased in age, the primary response did not correlate with protection to a re-infection. Increased capacity to mount a Th1 type of reaction to a primary infection with *E.maxima* did not result in a decreased amount of excretion of parasites or in an increased protection to re-infection. Finally, a low responsiveness to re-infection was found in animals that received a homologous *E.maxima* or *E.acervulina* challenge. No T-cell subsets were identified specifically playing a role in protection to re-infection. The reaction to a primary infection is diverse and robust, but the reaction to a secondary response is more subdued. A small subset of a measured cell types might be responsible for protection to re-infection. New cell type markers might reveal the cells responsible. Next to this a pathway analysis of the data generated with the micro-array might provide new information to our work.

Chapter 10

Nederlandse samenvatting

De parasiet *Eimeria* veroorzaakt de ziekte coccidiose. Deze ziekte is de meest voorkomende ziekte bij kippen en het gevolg is een grote economische schade, met name in de vleeskippen (broilers) industrie. *Eimeria* infecties in kippen worden wereldwijd gevonden. De drie *Eimeria* soorten die in Nederland het meest gediagnostiseerd worden, zijn: *E.acervulina*, *E.maxima* en *E tenella. Eimeria*, is een parasiet die in kippen de darm infecteert. Elke *Eimeria* soort infecteert een specifiek stuk darm. Zo infecteert *Eimeria acervulina* het duodenum, komt *E.maxima* alleen in het jejunum voor en vinden we *E.tenella* in de caeca (blindedarm). Tijdens de infectie met *Eimeria* bouwt de kip een immunologische bescherming op. Dit houdt in dat de eenmaal geïnfecteerde en herstelde kip niet meer ziek wordt na een volgende *Eimeria* infectie, zolang deze tenminste met dezelfde soort *Eimeria* geïnfecteerd wordt. *Eimeria* ontwikkelt zich in de darm en maakt in de darmcellen van de kip verschillende stadia door. Het eerste stadium is een a-sexuele vermenigvuldiging, gevolgd door een sexuele vermenigvuldiging en uiteindelijk uitscheiding van oöcysten, het stadium dat weer in het milieu terecht komt. Deze oöcysten kunnen buiten de kip lang overleven. Wanneer deze oöcysten weer opgepikt worden door dezelfde of een andere kip kan een nieuwe infectie plaats vinden. *Eimeria*'s zijn ingewikkelde parasieten, waarvan de verschillende ontwikkelingsstadia verschillen in uiterlijke verschijningsvorm. Het afweersysteem van de kip herkent die stadia aan de eiwitten en suikers die voorkomen aan hun buitenkant. De stadia die de *Eimeria* parasiet in de kippendarm doorloopt verschillen echter in de eiwitten en suikers die ze aan hun oppervlakte dragen. Hierdoor vormt de afweerreactie van de kip een complex patroon wanneer het in de tijd gemeten wordt. Ook tussen de *Eimeria* soorten zijn zulke grote verschillen in uiterlijke kenmerken, dat wanneer het immuunsysteem heeft geleerd om een soort te herkennen, dat wel leidt tot bescherming tegen die specifieke soort maar niet tot bescherming tegen andere *Eimeria*.

Het werk beschreven in dit proefschrift is onderdeel van een groter onderzoeksprogramma. Het doel van dit programma was om meer inzicht te krijgen in de binnen-dier dynamica (beschreven in dit proefschrift) van de *Eimeria* parasiet in kippen en deze te verbinden met de tussen-dier transmissie van *Eimeria* (wordt beschreven in het nog te verschijnen proefschrift van F.C. Velkers). Dit alles om uit te vinden hoe deze

processen samenhangen en zo begrip te krijgen in de verspreiding van de infectie, zowel in een koppel als tussen koppels dieren. Voor het echter mogelijk was een gezamenlijk transmissie experiment te doen, was het nodig om uit te vinden welke factoren de immuunresponsen van de gastheer beïnvloeden. Bij het bestuderen van het mechanisme van de opbouw van de afweerreactie tegen *Eimeria* is het van belang rekening te houden met de verschillen die zowel in eigenschappen van de kip, de gastheer, als in de eigenschappen van de *Eimeria*'s kunnen optreden. In dit proefschrift worden een aantal van de variabelen beschreven die van invloed kunnen zijn op deze *Eimeria*-kip interactie. De factoren die bestudeerd zijn, omvatten zowel variabelen van de parasiet (hoeveelheid *Eimeria* en soort *Eimeria*), alsook variabelen van de kip (leeftijd, type kip en eerder doorgemaakte *Eimeria* infecties). Als we meer kennis zouden hebben van het mechanisme dat tengevolge van infectie leidt tot bescherming zouden we mogelijk vaccins kunnen ontwikkelen die zonder schade of infectie in de kip bescherming opwekken.

De immuunrespons van de kip werd voornamelijk geanalyseerd op basis van de veranderingen in de frequentie van immuun-cel subpopulaties en cytokine expressieprofielen in de darm. De immuun-cellen zijn o.a. T-cellen die op hun beurt weer onderverdeeld kunnen worden in de T-helper (Th) 1 en T-helper 2 cellen, de CD4 cellen en in cytotoxische T cellen, de CD8 cellen. De lokale expressie van cytokinen, eiwitten die de afweerrespons sturen, in de darm wordt niet gemeten aan de eiwitten zelf, maar aan de hand van het genetisch materiaal dat voor de productie van deze cytokine wordt aangemaakt: het mRNA. Het cytokine expressieprofiel en het type en relatieve frequentie van de immuuncellen zijn een maat voor het type afweerrespons dat wordt opgewekt. Th1 cellen die een meer cellulaire afweer respons verzorgen reageren op intracellulaire indringers en zijn geassocieerd met een typerende set van 'Th1 cytokinen' die lokaal worden geproduceerd. De Th2 cellen zijn geassocieerd met specifieke antilichaam productie en met een set van 'Th2 cytokinen' en reageren voornamelijk op parasieten die tussen cellen in zitten. Parasitair materiaal werd gekwantificeerd aan de hand van het species specifieke *Eimeria* DNA in de kippendarm.

De eerste factor met een mogelijke invloed op de *Eimeria*-kip interactie die we onderzocht hebben was de variatie in de hoeveelheid *Eimeria* parasieten waarmee kippen geïnfecteerd werden. In een experiment beschreven in **hoofdstuk 2** infecteerden we kippen met een lage ($5 * 10^2$ oöcysten) of een hoge ($5 * 10^4$ oöcysten) dosis *E.acervulina*. De aanwezigheid van *Eimeria* DNA in de darm en ook de hoeveelheid hiervan is bepaald met een door ons ontwikkelde *Eimeria*-specifieke PCR. De complete infectieuze cyclus die *E.acervulina* doormaakt in de kip duurt ongeveer vier dagen. In kippen geïnfecteerd met een hoge dosis *E.acervulina* wordt met name na dit tijdstip lokaal een verhoogde frequentie van $CD8\alpha^+$ T-cellen en een toename in interleukine-8 (IL-8) mRNA in de darm gevonden. De afweer reactie op een infectie met een lage dosis was echter relatief zwak. Bij dieren geïnfecteerd met de lage dosis *E.Acervulina* werd na de periode van vier dagen geen verhoging van de frequentie van $CD8\alpha^+$ T-cellen gevonden in de darm, hoewel er wel een toename in IL-8 mRNA in de darm was. Er is dus een invloed van de hoeveelheid parasieten waarmee kippen geïnfecteerd worden op de immuunrespons van de kip, namelijk op de hoeveelheid immuuncellen in de darm. In beide gevallen zijn de kippen volledig beschermd tegen herinfectie.

De tweede pathogeen-afhankelijke factor die werd onderzocht, was het effect van verschil in soort *Eimeria* waarmee de gastheer geïnfecteerd is op het karakter van reactiviteit van het afweersysteem. Er zijn zeven soorten *Eimeria* bekend die kippen kunnen infecteren en elk van deze soorten infecteert een specifiek stuk van de darm. De meeste studies beperken zich tot e*en modelinfectie met één soort Eimeria, terwijl gastheren in het veld vaak met meerdere Eimeria* soorten tegelijk geïnfecteerd kunnen zijn. In **hoofdstuk 3** hebben we het verschil in effect op de immuunrespons vergeleken tussen een infectie met één soort *Eimeria* en het effect van een infectie met meerdere soorten tegelijk. In dit experiment hebben we drie soorten *Eimeria* gebruikt, *E.acervulina*, *E.maxima* en *E.tenella*. Deze veroorzaakten alle drie een soort-specifieke immuunrespons op de plaats van de infectie in de darm. *E.acervulina* wekte in het duodenum vooral een $CD8\alpha^+$ T-cel respons op en een verhoging van de lokale IL-18 cytokine productie (mRNA). De reactie op een *E.maxima* infectie was in het jejunum vooral een verhoging van de

hoeveelheid $CD4^+$ T-cellen en de cytokinen IFN-γ en IL-4. Een infectie met *E.tenella* tenslotte, zorgde voor een verhoogde hoeveelheid $CD4^+$ T-cellen en verhoging van IL-2 en IL-4 cytokine mRNA in de blinde darm. Een gecombineerde infectie van deze drie soorten veroorzaakte geen extra responsen, maar er was eerder sprake van een simultane expressie van verschillende responsen tegen de respectievelijke *Eimeria* species, ieder op hun eigen specifieke locatie. Ook wanneer drie typen parasieten tegelijk aanwezig zijn op verschillende locaties in de darm ondervind elk daarvan voornamelijk de soort-specifieke respons van de kip en worden er geen extra reacties vanuit de kip gezien.

Omdat ook gastheer afhankelijke factoren invloed kunnen hebben op de *Eimeria*-kip interactie, hebben we drie gastheer-afhankelijke-factoren onderzocht. Zo hebben we gebruik gemaakt van verschillende kippenlijnen en daarmee dus de genetische achtergrond van de kippen als variable onderzocht. Ook hebben we kippen met verschillende leeftijden gebruikt in ons onderzoek. Omdat het immuunsysteem een lerend systeem is en het door een infectie veranderd in tal van kenmerken is ook als variable onderzocht wat er gebeurt met infectie van kippen die al een eerdere infectie van *Eimeria* gekregen hadden.

Er worden voor de kippenvleesindustie genetisch verschillende kippenrassen gebruikt, bij kippen 'lijnen' genoemd. Door gebruik te maken van kippen met een verschillende genetische achtergrond kan er vlees van een verschillende kwaliteit geproduceerd worden. We hebben onderzocht of er een effect is van de genetische achtergrond van de kip op de afweerreactie na infectie met de *Eimeria* parasiet. Dit experiment hebben we in **hoofdstuk 4** beschreven. In het experiment hebben we twee verschillende kippenlijnen geïnfecteerd met *E.acervulina*. Deze twee lijnen waren een vleeskuikenlijn, geselecteerd op snelle groei, die na 7 weken het slachtgewicht bereikt en een langzaam groeiende vleeskuikenlijn die het slachtgewicht bereikt in een tijd van ongeveer 12 weken. De langzaam groeiende lijn uit dit experiment is een kippenlijn die gebruikt wordt in de biologische houderij. In dit experiment vonden we kippenlijn afhankelijke reactieprofielen op een *Eimeria* infectie. In snel groeiende kippen werd vroeg na infectie in de darm een verhoogde frequentie van $CD8\alpha^+$ en $TCR\gamma\delta^+$ T-cellen en een verhoogde hoeveelheid IL-8 en IL-18 cytokine mRNA gevonden. Bovendien was de hoeveelheid *E.acervulina* DNA die in

de darm van de conventionele kippen werd gevonden lager dan die in de langzaam groeiende lijn. Er is dus een invloed van de kippenlijn op de immuunrespons, namelijk op de snelheid waarop de respons opgewekt wordt. Een snelle respons zorgt ervoor dat er minder parasieten overblijven die zich in de kip vermenigvuldigen.

De dieren uit de experimenten beschreven in hoofdstukken 2 en 4 verschilden in leeftijd op het moment van infectie. Deze dieren met andere leeftijden vertoonden andere afweerpatronen op een *Eimeria* infectie. Vooral het ontbreken van de IFN-γ respons in dieren geïnfecteerd op een leeftijd van één dag terwijl er in dieren geïnfecteerd op dag zeven van leeftijd wel een IFN-γ respons gemeten werd, was opvallend. Dit suggereerde dat de gastheer-afhankelijke factor leeftijd van de kip ook van invloed is op de afweerreactie op een *Eimeria* infectie. Omdat de twee groepen niet uit hetzelfde experiment komen mochten ze niet vergeleken worden. In **hoofdstuk 5** beschrijven we dan ook een nieuw experiment waarin we de invloed van leeftijd van de kip op een *E.maxima* infectie onderzocht hebben. Vleeskippen werden op een leeftijd van één, zeven en eenentwintig dagen geïnfecteerd. Hoewel bij kippen na een *Eimeria* infectie gewichtsverlies kan ontstaan werd alleen in de kippen die op dag één geïnfecteerd waren gewichtsverlies gevonden. Er was dus sprake van een milde infectie.

T-helper (Th) cellen kunnen een cellulaire (Th1) of een humorale respons (Th2) ondersteunen. Om te bepalen of de kippen via een Th1 of een Th2 type respons reageerden op de *Eimeria* infecties hebben we onderzocht hoe de verhouding tussen IFN-γ en IL-10 mRNA expressie was. Aangenomen werd dat IFN-γ de Th1 kant vertegenwoordigde en IL-10 de Th2 type respons. Naarmate kippen ouder worden gaat de reactie op een primaire infectie meer de Th1 kant op. Hoewel in op dag één van leeftijd geïnfecteerde dieren de Th1 respons op een primaire infectie niet erg duidelijk aanwezig was, had dit geen gevolgen voor de het verloop van deze primaire infectie. De kippen die op dag één van leeftijd geïnfecteerd waren hadden namelijk dezelfde *Eimeria* hoeveelheid in hun darmen als kippen die op latere leeftijd geïnfecteerd waren. Het type respons waarmee de kip reageert heeft dus geen gevolgen voor de aanpak van de infectie die op dat moment gaande is. Daarnaast is ook gebleken dat het

type respons op een primaire infectie geen invloed heeft op de opbouw van immunologische bescherming tegen latere infecties.

Het is bekend dat kippen een immunologische bescherming ontwikkelen na een primaire infectie met *Eimeria*. Deze immunologische bescherming is *Eimeria* soort specifiek. Er is in het algemeen geen kruisimmuniteit tussen soorten. In **hoofdstuk 6** is het effect van een eerder doorgemaakte *E.maxima* infectie op de response tegen zowel een homologe (*E.maxima*) als een heterologe (*E.acervulina*) herinfectie beschreven. Er werden geen oöcysten meer uitgescheiden op de dag dat de dieren een herinfectie ondergingen. Maar ook na homologe herinfectie, dat wil zeggen herinfectie met dezelfde soort (*E.maxima*) als waar eerder de weerstand tegen werd opgewekt werd (*E.maxima*) bleek dat er geen oöcysten werden uitgescheiden en de dieren dus als verwacht volledig beschermd waren. De kippen bleken dus door immunologische reactie op de primaire infectie beschermd te zijn. Kippen die een heterologe herinfectie ondergingen, een infectie met een andere soort (*E.acervulina)* dan waar bescherming mee werd opgewekt (*E.maxima)*, waren niet immunologisch beschermd en bleven evenveel oöcysten uitscheiden als kippen die een primaire *E.acervulina* infectie kregen. Tegen de verwachting in vonden we in kippen na een homologe herinfectie een lage reactiviteit van T-cellen en cytokinen. Met andere woorden: de beschermende reactiviteit geeft geen aanleiding tot heftige immunologische responsen. De responsen zijn specifiek tegen de parasiet gericht en veroorzaken geen bijkomende schade aan de kip zelf. Verwacht werd, dat meer immunologische activiteit zou worden waargenomen bij de herinfectie omdat die leid tot bescherming. Aan de hand van de afname van het aantal parasieten in de darm kunnen we wel concluderen dat er immunologische activiteit bestaat. Maar we hebben het karakter van die activiteit niet nader kunnen preciseren omdat er in door ons getoetste parameters geen significante verandering optrad of omdat er sprake is van een activiteit die we met de gekozen methoden niet kunnen meten.

Micro-arrays zijn zogenaamde DNA 'chips', dat wil zeggen dat er kleine stukjes genetisch materiaal in lange rijen op een glasplaatje geprint zijn. Op een micro-array staan stukjes DNA die corresponderen met een bepaald gen of stuk uit het genoom. DNA kan een specifieke binding aan gaan, hybridisatie. Op deze manier kunnen specifieke stukjes genetisch

materiaal, RNA of DNA, in een test monster van weefsel of uitscheidingsproduct aan de chip binden en zo zichtbaar worden gemaakt. Het genoom van de kip is al volledig ontcijferd en van vele stukken van het DNA is ook al bekend voor wat voor genen ze coderen. Uit hybridisatie experimenten op arrays die synthetische stukjes bevatten van het gehele genoom van de kip, bleek dat er veel genen waren die in alle experimenteel geïnfecteerde groepen uit hoofdstuk 6 hoger tot expressie kwamen. Dat kan het gevolg zijn van een ontstekingsproces of het proces van weefselherstel. Naast deze "algemene" responsen op een *Eimeria* infectie werden er ook genen gevonden die alleen na een homologe herinfectie specifiek hoger tot expressie kwamen. Dit waren met name T-cel genen en eiwitaanmaak gerelateerde genen. Deze responsen spelen mogelijk een rol in de bescherming tegen herinfecties, maar dit moet nog verder onderzocht worden. Er is dus een invloed van eerder doorgemaakte infecties op de immuunrespons, welke zorgt voor een beschermende respons op een homologe herinfectie. Vaccins of vaccinonderdelen die in staat zijn dit proces op te wekken zijn mogelijk kandidaten voor toepassing in bestrijding van *Eimeria*.

Zoals beschreven in dit proefschrift kunnen zowel *Eimeria* als kip-afhankelijke factoren de afweer reacties van de kip op een *Eimeria* infectie beïnvloeden. De immuun-responsen van de gastheer beïnvloeden op hun beurt de uitscheiding van oöcysten en kunnen een invloed hebben op de transmissie van *Eimeria*. Transmissie is het overdragen van infectieuze kiemen van een geïnfecteerd dier naar een nog onbesmet dier. Met de kennis verkregen uit de experimenten beschreven in dit proefschrift en verkregen door F.C. Velkers waren we uiteindelijk in staat om de relatie tussen de verschillen in afweer reacties veroorzaakt door eerdere infectie met *Eimeria* en de transmissie van *Eimeria* te bepalen. Daarvoor hebben we een experiment ontworpen en uitgevoerd, zoals beschreven in **hoofdstuk 7**. In dit experiment hebben we zowel de gastheer immuun-responsen binnen het dier als de transmissie van *Eimeria* infecties tussen dieren onderzocht. Paren kippen zijn samen in een hok geplaatst. Van alle paren werd één dier geïnfecteerd. We hebben onderzocht wat de kans was dat het tweede dier geïnfecteerd raakt met *Eimeria*. Hieruit bleek dat overdracht van *Eimeria* verminderd was in paartjes van vleeskuikens bestaande uit een opzettelijk besmet infectieus dier en een contactdier dat

al eens eerder geïnfecteerd is geweest vergeleken met paartjes bestaande uit een opzettelijk besmet dier dat al eerder eenzelfde infectie doorgemaakt had en een contactdier. Ook hier bleek dat er wel sprake is van opgewekte beschermende activiteit maar we hebben het karakter van die activiteit niet nader kunnen preciseren omdat er in door ons getoetste parameters geen significante verandering optrad of omdat er sprake is van een activiteit die we met de gekozen methoden niet kunnen meten. De overdracht van een *Eimeria* infectie is dus afhankelijk van de immunologische achtergrond van de kip.

In **hoofdstuk 8** zijn de algemene bevindingen en conclusies uit dit proefschrift beschreven, samen met de suggesties voor verder onderzoek. Wanneer we de in dit proefschrift beschreven experimenten samenvatten, komen we tot de conclusie dat we in meerdere proeven dezelfde responsen vinden na een *Eimeria* infectie. Dit ondanks het feit dat alle geteste variabelen, zowel de variabelen in de parasiet (hoeveelheid *Eimeria* en soort *Eimeria*), als de variabelen in de kip (leeftijd, type kip en eerder doorgemaakte *Eimeria* infecties) de gastheer-responsen beïnvloeden. Een *Eimeria* infectie wekt een typische twee-fasen respons op die bestaat uit een vroege en een late respons. De vroege respons vindt plaats binnen 3 dagen na infectie en lijkt een reactie op de zich vermenigvuldigende parasiet in de darm. De late respons, die plaats vindt wanneer er geen *Eimeria* DNA meer in de darm gevonden wordt, lijkt een reactie op de celschade die ontstaat wanneer de parasiet de darmcellen verlaat. Het profiel van de reactie op een *Eimeria* infectie is Th1, vooral door de hoge hoeveelheid IFN-γ mRNA. Naarmate dieren ouder worden, zijn ze kennelijk beter in staat te reageren via een Th1 type respons. De verhoogde capaciteit van oudere dieren om via een Th1 respons te reageren, resulteerde echter niet in een verlaging van de uitscheiding van parasieten bij een primaire infectie. Hoewel de reacties op een primaire infectie een duidelijker Th1 profiel hadden naarmate de gastheer ouder was, was er ook geen relatie tussen het type respons op een eerdere infectie en later gevonden bescherming tegen een herinfectie. Tenslotte is er een lage reactiviteit gevonden op homologe herinfecties met zowel *E.maxima* als *E.acervulina*. Er zijn geen T-cellen of cytokinen gevonden die een rol lijken te spelen bij bescherming tegen herinfectie. Hoewel er een duidelijke bescherming aanwezig was, is de reactie op een herinfectie

meer ingetogen, daar waar de reactie op een primaire infectie divers en robuust is. Het is mogelijk dat slechts een deel van de T-cellen die we bekeken hebben verantwoordelijk is voor de bescherming. Nieuwe celmarkers kunnen de celtypen die verantwoordelijk zijn voor de gevonden bescherming wellicht onthullen. Daarnaast kan de data die met de analyse van de micro-array verkregen is ook nog nieuwe informatie verschaffen. Door te kijken in welke processen de gevonden genen een rol spelen, een zogenaamde pathway analyse van de verkregen data, kunnen er processen aan het licht komen die een rol spelen in bescherming. Modulering van deze processen kan een belangrijke rol spelen in een nieuw te ontwikkelen vaccinatie-strategie.

Curriculum vitae

Op 30 augustus 1978 werd ik, Willem Swinkels, geboren in Breda. In 1997 slaagde ik voor mijn VWO diploma in Breda. In datzelfde jaar ben ik begonnen met mijn studie Biologie aan de Katholieke Universiteit Nijmegen (thans Radboud Universiteit Nijmegen). Voor mijn eerste stage heb ik bij de afdeling Microbiologie van de Katholieke Universiteit Nijmegen onder begeleiding van Dr. Peter Steenbakkers 9 maanden onderzoek gedaan naar een microbieel serpine. Mijn tweede stage duurde 6 maanden en deze heb ik gedaan bij de afdeling Medische Microbiology van het Nijmegen Center of Molecular life Sciences onder leiding van Dr. Frank van Kuppeveld. Tijdens deze periode heb ik onderzoek gedaan naar de invloed van het Coxsackie B3 virus op Y-RNA's. In februari 2003 ben ik afgestudeerd en in oktober van datzelfde jaar ben ik begonnen als AIO bij de afdeling Landbouwhuisdieren van de Universiteit van Utrecht en was ik gedetacheerd in Lelystad bij de Animal Sciences Group van Wageningen UR (thans Centraal Veterinair Instituut van Wageningen UR). Hier heb ik gewerkt aan het onderzoek wat is beschreven in dit proefschrift. Per 1 mei 2008 ben ik als onderzoeker werkzaam bij de afdeling maag, darm en leverziekten van het Erasmus MC te Rotterdam.

Dankwoord

Een periode is ten einde. Bij mijn afstuderen in Nijmegen kreeg ik de mededeling dat ik nu net als bioloog uit het ei gekropen was. Als kuiken heb ik de afgelopen vier jaar in Lelystad onder de beschermende vleugels van mijn begeleiders heerlijk onderzoek kunnen doen. Dankzij hen heb ik vervolgens zelf ook een ei gelegd.

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Uiteraard is het ongepast om met andermans veren te pronken. Experimenten doe je niet alleen, daarom wil ik graag de mensen waarmee ik de experimenten en analyses uitgevoerd heb een grote pluim geven. Jacob, bedankt voor de eerste kennismaking met kippenimmunologie, FACS en celkleuringen. Jan, jouw grondige voorbereiding van de experimenten zorgde er voor dat alles zonder problemen verlopen is. Er bestaat waarschijnlijk geen protocol waarvan jij niet op de hoogte bent. Gebleken is, dat je met twee man op een project drie keer zo snel gaat als in je eentje. Ik hoop dat ik je beeld van AIO's iets heb veranderd, maar ben in ieder geval blij dat je bij de verdediging aan mijn zijde staat. Leo,

de nauwkeurigheid waarmee jij je experimenten uitvoert is ongekend. De array resultaten waren fantastisch mooi en ik hoop dat er na mij nog veel mensen mee aan de slag kunnen. Arjan bedankt voor de hulp bij de vele labzaken en zeker ook de gezelligheid! Astrid, je kan niet alleen kakelen als een kip zonder kop, ook array analyses gaan je goed af. Bedankt voor alle opbeurende woorden en de roze knuffels. Albert, naast proefdierverzorger, speelde je ook als adviseur in de opzet van dierproeven een grote rol. Ik heb erg fijn met je samengewerkt en verbaas me er nog steeds over dat zulke grote handen zulke kleine kuikens voorzichtig vast kunnen houden. Bas, bedankt voor de hulp bij de statistiek. Herman Peek bedankt voor de introductie in de wereld van *Eimeria* en de oocysten om mijn proeven mee te doen. Tenslotte wil ik de mensen die in de afgelopen tijd op vleugel 15 gewerkt hebben bedanken. Jullie hebben allen ook bijgedragen aan het tot stand komen van dit proefschrift en zijn daarnaast ook nog eens verantwoordelijk geweest voor de gezelligheid tijdens koffiepauzes en lunch. Zonder jullie was het verblijf in Lelystad niet te doen geweest.

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