Studies on T-cell Properties during Coccidiosis and a Vitamin E Supplement to an
in ovo Coccidiosis Vaccine

THESIS

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By

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This project studied 1. T-regulatory cells (Tregs) and immunosuppressive properties post-coccidial challenge and 2. the effect of vitamin E supplementation to an in ovo anti-coccidial vaccine. Parameters evaluated included key cytokines involved in a coccidia response, specific T-cell populations, macrophage nitric oxide (NO) production and T-cell proliferation.

For the first experiment, layer chickens were orally gavaged at three weeks of age with live oocysts to produce an active, lesion forming, infection or left uninfected to serve as the control. At 6 and 11 days post-challenge, spleen and cecal tonsils were collected and evaluated for cell proliferation, CD4⁺, CD8⁺ and CD25⁺ T cell populations and the cytokines IL-1, IFN-γ and IL-10. Successful infections with coccidiosis can be characterized by reduced weight gain and shedding of oocysts in the feces. Infected birds gained an average of 22.5g less weight over the 11 day trial period and shed an average of 1.5 x 10⁴ oocysts/g of feces 5 days post-infection. Infected birds had an increase in IL-10 transcripts in the cecal tonsils and a decrease of IFN-γ in the spleen after 11 days of infection. Also, 11 days after infection the population of Treg cells decreased in the spleen and increased in the cecal tonsils. During early infection, 6 days post-challenge, CD4⁺ and CD8⁺ cells increased in the cecal tonsils but there was no effect in the spleen.
At 11 days post-challenge, the CD4+ cells decreased in the cecal tonsils. T-cell populations depleted of Tregs had higher cell proliferation than populations containing Tregs. Overall, the coccidia infection caused an up regulation in Tregs, the immune suppressive cytokine IL-10, in the cecal tonsils and a decrease in IFN-γ in the spleen during late infection. These three factors together will suppress the response to the coccidia pathogen.

In a second experiment, fertile eggs were injected with vitamin E and/or a live anti-coccidial vaccine at 18 days of incubation. Chicks that were exposed to supplemental vitamin E in ovo had splenic macrophages that produced higher concentrations of NO 6 days post-hatch and higher serum levels of anti-coccidial IgG 12 days post-hatch. There was no effect of vitamin E on NO levels at day 12 or on serum anti-coccidial IgG concentrations at day 6.

This study demonstrated that IL-10 release by Tregs is a key component of the immune suppression brought on by a coccidial infection and that vitamin E could be a beneficial supplement to an in ovo anti-coccidial vaccine.
Dedication

Dedicated to my parents, grandparents and great-grandparents who have shown me the value of, and inspired me to pursue, my education.
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Chapter 1: Introduction

Coccidiosis in commercial poultry production is a disease that has great impact on the industry. Each year it causes a loss of about $800 million in the United States alone (Sharman et al., 2010). Current treatment protocols, such as the use of anticoccidial medications, are susceptible to resistance (Chapman, 1998) and vaccination by itself is not always enough to control the problem. Further research on how coccidia affects the avian immune system could lead to novel applications in preventing the disease.

Coccidia is an enteric protozoal pathogen of the genus *Eimeria* that replicates in the lining of the intestine causing lesions that prevent the proper absorption of nutrients, bloody hemorrhages and, at times, mortality (Lillehoj and Trout, 1996; Paris and Wong, 2013). T-cell-mediated immunity by intestinal intraepithelial lymphocytes is the primary component of protective immunity to *Eimeria* (Lillehoj and Lillehoj, 2000). Macrophages, T-helper cells (CD$^+$) and T-cytotoxic cells (CD$^+$) are also an important part of the immune response against coccidiosis. Tregs, an immune suppressive T-cell, secrete IL-10 that suppresses the activation and proliferation of CD$^+$ and CD$^+$ T cells (Dieckmann et al., 2001). Genetic lines of chickens that are more susceptible to coccidiosis have higher levels of IL-10 (Rothwell et al., 2004), suggesting that it would
be advantageous for pathogen survival to up regulate IL-10 or cells that produce IL-10, like Tregs.

IL-10 can also reduce the production of IFN-γ, which is crucial in the response to a coccidia infection. An anti-coccidial immune response increases following co-administration of IFNγ with a coccidial vaccine (Ding et al., 2004). Genetic differences between inbred lines have shown that IFN-γ levels are higher in *Eimeria*-resistant lines than in susceptible lines after an *Eimeria* challenge (Yun et al., 2000). Low levels of IL-10, an immunosuppressive cytokine, increase resistance, whereas high levels of IL-10 increase susceptibility to intracellular pathogens (Moore et al., 2001). Overall, IL-10 inhibits immune responses to the pathogen, while a strong IFN-γ driven anti-coccidial immune response is critical for the host. The first part of this research evaluates T-cell properties during a coccidia infection.

Vaccines can prepare a bird's immune system for future challenges from diseases like coccidiosis. Chickens are capable of developing immunity to homologous species of *Eimeria* where development of the sporozoite is inhibited, but invasion of the sporozoite into the enterocyte still occurs. Antibodies specific to coccida have been identified in the bile and can prevent them from invading host cells (Rose and Hesketh, 1987). An efficient and accurate way to deliver vaccines is by *in ovo* injection. This exposes the bird to the pathogen at an early life stage and gives it the ability to start mounting an immune response before it hatches.
Nutritional interventions can also be implemented to aid the immune response. Vitamin E has proven to be an effective dietary supplement to increase the immune response in general (Konjufca et al., 2004; Silva, 2011) and to certain challenges such as coccidiosis and LPS (Colnago et al., 1984; Muir et al., 2002; Kaiser et al., 2012). Vitamin E can also be beneficial when injected \textit{in ovo} by increasing the total antibody response, macrophage potential and nitrite production post-hatch (Gore and Qureshi, 1997).

Adjuvants, or supplements to vaccines can be used to increase the efficacy of a vaccine. Vitamin E has also been used as an adjuvant in a Newcastle viral vaccine where it decreased lesion healing time and increased titers of antibodies (Franchini et al., 1991). The second part of this study evaluates the use of vitamin E as a supplement to an \textit{in ovo} coccidia vaccine.

The objectives of the present study are as follows:

**Specific Aim 1**

To identify Treg numbers and immunosuppressive properties after a coccidial challenge

**Specific Aim 2**

To study the effects of an \textit{in ovo} vitamin E supplement to an \textit{in ovo} anti-coccidial vaccine
Chapter 2: Review of Literature

Species and Lifecycle of Avian Coccidiosis

Coccidiosis is an enteric protozoan infection. The general lifecycle of *Eimeria* species involves ingestion of coccidial oocysts by the chicken and replication in the enterocytes. This endothelial penetration and replication within the enterocyte damages the intestinal epithelium and causes lesions to form. The lesions decrease the bird’s ability to absorb nutrients and cause reduced body weight gain or weight loss (Lillehoj and Trout, 1996; Paris and Wong, 2013). This makes it an economically important disease for the poultry industry with losses estimated at more than $800 million annually in the United States (Sharman et al., 2010). External signs of a coccidial infection can include lethargy, lack of grooming behavior, blood in the feces and a decrease in feed and water intake (Lillehoj and Trout, 1996).

There are 7 different species of coccidia that belong to the genus *Eimeria*: *Eimeria tenella*, *E. acervulina*, *E. maxima*, *E. brunetti*, *E. mitis*, *E. necatrix* and *E. praecox*. Five of these species are more pathogenic and easy to clinically identify, but the other two (*E. praecox*, *E. mitis*) typically only produce clinical signs experimentally (Allen and Fetterer, 2002b). *E. tenella* and *E. necatrix* target the cecum while *E. acervulina* develops in the duodenum and *E. maxima* is found in the mid to posterior intestine (Chapman, 2014).
The lifecycle of *Eimeria* spp. begins as an unsporulated oocyst shed in the feces of an infected bird. This exposure to atmospheric oxygen triggers the oocyst to undergo meiosis and cell division giving rise to eight haploid sporozoites in approximately 24 hours (Johnson, 1923; Allen and Fetterer, 2002b; Lal et al., 2009). Infection begins when these sporulated oocysts are ingested by a susceptible host. Excystation, a process by which the oocyst is broken down, occurs through abrasion in the gizzard and factors in the upper intestine such as bile and trypsin (Allen and Fetterer, 2002; Lal et al., 2009). The released sporozoites will invade the intestinal epithelia locally (*E. brunetti*, *E. praecox*) or migrate (*E. tenella*, *E. maxima*) to different areas of the small intestine or ceca via intra-epithelial lymphocytes (Lawn and Rose, 1982; Al-Attar and Fernando, 1987; Allen and Fetterer, 2002b). The sporozoites then undergo schizogony, or the asexual reproduction of schizonts. The schizonts continue asexual reproduction to produce merozoites, which are motile. Cells containing merozoites will rupture and merozoites will reinvade locally to reproduce themselves and produce a smaller schizont (Lal et al., 2009).

The merozoites eventually differentiate into male (microgamonts) or female (macrogamonts) gametocytes. The macrogamonts give rise to one macrogamete and the microgamonts divide to produce many motile microgametes which exit the cell and use their flagella to penetrate other cells containing macrogametes to fertilize and produce oocysts (Lillehoj and Trout, 1996; Allen and Fetterer, 2002b). The oocyst matures and the host cell ruptures, expelling the oocyst from the body in the feces and a new round of infection begins.
Avian Immune Response to Coccidiosis

a.) Cytokines

T-cell-mediated immunity by intestinal intraepithelial lymphocytes and lamina propria lymphocytes confers the main component of protective immunity to *Eimeria*, an intracellular pathogen (Lillehoj and Lillehoj, 2000). The anti-coccidial activities of T cells are augmented by T helper-1 cytokines like IL-2 and IFNγ. Recombinant chicken IFNγ inhibits *Eimeria* development *in vitro* and reduces oocyst production and body weight loss following an *Eimeria* challenge (Lillehoj and Choi, 1998) and the anti-coccidial immune response increases following co-administration of IFNγ with a coccidial vaccine (Ding et al., 2004).

An inflammatory response is initiated by the invasion of sporozoites into the enterocyte and mediated by cytokines produced by activated macrophages and T cells such as IL-1β, IL-6, IL-17 and IFN-γ (Hong et al., 2006). TGF-β4 transcripts have also been found to increase in the intestinal IEL and spleen after primary infection with *E. acervulina*. TGF-β4 amounts decreased after secondary infection, but still remained above baseline. In chickens, TGF-β4 is concentrated in the tips of the villi, so it is hypothesized that it is involved with growth of the villi (Jakowlew et al., 1997).

IL-10 is an anti-inflammatory cytokine that will inhibit the synthesis of inflammatory cytokines and increase susceptibility to infection. IL-10 levels will increase after an exposure to *E. maxima* (Rothwell et al., 2004). The Hong et al. (2006) study also showed an increase in IL-10 levels after exposure to *E. acervulina* and *E. tenella*, parallel to increased amounts of inflammatory cytokines. This could
be an indication that levels are changing differently in certain areas of the gut, or that IL-10 does not have the same function in chickens as it does in mammals. Rothwell (2004), however, contended from their in vitro studies and a study with Marek’s disease by Heidari (2008) in ovo, that IL-10 does, in fact, inhibit inflammatory responses in chickens similar to what is observed in mammals (Heidari et al., 2008).

In mammals, IL-10 is a homodimer of two interpenetrating polypeptide chains, closely resembling the structure of IFN-γ (Zdanov et al., 1996). It is also similar in regards to its receptor. The IL-10 receptor contains two subunits that are part of the IFN receptor family (Liu et al., 1994). One mechanism to IL-10 anti-inflammatory properties was demonstrated in a study using human monocytes showing that IL-10 inhibits Nuclear Factor κB (NFκB). NFκB consists of two proteins (P65 and P50) and is normally inactive in the cytoplasm, being inhibited by IκB. Once the IκB is removed by phosphorylation, the active NFκB is translocated to the nucleus where it induces transcription of its target genes, including inflammatory cytokines. It is known that IL-10 inhibits this cytokine production in a dose-dependent manner by blocking NFκB activation (Wang et al., 1995). It is speculated that IL-10 interferes with the phosphorylation of IκB, leaving NFκB inactive in the cytoplasm (Ehrlich et al., 1998).

IFN-γ levels are crucial in resistance to Eimeria. (Lowenthal et al., 1997). It has been observed that IFN-γ mRNA increases 200-fold after exposure to E. tenella (Laurent et al., 2001) and the levels of IFN-γ will continue to increase with each subsequent infection. Yin et al. (2013) found that there was a significant increase in IFN-γ producing cells from the first to the third exposure of E. tenella (Yin et al.,
Birds that are given an injection of IFN-γ post-coccidial infection had reduced body weight loss due to the disease and recovered faster. A suggested mechanism is that IFN-γ can activate macrophages by inducing the release of IL-1 (Lowenthal et al., 1997). There are also differences in susceptibility to *Eimeria* infections across different lines of birds. More susceptible lines will have lower expression of IFN-γ during infection compared to more resistant chickens (Yun et al., 2000).

Genetic differences between inbred lines have shown that IFNγ levels are higher in *Eimeria*-resistant lines than in susceptible lines after an *Eimeria* challenge (Yun et al., 2000). In contrast, low levels of IL-10, an immunosuppressive cytokine, increase resistance, whereas high levels of IL-10 increase susceptibility to intracellular pathogens (Moore et al., 2001). IL-10 plays a crucial role in preventing the development of a strong IFNγ-driven anti-coccidial response. Susceptible birds upregulated IL-10 mRNA after *Eimeria* infection whereas resistant birds did not. *Eimeria*-susceptible birds had 43-fold higher splenic IL-10 mRNA levels than uninfected or resistant birds (Rothwell et al., 2004). This suggests that higher amounts of IL-10 may increase susceptibility to *Eimeria*. Chickens vaccinated with *Eimeria* Ag-pulsed dendritic cell exosomes and subsequently challenged with *Eimeria* had a lower number of IL-10-producing cells (del Cacho et al., 2012). In fact, restoration of IL-10 to normal levels has been proposed as a marker to evaluate the efficiency of anti-coccidial drugs (Haritova and Stanilova, 2012). Because IL-10 plays a crucial role in preventing the development of anti-coccidial responses and because IFNγ plays a crucial role in the development of anti-coccidial responses, studying how IL-10 and IFNγ are altered during coccidiosis is essential.
IL-10 antibodies (to neutralize IL-10) improved coccidial clearance and increased body weight (Sand, 2012).

**b. Innate Immunity**

The innate immune response is the first line of defense against a coccidia infection and includes cells such as heterophils, dendritic cells and macrophages/monocytes. The inflammatory cytokines and nitric oxide (NO) that activated macrophages release are significant mediators to the innate immune response against coccidiosis (Allen and Fetterer, 2002b). Following both primary and secondary *E. acervulina* infections, macrophages contain high numbers of sporozoites (Trout and Lillehoj, 1995). After exposure to *E. tenella* immune serum, phagocytosis of sporozoites by macrophages was enhanced (Onaga and Ishii, 1980). A high number of macrophage migration inhibitory factor (MIF) transcripts are present in *E. tenella* infected birds and this up regulates transcription of the inflammatory cytokines IL-6, IL-17 and TNFSF15 (Jang et al., 2011). Heterophil and monocyte-derived macrophages stimulated with heat killed *E. tenella* sporozoites upregulate the expression of toll-like receptor 4 (TLR), TLR 15 and the adapter protein MyD88. Pathogen-associated molecular pattern activated pathways will induce production of inflammatory cytokines such as TNF-α and IFN-γ (Zhou et al., 2013). TLRs are type 1 glycoproteins in that they are integrated into the membrane and can contain both intra and extracellular components. The extracellular domain is a ligand binding domain and the IL-1 receptor domain is located intracellularly (St Paul et al., 2013). Ten different TLR genes have been identified in chickens. Mammalian orthologues
are present, but chickens also have two unique toll-like receptors in TLR15 and TLR21. Activation of TLR3 and TLR21 induces the release of IL-10 and IFN-γ in chicken monocytes (He et al., 2012). This dual-release of both an inflammatory and anti-inflammatory cytokine could be a mechanism that prevents excessive inflammation.

c.) B and T cells

Lymphocytes consist of B cells and T cells. T cells are distinguished by different surface markers and functions. T cells begin development when hematopoietic stem cells from the bone marrow enter the thymus. T cells then undergo thymic selection consisting of positive selection, to ensure recognition of self-antigen presented by the major histocompatibility complex (MHC), and negative selection, to delete thymocytes with too high an affinity for self MHC complexes. The two main cell subsets are CD4+ and CD8+. If the T cell’s receptor (TCR) can bind MHC class II, it will also bind a CD4 molecule, selecting the cell for CD4+. If the TCR binds MHC class I, it will be selected for the CD8+ subset. CD8+ cells are also known as Cytotoxic T Lymphocytes (CTL). CD4+ cells are then further divided into five different groups: T-regulatory (Tregs) and T helper (TH) 1, 2, 9 or 17 cells. T cells have an important role as effector cells fighting coccidial infections and establishing immunity (Rose and Hesketh, 1979; Lillehoj, 1987). CD8+ T cells have also been implicated in the transport of sporozoites to different locales within the gut (Trout and Lillehoj, 1995) but are key players in resistance as CD8+ cell numbers increase in the intestinal epithelium following a secondary infection (Lillehoj and Trout, 1996) and
can be seen in tissue sections making direct contact with infected cells (Lillehoj and Bacon, 1991). Following a primary infection of *E. acervulina* in SC (Hy-line lineage) chickens, the number of CD8\(^+\) T lymphocytes in the duodenum significantly increased (Lillehoj, 1994). The comparative value of B and T cells in fighting coccidiosis were studied in athymic rats and bursectomized chickens. Rats with no thymus (no T cells) released three times as many oocysts in the feces than control rats during the primary infection and even more oocysts during the secondary infection. Bursectomized chickens (no B cells) released twice as many oocysts in the feces as control chicks during the primary infection but there was a 73 fold reduction after the secondary infection (Rose and Hesketh, 1979). Chickens are capable of developing immunity to homologous species of *Eimeria* where development of the sporozoite is inhibited, but invasion still occurs (Rose and Hesketh, 1987). While cell-mediated immunity is predominant against coccidiosis, humoral immunity also plays a role. Coccidial antigens are taken up by M cells located in enterocytes by pinocytosis and presented to antigen presenting cells leading to the activation of the adaptive response (Bockman and Cooper, 1973). Antibodies specific to sporozoites in bile prevent sporozoites from invading host cells. Fewer *E. tenella* sporozoites were recovered from homologous primed birds than controls, indicating that intraluminal destruction of the early invasive stages may be a mechanism to inhibit replication (Rose and Hesketh, 1987).

d.) Avian T Regulatory Cells

Tregs are a subset of T-cells that specialize in immune suppression. In response to an infection, pathogens are killed by activated cells of the immune system. Activated
immune cells, although essential for pathogen elimination, produce inflammatory cytokines and reactive oxygen species and cause undesirable host damage (Belkaid and Rouse, 2005). Tregs protect the host from an excessive immune response and maintain self-tolerance and mucosal tolerance (Workman et al., 2009). On the other hand, hyperactive Tregs can impair T cell, B cell, and other immune cell functions and, therefore, are implicated in impaired microbial defenses, pathogen persistence (Li et al., 2008b), and impaired vaccine responses (Stober et al., 2005). When Tregs are activated they will secrete cytokines such as transforming growth factor-β (TGF-β), IL-10 and upregulate Cytotoxic T-lymphocyte Antigen-4 (CTLA-4) and Lymphocyte Activation Gene-3 (LAG-3). TGF-β helps to repair any damage by inducing angiogenesis and contributing to connective tissue remodeling in chickens (Yang and Moses, 1990). CTLA-4 is located on the membrane of the cell and prevents the co-stimulation of CD28, negatively regulating T-cell activation (Kaur et al., 2013). LAG-3 is another surface molecule that binds to MHC II and negatively regulates T cell activation (Workman et al., 2002). IL-10 will help to prevent damage to the body by inhibiting reactive oxygen species production through inhibition of IFN-γ, which induces NO production (Rothwell et al., 2004). IL-10 will also suppress activation and proliferation of CD4+ and CD8+ T cells (Dieckmann et al., 2001).

Treg cells are distinguished from T cells by expressing CD4+, CD25+ (an IL-2R) and the transcription factor FoxP3 in humans (Li et al., 2008a). A FoxP3 analog is yet to be identified in chickens (Shack et al., 2008). Therefore, it is yet to be determined if the other properties (CD4+CD25+) are enough to characterize chicken CD4+CD25+
T cells as specifically Treg cells. A study by Shanmugasundaram (2011) addressed this question and found the properties to be similar. Chicken CD4⁺CD25⁺ cells produced IL-10 and TGF-β, both of which are signature cytokines of the mammalian Treg. Another property involves the need for IL-2 for proliferation and their inability to produce it on their own. This was observed to be true and supplemental IL-2 also ceased the suppressive properties of the CD4⁺CD25⁺ cells. Based on these results, it was concluded chicken CD4⁺CD25⁺ cells are analogous to the mammalian Treg (Shanmugasundaram and Selvaraj, 2011).

e.) Chemokines

Chemokines are small molecules that attract immune cells in a certain direction during infection as well as during homeostasis. Chemokine receptors are located on the immune cells and subsequently mediate migration. In chickens, there are a total of 12 chemokine receptors (Smith et al., 2004) and 23 different chemokines have been identified from the genome (Wang et al., 2005). A study by Annamalai and Selvaraj (2010) showed that expression of chemokine receptors on CD4⁺ and CD8⁺ cells depended on which organ they were homing to and at which developmental stage the immune cell was in. CCR7 mRNA transcripts were high in thymic CD4⁺ cells, which, in mammals, is important for intrathymic migration. CXCR5 was elevated in CD4⁺ cells from the bursa and thymus. CXCR5 in mammals is expressed on B lymphocytes so elevation of CXCR5 in the Bursa of Fabricious is not unexpected since this is the site of B cell maturation in chickens. (Annamalai and Selvaraj, 2010).
During an *Eimeria* infection, the expression of chemokines released by infected tissues changes to redirect immune cells to the site of infection. *E. tenella* is concentrated in the ceca and *E. Maxima* is localized in the jejunum, so MIP-1β and K203 (Both CC chemokines that attract Th1 CD4\(^+\) cells, macrophages and monocytes) are observed at higher levels in the mRNA at these sites during their respective infections (Laurent et al., 2001). Different species of *Eimeria* also elicit different levels of chemokine response. MIF, IL-8, Lymphotactin, MIP-1β and K203 chemokine transcripts were at higher levels after a primary *E. tenella* infection versus an *E. Acervulina* infection (Hong et al., 2006).

**Functional Assays for T cells**

Flow cytometry is a versatile technique that passes single cells or particles in suspension across a light source and detects the subsequent signals to gather information about the cells such as forward scatter (size), side scatter (granularity) and fluorescence. The lenses collecting this information are blocked from the direct light source so the light they record is only due to light being scattered or refracted by the cell passing by. Antibodies can also be conjugated to fluorescent dyes so cell surface proteins or antigens can be quantified (Hawley and Hawley, 2004). Common cell markers for T-cells include CD4, CD8 and CD25. CD4 surface proteins are associated with T-helper cells, CD8 correlates to T-cytotoxic cells and CD4 in combination with CD25 corresponds to T-regulatory cells in chickens. In humans, CD34 is used to identify hematopoietic stem cells in the laboratory (Chang et al., 2000).

Lymphocyte proliferation, or the amount of dividing cells, is another important factor in determining the extent of a T-cell response. A method to measure proliferation
is using tetrazolium salt 3-(4,5-dimethythiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT). The mitochondria of metabolically active cells convert the MTT into a purple insoluble formazan product which is measured by a spectrophotometer (Mosmann, 1983). A more sensitive, yet radioactive, assay is the use of tritiated thymidine. After the H-thymidine is added to incubated cells, and a subsequent labeling period, the H content can be measured through automated scintillation to determine the number of dividing cells (Nagorsen and Marincola, 2005). BrDU (5’-bromo-2’-deoxyuridine) can also be incorporated if using a radioactive material like H-thymidine is not an option. This method utilizes immunohistochemistry or flow cytometry to measure conjugated fluorescent antibodies to measure proliferation and can be used along with other flow cytometry techniques (Gratzner, 1982).

Management Practices to Control Coccidiosis

a.) Poultry House Management

There are several ways to control coccidiosis in a flock including management of the poultry house, anticoccidial medications and vaccination. Often, a combination of two to three of these methods is incorporated to reduce the incidence of coccidiosis. The sporulation of an oocyst in the litter is affected by ammonia from the feces, bacterial damage and is therefore degraded over time, affecting its viability (Williams, 1995). But, a majority of the oocysts remain viable and capable of re-infecting other birds after they are shed in the feces and remain in the local environment. To combat this, caked litter is removed and/or aerated every 2-3 weeks and fresh litter is placed on top of the old litter before new flocks are placed (Tewari...
and Maharana, 2011). These management practices alone are not enough to control infections so the use of anticoccidials and vaccines are important.

b.) **Anticoccidials**

Anticoccidials are typically products that are added to the feed that will affect the metabolism of the parasite in the chicken. They are split into two main categories: chemicals and ionophores. The chemicals affecting metabolism are nicarbazin, halofuginone, clopidol, decoquinate and amprolium. Ionophores are products that alter the ion transport of the protozoa and disrupt osmotic balance. They are frequently used today and include products such as monensin, narasin and salinomycin (Jeffers, 1997). Differing levels of resistance occur in all of these anticoccidial products, so rotation programs are frequently used within or between flocks using different products or rotating with vaccination (Chapman, 1998).

c.) **Vaccines**

There are three different types of vaccines used in the poultry industry: live unattenuated, live attenuated and recombinant subunit vaccines. There are several ways to administer vaccinations including aerosol application, *in ovo*, intra-nasal and orally.

Live vaccines are typically given orally, through the feed or water, or *in ovo* while the birds are still embryos (Tewari and Maharana, 2011). Live vaccines are given in low doses to facilitate a low level infection to establish immunity to the specific *Eimeria* strain/s given. There is always a risk with live vaccines in that it can result in an active infection. Administration through the oral routes of feed and water can result in unequal dosages among individual birds followed by a variable distribution
of oocysts in the flock (Williams et al., 1999). Recent improvements in administration have lessened the problems and improved the dispersion of oocysts (Danforth, 1998; Williams, 2002). Broiler breeder vaccines can include up to eight *Eimeria* species, while broilers only receive four.

Live attenuated vaccines pose less of a risk of establishing a harmful infection than a live unattenuated vaccine. They are immunogenic, but are less pathogenic and decrease oocyst production. Attenuation is achieved through cycling of oocysts through embryos or by selecting for a trait known as precociousness. The latter is more widely used today and is the process by which oocysts are selected for a faster life cycle (about 30 hours faster) than the typical oocyst from the same parent strain. (Long, 1972; Jeffers, 1975). Commercially available live attenuated vaccines include Paracox (Merck Animal Health, Millsboro, DE) and Livacox (BIOPHARM, Czech Republic). A unique vaccine that includes a precocious attenuated strain of *E. tenella* and non-attenuated live strains of *E. maxima* and *E. acervulina* is marketed as Supercox 1 (Osho Chemical Industries Ltd., Nairobi, Kenya; Tewari and Maharana, 2011).

After vaccination it takes about 4 weeks to develop immunity to *Eimeria*. This allows for oocysts to cycle approximately 2-3 times. With any live vaccine, attenuated or not, the use of anticoccidials should not be used during this initial phase of immunity development or it could interfere with the efficacy of the vaccine (Vermeulen et al., 2001). To work around this problem a vaccine was developed using strains of *E. tenella*, *E. maxima* and *E. acervulina* that were partially tolerant to ionophores (Merck Animal Health, Millsboro, DE). This allows the use of ionophores
for the first few weeks when the birds would otherwise be susceptible to field strains while they are developing immunity using the vaccine (TPM Schetters, 1999).

Recombinant subunit vaccines use antigens from certain stages of the life cycle of *Eimeria* parasites. Early subunit coccidiosis vaccines focused on the asexual part of the life cycle. Microneme proteins are involved in host cell adhesion and penetration (Ryan et al., 2000). EtMIC2 is a microneme protein that was shown to stimulate intestinal protective immunity when administered *in ovo* (Ding et al., 2005). Since proteins associated with the sexual stages only confer moderate protection, proteins associated with the sexual stages can also be utilized to create a transmission-blocking vaccine (Jenkins, 2001). Coxabic (Phibro Animal Health, Teaneck, NJ) uses sexual stage antigens from *E. maxima* to inhibit the development of macrogametocytes into a viable oocyst. It is injected into the breast muscle of broiler breeder hens who produce antibodies which are then passed on to their offspring through the yolk (Sharman et al., 2010).

d.) In Ovo Vaccines

A number of anti-coccidial vaccines have been developed and are routinely used to reduce the incidence and severity of a coccidial challenge. One delivery method involves introducing live oocysts into the embryonating egg (Weber et al., 2004). For example, the Embrex® Inovject® System (Zoetis) is designed to deliver a precise and uniform dose of anti-coccidial vaccine to 18-19 d old embryos. Small holes are mechanically punched in the top of the egg where a needle will then penetrate into the amniotic fluid and deliver its payload (Ricks et al., 1999). Eggs are transferred from the incubator to a hatcher around this time, so it is convenient for the producer to
vaccinate *in ovo* at this point. *In ovo* administration of live oocyst vaccines has several distinct advantages over conventional delivery methods, including increased accuracy and repeatability of vaccine delivery (Dalloul and Lillehoj, 2005).

Inovocox® (Zoetis, Florham Park, NJ) is a commercially available *in ovo* anti-coccidial vaccine that consists of live oocysts of *E. acervulina* and *E. tenella* and two strains of *E. maxima* (Lee et al., 2012) and has been approved by USDA for use in commercial poultry production. *In-ovo*-injected oocysts enter the intestinal tract of the embryo following a swallowing reflex, excyst in the intestine, and produce an active infection in the newly hatched chick (Chapman et al., 2002). *In ovo* injection of *E. maxima* oocysts induces moderate intestinal lesions, very similar to those of an *E. maxima* infection at 7 d post-hatch (Weber et al., 2004). In fact, moderate intestinal lesions are critical to the efficacy of the coccidial vaccine (Chapman et al., 2002) and any protocol that increases inflammation in the gut can improve vaccine efficacy.

Birds injected with *in ovo* coccidial oocysts will shed oocysts, which can still be present in the litter up to 42 d of age. This shedding can lead to reinfection of the birds. Hence, it is common to combine an *in ovo* coccidial vaccine with anti-coccidial drugs at 19 d post-hatch to reduce oocyst shedding and re-infection of birds (Lee et al., 2012). Any protocol that can decrease fecal oocyst shedding post-*in ovo* vaccination will decrease the probability of the birds ingesting coccidial oocysts from the litter and improve their productive performance.
**Vitamin E**

Vitamin E (VE) is an intracellular, fat soluble antioxidant and prevents oxidation of unsaturated lipids in the cell. VE is divided into two main subgroups that are further broken down into four analogs: saturated tocopherols (α, β, γ, δ) and unsaturated tocotrienols (α, β, γ, δ) (Singh et al., 2013). Oxidation occurs when a proton leaves the α-methylenic carbon of a triglyceride by oxidation. It can then be attacked by free oxygen to form a peroxide free radical. The free radical can induce more oxidation, causing a chain reaction (Sherwin, 1978).

VE can only be synthesized by photosynthetic organisms. It consists of a chromanol ring and a 15-carbon tail derived from homoglarinate and phytol diphosphate. Unlike tocopherols, tocotrienols contain three trans double bonds in the hydrocarbon tail (Sen et al., 2006). VE neutralizes peroxide radicals by donating an H from its phenolic hydroxyl group, resulting in a stable lipid species and a relatively stable VE free radical (Palozza et al., 2006). Tocopherols are found in the seeds and leaves of dicot plants while tocotrienols are predominantly found in the endosperm of monocot seeds (barley, wheat, rice) (Sen et al., 2006).

Consumption of oxidized oils can lead to a lack of growth and retention of energy, α-tocopherol and fat (Engberg et al., 1996). Birds that consume a diet high in fats that are prone to oxidation, such as poly-unsaturated fatty acids (Jung et al., 2010), will have a lower meat quality based on color, taste and drip loss (Gray et al., 1996). VE is responsible for radical chain breaking and inhibiting lipid peroxidation (Tappel, 1970).
and tocopherols are one of the antioxidants approved for use in edible fats and oils (Sherwin, 1978).

Supplemental dietary VE reduces mortality, increases body weight gain and increases the percentage of T helper cells during a coccidial challenge in chickens (Colnago et al., 1984; Muir et al., 2002). After an LPS challenge, birds fed supplemental natural type VE exhibited a reduced inflammatory response, gauged by a lower number of IL-6 transcripts compared with the control (Kaiser et al., 2012). In a contrasting study, birds supplemented with VE had elevated levels of the pro-inflammatory cytokine IL-1, compared to the control, 12 hours after an LPS injection (Zhao et al., 2011). VE also improves the phagocytic activity of macrophages (Konjufca et al., 2004) and improves the overall cellular immune response in birds (Silva, 2011). Birds under heat stress had improved antibody responses following SRBC injection and increased phagocytic macrophages when supplemented with dietary VE (Niu et al., 2009). VE, when used as an adjuvant in Newcastle virus vaccine, decreases lesion healing time and increases titers of antibodies (Franchini et al., 1991).

*In ovo* exposure of chicken embryos to 10 IU VE by injection into the amnion increases total antibody response against SRBC and increases macrophage phagocytic potential and nitrite production post-hatch (Gore and Qureshi, 1997). *In ovo* exposure to 10 IU VE by turkey embryos caused cultured monocytes stimulated by LPS to produce more Prostaglandin E$_2$ (PGE$_2$) than the control (0 IU VE). Chicken mononuclear phagocytic cells stimulated by LPS had higher production of PGE$_2$ and Thromboxane B$_2$ when treated with VE (Qureshi and Gore, 1997).
For the in ovo injections, d-\(\alpha\)-tocopheryl polyethylene glycol 1000 succinate (TPGS) was used. It is an amphipathic form that includes a lipophilic tocopherol connected to a polyethylene glycol 1000 chain by a succinate bridge (Johnson et al., 2002; Chang et al., 2011). The attached polyethylene glycol chain allows the normally lipophilic tocopherol to become water soluble. This makes it an ideal compound for delivering VE to an embryo through the amniotic fluid. No effects have been shown that the feeding of TPGS is beneficial to chickens versus the traditional source of VE, DL-\(\alpha\)-tocopherol acetate (TA). A level of 100 IU of TA increased serum levels of \(\alpha\)-tocopherol in 21 day old chickens, while additional TPGS in the diet had no effect (Soto-Salanova et al., 1993). The hydrophilic properties of TPGS have also been utilized to increase water solubility and as an adjuvant for human drugs (Somavarapu et al., 2005; Gao et al., 2008; Goddeeris et al., 2008).
Chapter 3: T-cell properties postcoccidia challenge

Abstract

Following a primary coccidia infection, the expression levels of key cytokines and T-cell populations were measured. IL-1, IFN-γ and IL-10 mRNA content, CD4+ (T-helper cells), CD8+ (T-cytotoxic cells) and CD4+CD25+ (Treg) cell percentages from the spleen and cecal tonsils were investigated along with cell proliferation from the cecal tonsils. At 6 days post-challenge there was a 5-fold increase (P=0.20) in IL-10 mRNA and an increase (P=0.30) in IFN-γ mRNA in the cecal tonsils from infected birds. At 11 days post-challenge, there was a 4-fold increase (P=0.09) in IL-10 mRNA content in the cecal tonsils from infected birds (P=0.10) in IFN-γ mRNA content in the spleen. At 11 days post-challenge, there was a decrease in the percentage of Tregs in the spleen (P=0.01). Conversely, at 11 days post-infection the percentage of Tregs in the cecal tonsils increased (P=0.06). At 6 days post-challenge, there was a significant increase in the percentage of CD4+ cells in the cecal tonsil (P<0.01) and an increase in the percentage of CD8+ cells (P<0.01). Conversely, at 11 days post-coccidial infection there was a decrease in the percentage of CD4+ cells in the cecal tonsil (P<0.01). At 6 days post-infection, the Treg depleted population of lymphocytes from the cecal tonsils of infected birds had increased proliferation (P=0.02) compared to the control. In
conclusion, Tregs are up regulated during a coccidia infection and suppress the immune system by release of IL-10.
Introduction

Tregs cells are a subset of T cells that specialize in immune suppression. In response to an infection, activated immune cells release reactive oxygen species and inflammatory cytokines essential for pathogen elimination, but if left unchecked, can lead to undesirable damage to the host (Belkaid and Rouse, 2005). Hyperactive Tregs can impair B cell, T cell and other immune cell functions (Li et al., 2008a). In chickens, Tregs are identified by the expression of CD4 and CD25 proteins on the cell surface and are further characterized by releasing high amounts of the anti-inflammatory cytokine IL-10 and low amounts of IL-2 (Shanmugasundaram and Selvaraj, 2011). The IL-10 secreted by Tregs will suppress the activation and proliferation of CD4+ and CD8+ T cells (Dieckmann et al., 2001).

The general lifecycle of coccidiosis, an enteric protozoan infection, begins with the ingestion of a sporulated oocyst, replication within the enterocytes and the eventual release back into the environment through the feces. The endothelial penetration and replication within the enterocyte damages the intestinal epithelium and causes lesions to form. The lesions lower the bird’s ability to absorb nutrients resulting in reduced body weight gain or weight loss (Lillehoj and Trout, 1996; Paris and Wong, 2013). This makes it an economically important disease for the poultry industry with losses estimated at more than $800 million annually in the United States (Sharman et al., 2010). There are 7 different species of coccidia that infect chickens and belong to the genus Eimeria: Eimeria tenella, E.acervulina, E. maxima, E. brunetti, E. mitis, E. necatrix and E. praecox (Allen and Fetterer, 2002b). Infection will occur at different locations based on
the species. *E. tenella* and *E. necatrix* target the cecum while *E. Acervulina* develops in the duodenum and *E. maxima* is found in the mid to posterior intestine (Chapman, 2014).

T-cell-mediated immunity by intestinal intraepithelial lymphocytes and lamina propria lymphocytes is the primary component of protective immunity to *Eimeria*, an intracellular pathogen (Lillehoj and Lillehoj, 2000). The anti-coccidial activities of T cells are augmented by T helper-1 cytokines like IL-2 and IFNγ. In vitro, recombinant chicken IFNγ will inhibit the development of *Eimeria* and following a challenge in vivo will reduce body weight loss and oocyst production (Lillehoj and Choi, 1998). An anti-coccidial immune response increases following co-administration of IFNγ with a coccidial vaccine (Ding et al., 2004). IFNγ levels are higher in *Eimeria*-resistant inbred poultry lines than in susceptible lines (Yun et al., 2000). In contrast, low levels of IL-10, an immunosuppressive cytokine, increase resistance, whereas high levels of IL-10 increase the susceptibility to intracellular pathogens (Moore et al., 2001). IL-10 plays a crucial role in preventing the development of a strong IFNγ-driven anti-coccidial response as evidenced by the fact that lines of chickens that are more susceptible to coccidiosis have higher levels of IL-10 (Rothwell et al., 2004). Our hypothesis is that an upregulation of Tregs, resulting in elevated levels of IL-10, is the cause of immune suppression during a coccidial infection making the bird more susceptible to a prolonged, more damaging, infection.
**Materials and Methods**

**Birds**

Specific-pathogen-free White Leghorn birds were provided ad libitum water and feed, housed in battery cages and raised using standard animal husbandry practices. Experiments were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

**Coccidial Infection**

One vial (8000 doses) of Inovocox, EM1 (Zoetis, Florham Park, NJ) was emptied into a 50ml tube using a 10ml syringe and an 18GTW needle. The empty vial was rinsed with 10ml 1x PBS to suspend any oocysts adhered to the walls. The PBS was then added to the tube containing the rest of the oocysts. The oocysts were then centrifuged at 630g for 10 min and the supernatant was removed. The oocysts were washed again in 10ml of PBS and resuspended in 10 ml PBS. One ml of the oocyst solution was diluted with 49ml PBS. The oocyst concentration was determined in a McMaster Slide chamber. Birds were gavaged with $4 \times 10^5$ oocysts/bird in 200μl of PBS at three weeks of age.

**Effects of a Coccidia Infection on CD4$^+$, CD8$^+$ and Treg Cell Percentages in Local and Systemic Immune Organs**

Single-cell suspensions of the spleen and cecal tonsils were collected from birds at 27 and 32 d of age (6 and 11 days post-coccidial challenge) from 5 birds per treatment (n=5). Cells were pooled from two different birds and concentrated for lymphocytes by density centrifugation over Histopaque (1.077 g/ml Sigma-Aldrich). Cells (1x10$^6$) were
incubated with 1:250 fluorescent-conjugated mouse anti-chicken CD4-FITC (CD4 Ab) only or CD4 Ab and 1:450 fluorescent-conjugated mouse anti-chicken CD8a-RPE (CD8 Ab) (Southern Biotechnology Associates, Birmingham, AL). After a 45 minute incubation, 10 μg/ml primary fluorescent linked mouse anti-chicken CD25 was added to samples that had anti-chicken CD4 and subsequently incubated for 15 minutes. Samples containing both CD4 and CD8 Ab were incubated for 20 minutes. Unbound primary antibodies were removed by centrifugation at 750g for 3 minutes. Cells were analyzed using a flow cytometer (Guava Easycyte; Millipore) and CD4+CD25+ cell percentages were expressed as a percentage of CD4+ cells.

Effects of Tregs on Lymphocyte Proliferation Post-coccidia Challenge

Single-cell suspensions of cecal tonsils were collected from 6 and 11 d old birds (n=5) post-coccidial challenge. Cecal tonsils were pooled in groups of two and a quarter of the total cells were incubated with 1μg/well PE-conjugated mouse anti-chicken CD25 for 45 minutes at 4⁰C. Unbound primary antibody was removed by centrifugation at 400g for 6 minutes and the removal of the supernatant. 40μl of anti-PE Microbeads (Miltenyi Biotec) were added and incubated 15 minutes at 4⁰C. Unbound beads were removed by centrifugation at 400g for 6 minutes. The cells with Ab and beads were passed through a MACS magnetic column (Miltenyi Biotec) to retain CD25+ cells. The flowthrough (CD25 depleted cecal tonsil cells) was collected. CD25 undepleted and CD25 depleted cecal tonsil cells (5.7 x 10⁶) were plated in triplicate on a 96 well plate (n=5) in 100μl RPMI-1640 (Sigma Aldrich) and supplemented with 10% chicken serum and 1%
penicillin, streptomycin and stimulated with 200ng/ml phorbol 12-myristate 13-acetate (PMI), 50ng/ml ionomycin (IM) and 1µg/ml CD28. The plate was incubated for 72 hours at 37°C in the presence of 5% CO2. The cells were incubated with MTT (Thiazolyl Blue Tetrazolium Bromide at 5mg/ml in 1x PBS) (Sigma-Aldrich) for three hours. The plate was then centrifuged at 400g for 5 minutes, supernatant removed and the cells were resuspended in MTT solvent (4 mM HCl, 0.1% Nondet P-40 in isopropanol). The plate was read using a BioTek ELx 800 absorbance microplate reader at 562 nm. Proliferation efficiency was reported as mean optical density blanked with the MTT solvent.

*Oocyst count in the feces*

Feces were collected and enriched for oocysts by using a salt flotation technique as previously described (Hein, 1975). Briefly, 1g of feces was dissolved in 10ml of 1x phosphate buffered solution (PBS) in a glass tube. The feces were centrifuged at 400g for 10 minutes and the supernatant was removed. The fecal pellet was resuspended in 10ml of solution A (151g NaCl in 1 L dH2O). After a 10 minute incubation, 1 ml of solution, collected from the top, was suspended in 9 ml of solution B (311g NaCl in 1 L dH2O) followed by a five minute incubation. 150µl, taken from the top of the feces in solution B, was transferred to both the left and right sides of a McMaster slide. A cover slip was placed on top of each side and oocysts were counted under a light microscope at 40x magnification (Levine et al., 1960).
**RNA Extraction from Spleen and Cecal Tonsils**

Single-cell suspensions of cecal tonsils and spleens were collected from 6 d and 11d birds (n=5) post-coccidial challenge. Cecal tonsils were pooled in groups of two and a quarter of the total cells were suspended in 750μl of Tri Reagent (Molecular Research Center, Cincinnati, OH). Chloroform (200μl) was added to the solution and after a 5 minute incubation the sample was centrifuged at 11,000g for 15 minutes. The solution was split into three layers after centrifugation and the top aqueous layer was removed and added to 500μl isopropyl alcohol to precipitate the RNA. After a 10 minute incubation, the sample was centrifuged at 13,000g for 10 minutes. The supernatant was removed and the pellet was washed in 250μl of 75% ethanol. After mixing, the pellet in ethanol was centrifuged at 5,000g for 5 minutes. The ethanol was removed and the pellet was air dried for 10 minutes or until all of the visible ethanol was evaporated. Based on the size of the pellet, it was dissolved in 20μl – 200μl of TE buffer. The pellet was then resuspended by vortexing and the RNA concentration determined using the NanoDrop (Thermo Scientific).

**Effects of a Coccidia Infection on Inflammatory and Anti-Inflammatory Cytokine mRNA content**

Total RNA was collected from cecal tonsils and spleen at d6 and d11 postcoccidial challenge and reverse transcribed into cDNA (Selvaraj and Klasing, 2006). mRNA was analyzed for IFN-γ (5’-gtgaagaaggtgaagatcatga-3’ and 5’gctttgcgctggattctca-3’), IL-10 (5’-catgctgctgggcctgaa-3’ and 5’-cgtctccttgatctgcttgat-3’), IL-1 (5’-gcataaggctacaagctc-3’ and 5’-caggcgtagaagatgaagc-3’) and normalized with β-Actin.
(5’-tctggtgaggaat-3’ and 5’-gactgctgacaccttgca-3’) mRNA using SyBr Green (Shanmugasundaram and Selvaraj, 2010). The annealing temperature for IFN-γ was 60°C, for IL-10 was 58°C, for IL-1 was 57.5°C and for β-Actin was 57°C. Fold change from the reference gene was determined as $2^{(Ct \text{ Sample} - \text{housekeeping})/2(Ct \text{ Reference} - \text{housekeeping})}$, where Ct represents the threshold cycle. The Ct value was calculated using the iQ5 software (Biorad, Hercules, Ca) at the point the fluorescence rose exponentially 2-fold above the background.

**Statistical Analysis**

A one-way ANOVA (JMP, SAS Institute INC., Cary, NC) was used to determine the effect of a coccidial infection on the dependent variables. When the main effect was significant (P < 0.05), differences between means were analyzed using Tukey’s least squares means comparison.
**Results**

*Effects of a Coccidia Infection on CD4⁺, CD8⁺ and Treg Cell Percentages in Local and Systemic Immune Organs*

There was a decrease in the percentage of Tregs in the spleen (P=0.01) at 11 days post-coccidial challenge (Figure 7). The percentage of Tregs in the cecal tonsils increased (P=0.06) at 11 days post-challenge (Figure 5). At 6 days post-coccidial challenge there was a significant increase in the percentage of CD4⁺ cells in the cecal tonsil (P<0.01) (Figure 1). Conversely, at 11 days post-coccidial challenge there was a decrease in the percentage of CD4⁺ cells in the cecal tonsil (P<0.01) (Figure 4). At 6 days post-challenge there was an increase in the percentage of CD8⁺ cells in the cecal tonsil (P<0.01) (Figure 1).

*Effects of a Coccidia Infection on Inflammatory and Anti-Inflammatory Cytokine mRNA content*

At 6 days post-challenge there was a 5-fold increase (P=0.20) in IL-10 mRNA content in the cecal tonsil’s of infected birds compared to the control (Figure 14). Similarly, at 11 days post-coccidial challenge there was a 4-fold increase (P=0.09) in IL-10 mRNA content in the infected bird’s cecal tonsils (Figure 15). At 6 days post-challenge there was a 2.5 fold increase (P=0.30) in IFN-γ mRNA content in the cecal tonsils of infected birds compared to the control (Figure 12). At 11 days post-challenge there was half as much (P=0.10) IFN-γ mRNA content in the spleen of infected birds compared to the control (Figure 13).
Effects of Tregs on Lymphocyte Proliferation Post-coccidia Challenge

At 6 days post-coccidial challenge, the CD25 depleted cecal tonsils of infected birds had increased proliferation (P=0.02) compared to the control (Figure 17).

Discussion

Eimeria infections are localized to specific regions of the gut, depending on the species of the Eimeria pathogen. Birds in this experiment were infected using the live vaccine, Inovocox® (Zoetis, Florham Park, NJ). Inovocox® contains three species of Eimeria: E. tenella, E. acervulina and E. maxima. E. tenella targets the cecum, E. acervulina develops in the duodenum and E. maxima is found in the mid to posterior intestine (Chapman, 2014). The secondary lymphoid organ, the cecal tonsils, was collected to evaluate the local immune response.

T-cell-mediated immunity by intestinal intraepithelial lymphocytes confers the main component of protective immunity to Eimeria infections (Lillehoj and Lillehoj, 2000). This includes the T-helper (CD4⁺) and T-cytotoxic (CD8⁺) cells. CD8⁺ cells are important due to their ability to fight intracellular pathogens, such as coccidia. In the present study, there were no changes in the CD8⁺ cell population in the spleen, but an increase was observed at day 6 in the cecal tonsil. Systemically, there was no change, but locally the T-cytotoxic cells were important during an early infection. CD8⁺ cells have been suggested to play a role in the transport of the sporozoite (Trout and Lillehoj, 1995). When the oocyst is ingested and sporozoites are released, they still must migrate to their
preferred location of the gut. In this study, T-cell percentage measurements were made in the cecal tonsils, the preferred location of \(E. \text{tenella}\). CD8\(^{+}\) cells migrating to the cecal tonsil with \(E. \text{tenella}\) could account for the higher concentrations during early infection. Perhaps if other parts of the intestine were sampled, similar results could be seen for the preferred locales of \(E. \text{maxima}\) and \(E. \text{acervulina}\). CD4\(^{+}\) cells, like CD8\(^{+}\), were not different between treatment groups in the spleen (\(P < 0.05\)) but increased at day 6 and decreased at day 11 in the cecal tonsils of infected birds. T-helper cells were up regulated during an early infection but down regulated later. Consistent with our results, previous studies have shown that following a primary infection, CD8\(^{+}\) and CD4\(^{+}\) cells increased shortly after primary infection, but decreased during later infection in the small intestine (Lillehoj, 1994).

Tregs play a key role in the immune response to a coccidiosis infection. Tregs are a major producer of IL-10 (Dieckmann et al., 2001). IL-10 is an immune suppressive cytokine that inhibits NFKB (Wang et al., 1995) preventing the production of inflammatory cytokines, such as IFN-\(\gamma\) (Moore et al., 2001), by blocking the phosphorylation of the P50/P65 NFKB complex (Ehrlich et al., 1998). Immune suppressive cytokines, such as IL-10, have been observed at elevated levels following exposure to \(E. \text{maxima}\) (Rothwell et al., 2004), \(E. \text{acervulina}\) and \(E. \text{tenella}\) (Hong et al., 2006). In the present study, the cecal tonsils during late infection had higher levels of IL-10 mRNA transcription and a higher percentage of Tregs. This is even more apparent in susceptible lines of birds (Yun et al., 2000; Rothwell et al., 2004) where IL-10 transcription is significantly higher in susceptible versus resistant lines of chickens. A decrease in Tregs was observed in the spleen and a 2-fold decrease in IL-10 during late
infection. The coccidiosis infection resulted in up regulation of Tregs locally and down regulation systemically. Previous studies have indicated an increase in IL-10 mRNA in both the spleen and small intestine in coccidia susceptible lines of birds but only in the small intestine in resistant birds (Rothwell et al., 2004). This suggests that the birds used in the current study were inherently resistant to coccidial infections since IL-10 increased locally but not systemically. In contrast to the conclusion that the birds used in the current experiment were resistant was the observation that there were no significant differences in IFN-γ mRNA amounts in either the spleen or cecal tonsils.

To determine if the Tregs were suppressive, lymphocyte proliferation was determined using two populations of cells from the cecal tonsils: Treg depleted and whole lymphocyte population. The only significant effects were observed at 6 days post-infection when the Treg depleted population from lymphocytes of the infected birds had increased proliferation. Removal of the suppressive Tregs allowed for increased lymphocyte proliferation. The proliferation assay measures coccidial antigen specific proliferation. The cecal tonsils from control birds were never exposed to the coccidial antigen before stimulation in vitro, unlike cecal tonsils collected from the treatment group. This lack of prior exposure could explain why there was no difference in proliferation between the Treg depleted group and Treg un-depleted group in the cecal tonsils taken from birds that were previously uninfected.

We conclude that Tregs are up regulated during a coccidia infection and suppress the immune system by release of IL-10.
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Chapter 4: Vitamin E Supplementation to an *in ovo* Coccidiosis Vaccine

*Abstract*

Following an *in ovo* vaccination for coccidiosis supplemented with VE, nitric oxide (NO) concentrations, serum anti-coccidia IgG concentrations and fecal oocyst shedding were determined. At 6 days post-hatch, birds injected *in ovo* with a vaccine supplemented with vitamin E had higher (P =0.14) concentrations of splenic macrophage nitric oxide than birds that only received the *in ovo* vaccine. There was no difference in nitric oxide concentrations 12 days post-hatch. At 6 d post-hatch there was no difference in serum anti-coccidial IgG concentrations whereas at 12 d post-hatch, birds injected *in ovo* with the vaccine supplemented with vitamin E had significantly higher (P < 0.01) levels of serum anti-coccidia IgG than the birds only injected *in ovo* with the coccidia vaccine at both 1:10 and 1:20 serum dilutions. Vitamin E increased NO concentrations 6 d post hatch and serum anti-coccidia IgG at 12 days post-hatch.
Introduction

Coccidiosis is an enteric protozoal disease that causes an estimated $800 million annual loss to the US poultry industry (Sharman et al., 2010). The protozoa penetrate the lining of the intestine causing lesions that decrease nutrient absorption resulting in lower weight gain (Lillehoj and Trout, 1996; Paris and Wong, 2013). Producers combat coccidial infections through supplemental anti-coccidial products or vaccination. Anti-coccidial products work well to prevent coccidial out breaks in a flock, but resistance to these products can develop over time (Chapman, 1998). Effective and economic vaccine protocols are becoming more important as certain anti-coccidial products become less effective.

Live vaccines are typically given orally, through the feed or water, or in ovo while the birds are still embryos (Tewari and Maharana, 2011). Live vaccines are given in low doses in order to induce minor lesions that induce an immune response to the specific strain/s given. There is always a risk with live vaccines of inducing an active infection. The administration through the oral routes of feed or water, however, can result in an unequal distribution of oocysts to the flock (Williams et al., 1999). Recent improvements in administration have somewhat lessened the application problems and improved the dispersion of oocysts (Danforth, 1998; Williams, 2002). Broiler breeder vaccines can include up to eight species of Eimeria, while broilers only receive four. Commercial vaccines include Coccivac B/D (Merck Animal Health, Millsboro, DE), Immucox C1/2 (Ceva Animal Health, Lenexa, KS) and Inovocox (Zoetis, Florham Park, NJ) (Tewari and Maharana, 2011).
Administration of vaccines using *in ovo* techniques is a consistent way to deliver oocysts accurately to each egg. For example, the Embrex® Inovoject® System (Zoetis) is designed to deliver a precise dose of anti-coccidial vaccine to 18-19 d old embryos. The payload is delivered into the amniotic fluid by penetration of a needle through a pre-punched guide hole (Ricks et al., 1999). It is convenient for the producer to vaccinate *in ovo* at 18-19 d because eggs are transferred from the incubator to the hatcher at this stage of incubation. Accuracy and repeatability are increased when using *in ovo* administration of live oocyst vaccines over more conventional methods (Dalloul and Lillehoj, 2005).

Inovocox® (Zoetis, Florham Park, NJ) is a commercially available *in ovo* anti-coccidial vaccine containing live oocysts of *E. acervulina* and *E. tenella* and two strains of *E. maxima* (Lee et al., 2012) and has been approved by the USDA for use in commercial poultry production. *In-ovo*-injected oocysts enter the intestinal tract of the embryo following a swallowing reflex, excyst in the intestine, and produce an active infection in the newly hatched chick (Chapman et al., 2002).

Vitamin E has proven to be an effective dietary supplement to increase the immune response in general (Konjufca et al., 2004; Silva, 2011) and to certain challenges such as coccidiosis and LPS (Colnago et al., 1984; Muir et al., 2002; Kaiser et al., 2012). Vitamin E injected *in ovo* has also benefitted the immune system by increasing the total antibody response, macrophage potential and nitrite production post-hatch (Gore and Qureshi, 1997). Vitamin E has also been used as an adjuvant in a Newcastle viral vaccine where it decreased lesion healing time and increased titers of antibodies (Franchini et al., 1991).
D-\(\alpha\)-tocopheryl polyethylene glycol 1000 succinate (TPGS) is the form of VE used for \textit{in ovo} injections. It is an amphipathic form that includes a lipophilic tocopherol connected to a polyethylene glycol 1000 chain by a succinate bridge (Johnson et al., 2002; Chang et al., 2011). The attached polyethylene glycol chain allows the normally lipophilic tocopherol to become water soluble making it an ideal compound for delivering vitamin E to an embryo through the amniotic fluid. Since vitamin E is an effective immune modulator, we hypothesized that an \textit{in ovo} vitamin E supplementation to a live \textit{in ovo} coccidial vaccine will improve the birds immune response by increasing the macrophage and antibody response post-hatch.
Materials and Methods

Birds

Specific-pathogen-free White Leghorn birds were provided water and ad libitum feed, housed in battery cages and raised under standard animal husbandry care. Experiments were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Vitamin E and Coccidiosis Vaccine In Ovo Injection

1000 IU (0.67g) of d-α-tocopherol polyethylene glycol 1000 succinate (TPGS) (Sigma-Aldrich) was autoclaved at 121°C for 15 minutes. The TPGS was then dissolved in 10ml of sterile distilled water to obtain a 100 IU/ml d-α-tocopherol solution. The live coccidia vaccine, Inovocox (Zoetis, Florham Park, NJ), was used and the manufacturer’s instructions were followed for vaccinating each egg. The test materials were injected into the egg through the amniotic route as previously described (Sharma, 1985; Weber and Evans, 2003). Eggs were prepared by swabbing with 70% ethanol and a hole large enough for injection using a 1-inch 20G needle was drilled into the middle of the large end of a fertilized egg at 18d of incubation. Eggs were injected with either 0 or 100μl of TPGS in distilled water and 0 or 2.0 x 10^5 oocysts in 100μl of 1x PBS.

Oocyst count in the feces

Feces were collected and enriched for oocysts by using a salt floatation technique as previously described (Hein, 1975). Briefly, 1g of feces was dissolved in 10ml of 1x
phosphate buffered solution (PBS) in a glass tube. The feces were washed in PBS by centrifugation at 400g for 10 minutes and the supernatant was removed. The fecal pellet was resuspended in 10ml of solution A (151g NaCl in 1 L dH2O). After a ten minute incubation, 1 ml of solution, collected from the top, was suspended in 9 ml of solution B (311g NaCl in 1 L dH2O) followed by a five minute incubation. 150μl, taken from the top of the feces in solution B, was transferred to both the left and right sides of a McMaster slide. A cover slip was placed on top of each side and oocysts were counted under a light microscope at 40x magnification (Levine et al., 1960).

**ELISA: Serum anti-coccidia IgG**

Blood was collected from birds at 6d and 12d post-hatch by cardiac puncture. Serum was collected by centrifugation of the blood at 326g for 6 minutes and stored at -20°C until testing. 100μl of 10μg/ml coccidia antigen in carbonate buffer was added to each well in a 96 well plate. The plate was incubated at 4°C for 14 hours for antigen adherence. The plate was then washed three times with PBS-Tween 20 (0.05% Tween 20 in PBS, pH = 7.4). 200μl 8% non-fat dry milk in PBS-Tween was added to each well and incubated for 90 minutes. The plate was washed three times with PBS-Tween. Serum samples were then diluted 1:10 in 5% non-fat dry milk in PBS-Tween and 100μl/well were added in triplicate to the plate and incubated for 90 minutes. The plate was then washed three times with PBS-Tween. 100μl HRP labeled anti-chicken IgG (Southern Biotech, Birmingham, AL) 1:8000 in 5% non-fat dry milk in PBS-Tween was added to each well and incubated for 30 minutes. The plate was washed three times with PBS-
Tween. 100μl of 3,3,5,5-tetramethylbenzidine substrate was added to each well and incubated for 10 minutes. The reaction was stopped by adding 100μl/well 1N HCL. The plate was read using a spectrophotometer at 450nm. Results are reported as mean optical density.

**NO assay**

Spleens were collected from birds at 6d and 12d post-hatch, single-cell suspensions were made and macrophages were concentrated by density centrifugation at 400g for 15 minutes over Histopaque (1.077 g/ml Sigma-Aldrich). Cells were plated from each spleen in separate petri dishes with 15ml of growth media (4% chicken serum, 2% fetal bovine serum, 2% penicillin plus streptomycin in RPMI). Cells were incubated for 24 hours at 37°C in the presence of 5% CO2. Non-adherent cells were removed by washing with PBS. 5ml RPMI was added to each petri dish and a cell scraper was used to suspend the attached monocytes. 1 x 10⁷ cells were plated in a 96 well plate in 100μl of growth media containing 1μg/ml LPS. Cells were incubated for 24 hours at 37°C in the presence of 5% CO2. The plates were centrifuged at 326g for 3 minutes and the supernatant was removed from each well. The nitrite concentrations of the supernatant were determined using a sulfanilamide/N-(1-Naphthyl) ethylenediamine dihydrochloride solution (#R223500, Ricca Chemical Company, Arlington, TX) following the manufacturer’s instructions. Concentrations of Nitrite were determined using a standard curve.
Results

**Effect of Vitamin E supplement to a Coccidia Vaccine on Macrophage Nitric Oxide Production**

At 6 days post-hatch, splenic macrophages collected from birds injected *in ovo* with the anti-coccidial vaccine supplemented with vitamin E produced 44% more (P = 0.13) nitric oxide than birds injected with the anti-coccidial vaccine and 0 IU vitamin E (Figure 19). There were no significant (P > 0.05) differences in nitric oxide concentrations between treatment groups at 12 days post-hatch (Figure 20).

**Effect of Vitamin E Supplement to an Anti-Coccidial Vaccine on Serum Anti-coccidia IgG**

At 6 days post-hatch there were no differences between treatments in serum anti-coccidia IgG concentrations (Figure 21). At 12 days post-hatch, birds injected *in ovo* with the vaccine supplemented with vitamin E had 42% more (P < 0.01) serum anti-coccidia IgG than the birds injected *in ovo* with the anti-coccidial vaccine alone at both 1:10 and 1:20 serum dilutions (Figure 22).

**Effect of Vitamin E Supplement to a Coccidia Vaccine on Oocyst Shedding**

No oocysts were observed in the feces at 3, 7 or 10 days post-hatch for both the vitamin E supplemented and unsupplemented birds.
Discussion

Innate and adaptive immunity are the two parts of the immune system. In our experiment, the parameters measured fell into the innate category (macrophage production of NO) and adaptive (anti-coccidia IgG). NO concentrations produced by splenic macrophages were elevated in the VE group at day 6, but not at day 12. Also, serum levels of anti-coccidia IgG were elevated in the VE group after day 12, but not day 6. VE enhanced the innate immune system at day 6 and the adaptive immune system at day 12.

In a study by Gore and Qureshi (1997), the effect of in ovo VE on IgG antibody response was evaluated by injecting sheep red blood cells (SRBC) intravenously at days 7 and 14, and measuring anti-SRBC antibody titers at 7 and 14 days post-injection. There were no differences in IgG titers after the primary injection but there was an increase 7 days (P = 0.08) and 14 days (P = 0.04) after the secondary injection (Gore and Qureshi, 1997). In our study, the birds showed an increase in IgG after the primary infection and were not exposed to a secondary infection as in the previous study.

The Gore and Qureshi (1997) study also analyzed macrophage function by measuring nitrite levels in the supernatant of LPS stimulated macrophage cultures. Consistent with their results, macrophages from birds supplemented in ovo with 10 IU VE had higher levels of nitrite in the supernatant (P = 0.04) than the control (Gore and Qureshi, 1997). Higher levels of nitrite are an indicator of higher macrophage activity, as macrophages release nitric oxide, which quickly reduces to nitrite and is more detectable. Previous studies have also shown that dietary VE increases the phagocytic activity of
macrophages and the overall cellular immune response in birds (Konjufca et al., 2004; Silva, 2011).

There are a couple ways that this study could have been improved. Dietary VE has shown little effect on the quantity of oocysts shed in the feces after a coccidial infection (Allen and Fetterer, 2002a). This study was originally designed to look at the number of oocysts in the feces at 3, 7 and 10 days post-hatch to verify that there was, in fact, an infection and see if there were any effects due to VE on oocyst shedding. We did not observe any oocysts in the feces and this could be due to an insufficient dose needed to establish an active clinical infection or oocyst shedding had not begun yet.

It would also be of use to have a more controls in this study. There were issues with egg fertility so the amount of birds obtained was not always ideal and did not allow for treatments including the vehicle alone or negative control. Future directions for a project like this should include a secondary challenge infection to evaluate whether the vaccine was effective in protecting against coccidiosis and if the VE conferred any extra protective properties.

We conclude that VE can potentially benefit the response to a coccidiosis vaccine by modulating both the innate and adaptive immune systems.

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Chapter 5: Conclusions

Tregs are present in the body to prevent excess inflammation and harm to the host from the immune system. Studies in chickens have found Tregs to be present and play a similar role as they do in other species. Cell mediated immunity, including T-cells, is crucial in resistance to protozoan pathogens such as coccidiosis. Evaluating how Tregs, and other T-cells such as T-helper and T-cytotoxic cells, are affected by a coccidial infection could lead to novel treatments to control the disease.

One common treatment option is to vaccinate birds against coccidial infections through in ovo techniques. In ovo vaccination is an efficient and accurate way to introduce the antigen at the earliest possible life stages. Supplements to these vaccines can make them more effective by helping the bird mount a full immune response to gain maximum benefit from the vaccine. In ovo vitamin E has been shown to be effective in boosting cell-mediated immunity in chickens post-hatch and after a secondary infection. Combining vitamin E with a coccidial vaccine could increase the efficacy of this treatment.

The objectives of this thesis were to

1. Identify Treg numbers and immunosuppressive properties post-coccidia challenge
2. Study the effects of an in ovo vitamin E supplement to an in ovo coccidia vaccine
The study presented in chapter 3 identified relative T-cell percentages, proliferation efficiencies and cytokine levels isolated from the immune tissues of chickens infected with coccidiosis. The objectives of the study were to evaluate, (1) the effects of a coccidia infection on CD4⁺, CD8⁺ and Treg cell percentages in local and systemic immune organs, (2) the effects of a coccidia infection on inflammatory and anti-inflammatory cytokine profiles and (3) the effects of Tregs on lymphocyte proliferation post-coccidia challenge.

Treg percentages in the cecal tonsil increased during coccidial infection during early infection and significantly during late infection. In the spleen, Treg percentages were similar between treatments during early infection but lower in infected birds during late infection. T-helper cells (CD4⁺) in the cecal tonsils were at higher amounts in infected birds in the early stages of infection but decreased in number compared to the control during late infection. Conversely, CD4⁺ cells in the spleen decreased during early infection and increased during late infection. T-cytotoxic cells (CD8⁺) in the cecal tonsils increased six days post-infection but did not vary between treatments during later infection or in the spleen during both time points.

The anti-inflammatory cytokine, IL-10, increased 5-fold during early infection and significantly increased during late infection in the cecal tonsils and did not change between treatments in the spleen. The inflammatory cytokines, IL-1 and IFN-γ, were largely unchanged between treatments, although IFN-γ decreased during late infection in the spleen.

Treg-depleted and Treg-undepleted cell populations did not differ in cell proliferation in cecal tonsils collected from uninfected birds. Cecal tonsil cells collected
from coccidia-infected birds and depleted of Treg cells had higher proliferation at 6 days post-infection than cecal tonsil cells from coccidia-infected birds not depleted of Tregs but no difference was seen 11 days post-infection. The removal of Tregs eliminates the suppression they cause, hence increased proliferation in Treg-depleted cell populations. Differences may not have been present in cecal tonsils taken from uninfected birds due to a lower amount of Tregs being present. Birds infected with coccidia had a higher amount of Tregs than uninfected birds, so depleting Tregs from cecal tonsils collected from infected birds could be expected to have a larger effect on proliferation.

Both Treg numbers and IL-10 amounts increased significantly at day 11 post-infection in the cecal tonsils. This correlation can lead to the inference that Tregs were the source of this IL-10 and caused a local immune suppression in the cecal tonsils of coccidian-infected birds. Systemically, Treg numbers decreased during the same time point as evidenced by the decrease in Tregs in the spleen. IFN-γ mRNA amounts decreased at day 11 post-infection in the spleen, indicating the impaired ability of the bird to mount an efficient response to the infection.

The study presented in chapter 4 evaluated the effects of a vitamin E supplement to an in ovo coccidia vaccine. The objectives of the study were to evaluate the effect of an in ovo vitamin E supplement to a coccidia vaccine on, (1) oocyst shedding post-infection, (2) splenic macrophage NO production and (3) serum anti-coccidia IgG content.

Macrophages collected at 6 days post-hatch from birds supplemented with vitamin E produced higher amounts of NO in culture than birds that were unsupplemented with vitamin E. There was no difference in macrophage nitric oxide production between treatment groups at 12 days post-hatch. This difference could be due
to the fact that macrophages would be more active during early infection than late infection. Vitamin E increased the macrophage’s ability to fight infection during a critical time for the innate immune system.

Serum anti-coccidia IgG amounts were higher in birds supplemented with vitamin E in serum collected 12 days post-hatch. There was no difference in serum anti-coccidia IgG amounts between treatments at 6 days post-hatch.

In conclusion,

1. Coccidiosis causes an up regulation of IL-10 in the cecal tonsils during the late stages of a coccidial infection.
2. Coccidiosis causes an increase in Tregs and CD4$^+$ cells in the cecal tonsil during late infection.
3. Tregs decrease in the spleen during late coccidial infection.
4. A vitamin E supplement to a coccidial vaccine increases splenic macrophage NO production during early coccidial infection.
5. A vitamin E supplement to a coccidial vaccine increases serum anti-coccidia IgG during the later stages of a coccidial infection.
References


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Figure 1. Effect of coccidial infection on cecal tonsil CD4⁺ and CD8⁺ cell percentages at 6 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 x 10⁵ live coccidial oocysts (treatment). Cecal tonsil cells were concentrated for lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD8 mAb and analyzed using flow cytometry. Bars (mean of 5 birds ± SEM) with an asterisk (*) differ significantly (P < 0.05) between control and treatment.
**Figure 2.** Effect of coccidial infection on cecal tonsil CD4\(^+\)CD25\(^+\) cell percentages at 6 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 x 10\(^5\) live coccidial oocysts (treatment). Cecal tonsil cells were concentrated for lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD25 mAb and analyzed using flow cytometry. The Treg population is represented by CD4\(^+\)CD25\(^+\) cells as a percent of CD4\(^+\) cells. Bars (mean of 5 birds ± SEM) do not differ significantly within a day (P < 0.05).
Figure 3. Effect of coccidial infection on spleen CD4+ and CD8+ cell percentages at 6 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 x 10^5 live coccidial oocysts (treatment). Spleen cells were concentrated for lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD8 mAb and analyzed using flow cytometry. Bars (mean of 5 birds ± SEM) do not differ significantly within a day (P < 0.05).
Figure 4. Effect of coccidial infection on cecal tonsil CD4\(^+\) and CD8\(^+\) cell percentages at 11 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 \(\times\) 10\(^5\) live coccidial oocysts (treatment). Cecal tonsil cells were concentrated for lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD8 mAb and analyzed using flow cytometry. Bars (mean of 5 birds ± SEM) with an asterisk (*) differ significantly within a day (P < 0.05).
Figure 5. Effect of coccidial infection on cecal tonsil CD4+CD25+ cell percentages at 11 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 x 10^5 live coccidial oocysts (treatment). Cecal tonsil cells were concentrated for lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD25 mAb and analyzed using flow cytometry. The Treg population is represented by CD4+CD25+ cells as a percent of CD4+ cells. Bars (mean of 5 birds ± SEM) with an asterisk (*) differ significantly within a day (P < 0.05).
Figure 6. Effect of coccidial infection on spleen CD4\(^+\) or CD8\(^+\) cell percentages at 11 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 \( \times 10^5 \) live coccidial oocysts (treatment). Spleen cells were concentrated for lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD8 mAb and analyzed using flow cytometry. Bars (mean of 5 birds ± SEM) do not differ significantly within a day (\( P < 0.05 \)).
Figure 7. Effect of coccidial infection on spleen CD4^+CD25^+ cell percentages at 11 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 x 10^5 live coccidial oocysts (treatment). Spleen cells were concentrated for lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD25 mAb and analyzed using flow cytometry. The Treg population is represented by CD4^+CD25^+ cells as a percent of CD4^+ cells. Bars (mean of 5 birds ± SEM) with an asterisk (*) differ significantly within a day (P < 0.01).
Figure 8. Effect of coccidial infection on spleen and cecal tonsil TNF-α mRNA content at 6 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or $4 \times 10^5$ live coccidial oocysts (treatment). At 6 d post-coccidial challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (mean of 5 birds ± SEM) do not differ significantly within a day ($P < 0.05$).
Figure 9. Effect of coccidial infection on spleen and cecal tonsil TNF-α mRNA content at 11 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or $4 \times 10^5$ live coccidial oocysts (treatment). At 11 d post-coccidial infection, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (mean of 5 birds ± SEM) do not differ significantly within a day (P < 0.05).
Figure 10. Effect of coccidial infection on spleen and cecal tonsil IL-1 mRNA content at 6 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 x 10^5 live coccidial oocysts (treatment). At 6 d post-coccidial challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (mean of 5 birds ± SEM) do not differ significantly within each organ (P < 0.05).
Figure 11. Effect of coccidial infection on spleen and cecal tonsil IL-1 mRNA content at 11 days post-coccidial infection. At 21 d of age, birds were challenged with 0 (control) or 4 x 10⁵ live coccidial oocysts (treatment). At 11 d post-coccidial challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (mean of 5 birds ± SEM) do not differ significantly within each organ (P < 0.05).
Figure 12. Effect of coccidial infection on spleen and cecal tonsil IFN-γ mRNA content at 6 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or $4 \times 10^5$ live coccidial oocysts (treatment). At 6 d post-coccidial challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (mean of 5 birds ± SEM) do not differ significantly within a day ($P < 0.05$).
Figure 13. Effect of coccidial infection on spleen and cecal tonsil IFN-γ mRNA content at 11 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or $4 \times 10^5$ live coccidial oocysts (treatment). At 11 d post-coccidial challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (mean of 5 birds ± SEM) do not differ significantly within a day (P < 0.05).
Figure 14. Effect of coccidial infection on spleen and cecal tonsil IL-10 mRNA content at 6 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 x 10⁵ live coccidial oocysts (treatment). At 6 d post-coccidial challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (mean of 5 birds ± SEM) did not differ significantly within a day (P < 0.05).
Figure 15. Effect of coccidial infection on spleen and cecal tonsil IL-10 mRNA content at 11 days post-coccidial challenge. At 21 d of age, birds were challenged with 0 (control) or 4 x 10^5 live coccidial oocysts (treatment). At 11 d post-coccidial challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (mean of 5 birds ± SEM) did not differ significantly within a day (P < 0.05). P-values: Cecal Tonsil, P = 0.09
Figure 16. Effect of Tregs on control cecal tonsil lymphocyte proliferation. Cecal tonsils were pooled in groups of two and a quarter of the total cells were incubated with mouse anti-chicken CD25 conjugated with PE and subsequently with anti-PE microbeads (Miltenyi Biotec). These cells were run through a MACS magnetic column (Miltenyi Biotec) and the flow through was collected. The flow through represents the CD25– population or Treg depleted population (whole = entire lymphocyte population of the cecal tonsil, including Tregs). At 6 and 11 d post-coccidial challenge, cecal tonsil cell proliferation efficiencies were measured using the thiazolyl blue tetrazolium bromide (MTT) colorimetric assay. Bars (mean of 10 birds ± SEM) do not differ significantly within a day (P < 0.05).
Figure 17. Effect of Tregs on coccidial infected cecal tonsil lymphocyte proliferation.

Cecal tonsils were pooled in groups of two and a quarter of the total cells were incubated with mouse anti-chicken CD25 conjugated with PE and subsequently with anti-PE microbeads (Miltenyi Biotec). These cells were run through a MACS magnetic column (Miltenyi Biotec) and the flow through was collected. The flow through represents the CD25⁻ population or Treg depleted population (whole = entire lymphocyte population of the cecal tonsil, including Tregs). At 6 and 11 d post-coccidial challenge, cecal tonsil cell proliferation efficiencies were measured using the thiazolyl blue tetrazolium bromide (MTT) colorimetric assay. Bars (mean of 10 birds ± SEM) with an asterisk (*) differ significantly within a day (P < 0.01).
Figure 18. Effect of coccidial infection on body weight gained. At 21 d of age, birds were challenged with 0 (control) or $4 \times 10^5$ live coccidial oocysts (treatment). 6 and 11 d post-coccidial challenge, birds were weighed and the differences between the two days were calculated. Bars (mean of 10 birds ± SEM) do not differ significantly within a day ($P < 0.05$).
Figure 19. Effect of TPGS vitamin E (VE) on NO concentrations produced by splenic macrophages. At 18 d of incubation, eggs containing embryos were injected with 10IU TPGS VE (Cocci + VE) and/or vaccinated with $2.0 \times 10^5$ live coccidial oocysts (Cocci). 6d post-hatch spleens were removed and macrophages were cultured in media containing LPS. The supernatant was analyzed for NO concentration. Eggs injected with TPGS VE had higher ($P = 0.14$) concentrations of NO than the control. Bars (mean of 5 birds ± SEM) with an asterisk (*) differ significantly ($P < 0.05$).
Figure 20. Effect of TPGS vitamin E (VE) on NO concentrations produced by splenic macrophages. At 18 d of incubation, eggs containing embryos were injected with 10IU TPGS VE (Cocci + VE) and/or vaccinated with $2.0 \times 10^5$ live coccidial oocysts (Cocci). 12d post-hatch spleens were removed and macrophages were cultured in media containing LPS. The supernatant was analyzed for NO concentration. Bars (mean of 5 birds ± SEM) do not differ between the treatments ($P < 0.05$)
Figure 21. Effect of TPGS vitamin E (VE) on serum concentrations of anti-coccidia IgG. At 18 d of incubation, eggs containing embryos were injected with 10IU TPGS VE (Cocci + VE) and/or vaccinated with $2.0 \times 10^5$ live coccidial oocysts (Cocci). 6 d post-hatch blood was collected by cardiac puncture and serum separated by centrifugation. Anti-coccidia serum IgG ELISA was performed at a 1:10 and 1:20 dilution with 5% non-fat dry milk in PBS-Tween. Bars (mean of 5 birds ± SEM) did not differ significantly within a dilution ($P < 0.05$).
Figure 22. Effect of TPGS vitamin E (VE) on serum levels of anti-coccidia IgG. At 18 d of incubation, eggs containing embryos were injected with 10IU TPGS VE (Cocci + VE) and/or vaccinated with $2.0 \times 10^5$ live coccidial oocysts (Cocci). 12 d post-hatch blood was collected by cardiac puncture and serum separated by centrifugation. Anti-coccidia serum IgG ELISA was performed at a 1:10 and 1:20 dilution with 5% non-fat dry milk in PBS-Tween. Bars (mean of 5 birds ± SEM) with an asterisk (*) differ significantly within a dilution (P < 0.01).