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# Expression of perforin, granzyme A and Fas ligand mRNA in caecal tissues upon *Eimeria tenella* infection of naïve and immune chickens

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ABSTRACT

Cytotoxic cells of the immune system may kill infected or transformed host cells via the perforin/granzyme or the Fas ligand (FasL) pathways. The purpose of this study was to determine mRNA expression of perforin, granzyme A and FasL in *Eimeria tenella* infected tissues at primary infection and infection of immune chickens as an indirect measure of cytotoxic cell activity. Chickens were rendered immune by repeated *E. tenella* infections, which was manifested as an absence of clinical signs or pathological lesions and significantly reduced oocyst production upon challenge infection.

During primary *E. tenella* infection perforin, granzyme A and FasL mRNA expression in caecal tissue was significantly increased at 10 days after infection, compared to uninfected birds. In contrast at infection of immune birds perforin and granzyme A mRNA expression in caecal tissue was significantly increased during the early stages of *E. tenella* challenge infection, days 1-4, which coincided with a substantial reduction of parasite replication in these birds.

These results indicate the activation of cytotoxic pathways in immune birds and support a role for cytotoxic T-cells in the protection against *Eimeria* infections.

INTRODUCTION

*Eimeria tenella* is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa (reviewed in [1-3]). The life cycle of *Eimeria* species comprises three phases: sporulation of oocysts that occur outside the host, schizogony, that is, asexual replication involving several repeated generations, and gametogony, sexual replication, that occur inside the host. The majority of *Eimeria* species infect and replicate in host intestinal epithelial cells and may cause coccidiosis, a severe disease in many domestic species, for example poultry, cattle and sheep. In modern poultry production coccidiosis is one of the most economically important infectious diseases [1-3]. *Eimeria tenella* replicates exclusively in caecal tissue and is one of the most pathogenic of the seven *Eimeria* species that infect domestic fowl. In the *E. tenella* life cycle sporozoites invade epithelial cells at the tip of the caecal folds and then migrate through the *lamina propria* to invade epithelial cells in the crypts of Lieberkühn where they undergo the first asexual replication and develop into first generation schizonts (reviewed in [4]). Merozoites from the first generation schizonts invade further crypt epithelial cells which migrate through the basement membrane into the *lamina propria* where second generation schizonts develop resulting in devastating pathology. Merozoites from the second generation schizonts infect epithelial cells on the tips of the caecal folds and a third asexual

replication occurs prior to the sexual replication that also takes place in the caecal epithelium. For the Houghton strain of *E. tenella* used in the present study first, second and third generation schizonts start to appear 48, 84 and 108 h post infection, respectively, and are present at maximum numbers at 60, 96 and 114 h, respectively (reviewed in [5]). The first oocysts appear in faeces approximately 132 h post infection, peak oocyst excretion occurs around day 8 post infection and oocysts can be detected in faeces up to 14 days after infection.

Species-specific immunity is elicited in chickens that recover from one or more infections with the same *Eimeria* species [6-13]. Studies of parasite development in *E. tenella* immune chickens have shown that sporozoites readily penetrate the caecal fold tip epithelium in immune birds [6, 8, 11, 14, 15]. Some of these reports indicated reduced transport of sporozoites through the *lamina propria* of immune birds and all agree that intracellular development of parasites that did reach the crypt epithelium is arrested at the trophozoite stage [6, 8, 11, 14, 15]. These intracellular stages are thus a primary target for protective immunity. Moreover, CD8 expressing T-cells may have a role in the limitation of *Eimeria* infections in immune chickens (reviewed in [16-18]). For example, adoptive transfer of immunity was shown using spleen and blood leukocytes from *E. maxima* immune birds [19], and *in vivo* depletion of CD8+ or TCR$\alpha/\beta$+ cells abrogated immunity upon re-infection of chickens with *E. tenella* [20]. Furthermore, infection of immune chickens with *E. acervulina* elicited increased numbers of CD8+ and TCR$\alpha/\beta$+ cells in the infected duodenal mucosa [21, 22] and CD8+ cells were frequently observed in contact with infected epithelial cells [22]. Moreover, TCR$\alpha/\beta$+CD8+ cells accumulated in caecal tissues during the early stages of *E. tenella* infection of immune birds [23] and CD8+ or TCR$\alpha/\beta$+ cells were located near sporozoites to a higher degree in immune compared to in primary infected birds.

The TCR$\alpha/\beta$+CD8+ T-cells are classically considered to be cytotoxic T-cells (CTL) that have the ability to kill infected/transformed host cells as one important effector function. However, the actual role of TCR$\alpha/\beta$+CD8+ cells in protection against *Eimeria* spp. remains undefined. To gain more insight into potential cytotoxicity in the context of *E. tenella* immunity we studied mRNA expression of components of the two main pathways of cytotoxicity, granule-mediated and ligand-mediated cytotoxicity [24]. We compared the levels of perforin, granzyme A and Fas ligand (FasL) mRNA in uninfected chickens with those in chickens undergoing primary or tertiary *E. tenella* infection, the latter birds exhibit considerable immunity. Focus was on the first 96 h of infection because inhibition

of the asexual replication stages, particularly the second generation schizonts, is crucial for limiting tissue damage caused by *E. tenella*.

## MATERIALS AND METHODS

### Chickens and experimental design

The experiment was approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden, (C67/05). Eighty-six female Bovans Robust chickens, a commercial Leghorn layer hybrid purchased from Swedfarm AB (www.swedfarm.se), were used. From one day old, birds were reared under SPF-conditions at the National Veterinary Institute, Uppsala, Sweden. Chickens were weighed and blood samples were collected at 17 days of age and the birds were subsequently allocated to 15 sampling groups of 5-6 chickens each (Table 1) with respect to an even distribution of weight and levels of maternal antibodies to *E. tenella* in serum. Five sampling groups were allocated to each of three status groups designated immune, primary and uninfected, respectively (Table 1). Chickens in the immune status group were inoculated with *E. tenella* at 19 days old, 1$^{st}$ infection, at 31 days old, 2$^{nd}$ infection, and at 44 days old, challenge infection (Table 1). All inoculations were with 1000 live *E. tenella* oocysts per bird. Chickens in the primary status group were inoculated with *E. tenella* at 44 days old, challenge infection, and chickens in the uninfected status group were kept uninfected throughout the experiment. Uninfected and *E. tenella* infected chickens were housed in separate rooms under positive pressure ventilation.

### Sample collection

Chickens were reared in specially designed cages (1 sample group/cage) with interchangeable flooring. Most of the time chickens were kept on solid floors with wood shavings as bedding. For faecal collection chickens were kept on wire floors without bedding and faeces were collected onto plastic sheeting in trays underneath the flooring. All faeces produced during 24 h intervals were collected from day 5, 120 h, post-infection to day 9, 218 h, post infection.

Blood samples for *E. tenella* serology were collected from the jugular vein into plastic micro centrifuge tubes. All birds were blood sampled at 17 days of age (prior to the 1$^{st}$ infection), at 30 days of age (prior to the 2$^{nd}$ infection), and at 44 days of age (prior to the challenge infection). At 53 days of age (on day 9 after the challenge infection), the remaining birds, n=15, were blood sampled.

After the challenge infection birds in the indicated sample groups (Table 1) were euthanized by cervical dislocation and the caeca were collected. The two caeca and caecal tonsils were collected from each bird. Approximately 1 cm of the proximal part of the caeca containing the caecal tonsils were cut off, this piece was cut open longitudinally and each caecal tonsil was dissected from the surrounding intestinal tissue and placed in 250 µl RNAlater (RNAlater® stabilization solution, Invitrogen, ThermoFisher Scientific www.thermofisher.com). The remaining caeca were cut open longitudinally and caecal contents were thoroughly rinsed off with phosphate buffered saline (pH 7.2; PBS) then the ceacal tissue was cut into 0.5 cm wide longitudinal strips. From each caeca, strips of tissue constituting a volume of 1 ml were placed in 5 ml RNAlater. Samples were collected sequentially from each bird and care was taken to minimise time between euthanasia and samples being submerged in RNAlater. Samples in RNAlater were subsequently stored at 4°C for 24 h and thereafter stored at -20°C until further processing.

**Preparation of *E. tenella* inoculum and detection of oocysts in faecal samples**

A pure *E. tenella* Houghton strain [5] isolate was maintained by minimum twice yearly passage in chickens. Oocysts were purified from faeces using a saturated sodium chloride flotation method [25]. Purified oocysts were resuspended in 2 % aqueous potassium dichromate solution and incubated in Petri dishes at 27 °C for sporulation. When 95 % sporulation was achieved oocysts were stored at 4 °C until use. All oocysts used in this experiment were from the same batch and had been stored for 4 months at the first inoculation. At inoculation oocysts were washed, resuspended in water and counted. At all experimental infections each bird in the present study was inoculated orally with 1000 live *E. tenella* oocysts.

From day 5, 120 h, and until day 9, 218 h, after inoculations all faeces produced by the birds in each sampling group (Table 1) during each 24 h period were collected, weighed and thoroughly mixed. Numbers of oocysts per gram faeces (OPG) were determined using a modified McMaster technique described in [26]. For samples collected prior to the challenge infection OPG results were presented as mean values of n=5 (1/sampling group) in each status group. After the challenge infection OPG was determined in n=3 replicate samples from faeces collected from each of the three remaining sampling groups. A sensitive centrifugal flotation method [27] to detect small numbers of oocysts was also applied on all samples and *Eimeria* species identity was verified using DNA-typing according to previously described methods [28].

**ELISA for detection of *E. tenella* antibodies**

An in house ELISA was used for detection of *E. tenella* antibodies in chicken serum. The coating antigen was prepared from purified *E. tenella* oocysts of the Houghton strain. Oocysts were suspended in PBS with proteinase inhibitor (0.1 mM phenylmethanesulfonyl fluoride, Sigma-Aldrich, www.sigmaaldrich.com) and 0.1 mM EDTA and disrupted using glass beads (1 mm ∅) and repeated vortexing. The material was then further disrupted by sonication and subsequent incubation with detergent (2 % N-Decanoyl-N-methylglucamine in PBS, MEGA-10, Sigma-Aldrich). The lysate was dialysed against PBS where after particulate matter was removed by centrifugation (10000 x g). The coating antigen was used at a protein concentration of 1 µg/ml in 0.05 M $Na_2CO_3$/$NaHCO_3$ coating buffer, pH 9.6, in flat-bottomed 96-well plates (PolySorp, Nunc™, ThermoFisher Scientific). PBS with 0.05 % Tween 20 (Sigma-Aldrich) was used for blocking, as diluent and as wash buffer. Chicken sera were tested at dilution 1:100 in duplicate and known positive and negative control serum samples were included on every plate. Horseradish peroxidase conjugated polyclonal goat anti chicken IgG (IgY)-Fc antibodies (#A30-104P, Bethyl Laboratories, Inc; www.bethyl.com) were used as tracer and an in house substrate buffer (1 mM 3,5,3',5'-tetrametylbenzidine in 0.1 M potassium citrate, pH 4.2, with 0.007 % $H_2O_2$) was used for visualisation of antibody binding. This reaction was stopped at a standardised time point with 2 M $H_2SO_4$ and the $A_{450}$ was measured in an ELISA reader.

**DNA isolation and real time PCR for detection and quantification of *E. tenella* DNA**

DNA was isolated from caecal tissues preserved in RNAlater by thawing samples to room temperature and shaking 350 mg sample in 1200 µl standard TE-buffer pH 8.0 (in house) with approx. 30 zirconia beads, 2 mm ∅, in a TissueLyser (Qiagen, www.qiagen.com), at 3000 rpm for 1 min repeated three times. Subsequently, nucleic acids were extracted using magnetic bead capture using the Boom method [29] with the Nordiag Vet Viral NA kit in a Magnetic Biosolutions Magnatrix 8000+ extraction robot (DiaSorin, www.diasorin.com).

The real time *E. tenella* PCR was earlier described by Blake et al (2008) [30] and *E. tenella* primers are described there while for chicken reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the following primers and probe were used: CGCAAGGGCTAGGACGG (forward primer), GCGCTCTTGCGGGTACC (reverse primer) and probe [AminoC6+JOE]CCGCGCCAGACGCCGTTCTC[BHQ1a~JOE] (Eurofins, www.eurofins.com/genomic-services.aspx). In brief, PCR reactions contained 12.5 µl 2xRT-PCR buffer, 1 µl RT-PCR enzyme mix

(AgPath-ID™ OneStep RT-PCR Kit, ThermoFisher Scientific), 1.25 µl 10 µM of respective primer (final concentration 500 nM) and 0.625 µl 10 µM probe (final concentration 250 nM), 3 µl template and 5.375 µl nuclease free water to a final volume of 25 µl. Samples and standards were tested in triplicates in 96-well PCR-plates (Applied Biosystems, ThermoFisher Scientific) and the cycling conditions were 45°C for 10 min, 95°C for 10 min followed by 48 cycles of 95°C for 15 sec and 60°C for 45 sec. Amplification, detection, and data analysis were performed using a 7500 Fast Real-Time PCR System with 7500 Software v.2.0.5 (Applied Biosystems, ThermoFisher Scientific). Serial dilutions of *E. tenella* sporozoite DNA and chicken DNA, respectively, were used as standards in the PCR. The amounts of parasite and chicken DNA in samples were calculated by linear regression of Ct values on to the standard curves for the respective genes. The amount of *E. tenella* DNA in the samples was normalized to the amount of GAPDH DNA and presented as the ratio between these two parameters.

**Total RNA isolation, cDNA synthesis and real time reverse transcriptase PCR (real time RT PCR***)*

Total RNA was isolated from tissues preserved in RNAlater as described above for DNA but shaking up to 350 mg sample in 1200 µl AVL-buffer (Qiagen). Approximately 1 µg of the extracted RNA was treated with RQ1 RNase-Free DNase (Promega, www.promega.com) to remove contaminating DNA prior to cDNA synthesis, which was performed using High Cap RNA-cDNA (Applied Biosystems, ThermoFisher Scentific) using approx. 0.5 µg total RNA in a final volume of 20 µl. All methods were performed according to the manufacturer's recommendations. The cDNA samples were stored at -70° until PCR analysis.

All primers and TaqMan® probes used for real time RT PCR (synthesised by Eurofins) are listed in Table 2. All probes utilised the non-fluorescent Black Hole Quencher Dye at the 3´end. Primers and probes were designed using Eurofins online qPCR primer and probes design tool (http://www.eurofinsgenomics.eu/). To minimize the risk of amplifying genomic DNA at least one of the primers or the probe for each gene was designed to span an intron with the exception of the perforin PCR because the complete chicken perforin gene sequence [31] was not available at the time.

Oligonucleotides for each amplified region (cDNA level) were synthesised and cloned into plasmids, pCR®2.1 (Eurofins), for use as plasmid standards in the PCR assay. Glycerol stocks with plasmids were amplified in *E.coli* bacteria and purified using ®*Plus* Midiprep (Promega) following the manufacturer's

instructions. The plasmid DNA concentration was measured by NanoDrop ND-1000 (NanoDrop Technologies, www.nanodrop.com).

The real-time PCR assays were optimised separately for each gene to determine optimal annealing temperatures and concentration of primers and probes, by using cDNA from chicken tissue samples. Moreover, five- or ten-fold dilutions of template were used to determine PCR reaction efficiency. For duplex reactions, the primer-probe combinations for the two genes were optimised independently before they were combined and reaction efficiency was evaluated. For standard curves, *in vitro* transcribed purified plasmid DNA was used. Real-time PCR reactions contained 12.5 µl ABsoluteTM QPCR Mix (ThermoFisher Scientific), primer and probe concentrations according to Table 2, 2 µl template and water to a final volume of 25 µl. Samples and standards were tested in triplicates in PCR plates (MicroAmp Fast optical 96-well reaction plate, #4346906, Applied Biosystems, ThermoFisher Scientific) and the cycling conditions were 2 min at 94°C, followed by 30 cycles of 60 s at 94°C, 30 s at 60°C (data collected) and 60 s at 72°C. Amplification, detection, and data analysis were performed using a 7500 Fast Real-Time PCR System with 7500 Software v.2.0.5 (Applied Biosystems, ThermoFisher Scientific). The granzyme A and hexose-6-phosphate dehydrogenase (H6PD), FasL and H6PD and perforin and β-actin PCR reactions, respectively, were run as duplex reactions. Serial dilutions of the plasmids containing cloned target genes were included as standards in each run as well as non-template controls. The levels of gene expression in samples were calculated by linear regression of Ct values on to the standard curves for the respective genes. Granzyme A, perforin and FasL mRNA expression, respectively, in tissue samples was normalized to mRNA expression of reference genes GAPDH, β-actin and H6PD and presented as the ratio between the amount of respective parameter mRNA and the amount of GAPDH, β-actin and H6PD mRNA in the sample.

**Data presentation**

Data were presented as group mean values ± 95% confidence intervals (CI) where non-overlapping CI indicates statistically significant differences. Since the CI of mRNA expression ratios (amount of gene of interest mRNA/ amount of housekeeping gene mRNA) were asymmetrical and thus non-normally distributed, the arithmetic means and CI were estimated based on square-root transformation using a Microsoft Excel spreadsheet that implements the method of Land [(32)].

RESULTS

**Outcome of the infections**

The outcomes of all experimental *E. tenella* infections were monitored with respect to clinical signs and oocyst excretion in faeces. At caecal sample collection during the final challenge infection the macroscopic appearance of caecal tissues was also recorded.

The health status of chickens was recorded daily throughout the experiment and blood in faeces was observed on day 5 to day 7 after the first *E. tenella* infection of birds, that is the 1st infection for the immune group and the challenge infection for the primary group, respectively (Table 1). This was however, not observed after the 2nd or the challenge infections of birds in the immune status group. No other clinical signs of disease were observed during the experiment.

The total excretion of oocysts per bird from day 5 until day 9 after *E. tenella* infection is shown in Figure 1A. Birds in the immune status group excreted slightly smaller numbers of oocysts upon the 2nd infection compared to the 1st infection but this was not statistically significant. However, upon the challenge infection (3rd infection) birds in the immune status group excreted very few oocysts, significantly lower levels than those recorded at the 1st and 2nd infections as well as significantly lower levels than those recorded for the primary status group at challenge. Birds in the primary status group were infected for the first time with *E. tenella* at the challenge infection and their total oocyst excretion was lower than that recorded for birds in the immune status group at the 1st infection but this was not statistically significant. No oocysts were detected in the faeces of birds in the uninfected status group at any time during the experiment. The kinetics of oocyst excretion during the sampling period (Figure 1B) showed peak oocyst numbers in the samples collected at day 7 post infection in the birds infected with *E. tenella* for the first time, that is the 1st infection for the immune group and the challenge infection for the primary group, respectively. However, in birds infected repeatedly, that is the 2nd and the challenge infections of birds in the immune status group, the highest oocyst numbers were recorded in the sample collected at 6 days post infection.

Macroscopic examination of caecal tissue at the challenge infection of birds in the primary status group showed no pathological changes in caeca collected on days 1 to 4 post infection. At day 10 post infection however all birds in the primary status group displayed lesions in at least one caeca/bird. These lesions comprised thickening of the ceacal mucosa (5 of 5 birds), petechiae on the

caecal serosa (1 of 5 birds), a core of coagulated blood (3 of 5 birds), a core of white necrotic material (1 of 5 birds) and viscous blood in the caecal content (1 of 5 birds). In the immune status group two birds sampled on day 2 post challenge infection showed a slight thickening of the caecal mucosa and a small amount of hard white core material in the caecal content, and two birds sampled on day 3 post challenge infection showed a slight thickening of the caecal mucosa with normal or no caecal content, respectively. All other birds in the immune status group as well as all birds in the uninfected status group showed no pathological changes in the caeca.

Taken together, results show that primary *E. tenella* infections were established, induced moderate levels of characteristic clinical signs and pathological lesions and a considerable oocyst excretion. Repeated infections resulted in alterations of oocyst excretion kinetics and elimination of clinical signs. Upon the 3$^{rd}$ *E. tenella* infection (challenge) oocyst excretion was significantly reduced, by approximately 1000-fold, and no clinical signs or pathologic lesions were induced by the 3$^{rd}$ infection indicating that protective immunity had developed.

**Circulating antibodies to *E. tenella***

The levels of *E. tenella*-specific antibodies were monitored in serum samples collected before each of the three experimental infections and nine days after the final infection (Figure 2). Prior to the first infection many chickens had low to moderate levels of maternal *E. tenella* antibodies and care was taken to obtain an even distribution of seropositive chickens in the sampling groups at this time point. At the second sampling occasion, two weeks later, chickens in the uninfected and primary status groups were seronegative and remained so during the rest of the experiment. Chickens in the immune status group however had similar levels of antibodies to *E. tenella* as prior to the 1$^{st}$ infection when tested 12 days after infected with the parasite for the first time. When tested again 13 days after the 2$^{nd}$ infection *E. tenella* antibody levels had increased compared to before infections and remained at this level until the end of the experiment.

Thus, chickens in the immune status group showed a specific antibody response to *E. tenella* and were clearly seropositive at the challenge infection while the chickens in the primary status group did not seroconvert within nine days after their first parasite infection (at challenge infection).

### *Eimeria tenella* DNA in caecal tissues

The amount of *E. tenella* DNA was quantified using real time PCR-methodology in caecal tissues collected during the challenge *E. tenella* infection (Table 3). For primary infected chickens all samples were negative for *E. tenella* DNA on day 1 and only one sample was positive on day 2 post infection. On day 3 post infection 5 out of 6 chickens in the primary sampling group were positive for *E tenella* DNA in both caeca and on days 4 and 10 post infection all samples were positive. For primary infected chickens the mean amount of *E. tenella* DNA increased approximately 10-fold from day 3 to day 4 post infection and remained at this level on day 10 post infection. For immune chickens 1, 3 and 3 chickens were positive for *E. tenella* DNA on days 1, 2 and 3 post infection, respectively, while only one sample was positive on day 4 post infection. In these chickens the highest amount of *E. tenella* DNA was recorded in samples collected at day 3. At day 10 post infection 4 out of 5 immune chickens were positive for *E. tenella* DNA although most of them only in one ceaca and the amounts of parasite DNA were in mean 1000-fold lower than those detected in primary infected birds at the same time point (Table 3).

Hence, in primary birds the main amplification of *E. tenella* DNA during day 1-4 post infection occurred at days 3-4. On the contrary, in immune birds there was no evidence of *E. tenella* amplification during this period although small amounts of parasite DNA was detected throughout the experiment.

### Expression of preforin, granzyme A and FasL mRNA in caecal tissue and caecal tonsils

The expression of perforin, ganzyme A and FasL mRNA was determined in caecal tissue and caecal tonsils sequentially collected after the challenge *E. tenella* infection (Figure 3). In caecal tissues from birds in the immune status group, perforin and granzyme A mRNA expression was significantly increased early (days 2-4 for perforin; days 1-3 for granzyme A) after the challenge infection compared to that observed for uninfected birds. For FasL a similar trend was evident, with increased mRNA expression in caecal tissues from immune birds early after challenge infection. Even so, in uninfected birds FasL showed a relatively high background expression and there was a large variation between individuals, hence the changes in immune birds were not statistically different from the uninfected birds. In caecal tissues from birds in the primary status group perforin, granzyme A and FasL mRNA expression did not differ from that in uninfected birds at days 1-4 post infection. In contrast, all three targets were significantly increased in caecal tissues from primary infected birds at 10 days post *E. tenella* infection compared to uninfected birds.

In caecal tonsil tissues from birds in the immune status group perforin mRNA expression tended to be increased at days 2-4 post infection but was not significantly different from that observed for uninfected birds. For birds in the primary status group perforin mRNA expression tended to be increased at day 10 post infection compared to the uninfected birds. However, perforin mRNA expression in caecal tonsils was low and showed large variation between individuals and the expression patterns observed in response the *E. tenella* infection were less clear than those observed in the corresponding caecal tissue samples. Granzyme A and FasL mRNA expression in caecal tonsil tissues showed a large variation between individuals and no clear differences between the three chicken groups were observed.

Thus, in the caeca from immune chickens granzyme A, perforin and FasL mRNA expression was increased during the early stages of the *E. tenella* challenge infection while primary infected chickens showed an increased expression of these parameters only at the late, day 10, sampling occasion.

## DISCUSSION

In the present study immunity to *E. tenella* was induced in chickens by repeated infection with the parasite and the major finding was that an increased expression of genes associated with immune cell cytotoxicity was observed in immune chickens at the same time as the parasite replication was inhibited. This observation suggests that cytotoxic functions and/or the cells expressing them are involved in protective immunity.

In general terms, the perforin/granzyme- and FasL-pathways' main roles in the immune system are in the cytotoxic cells, that is CTL and NK-cells. Our results indicate that cells with cytotoxic capacity accumulate in *E. tenella* infected tissues and open up the potential for cytotoxicity as an effector mechanism in immune birds. In view of the reports that show involvement of TCR$\alpha/\beta$+CD8+ cells in *Eimeria* induced responses of immune chickens [20, 21, 23] one may speculate that CTL cytotoxic activity contributed in inhibition of parasite replication. For other intracellular parasites killing of infected host cells by CD8+ T-cells in an antigen specific, MHC restricted manner has been shown for example for *Toxoplasma gondii* [33-36], *Trypanosoma cruzi* (reviewed in [37]), and *Encephalitozoon cuniculi* [38]. In the case of *E. cuniculi* protective immunity in mice was also shown to be dependent on the perforin gene [38]. However, if CTL killing of *Eimeria* infected cells occurs and if it is of importance for immunity in chicken coccidiosis still remains to be proven.

Among the parameters monitored in the present study, perforin is a pore-forming protein with a central role in granule-mediated cytotoxicity as the key enabler of granzyme induced apoptosis of target cells (reviewed in [24, 39]). Chicken perforin differs from other vertebrate perforins by an elongated C-terminus [31]. Chicken perforin function has not yet been specifically studied but increased perforin mRNA expression has been shown during immune responses to several viral infections in chickens [40-43]. Moreover, by immunohistochemistry accumulation of perforin protein expressing CD8α+ chicken lymphocytes has been monitored in virus infected tissues [41, 42]. Our results showed that perforin, and granzyme A, mRNA expression were up-regulated early during the *E. tenella* infection of immune chickens and at a later stage in primary infection of chickens. This indicates that granule-mediated cytotoxicity may play a role in immunity to this parasite. Based on earlier reports [21, 23] recruitment of TCRα/β+CD8+ lymphocytes to *Eimeria* infected tissues would also be expected at these time points at least in the immune birds.

Granzymes belong to the family of granule associated serine proteases of immune defence [44] and their expression is, at least in mammals, generally restricted to cells of the lymphoid lineage (reviewed in [24]). In humans and mice granzyme B has the primary role in cytotoxic cell mediated killing while this function, especially *in vivo*, has been questioned for the other granzymes (reviewed in [24, 39, 45]). The latter granzymes have instead been suggested to have their major role in the inflammatory response. However, studies in mice have shown that in the absence of granzyme B, granzyme A may mediate NK-cell killing of target cells albeit slower than granzyme B (reviewed in [39]). Chickens only have two granzyme genes, granzyme A and K [44] and their specific functions have not yet been studied in depth. Nonetheless, increased expression of granzyme A mRNA has been observed during immune responses to different virus infections of chickens [40-43, 46-48] and in some of these cases granzyme A expression also correlated in time with indications of CTL responses [41, 42, 47, 48]. For example in a recent report [49], the mRNA expression of granzyme A, CD8α and several proinflammatory cytokines was monitored in the airways of infectious bronchitis virus (IBV) infected chickens. The results showed that granzyme A and CD8α mRNA expression was up-regulated 7 days post IBV infection while expression of proinflammatory cytokines, IL-6, IL-1β and IFN-γ, was up-regulated earlier during the infection. This could support that chicken granzyme A has a role in cellular cytotoxicity rather than in proinflammatory responses. Our results, where granzyme A mRNA expression coincided with increased perforin and FasL mRNA expression both in immune and primary infected chickens, are also in line with this hypothesis but more knowledge is needed before the function of granzyme A in the chicken is fully elucidated.

Studies of murine cells show that the majority of CTL express both perforin/granzyme and FasL [50-52]. The two killing pathways differ in time-to-kill, perforin/granzyme is fast-acting while FasL is slower, and individual CTL may employ one of the pathways to a greater or lesser degree but in general they have been reported to work in concert [52]. In the present *E. tenella* infected chickens FasL mRNA expression in caeca followed the same pattern as perforin and garnzyme A mRNA expression albeit FasL showed a relatively lower level of expression. It is therefore likely that chicken killer cells express both pathways. Activation of both perforin/granzyme and FasL pathways has also been reported in response to infectious bursal disease virus infections of chickens [42].

In the present study the induction of protective immunity to *E. tenella* was achieved by repeated infections with the parasite. It is well know that *Eimeria* infections induce protection against re-infection with homologous *Eimeria* species [6-13]. The number of immunising infections that are required before protection is achieved seem to depends on factors including *Eimeria* species, infection dose and time before re-infection [6-10, 12, 13]. Our results with strong immunity observed at a third infection after two infections with moderate numbers of oocysts are comparable with what has been reported by others. Likewise, our observations that a major bout of parasite replication in primary infected birds occurred days 3 to 4 corresponds well with the beginning and peak of the second generation schizonts reported for the *E. tenella* Houghton strain (reviewed in [5]). That the parasite replication was inhibited in the schizont phase and that some oocysts were produced even though very few or no replicative stages were observed in caecal tissue have also been observed earlier [6, 14]. To our knowledge there are however no comparable reports on the kinetics of oocyst production in immune birds. Nonetheless, Rose and Hesketh (1987) [12] state that *E. tenella* patency was shorter in immune birds. Our results show that peak oocyst production occurred earlier in immune and “semi immune” birds, which could indicate shifts towards parasites with a shorter pre-patent period. Studies on *E. tenella* vaccine strains have shown that precocious strains of this species mainly develop smaller second generation schizonts that contain fewer merozoites and have a faster maturation time but one strain lacking second and third generation schizonts has also been reported (reviewed in [18]). Hypothesising that protective immune reactions are mainly directed to the intracellular replication stage [14] the capacity to alter the length of this stage may be an adaptation to growth in the face of immunity with parasites spending a shorter time in the replicative phase having a greater chance to escape defences in an immune chicken. Hence, selection of precocious parasites for vaccines may be exploiting this natural capacity to modify the life cycle in immune birds.

To conclude our results support the role of CTL in chicken immunity to *Eimeria* infections and indicates that CTL killing of parasite infected cells may contribute to protection. In addition, results from the present and previous studies on *E. tenella* replication in immune chickens corroborate the belief that inhibition of the early intracellular stages is a critical part of protective immunity.

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EW planned and performed experiments, analysed the data and wrote the paper; AL and PT planned experiments, performed laboratory work and revised the paper; SEM, KN and ÅH established and validated methodology, performed laboratory work and revised the paper; ALS provided input into study design and methodology and revised the paper.

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LEGENDS TO THE FIGURES

Figure 1. A) The total number of oocysts excreted per bird. Results are from day 5 to day 9 after experimental infections with *E. tenella* (immune status group - light grey bars; primary status group – dark grey bars). During the 1st and 2nd infections results are shown from faeces collected from 5 groups of birds (total 29 birds) and during the challenge infection results are shown from faeces collected from one group of 5 birds each of immune and primary infected birds (for details see Table 1). Results are mean values ± 95% CI for replicate sample preparations, n=5 for 1st and 2nd infections and n=3 for the challenge infection. Non-overlapping confidence intervals indicate statistical difference.

B) Kinetics of oocyst excretion. Results are from day 5 to day 9 after experimental infections with *E. tenella* (immune status group: 1st infection - black squares, 2nd infection - black and white squares, challenge infection - open squares; primary status group challenge infection – black circles). Results show the mean number of oocysts excreted per bird from faeces collected for 24 h intervals during the 1st and 2nd infections (5 groups of birds, total of 29 birds) and during the challenge infection (one group each 5 birds of immune and primary infected birds; for details see Material and methods and Table 1). Results are mean values ± 1 standard error for replicate sample preparations, n=5 for 1st and 2nd infections and n=3 for the challenge infection.

Figure 2. Levels of antibodies to *E. tenella* in chicken sera from chickens expressed as absorbance at 450 nm. Results are mean values ± 95% CI for the status groups immune (black squares), primary infected (black circles) and uninfected (open diamonds). Experimental *E. tenella* infections are indicated with arrows. At the three first sampling occasions n=29 birds for the immune and primary status groups, respectively, and n=24 birds for the uninfected group. At the fourth and final sampling occasion all groups comprised n=5 birds. Non-overlapping confidence intervals indicate statistical difference. For details see Material and methods and Table 1.

Figure 3. Relative perforin (A, D), granzyme A (B, E) and FasL (C, F) mRNA expression in caecal tissue (A, B,C) and caecal tonsils (D, E, F) from the status groups immune (light grey bars), primary infected (dark grey bars) and uninfected (open bars). Samples were collected at the indicated days after *E. tenella* challenge infection. Results are mean values ± 95% CI for each sample group (for details see Table 1), n=12 or n=10 according to Table 1. Non-overlapping confidence intervals indicate statistical difference. Note that CI for proportions are asymmetrical, for details see Materials and Methods.

Table 1. Experimental design: description of status and sampling groups and age of birds at the different infections with *E. tenella,* and presence of clinical signs post infection. At the 3rd infection, challenge, the birds in designated sampling groups were euthanized at the indicated days after that infection.

| | **1st infection** | | **2nd infection** | | **3rd infection, challenge** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Birds 19 days old | | Birds 31 days old | | Birds 44 days old | | **Sampling groups** (number of birds) | | | | |
| **Status** | *E. tenella* | Clinical signs | *E. tenella* | Clinical signs | *E. tenella* | Clinical signs | Day 1 | Day 2 | Day 3 | Day 4 | Day 10 |
| Immune | + | yes[a] | + | no | + | no | 6 | 6 | 6 | 6 | 5[b] |
| Primary | - | no | - | no | + | yes[a] | 6 | 6 | 6 | 6 | 5[b] |
| Uninfected | - | no | - | no | - | no | 6 | 6 | 6 | 5[b] | 5[b] |

+ orally infected with approx. 1000 live sporulated *E. tenella* oocysts/bird. – not infected.

a The clinical signs observed were blood in faeces days 5-7 post-infection.

b Due to technical reasons we did not have enough birds for n=6 in all groups.

Table 2. Primer sequences and assay conditions for real time RT-PCR and GenBank accession numbers for the indicated chicken genes

| Gene | Primer/probe | Sequence 5'-3' | 5' labelling | Concentration (µM) | Product size (bp) | Accession # |
|---|---|---|---|---|---|---|
| Granzyme A | FWD | atc taa gag agg aga caa gga c | - | 0.2 | 94 | NM_204457 |
| | REV | ctt ccc aaa aga agt gat gcc | - | 0.2 | | |
| | Probe | cga ggg gat tca ggt gga cct tta ata | FAM | 0.2 | | |
| Perforin | FWD | gcg ttc agc gcc atg tc | - | 0.2 | 59 | KC551799.1 |
| | REV | ctg tgg gca ctg ttt ttg ga | - | 0.2 | | |
| | Probe | cgc gtg ccg caa agc caa | FAM | 0.2 | | |
| FasL | FWD | gca tgt ttc aga ttt tcc acc | - | 0.4 | 135 | AJ890143.1 |
| | REV | gaa aaa gga agc aag gga ggc | - | 0.4 | | |
| | Probe | agg aac tgg ctg aac tca gag agt ctg tca a | FAM | 0.2 | | |
| GAPDH | FWD | ggc att gca ctg aat gac c | - | 0.2 | 89 | NM_204305 |
| | REV | gac cat caa gtc cac aac ac | - | 0.2 | | |
| | Probe | ttc ctg gta tga caa tga gtt tgg ata cag ca | JOE | 0.2 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| β-actin | FWD | cca aag cca aca gag aga ag | - | 0.3 | 134 | NM_205518.1 |
| | REV | acc aga gtc cat cac aat acc | - | 0.3 | | |
| | Probe | tga cac aga tca tgt ttgaga cct tca aca ccc c | JOE | 0.2 | | |
| H6PD | FWD | aac cga cat cat gtg gaa ag | - | 0.3 | 94 | XM_425746.4 |
| | REV | cgg atg act cca tac tgc tc | - | 0.3 | | |
| | Probe | gag atc gtc ttg aag gag att gtg gat gct aaa | JOE | 0.3 | | |

bp – base pairs; FWD – forward; REV – reverse; FAM - 6-carboxyfluorescein; JOE - 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluoresceine

Table 3. *Eimeria tenella* DNA detected by real time PCR-methodology. Results are expressed as the ratio between the amount of *E.tenella* DNA and the amount of chicken GAPDH DNA in caecal tissue collected at the indicated days post infection

| | Day post infection | | | | |
|---|---|---|---|---|---|
| **Primary** | **1** | **2** | **3** | **4** | **10** |
| Mean ratio ($x10^{-3}$) | - | 0.36 | 530 | 8041 | 7304 |
| Range ($x10^{-3}$) | - | - | 4.3-2555 | 5.5-85610 | 53.9-34289 |
| Positive samples/total samples | 0/12 | 1/12 | 10/12 | 12/12 | 10/10 |
| Positive chickens/total chickens | 0/6 | 1/6 | 5/6 | 6/6 | 5/5 |
| **Immune** | | | | | |
| Mean ratio ($x10^{-3}$) | 1.5 | 7.1 | 21.0 | 0.00072 | 5.1 |
| Range ($x10^{-3}$) | 0.02-3.0 | 0.17-20.9 | 0.58-34.5 | - | 0.90-19.1 |
| Positive samples/total samples | 2/12 | 5/12 | 5/12 | 1/12 | 5/10 |
| Positive chickens/total chickens | 1/6 | 3/6 | 3/6 | 1/6 | 4/5 |
| **Uninfected** | | | | | |
| Mean ratio ($x10^{-3}$) | - | - | - | - | - |
| Range ($x10^{-3}$) | - | - | - | - | - |
| Positive samples/total samples | 0/12 | 0/12 | 0/12 | 0/10 | 0/10 |
| Positive chickens/total chickens | 0/6 | 0/6 | 0/6 | 0/5 | 0/5 |

- Not detected