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Research article

Analysis of local immune responses in chickens infected with *Eimeria tenella*

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Abstract

Eimeria tenella is a major coccidian parasite in poultry, causing severe economic losses. Understanding the local immune response during *E. tenella* infection is essential for developing effective control strategies. This study investigated the transcriptional responses of T cell markers (CD3 and CD4), cytokines (IL-10, IL-12, IL-18, IL-22, IFN-γ, TGF-β1), and the chemokine K203 in experimentally infected chickens. Gene expression was analyzed using quantitative PCR, and histopathological changes were evaluated in hematoxylin and eosin-stained cecal sections. The expression of CD3 and CD4 genes showed an upward trend in infected chickens, suggesting potential involvement of T cells in the immune response, although this was not statistically significant. Several cytokines, including IFN-γ, IL-10, IL-18, IL-22, and TGF-β1, were significantly upregulated ($p < 0.05$), indicating a complex transcriptional response involving both pro-inflammatory and regulatory pathways. K203 expression increased markedly (approximately 60-fold, $p < 0.001$), consistent with enhanced chemotactic signaling and immune cell recruitment to the infection site. Histopathological examination revealed epithelial disruption and inflammatory cell infiltration in the infected group, correlating with the observed gene expression changes. Overall, this study provides insights into the transcriptional modulation of local immune mediators during *E. tenella* infection. While the results highlight possible involvement of T cell-associated and cytokine-mediated responses, further studies, particularly protein-level analyses, are needed to confirm the functional activation of immune cells and their specific roles in host defense.

Keywords: Cytokine, Chemokine, *Eimeria tenella*, Gene expression, Immune responses.

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INTRODUCTION

Infections by intestinal parasites in domesticated food animals pose a significant global veterinary health challenge. Avian coccidiosis, a specific intestinal disease caused by apicomplexan protozoa, exemplifies this issue. The most important poultry *Eimeria* species are *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. mitis*, and *E. praecox* (López-Osorio et al., 2020), which are highly virulent etiological agents. Various strategies have been employed to mitigate the harmful effects of coccidiosis, including the use of anticoccidial medications as preventative measures, the selection of chicken strains resistant to the disease, and efforts to enhance immunity. These approaches have been explored in studies by (Yun et al., 2000; Lee et al., 2012; Eyerich et al., 2017; Venkatas and Adeleke, 2019). However, it is crucial to understand that each methods has its own limitations. Coccidiosis alone has been estimated to result in annual financial losses exceeding $3 billion. Roughly 80% of these losses can be attributed to the direct impact on factors such as loss weight gain, mortality, and feed conversion, while the remaining 20% is associated with the costs of chemoprophylaxis (Blake et al., 2011).

Live vaccines are widely regarded as the most effective method for inducing long-lasting protective immune responses, largely because they mimic natural infections. Coccidiosis vaccination depends on the development of immunity through exposure to *Eimeria* parasite components. Several types of vaccines have been utilized to combat *Eimeria*, including live attenuated (or live virulent) vaccines, genetically engineered subunit vaccines, and non-infective parasite derivatives (Lillehoj and Erik, 2000; Yun et al., 2000; Dalloul and Lillehoj, 2006). Both vaccination and infection with coccidian parasites stimulate a specific immune response mediated by T cells, which are primarily responsible for resistance to coccidiosis. These immune responses also trigger the production of a diverse range of cytokines and chemokines in chickens, playing critical roles in developing protective immunity, as demonstrated by research from (Shini et al., 2010). Identifying the molecular profile of early host cellular immune responses offers valuable insights into the processes following *Eimeria* infection.

There has been limited knowledge of cytokines in chickens. Early cytokines identified and characterized include interferon (IFN)-γ, interleukin (IL)-2, and transforming growth factor (TGF). However, with the advent of the chicken genome project, many chicken cytokine and chemokine genes have been discovered through the work of various researchers, including (Hughes and Bumstead, 2000; Min and Lillehoj, 2002, 2004; Rothwell et al., 2004; Schneider et al., 2004; Kaiser et al., 2005; Read et al., 2005; Hong et al., 2006). The cloning of these genes has enabled the development of a wide range of tools for studying avian innate and adaptive immune responses at both the molecular and cellular levels. This capacity was unavailable just a few years ago.

This study aims to perform an initial gene expression screening of immune-related markers involved in the local immune response during *Eimeria tenella* infection. By analyzing the transcriptional profiles of genes associated with pro-inflammatory, anti-inflammatory, T helper (Th)1, and Th2 cytokines, as well as chemokines in experimentally infected chickens, we seek to identify genes showing altered expression patterns in response to coccidiosis. These preliminary findings provide a foundation for future studies employing protein-level and cellular analyses to elucidate the underlying immune mechanisms. Understanding these transcriptional responses is important for guiding the development of strategies to mitigate the impact of the disease on poultry production and improve animal health and welfare.

MATERIALS AND METHODS

Parasite

A virulent *E. tenella* isolate from local chickens in Thua Thien Hue province was maintained at the Parasitology Laboratory, Department of Veterinary Medicine, University of Agriculture and Forestry, Hue University (HUAF), Vietnam. The oocysts were collected using the sugar flotation technique, sporulated in 2.5% potassium dichromate at 28°C for 48 hours, and stored at 4°C for up to one month before use.

Experimental design and tissue collection

Chicken eggs (3F Vietnamese chickens, a local commercial hybrid breed commonly used for meat production in central region, Vietnam), supplied by 3F Vietnam Joint Stock Company (Thua Thien Hue, Vietnam), were incubated at 37.7 ± 1°C. The chicks were housed in a coccidia-free room under controlled conditions, with a constant temperature of 27 ± 1°C, a 12-hour light/dark cycle, and provided with ad libitum access to food and water (Pham et al., 2021). The necessary ethical approval to work on animals was obtained by the Animal Ethic Committee of Hue University (HUVN0040, May 20th, 2024). The experiments adhered strictly to the Policy on the Care and Use of the Laboratory Animals (HUAF).

Sixty chicks were randomly divided into two groups, control (C) and treatment (T), with 30 chicks in each group. At 14 days old, each chick in the treatment group was orally inoculated with 10^4 mature sporulated *E. tenella* oocysts.

Sample collection

Ceca were collected from 15 randomly selected chicks per group by cervical dislocation five days post-infection (dpi). One cecum, designated for gene expression analysis, was immediately frozen at -80°C. The section of the remaining cecum was fixed in 10% formaldehyde for subsequent histopathological examination (Ho et al., 2021).

Histopathological

Histopathological analysis was conducted on the formaldehyde-fixed ceca, which were embedded in paraffin. The tissue was sectioned into 5 µm thick slices, deparaffinized, and stained with hematoxylin and eosin (HE). Six specimens per chick were examined at 200 µm intervals to assess parasite load and histological changes using 200× and 400× magnification on a light microscope (Olympus FSX100, Olympus, Tokyo, Japan). This analysis aimed to observe alterations at the cellular level, particularly regarding histological changes associated with *E. tenella* infection.

Gene expression analysis

Once the total RNA is extracted from ceca using Freezol Reagent (Nanjing Vazyme Biotech Co., Ltd), Nanodrop Lite Spectrophotometer (Thermo scientific) was deployed to quantify the RNA concentration. The cDNA was prepared from one microgram of total RNA by reverse transcription with oligo (dT_{18}) primers using the FIREScript RT cDNA Synthesis MIX (Solid Biodyne, Estonia), then diluted with nuclear-free water in the ratio 1:49 according to instruction before storing at -20 ˚C until used

The quantitative real-time polymerase chain reaction (qRT-PCR) was run in the QuantStudioTM 5 Real-Time PCR System (Thermo Fisher Scientific) using the HOT FIREPol® EvaGreen® qPCR Supermix Kit (Solid Biodyne, Estonia). The gene expression levels were first normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) within each sample and subsequently compared to the control group for final normalization. The primer pairs used in these reactions are shown in Table 1. The qRT-PCR was performed with the initial denaturation at 95

˚C (5 min), followed by 40 cycles of 95 ˚C/15 sec and 60 ˚C/1 min (Pham and Hatabu, 2021). Three biological replicates were included for each experimental group. Each replicate represented RNA isolated from the cecal tissue of one individual chicken. These chickens were randomly selected from the infected and control groups to ensure biological variability and minimize sampling bias. Each sample was tested in triplicate on the same reaction plate. The relative mRNA level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Data analysis was performed using SPSS 26.0 software for MacOS. Results were presented as mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Graphs were generated using GraphPad Prism version 10 for MacOS.

Table 1. Sequence of primer pairs used for amplification of target genes

Gene Name	Primer sequences (5' to 3')		Genbank
	Forward	Reverse	Accession No.
Cell markers			
CD3	GGGACCACAGTGACAATCACAT	AGTTTGCACACACTTTGGCAATT	XM_015296812.2
CD4	CAAAAGTGGAGGTGAACGTCGA	ACATGAGCTTCCTCCACGGTAT	NM_204649.1
Cytokines			
IL-10	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC	AJ621614
IL-12	ATGGAACTGTGACCTGGACAT	TGGAATCTGAATAGACTGCTCATCA	XM_015293642.2
IL-18	AGGTGAAATCTGGCAGTGGAAT	TGAAGGCGCGGTGGTTT	XM_015297948.2
IL-22	TCAACTTCCAGCAGCCCTACAT	TGATCTGAGAGCCTGGCCATT	XM_025147965.1
IFN-γ	CACTGACAAGTCAAAGCCGC	ACCTTCTTCACGCCATCAGG	NM_205149.1
TGF-β1	GAGCATTGCCAAGAAGCACC	TGCGGAAGTCGATGTAGAGC	XM_025144453.1
Chemokine			
K203	ACCACGAGCTCCTGACACA	TTAAATGCCCTCCCTACCAC	Y18692
Internal controls			
GAPDH	CAACCCCAATGTCTC	TCAGCAGCAGCCTTCA	NM_204305.1

RESULTS

Gene expression level

The purpose of gene expression analysis is to elucidate the molecular mechanisms underlying the immune response during *E. tenella* infection, allowing for the identification of key regulatory pathways and potential therapeutic targets for enhancing host defense and managing coccidiosis in poultry. In this study, we investigated the expression of various genes associated with T cell markers, specifically cluster of differentiation CD3 and CD4, as well as cytokines such as IL-10, IL-12, IL-18, IL-22, IFN-γ, and the regulatory cytokine TGF-β1. Additionally, we examined the expression of the chemokine K203.

Our findings revealed that the relative expressions of both CD3 and CD4 were elevated in the *E. tenella*-infected group compared to the uninfected controls, with each marker exhibiting approximately a twofold increase in expression levels. However, it is important to note that this difference did not reach statistical significance ($p > 0.05$) (Figure 1). In contrast, the expression of TGF-β1 was significantly elevated in the infected group, with levels increasing by about 3.2 times when compared to the uninfected controls ($p < 0.05$) (Figure 2). Similarly, the expression of IFN-γ was markedly upregulated, demonstrating a significant thirtyfold increase in the infected birds ($p < 0.01$) (Figure 3).

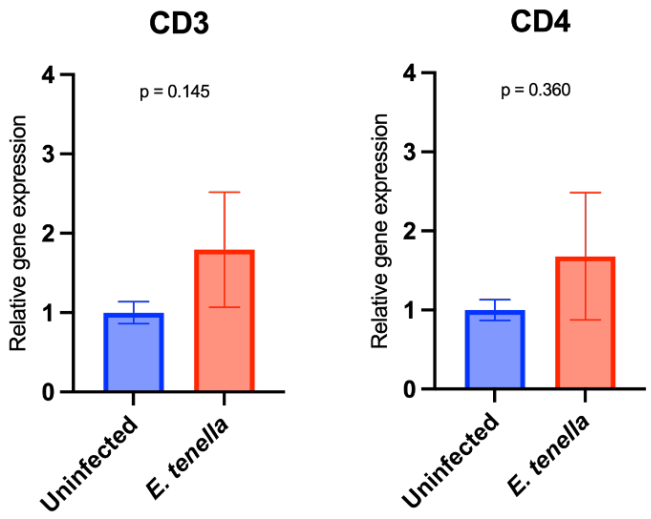

Figure 1 mRNA expression levels of CD3, CD4 in cecal tissues. At 5 dpi, the chickens were euthanized (15 chickens/group), and their cecal tissues were collected for mRNA expression level checking by qRT-PCR.

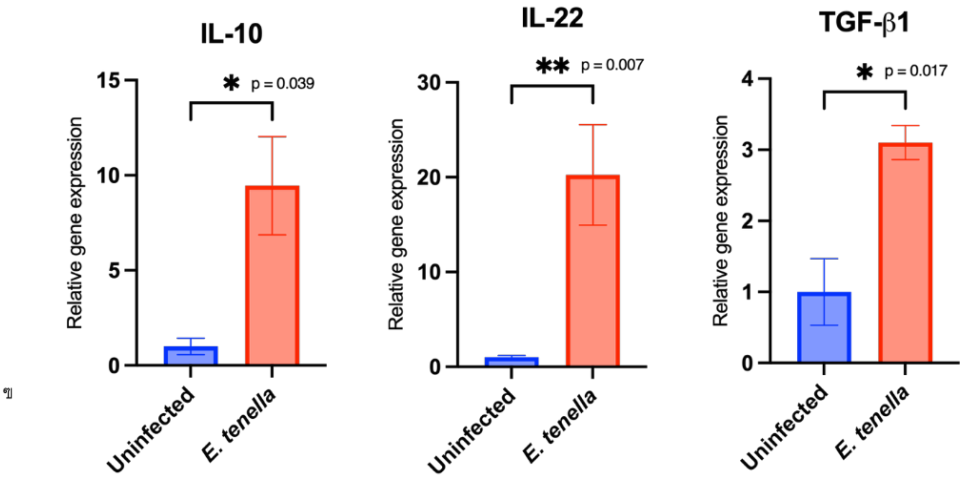

Figure 2 mRNA expression levels of IL-10, IL-22, and TGF-β1 in cecal tissues. At 5 dpi, the chickens were euthanized (15 chickens/group), and their cecal tissues were collected for mRNA expression level checking by qRT-PCR. * $p < 0.05$; ** $p < 0.01$.

Among the various interleukin genes assessed, IL-22 showed the most pronounced elevation, with expression levels approximately thirty times higher in the infected birds ($p < 0.01$) (Figure 2). Furthermore, IL-10 expression was found to be nine times higher in the infected group, this change also reaches statistical significance ($p < 0.05$) (Figure 2). IL-18 and IL-12 also displayed increases in expression (Figure 3); IL-18 exhibited a modest 2.8-fold rise ($p < 0.05$), while IL-12 showed an increase of approximately 2.5 times compared to controls, although this difference was not statistically significant ($p > 0.05$). Collectively, these findings highlight a broad activation of cytokine pathways in response to *E. tenella* infection.

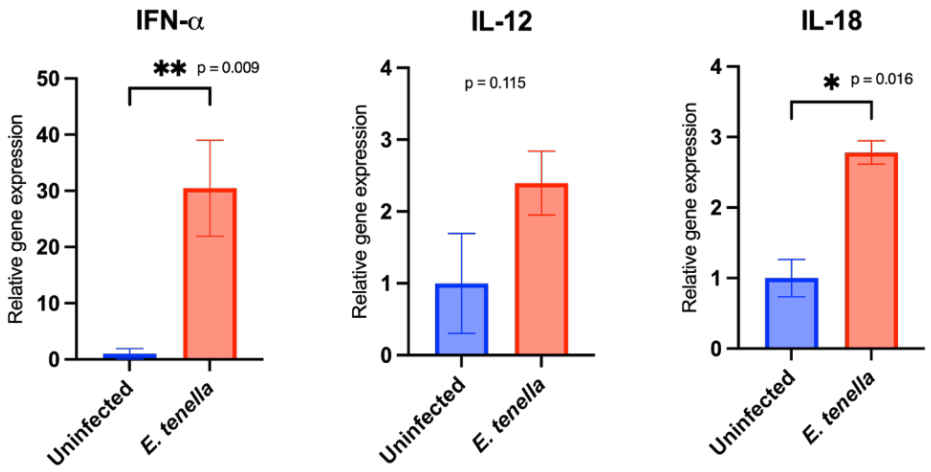

Figure 3 mRNA expression levels of IFN-γ, IL-12, and IL-18 in cecal tissues. At 5 dpi, the chickens were euthanized (15 chickens/group), and their cecal tissues were collected for mRNA expression level checking by qRT-PCR. * $p < 0.05$; ** $p < 0.01$.

In terms of chemokine expression, the relative expression of K203 demonstrated a substantial increase in the infected group (Figure 4), reaching approximately 60 times that of the uninfected controls ($p < 0.001$). This remarkable upregulation of K203 indicates its potential significance in the immune response against *E. tenella* infection, reinforcing the notion that chemokines play a vital role in modulating local immune responses during parasitic infections.

Histopathological observation

The histological figure depicts tissue sections from uninfected and infected group with *E. tenella*, stained with hematoxylin and eosin (H&E) and viewed at 200x and 400x magnification. In the uninfected group, both magnifications show intact epithelial layers and a healthy structure without abnormalities. In contrast, the infected group at 200× shows inflammation with increased cellularity and disrupted epithelial layers, while at 400×, the inflammation is more pronounced, with numerous inflammatory cells such as neutrophils and eosinophils, along with severe epithelial damage, necrosis, and ulceration (Figure 5).

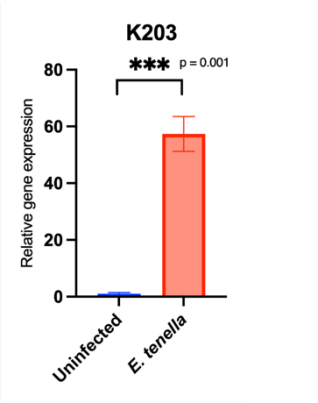

Figure 4 mRNA expression levels of K203 in cecal tissues. At 5 dpi, the chickens were euthanized (15 chickens/group), and their cecal tissues were collected for checking mRNA expression levels by qRT-PCR. *** $p < 0.001$.

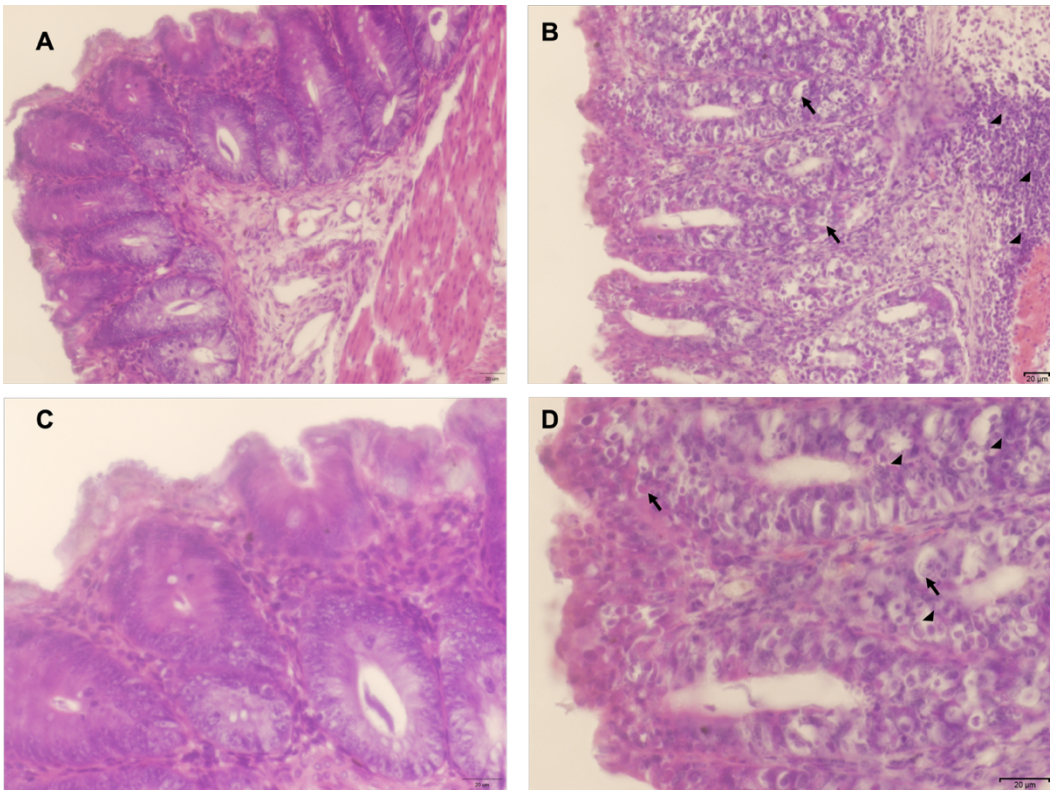

Figure 5 Histopathological examination of H&E-stained cecum sections from infected and control chickens at 5 dpi. Sections were observed under light microscopy at 200× and 400× magnifications. (A) Control group (200×): Normal cecal architecture with intact epithelial lining, well-organized crypts, and no evidence of inflammation or tissue damage. (B) Infected group (200×): Marked disruption of mucosal structure, with extensive immune-cell infiltration in the lamina propria (arrowheads) and presence of intracellular parasites within epithelial cells (arrows). (C) Control group (400×): High-magnification view showing normal mucosal epithelium, intact goblet cells, and absence of inflammatory lesions. (D) Infected group (400×): Severe epithelial degeneration and dense infiltration of mononuclear inflammatory cells (arrowheads). Intracellular parasites are visible within damaged epithelial cells (arrows).

DISCUSSION

Local immune responses are crucial for safeguarding chickens against infections, particularly those instigated by *E. tenella*, a major coccidian parasite that significantly endanger poultry health (Conway and McKenzie, 2008). The regulation of various immune-related genes, including cytokines and other inflammatory markers, is vital for shaping the immune response and influencing the outcome of the infection (Cronkite and Strutt, 2018). Cluster of Differentiation (CD)3 and CD4 are pivotal components in the adaptive immune system, particularly within T cell-mediated responses. The regulation of these markers in response to *E. tenella* infection highlights the intricate interplay between T cell activation and cytokine signaling (Kim et al., 2019). CD3 acts as a crucial signaling component for T cell activation and function (Menon et al., 2023), while CD4 is primarily involved in antigen recognition (Luckheeram et al., 2012). Although no significant differences were observed in the expression of CD3 and CD4, the upward trend noted in the *E. tenella*-infected groups underscores the importance of T cell-mediated immunity in combating this parasite.

Cytokines associated with T helper (Th)1 responses, such as Interferon (IFN)-γ and Interleukin (IL)-12, are predominantly involved in cell-mediated immunity and play a crucial role in controlling intracellular pathogens like *E. tenella* (Kim et al., 2019). IFN-γ is essential for activating macrophages and enhancing antigen presentation (Castro et al., 2018), while IL-12 promotes the differentiation of naive T cells into Th1 cells (Powell et al., 2019). In this study, the significant upregulation of IFN-γ ($p < 0.05$) and a moderate increase in IL-12 expression ($p > 0.05$) suggest a robust Th1 response critical for controlling the infection. These cytokines are fundamental in the clearance of intracellular pathogens, contributing to the immune response against the parasite. However, excessive levels of these genes can lead to heightened inflammation and potential tissue damage (Al-Qahtani et al., 2024).

Additionally, IL-18 serves as a pro-inflammatory cytokine closely linked to the activation of Th1-type responses, amplifying IFN-γ production, which is crucial for the immune response against intracellular pathogens like *E. tenella* (Ihim et al., 2022). The significant upregulation of IL-18 observed in this study ($p < 0.05$) indicates its supportive role in enhancing the Th1 immune response by stimulating further IFN-γ production. This amplification promotes macrophage activation and overall defense against the parasite. The elevated levels of IL-18 align with the upregulated IFN-γ, suggesting a well-coordinated response aimed at clearing the infection.

In contrast, Th2 cytokines, such as IL-10 and IL-22, are generally involved in humoral immunity and anti-inflammatory responses (Alcorn, 2020; Rasquinha et al., 2021). While IL-10 showed a notable increase ($p < 0.05$), implying a more nuanced role, potentially regulating the intensity of the Th1 response to prevent excessive inflammation (Iyer and Cheng, 2012). IL-22, which promotes tissue repair and epithelial barrier integrity, exhibited significant upregulation ($p < 0.01$), indicating its role in mitigating tissue damage caused by the infection (Ouyang and Valdez, 2008; Barone et al., 2015).

Transforming Growth Factor (TGF)-β1 is a regulatory cytokine known for its anti-inflammatory properties and ability to modulate the immune response to prevent excessive tissue damage (Sanjabi et al., 2017). In the context of *E. tenella* infection, the significant upregulation of TGF-β1 ($p < 0.05$) suggests its role as a regulatory mechanism controlling the immune response's intensity, preventing it from becoming overly aggressive and damaging host tissues. While Th1 cytokines like IFN-γ drive the fight against the parasite, TGF-β1 counterbalances this by limiting inflammation and facilitating tissue repair in the gut. This regulation maintains a delicate equilibrium between effectively combating the parasite and minimizing host damage (Huang et al., 2022; Obeagu, 2024).

The pronounced upregulation of IL-22 in response to *E. tenella* infection suggests that vaccine strategies promoting mucosal IL-22 production might be

particularly advantageous. For instance, mucosal or oral vaccine formulations containing adjuvants that specifically boost IL-22 responses in the gut could strengthen epithelial barrier integrity, reduce tissue pathology, and support parasite clearance. This concept aligns with previous findings that oral vaccination tends to stimulate strong local mucosal immunity, including cytokine-mediated epithelial protection, in poultry (Varmuzova et al., 2016). Concurrently, the regulatory role of TGF-β1 implies that fine-tuning—rather than wholesale suppression—of the inflammatory response is critical. Thus, adjuvants or immunomodulators that modulate TGF-β1 expression may allow effective anti-parasite immunity without excessive tissue damage. Recent integrative immunometabolic studies have also highlighted that balanced cytokine regulation is essential for optimal immune and metabolic adaptation during *Eimeria*infection (Fries-Craft et al., 2023).

Moreover, our findings can be contextualized with those of Ho et al. (2023), who reported that varying *E. tenella* infection dosages (10^2–10^5 oocysts) influenced oocyst excretion patterns but had minimal effects on epithelial infection kinetics in the ceca. Their work, supports the concept that local immune activation—including cytokine responses such as IL-22 and TGF-β1 may follow conserved temporal dynamics largely independent of infection dose. This indicates that mucosal immune regulation reflects intrinsic host programs rather than being purely dose-dependent.

In summary, the findings highlight the intricate interplay between pro-inflammatory cytokines like IFN-γ and IL-18, which are significantly upregulate during infection and are vital for activating immune cells against the parasite (Al-Qahtani et al., 2024). At the same time, anti-inflammatory cytokines, such as IL-10, IL-22, and TGF-β1, modulate this immune response, ensuring it remains controlled and does not escalate uncontrollably (Sanjabi et al., 2009). This dynamic balance allows the host to clear the infection while limiting collateral damage to its tissues. Additionally, the regulation of immune responses during *E. tenella* infection underscores the importance of maintaining a delicate balance between effectively combating the parasite and preventing excessive host damage, thereby avoiding complications such as a cytokine storm, which can result in severe inflammation and tissue damage (Al-Qahtani et al., 2024).

Chemokines, such as K203, also known as chemokine (C-C motif) ligand 20 (CCL20), play a pivotal role in recruiting immune cells to the infection site (Skovdahl et al., 2018; Sick et al., 2000). They orchestrate local immune responses by attracting T cells, macrophages, and other immune cells to infected tissues, ensuring a targeted and effective immune reaction (Djeraba et al., 2002). The substantial upregulation of K203 expression in chickens infected with *E. tenella*, reaching approximately 60 times higher than in uninfected controls ($p < 0.001$), emphasizes its critical role in the immune response. Considering the histopathological evidence of heterophil infiltration, the elevated K203 expression likely reflects enhanced recruitment of heterophils and other innate immune cells to the cecal mucosa, contributing to early inflammatory defense mechanisms. This finding aligns with the concurrent increase in pro-inflammatory cytokines such as IFN-γ and IL-18, which together promote local immune activation and containment of the infection. Nonetheless, excessive chemokine expression must be carefully regulated to prevent tissue damage caused by overactive inflammation (Kaňková et al., 2016). Overall, K203 appears to function as a key mediator linking early innate immune recruitment with subsequent adaptive immune modulation during *E. tenella* infection.

Together, the interplay between these cell markers, cytokines, and chemokines demonstrates a coordinated immune response during *E. tenella* infection. Th1 cytokines drive the defense against the parasite, while Th2 cytokines and chemokines assist in modulating the immune response, preventing collateral tissue damage, and promoting repair.

The histological examination of tissue sections from both the uninfected and *E. tenella*-infected groups provides critical insights that correlate with the molecular

findings discussed earlier. In the uninfected group, the histological images reveal intact epithelial layers and a healthy tissue structure, indicating robust gut barrier function. Conversely, the infected group exhibits significant histopathological changes, including inflammation characterized by increased cellularity, disrupted epithelial layers, and the presence of various inflammatory cells such as neutrophils and eosinophils. At higher magnification, the severity of the inflammation is accentuated, showcasing extensive epithelial damage, necrosis, and ulceration. These histological changes align with the observed upregulation of pro-inflammatory cytokines, including IFN-γ and IL-18, as well as the substantial increase in K203 expression associated with chemotactic signals that recruit immune cells to the infection site. The inflammatory response observed histologically reflects the underlying immune mechanisms activated during *E. tenella* infection, suggesting that while the immune response is necessary for controlling the infection, excessive inflammation can lead to significant tissue damage. This highlights the importance of regulatory pathways, such as TGF-β1, in moderating this response and maintaining gut integrity (Sanjabi et al., 2017).

This study provides evidence of transcriptional modulation of immune-related genes in the cecal tissues of chickens experimentally infected with *Eimeria tenella*. The significant upregulation of cytokine genes, including IFN-γ, IL-18, IL-10, IL-22, TGF-β1, and the chemokine K203 (CCL20), indicates a multifaceted immune response involving both pro-inflammatory and regulatory pathways. The observed increase in CD3 and CD4 gene expression, although not statistically significant, suggests possible engagement of T cell-associated responses at the infection site.

Histopathological findings, such as epithelial disruption and inflammatory cell infiltration, correlated with these molecular changes, supporting the presence of active local immune processes during infection. However, because this study focused solely on mRNA expression, further investigation at the protein level (e.g., cytokine quantification or T cell activation markers such as CD69) is necessary to confirm the functional activation and cellular sources of these mediators.

Overall, these results advance our understanding of local immune gene expression patterns in *E. tenella* infection and provide a foundation for future studies aimed at elucidating the cellular mechanisms underlying protective and regulatory immune responses in chickens.

CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

AUTHOR CONTRIBUTIONS

Pham Hoang Son Hung: Conceptualization, methodology, data analysis, writing – original draft.
Tran Nguyen Thao: Laboratory investigation, molecular analysis, writing – review & editing.
Nguyen Thi Hoa: Sample collection, animal handling, histological examination.
Le Dinh Phung: Supervision, project administration, funding acquisition.
Ho Thi Dung: Conceptualization, validation, writing – review & editing, corresponding author.

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