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Research paper

Investigating the role of interleukin 10 on *Eimeria* intestinal pathogenesis in broiler chickens

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ABSTRACT

Eimeria species are intestinal protozoan parasites that cause lack of production, malabsorption and mortality in floor raised chickens. Administering an oral antibody to interleukin 10 (aIL-10) reduces the symptoms of coccidiosis in broilers, indicating interleukin 10 (IL-10) is key to *Eimeria* pathology. IL-10 is an anti-inflammatory cytokine and acts as a stand down signal to reduce inflammation and host pathology during disease. Related protozoan parasites exploit IL-10 to reduce pathogen-damaging host inflammatory responses. We hypothesize that IL-10 is increased during *Eimeria* infection through an unknown host-pathogen interaction, and by feeding aIL-10 to neutralize excess IL-10 the bird is allowed to mount an effective immune response to *Eimeria.* To determine the effects of aIL-10 during the intestinal immune response, intestinal pathology and the relationship between IL-10, interferon gamma (IFNγ) and *Eimeria* infection were evaluated in this study.

In both experiments, broilers were administered either a 10x dose of Advent® *Eimeria* vaccine or saline. Duodenum, jejunum and cecum samples were collected, processed, stained and examined under a microscope. Evaluation of intestinal histomorphology during aIL-10 administration showed minimal differences in birds fed aIL-10 during infection compared to animals fed a control antibody during *Eimeria* infection. To further evaluate aIL-10's positive effect during infection, immunofluorescent histochemistry was performed on chicken intestines days 3-7 post *Eimeria* infection for IL-10 and IFNγ presence in intestinal mucosa in control and infected birds, in regions with and without visible *Eimeria* burden. IL-10 and IFNγ had significant changes between days 4.5-7 post-infection in birds fed aIL-10 compared to animals fed a control antibody. Overall we found that the duodenum had increased IL-10 presence and increased IFNγ presence, and the jejunum and cecum had decreased IL-10 presence and decreased IFNγ presence. These differences in spatial regulation of IL-10 and IFNγ may indicate *Eimeria* species induce slightly different cytokine responses.

1. Introduction

Eimeria spp. are a genus of intestinal parasitic pathogens that cause a disease termed coccidiosis, highly prevalent in floor raised poultry. *Eimeria* infection causes intestinal cell rupture, which leads to reduced production and mortality in both layer and broiler chickens. *Eimeria* infection causes an increase in host intestinal and systemic interleukin 10 (IL-10) levels (Hong et al., 2006a, b; Rochell et al., 2016; Arendt et al., 2016). IL-10 is a potent anti-inflammatory cytokine that acts as a 'stand down' signal for cells of myeloid origin. IL-10 controls the host immune response to limit off-target host cell damage during inflammation by, for example, inhibiting pro-inflammatory cytokines such as IL-12 and interferon gamma (IFNγ), preventing major histocompatibility II expression and suppressing nitric oxide species production (Moore et al., 2001). Protozoan parasites can use IL-10 to downregulate host immunity and reduce pathogen-damaging inflammatory responses (Cyktor and Turner, 2011). We previously found oral IgY antibodies to IL-10 (aIL-10) are effective at alleviating the negative effect of coccidiosis on body weight in broiler chickens (Sand et al., 2016). The benefit of aIL-10 during *Eimeria* infection suggests that *Eimeria* also use host IL-10 to dampen the chicken immune response. The direct relationship between *Eimeria* hijacking host IL-10 to benefit its pathogenesis has not been established.

To delve into the mechanism of aIL-10 as a therapeutic for

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coccidiosis, more needs to be known about IL-10's role during *Eimeria* infection. Specifically, when and where in the intestine IL-10 is increased during infection and how does this affect inflammation. If IL-10 is increased for succinct periods of time after exposure to *Eimeria*, poultry producers can shorten the time aIL-10 is administered after *Eimeria* vaccination to reduce costs associated with treatment. Intestinal luminally-secreted IL-10 levels during *Eimeria* infection were previously evaluated by ELISA, and an increase was found on days 4 in the cecum and 7 in the jejunum compared to uninfected chickens (Arendt et al., 2016).

In this study, we use immunofluorescent histochemistry (IHC) to measure IL-10 and IFNγ in intestinal mucosal microenvironments where *Eimeria* are or are not present compared to uninfected chickens. We chose to use IHC because reviewing our past studies, which used an ELISA to quantify luminal IL-10 protein, as well as related literature in which IFNγ and IL-10 were quantified using qPCR and ELISA, we noted the precise intestinal locations and timing of *Eimeria* induction of host intestinal cytokines was missing. IHC provided us qualitative insight to answer the question: Does *Eimeria* interact with host cells to create a pathogen-beneficial microenvironment enriched with anti-inflammatory cytokines, such as IL-10 and depleted of pro-inflammatory cytokines, such as IFNγ? By evaluating intestinal microenvironments with IHC rather than quantitative measures on whole intestine, we are able to determine if IL-10 production is homogeneously affected throughout the intestinal mucosa or only where *Eimeria* is present.

IL-10 antagonizes the inflammatory response by acting on macrophages and dendritic cells to inhibit macrophage activation and the production of proinflammatory cytokines such as IL-12 and IFNγ (Moore et al., 2001). IFNγ has previously been shown to be important in the protective immune response against *Eimeria*; IFN-γ levels and fecal oocyst shedding were inversely correlated (Dimier-Poisson et al., 2004; Kogut and Lange, 1989; Lillehoj and Choi, 1998; Yun et al., 2000). Therefore, we used IFNγ as a marker to measure the intestinal pro-inflammatory response. We hypothesize that *Eimeria* upregulate the production of IL-10 in surrounding microenvironments to allow for evasion of the host immune response and down regulation of IFNγ.

The increase of IL-10 during *Eimeria* infection and subsequent reduced host immunity hypothetically may aid in parasite replication. However, using aIL-10 as an anti-coccidial does not decrease *Eimeria* oocyst shedding in feces (Sand et al., 2016). The lack of an effect on oocyst shedding provides evidence that *Eimeria* are able to cycle and replicate normally. We hypothesize that aIL-10 allows the chicken to mount an adequate immune response to *Eimeria* and improves intestinal barrier integrity. In this study, measurements of intestinal histomorphology are used to determine if aIL-10 reduces *Eimeria*-associated intestinal damage, such as widened and shortened villi and increased crypt depth (Kim et al., 2017).

2. Materials and methods

Experiments involving chickens were approved by the College of Agricultural and Life Sciences Animal Care Committee at the University of Wisconsin-Madison.

2.1. Intestinal IL-10 and IFNγ mucosal microenvironments during Eimeria infection

2.1.1. Chick experimentation

Sixty-three, one-day-old straight run Cornish rock Cobb broiler chicks from Welp Hatchery (Bancroft, Iowa) were raised on a commercial chick diet until 19 days of age to ensure the presence of mature secondary lymphoid organs. At 19 days of age, chicks were divided into two treatment groups; chicks were randomly selected to be orally administered either *Eimeria* challenge by oral gavage with 10x dose of Advent® coccidia vaccine [infected group, n = 36] (Huvepharma, Sofia, Bulgaria) or phosphate buffered saline [control group, n = 27]. The

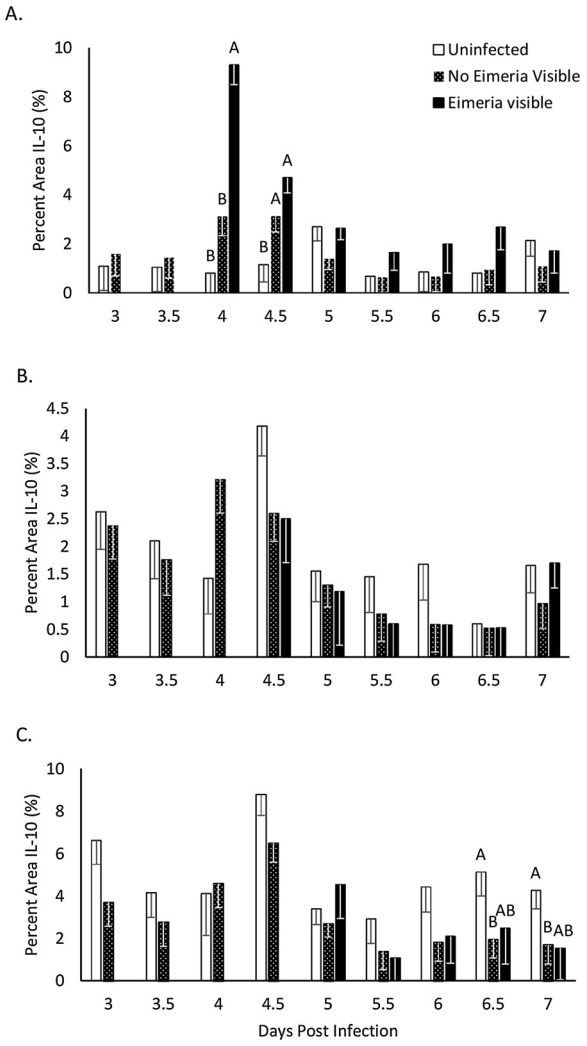

Fig. 1. Interleukin 10 Quantification in Intestinal Sections.
Interleukin 10 quantified with Image J as a percent area of fluorescence of the imaged intestinal section. Chickens were infected at day 19 of age and intestinal sections were collected on every 12 h days 3–7 post infection. *Eimeria* visible samples are not present early in the infection because only gametogony stages of *Eimeria* infection were assessed. Each data point shows the average percent area of IL-10 fluorescence and error bars show SEM, n = 3 uninfected chicks or 4 infected chicks. For intestinal sections that had a significant main effect of *Eimeria* infection on IL-10 presence, the differences of least squared means were used to determine significant differences between infection vs. non-infection values within time points. ^{A,B} Indicates significant difference at $P < 0.05$.

Advent® coccidia vaccine consists of three *Eimeria* species, *E. acervulina, maxima* and *tenella*, which each have tropism to a distinct intestinal section, the duodenum, jejunum and cecum, respectively. Each section of the intestine was collected at 12 h intervals due to the short, 60 min half-life of IL-10 and to accurately assess the three *Eimeria species* (Le et al., 1997). At 12 h intervals between 3–7 days post infection, three uninfected birds and four infected birds were randomly selected for sample collection. Samples were taken from the duodenal loop, jejunum proximal to Meckel's diverticulum, and the blind end of the cecum. The ileum was not collected because *Eimeria* strains used do not infect the ileum. Intestinal segments were fixed in 10% formalin solution before embedded into paraffin wax and sectioned.

2.1.2. Immunohistochemistry staining

Paraffin sections were incubated overnight at 60 °C before being deparaffinized with three changes of Xylene for 10 min each, and rehydrated with isopropyl alcohol at two changes of 100% alcohol, two

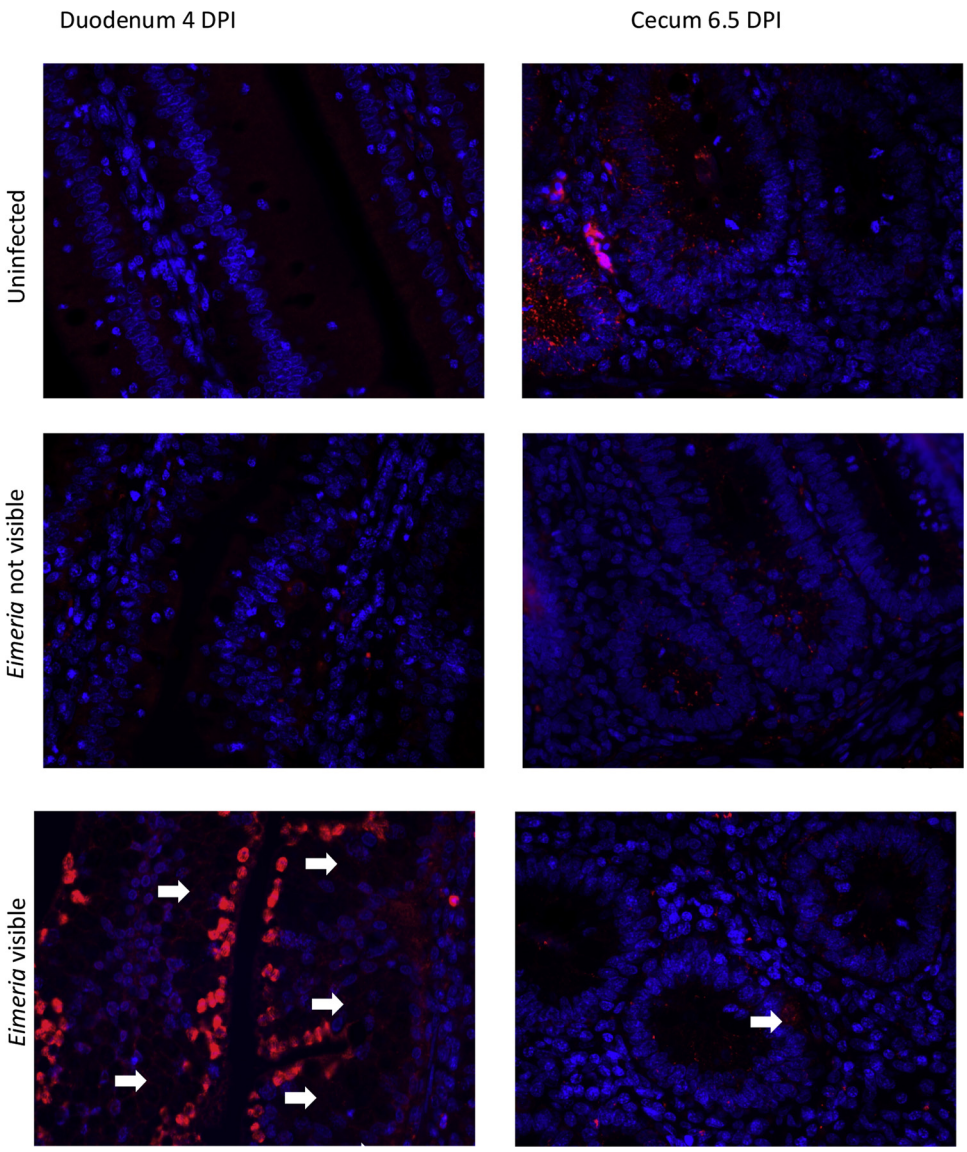

Fig. 2. Interleukin 10 Immunofluorescent Histochemistry Images.
Immunofluorescent histochemistry sections demonstrating IL-10 presence in uninfected and *Eimeria* infected chicks. Chicks at 19 days of age were infected with an avirulent coccivaccine (10X dose) that contained *E. acervulina*, *E. maxima* and *E. tenella*. Images shown are at time points in each section of the intestine when the largest difference of IL-10 presence was evident. Immunohistochemical staining of IL-10 protein (red) and cellular nuclei (blue) in control (top), null (middle) and infected (bottom) chicken mucosa. The intestinal lumen is located at the top of each photo. White arrows indicate areas where *Eimeria* are present.

changes of 95% alcohol, one change of 75% alcohol, and one change of distilled water for 1 min each change. Slides underwent heat induced epitope-retrieval in Tris Urea solution. After rinsing with Tris Buffered Saline solution three times, an ImmEdge ™ Hydrophobic Barrier pen (Vector Laboratories, Inc., Burlingame, CA) was used to isolate tissue sections and slides were submerged with blocking buffer for 1 h in a humidified chamber (Bobeck et al., 2015).

To stain IL-10, tissues were coated in rabbit aIL-10 polyclonal antibody (Bioss Inc., Boston, MA) at 1:300 dilution in blocking buffer overnight. To stain IFNγ, a contiguous intestinal section was coated in rabbit anti- chicken IFNγ polyclonal antibody (My Biosource Inc., San Diego, CA) at 1:100 dilution in blocking buffer overnight at 4 °C in a humidified dark enclosure. Slides were stained with 1:100 diluted Donkey anti-rabbit Dylight®594 (Bethyl, Montgomery, TX) for one hour in a humidified chamber. Nuclei were highlighted by 4′,6-diamidino-2-phenylindole (DAPI) in Fluoro-Gel with tris buffer solution (Electron Microscopy Sciences, Hatfield, PA).

2.1.3. Slide imaging and analysis

Slides were imaged at 200 X with a Nikon Eclipse E600 microscope with Y-FL fluorescence attachment (Nikon Instruments Inc., Melville, NY). *Eimeria* gamete and oocyst wall autofluorescence at 495 nm was used to consistently identify *Eimeria* infected regions (Beer et al., 2018). Three images per intestinal section per bird were taken systematically; one image of the tip, one image of the middle and one image of the crypt, each from three different random villi in a sample. When imaging slides from birds infected with *Eimeria*, three images were taken in a similar manner from villi not containing visible *Eimeria* (null) and three images were taken from villi where *Eimeria* were visible and easily identified (*Eimeria* visible) to evaluate if the microenvironment interaction between *Eimeria* and intestinal cells undergo differing cytokine interactions compared to the surrounding mucosal environment. Image J quantified the percent tissue area of IL-10 and IFNγ presence (Schindelin et al., 2015).

2.1.4. Statistical analysis

The experimental design is a completely randomized design with subsampling. Data analysis was done by a linear mixed effects model on SAS 9.4. For data that had a significant main effect the differences of least squared means were used to determine significant differences between infection vs. non-infection values within time points. Statistical significance was declared at a P-value of ≤ 0.05.

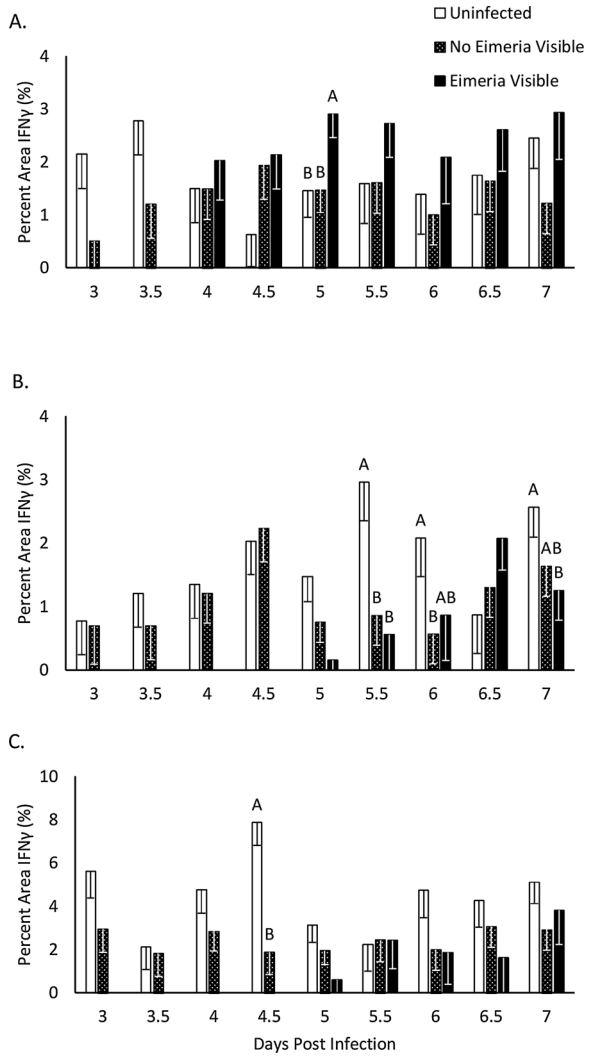

Fig. 3. Interferon Gamma Quantification in Intestinal Sections.
IFNγ quantified with Image J as a percent area of fluorescence of the imaged intestinal section. Chickens were infected at day 19 of age and intestinal sections were collected on every 12 h days 3–7 post infection. *Eimeria* visible samples are not present early in the infection because only gametogony stages of *Eimeria* infection were assessed. Each data point shows the average percent area of IFNγ fluorescence for 3 uninfected chicks or 4 infected chicks. $^{A, B}$ * Indicates significant difference at $P < 0.05$.

2.2. Intestinal histomorphology during coccidiosis with aIL-10 treatment

2.2.1. Chick experimentation

The experiment was set up in a 2 × 2 factorial design. Chicks were either orally gavaged with saline or a 10x dose of coccidia vaccine (Advent ®, consisting of a proprietary blend of live non-attenuated *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts, Huvepharma, Sofia, Bulgaria), on day 3 of life and fed control antibody or aIL-10 throughout the study. Control and IL-10 egg yolk antibody was prepared as previously described, lyophilized and fed at 0.341 g/Kg feed (Arendt et al., 2016; Sand et al., 2016). Thirty-six chicks were randomly separated into nine chicks per treatment, and randomly selected for intestinal sampling on 4, 7 and 13 days post infection (DPI). Intestinal sections were collected from the duodenum, jejunum and ceca and promptly placed in formalin. Intestines underwent paraffin sectioning and hematoxylin and eosin staining to evaluate intestinal morphology.

2.2.2. Intestinal histology measurements

Each sample had measurements taken of villi height, villi width, crypt depth, and muscularis height on an Axio Vert.A1 microscope (Zeiss, Oberkochen, Germany) with QCapture (QImaging, Surrey, British Columbia, Canada). Samples were blinded, and all measurements were taken by the same individual. To obtain an accurate mean, villi height, villi width, and crypt depth were measured three times per sample, and the muscularis height was measured five times per sample.

2.2.3. Statistical analysis

The trial was a single-blinded randomized controlled trial, and collected data were analyzed using ANOVAs PROC MIXED of SAS 9.4 (SAS Institute Inc., Cary, NC). The least significant difference test was used for multiple treatment comparisons using the least squares means statement of SAS 9.4 with letter grouping obtained using the SAS pdmix800 macro (pairwise mean comparisons). For the different statistical tests, significance was declared at a P-value of ≤ 0.05.

3. Results

3.1. Intestinal IL-10 and IFNγ mucosal microenvironments during Eimeria infection

After infection and immunofluorescent staining, we measured the percentage of red pixels for each intestinal section for infected and uninfected birds. In the duodenum, uninfected birds had a narrow range of 0.7 to 2.7 percent area of IL-10 fluorescence. For infected birds, duodenal IL-10 presence remains within this range except for 4 and 4.5 days post infection (DPI). IL-10 presence significantly peaked at 9.3% in microenvironments where *Eimeria* infection was visible on 4 DPI compared to uninfected birds at 0.8% and infected birds where *Eimeria* were not visible at 3.1% ($p < 0.05$, Fig. 1A). Immunofluorescent imaging shows the increased IL-10 appears to be cellular-associated and located along the apical surface of intestinal epithelial cells in regions heavily infected with *Eimeria* (Fig. 2). On 4.5 DPI in the duodenum, IL-10 presence is increased in *Eimeria* infected birds both in regions where *Eimeria* are and are not visible, at 4.7% and 3.1% respectively, compared to uninfected birds at 1.16% ($p < 0.05$, Fig. 1A). In the jejunum, no significant differences in IL-10 presence are observed (Fig. 1B); however, there was greater variation in IL-10 percent area between days, ranging from 0.6 to 4.2%. Similarly, the cecum had a wide range of percent area of IL-10 in uninfected birds from 2.91% on 5.5 DPI and 8.8% on 4.5 DPI. The increase of IL-10 presence in the duodenal mucosa is in contrast to the effect of *Eimeria* infection in the cecum. IL-10 presence is significantly decreased in infected birds where no *Eimeria* are visible on days 6.5 and 7 in the cecum ($p < 0.05$, Fig. 1C). IL-10 presence in the intestinal epithelial cells of uninfected birds is concentrated intracellularly at the crypt (Fig. 2).

IFNγ is increased in *Eimeria* infected duodenal mucosa at 5 DPI in microenvironments where *Eimeria* are visible ($p < 0.05$, Fig. 3A). Immunofluorescent images of *Eimeria* infected regions of the intestine demonstrate IFNγ surrounding *Eimeria* gametocytes and zygotes (Fig. 4). However, regions where *Eimeria* are not visible have an IFNγ presence equivalent to that of an uninfected bird. In the jejunum, IFNγ presence is decreased on days 5.5, 6 and 7 ($p < 0.05$, Fig. 3B). In the cecum, IFNγ presence is significantly decreased only on day 4.5 post infection ($p < 0.05$, Fig. 3C). Similar to IL-10, IFNγ presence in uninfected birds is located within intestinal epithelial cells (Fig. 4). The range of IFNγ percent area of uninfected birds is 0.6–2.8% in the duodenum, 0.8–2.9% in the jejunum and 2.1–7.9% in the cecum. No other significant differences were observed.

3.2. Intestinal histomorphology during coccidiosis with aIL-10 treatment

As a measure of intestinal pathology, we measured villi height, width, crypt depth and muscularis (Fig. 5). Villi height was significantly

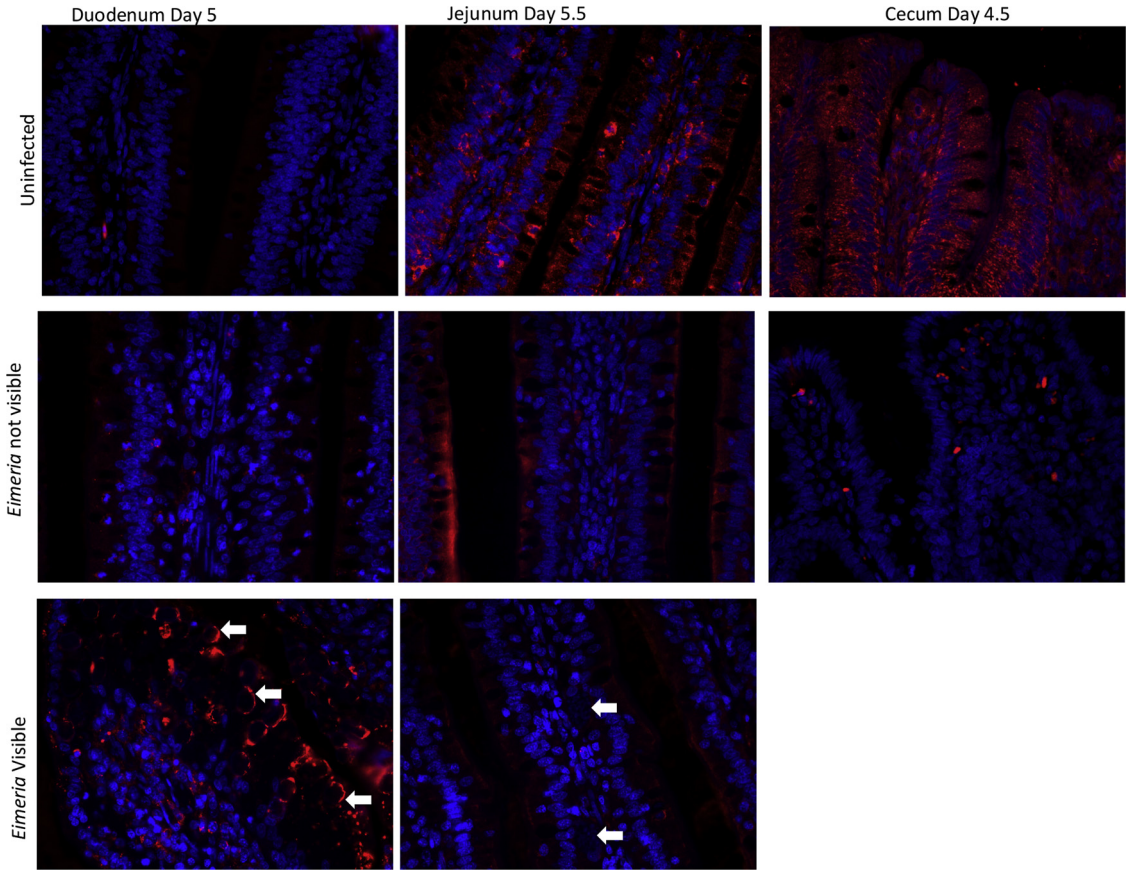

Fig. 4. Interferon Gamma Immunofluorescent Histochemistry Images.
IFNγ presence is quantified using image J to calculate the percent staining of the imaged intestinal section. Chicks were orally gavaged with a 10X dose of an avirulent coccidia vaccine that contained *E. acervulina*, *E. maxima*, and *E. tenella*. Images shown are at time points in each section of the intestine when the largest difference of IFNγ presence was evident. Immunohistochemical staining of IFNγ protein (red) and cellular nuclei (blue) in control (top), null (middle) and infected (bottom) chicken mucosa. White arrows indicate areas where *Eimeria* are present. The intestinal lumen is located at the top of each photo. On day 4.5, identifiable *Eimeria* stages were not visualized in the cecum, therefore only uninfected and null images are shown.

decreased on 4 DPI in aIL-10 fed, uninfected chicks (Fig. 6), but this decrease in villi height is recovered by 7 DPI. While aIL-10 fed uninfected chicks had decreased villi height, aIL-10 fed infected chicks had an even greater decreased villi height (Fig. 6). No significant differences were observed on villi height in the jejunum; however, aIL-10 diet had a positive effect on jejunal villi height on 13 DPI with a mean height of 738.5 μm compared to 582.5 μm in control fed birds ($p < 0.05$). No significant changes were observed in intestinal villi width among any of the treatments (Supplemental Fig. 1).

Eimeria infection increases crypt depth in the duodenum and jejunum at 4 and 13 DPI (Fig. 7). No significant change was seen on 7 DPI. On 13 DPI, the crypt depth of aIL-10, uninfected chicks is 105 μm, which is significantly greater than the crypt depth of control fed chicks at 74 μm ($p < 0.05$). Jejunal muscularis thickness was significantly increased by 43% in *Eimeria* infected chicks regardless of diet treatment on 4 DPI (Fig. 8). On 10 DPI, the duodenal muscularis decreased from 175 μm in uninfected chicks to 153 μm in infected chicks. The cecal muscularis of infected chicks significantly increased in size by 40% compared to uninfected birds. No other significant changes were observed.

4. Discussion

The purpose of this study was to determine the effects of *Eimeria* infection on intestinal IL-10 and IFNγ. We found that IL-10 and IFNγ were both present during infection and both increased in the duodenum compared to uninfected birds. In the jejunum, IFNγ was decreased and both IL-10 and IFNγ were decreased in the cecum at various time points. The decrease in cytokine presence compared to uninfected birds in the jejunum and cecum was not expected; however, it may indicate why there were minimal effects on intestinal histomorphology with aIL-10 treatment during *Eimeria* infection. IFNγ and IL-10 levels increased or decreased in conjunction and were not inversely related as we had hypothesized. Furthermore, our hypothesis that aIL-10 works by increasing IFNγ does not align with the lack of impact of aIL-10 on oocyst production. If aIL-10 supported an increase in intestinal IFNγ, our past production studies would show a decrease in *Eimeria* oocyst production as observed by Yun et al. (2000), but no effect of aIL-10 on oocyst excretion was observed (Sand et al., 2016). If aIL-10 targets luminal IL-10 and not mucosal IL-10, it may have more of an effect on microbes within the lumen rather than intestinal pathology in the jejunum and the cecum.

We previously found an increase in IL-10 on day 7 in the jejunal luminal contents and day 4 in the cecal luminal contents (Arendt et al., 2016). The increase of cytokines in the luminal contents and decrease of cytokines in the mucosa suggests that enterocytes secrete cytokines into the lumen of the intestine in the jejunum and the cecum. The IL-10 receptor is expressed on the apical side of intestinal epithelial cells, and the presence of luminal IL-10 suggests IL-10 has an extracellular role in the lumen of the intestine (Kominsky et al., 2014). The interaction between the microbiome and the mucosal immune system is an emerging field of study, and the effect of luminal cytokines are largely unknown (Shi et al., 2017). Our uninfected samples had variability in cytokine percent area fluorescence day to day despite uniform sampling

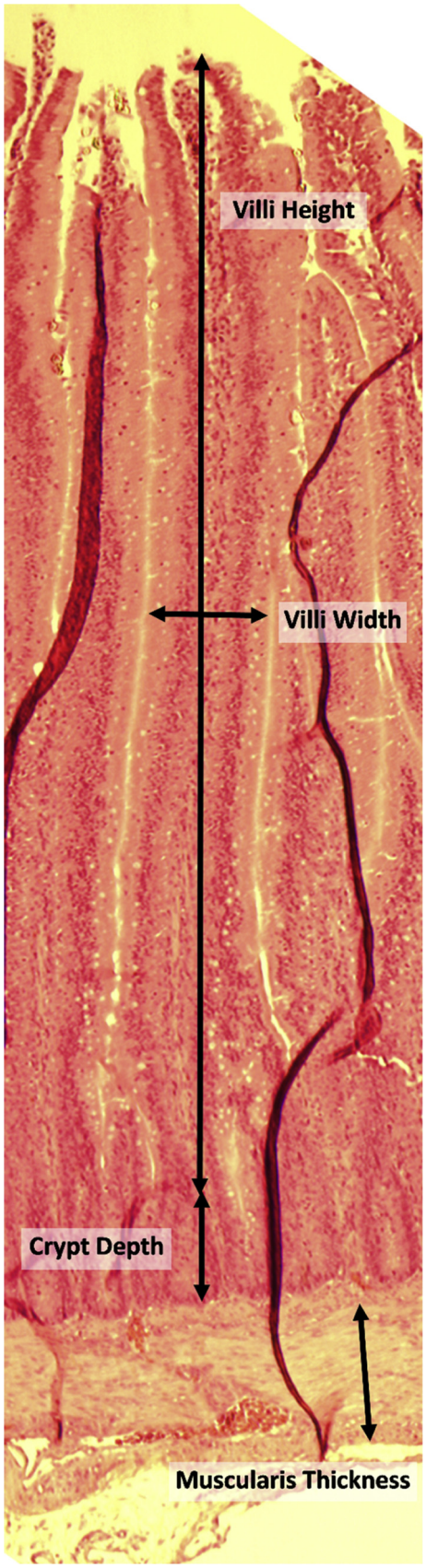

Fig. 5. Intestinal Histomorphology Measurement Diagram.
Intestinal histomorphology measures were taken on villi where the red blood cells could be visualized in the lamina propria and epithelial cell presence at the tip of the villus to ensure measurement of a complete villi. Measurements taken included villus height, villus width, crypt depth, and muscularis thickness.

and processing techniques. The normal variation in uninfected bird intestinal mucosal cytokine presence may be due to differing microbiome interactions.

IL-10 and IFNγ mRNA presence during *Eimeria* infection have been evaluated in previous literature. Each species has been evaluated individually in their respective section of intestine. *Eimeria acervulina* infection of the duodenum results in an increase in IL-10 mRNA on 5 and 6 DPI, and in IFNγ mRNA on 6, 7 and 13 DPI (Rochell et al., 2016; Hong et al., 2006a, Hong, et al. 2006b). *E. maxima* infection of the jejunum causes an increase in IL-10 mRNA on 4, 6 and 9 DPI and in IFNγ on 4, 6 and 9 DPI (Hong et al., 2006a; Rothwell et al., 2004). *E. tenella* infection of the cecum resulted in an increase in IL-10 mRNA on 6, 9 and 10 DPI and in IFNγ on 6, 7, 8 DPI (Hong et al., 2006b; Yun et al., 2000). We found an increase in cytokine presence in the jejunum and cecum in uninfected compared to infected birds. Our results may be different because we measured IL-10 protein in the intestinal epithelial cells, whereas the other studies measured mRNA in intestinal epithelial lymphocytes or intestinal tissue which would include luminal mucus and secretions. Additionally, our research has shown that IL-10 presence is not homogenous in the lumen, mucosa and lamina propria (Arendt et al., 2016).

Intestinal epithelial cell expression of IL-10 is important in maintaining intestinal homeostasis and barrier integrity (Hyun et al., 2015). In uninfected birds IL-10 increased in the distal intestine, which correlates with increased bacterial presence. These differences in baseline epithelial IL-10 levels could play a role in the variation in *Eimeria* species specific immune response, pathology or intestinal section specificity. Differences in cytokine presence observed in each section of the intestinal tract, may be due to either the *Eimeria* species specific immune response as the three *Eimeria* species used in this study have a tropism for different sections of the gut. Differences in cytokine response to each *Eimeria* species were explored by Hong et al. (2006a, 2006b). Another explanation may be that the *E. acervulina* strain in the Advent® vaccine may be more virulent than the *E. maxima* and *E. tenella* vaccine strains and elicits the increase in IL-10 and IFNγ seen in the duodenum. The different cytokine responses observed in distinct regions of the intestine suggests that the intestinal immune response may be compartmentalized to microenviroments of the gut surrounding infection.

Known pathology associated with *Eimeria* infection include shorter villi due to increased intestinal epithelial cell sloughing and widened villi with increased crypt depth due to leukocyte infiltration (Kim et al., 2017). However, we did not see a significant difference in villi height and width due to *Eimeria* infection, because of the use of less pathogenic vaccine *Eimeria* strains to avoid bird mortality. *Eimeria* infection caused an expected increase in crypt depth, which was observed in the duodenum on day 13 post infection and in the jejunum on days 4 and 13 post infection. The increase in crypt depth due to *Eimeria* infection indicates leukocyte infiltration and increased intestinal epithelial cell turnover. aIL-10 had no effect on crypt depth in the duodenum and the jejunum. IL-10 reduces the recruitment of leukocytes, such as neutrophils. We hypothesized that aIL-10 would result in increased leukocyte presence and increased crypt depth, which was seen on day 13 post infection in the cecum, aIL-10 increased crypt depth in uninfected birds when compared to control fed birds (Sun et al., 2009).

aIL-10 had a negative impact on duodenal villi height in uninfected birds at 7 days of age or 4 DPI. This result is likely due to an overdose of aIL-10 because the chicks consume two times their body weight during the first week of life. The decrease in villi height may be due to the effect of aIL-10 on the intestinal microbiota or gut associated lymphoid tissue development. Germ free chickens have shorter and wider villi, while chickens fed beneficial probiotics have longer villi (Pan and Yu, 2014). The mild negative effect in the duodenum of aIL-10 during the first week of life is recovered by day 10 in uninfected birds or at 7 DPI. At 2 weeks of age aIL-10 has a positive impact on villi height in the jejunum. The positive effect of aIL-10 on jejunal villi height on 16 days

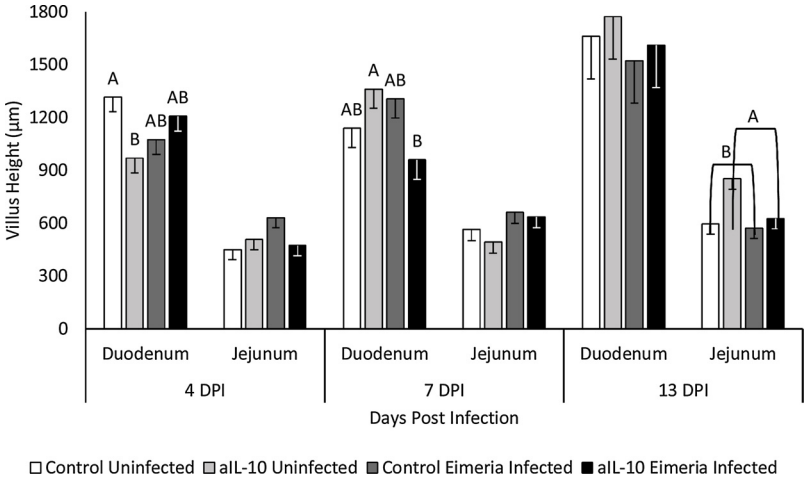

Fig. 6. Villus Height.
Chickens were infected with *Eimeria* at 3 days of age and samples were taken at 4, 7 and 13 DPI. Villus height is measured from tip of villi to start of crypt in micrometers. Cecal measurements are not included because by definition, the ceca lack villi. Each bar represents the mean villus height of 3 chicks. Error bars indicate SEM. [A,B] Indicates significant difference, $P < 0.05$ for each intestinal section and day post infection.

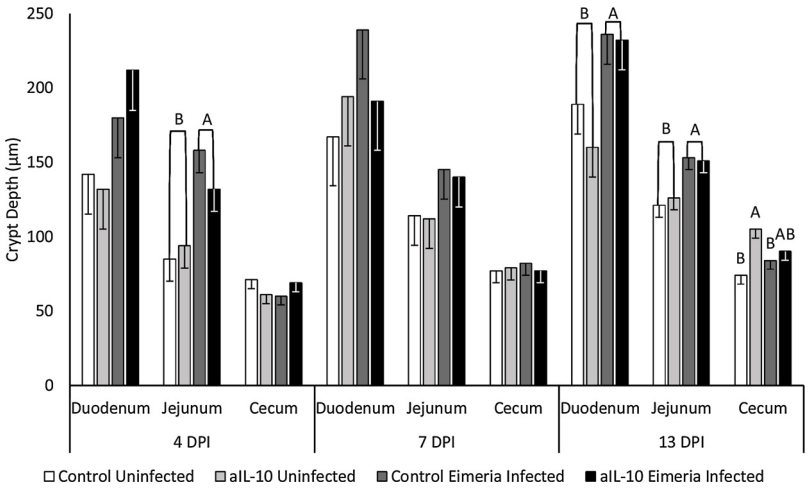

Fig. 7. Crypt Depth.
Chickens were infected with *Eimeria* at 3 days of age and samples were taken at 4, 7 and 13 DPI. Crypt depth is measured from start of crypt to muscularis in micrometers. Each bar represents the mean crypt depth of 3 chicks. Error bars indicate SEM. [A,B] Indicates significant difference, $P < 0.05$ for each intestinal section and day post infection.

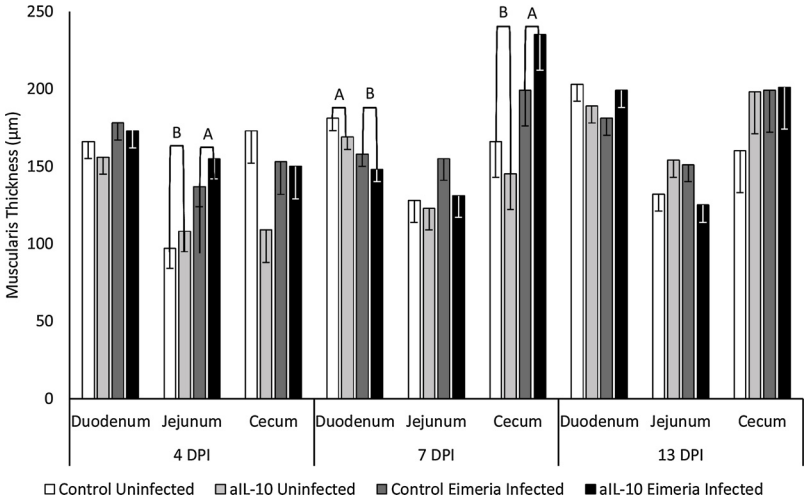

Fig. 8. Muscularis Thickness.
Chickens were infected with *Eimeria* at 3 days of age and samples were taken at 4, 7 and 13 DPI. Muscularis thickness is measured as the thickness of both muscular layers that make up the wall of the intestine. Each bar represents the mean muscularis thickness of 3 chicks. Error bars indicate SEM. [A,B] Indicates significant difference, $P < 0.05$ for each intestinal section and day post infection.

of age regardless of *Eimeria* infection offers support for a beneficial effect of aIL-10 on the gut microbiome. IL-10 is vital to intestinal homeostasis, in fact, IL-$10^{-/-}$ mice are used as a model for inflammatory bowel disease and have decreased villus height to crypt depth ratio compared to wild type mice (Gomes-Santos et al., 2012). In a healthy intestine, IL-10 promotes homeostasis by reducing the presence of pro-inflammatory cytokines, such as Tumor Necrosis Factor alpha (TNFα). TNFα increases intestinal epithelial shedding during intestinal inflammation. An increase in TNFα due to the inactivation of IL-10 by aIL-10, may be why we see a decrease in villi height in the duodenum on day 7 of life in aIL-10 treated, uninfected chicks (Blander, 2016; Oliveira et al., 2014). Overall aIL-10 had limited positive effects on intestinal pathology during *Eimeria* infection, but the increase in villi height at 14 days of age may indicate aIL-10 has beneficial effects on the intestinal microbiome during disease. aIL-10 may aid to prevent the growth of *Clostridium perfringens*, a key organism in *Eimeria* infection sequelae, necrotic enteritis. *Clostridium perfringens* stimulates an increase in IL-10 production in chicken intestinal epithelial cells (Lee

et al., 2018).

This study found that aIL-10 has minimal effects on the intestinal histopathology during *Eimeria* infection. Results from this study, in addition to our previous work, indicate the role of IL-10 during *Eimeria* infection is likely more important in the mucosa in *E. acervulina* as its presence is increased in the mucosa rather than the luminal contents of the duodenum (Arendt et al., 2016). In contrast, during *E. maxima* and *E. tenella* infection, IL-10 presence is increased in luminal contents, rather than mucosal contents. This distinction is important as it may be related to the pathogenicity of each strain. The demonstrated difference in quantity of cytokine in mucosal and luminal samples indicates that cytokines are secreted into the lumen of the intestine and act on apical receptors. This difference underlines the importance of collecting luminal and mucosal samples to gain an accurate understanding of cytokine presence during intestinal infections. Moving forward, the findings presented in this study indicate future research on the microbiome during aIL-10 *Eimeria* infection and the role of intestinal luminal cytokines is needed to improve the understanding of intestinal immunity.

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Declaration of Competing Interest

M. Cook had a patent registration for aIL-10 and had an ownership interest in AbE Discovery, LLC, which has licensed technology reported in this publication. All of the remaining authors have no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109934.

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