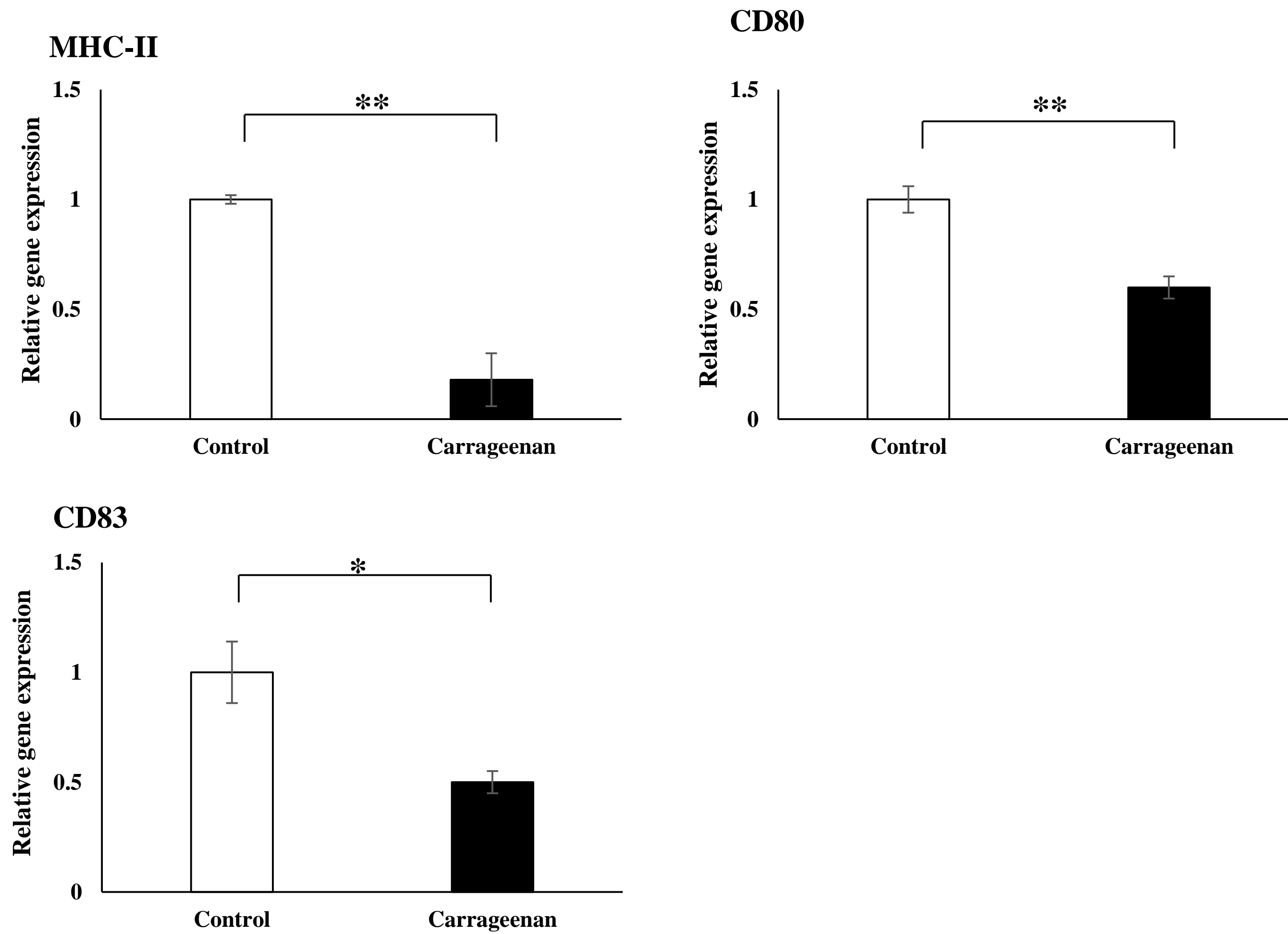
High light

Title: Reduction of macrophages by carrageenan decreases oocyst output and modifies local immune reaction in chick cecum with *Eimeria tenella.*

- Macrophages reduction mitigates the inflammation in ceca of chicken with *E. tenella*
- Macrophages may suppress the other type of immune cells during early infection time
- Macrophages may be helping the parasite proliferation

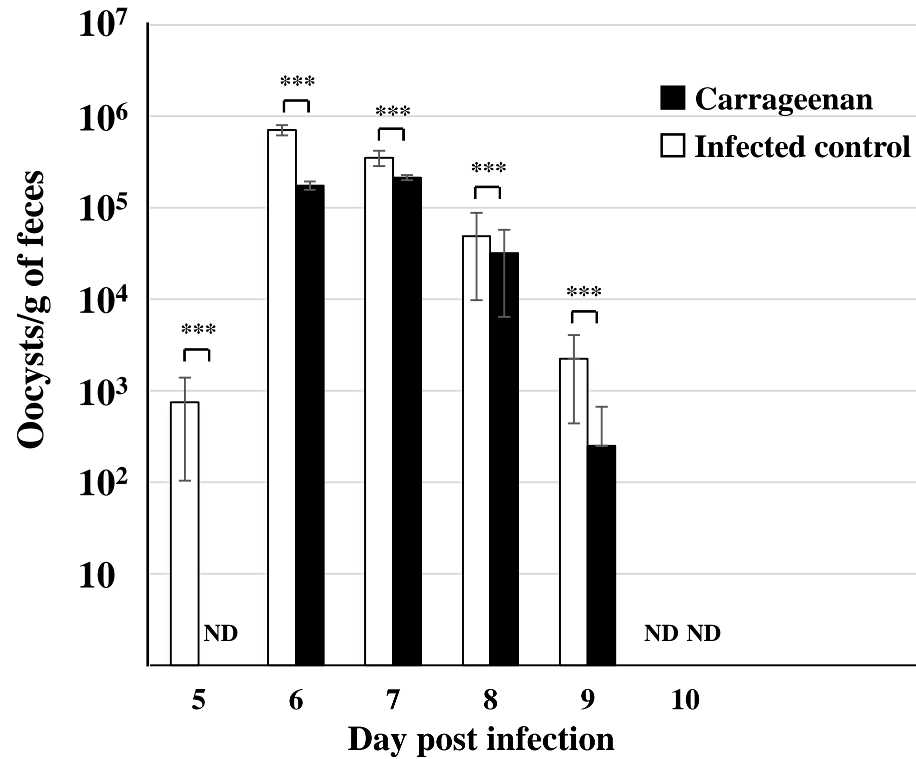
Graph

Ho DT et al., Fig. 1



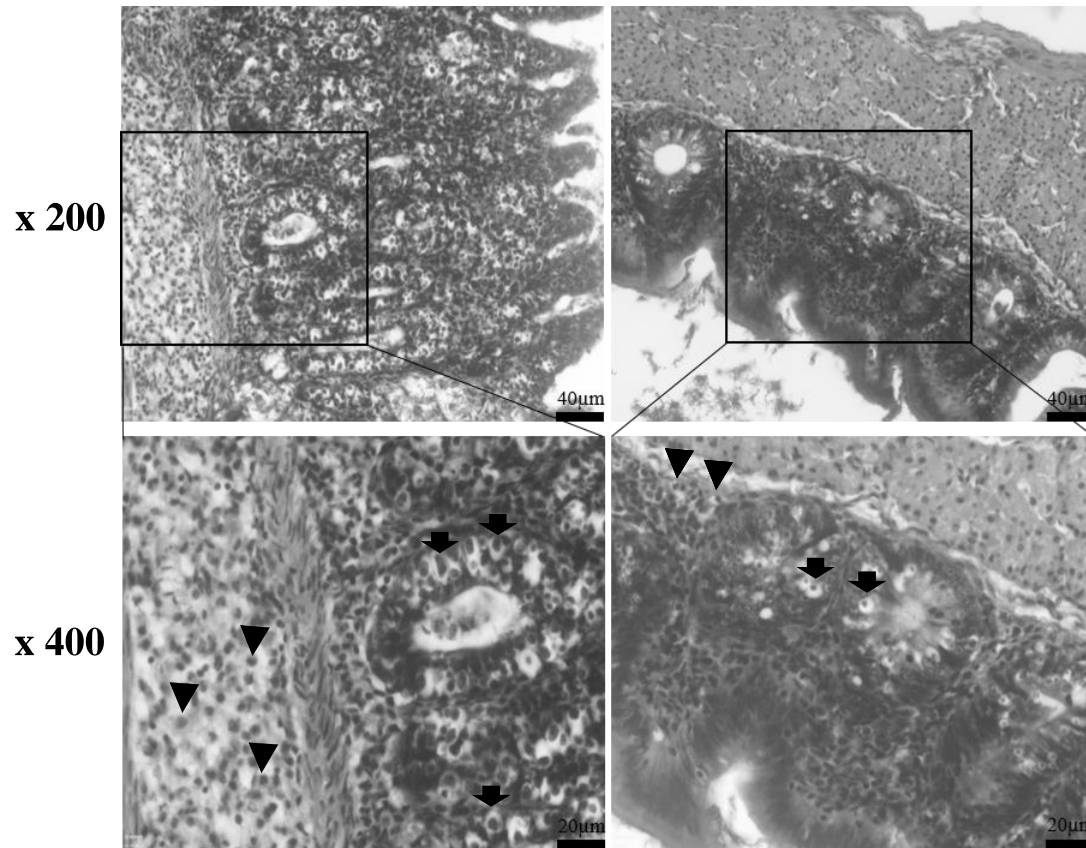
(* p < 0.05, **p < 0.01)

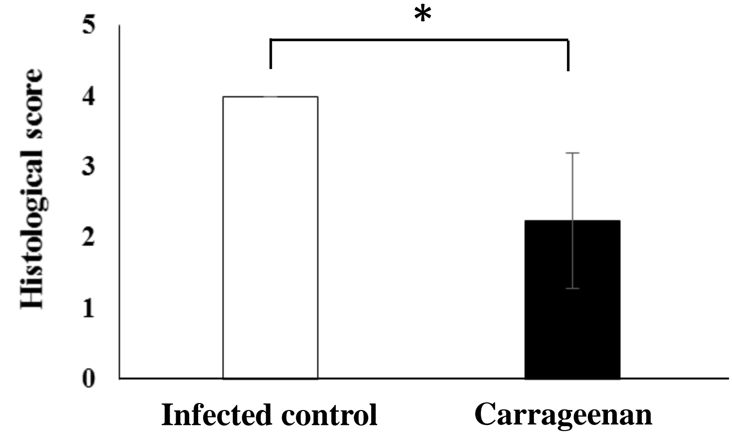
Ho DT et al., Fig. 2

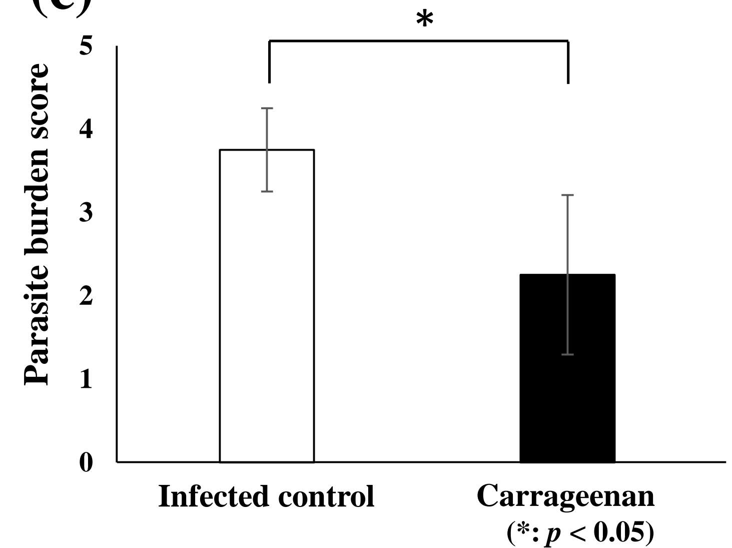


(***: $p < 0.001$, ND: not detected)

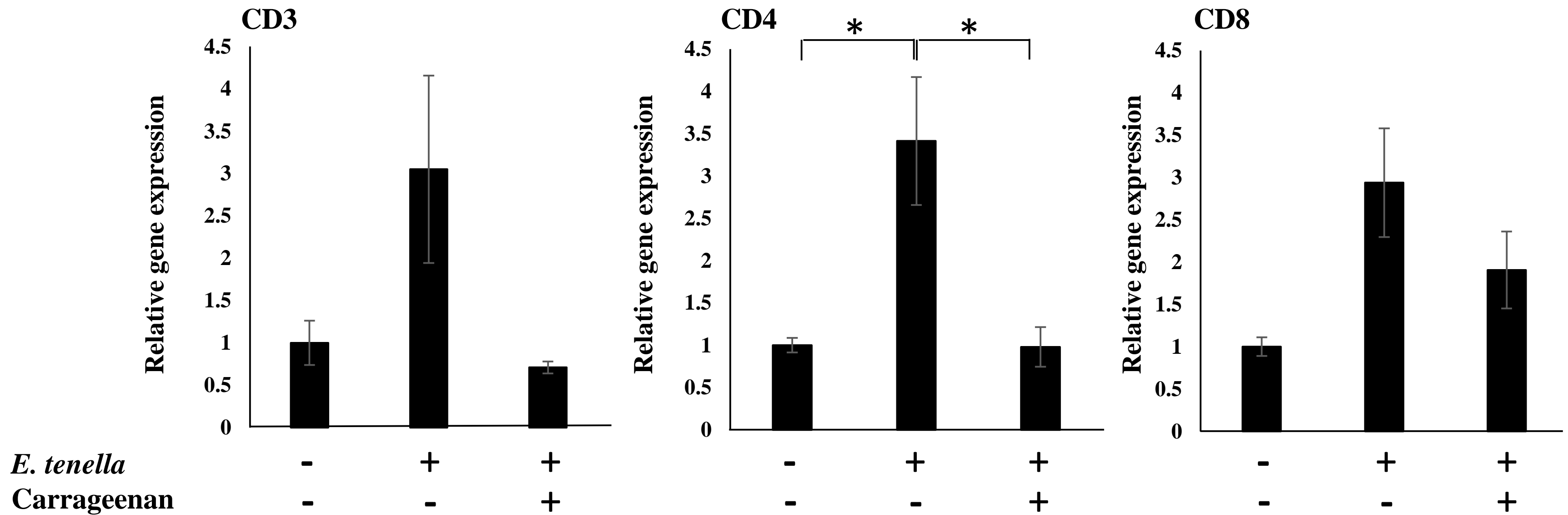
Ho DT et al., Fig. 3





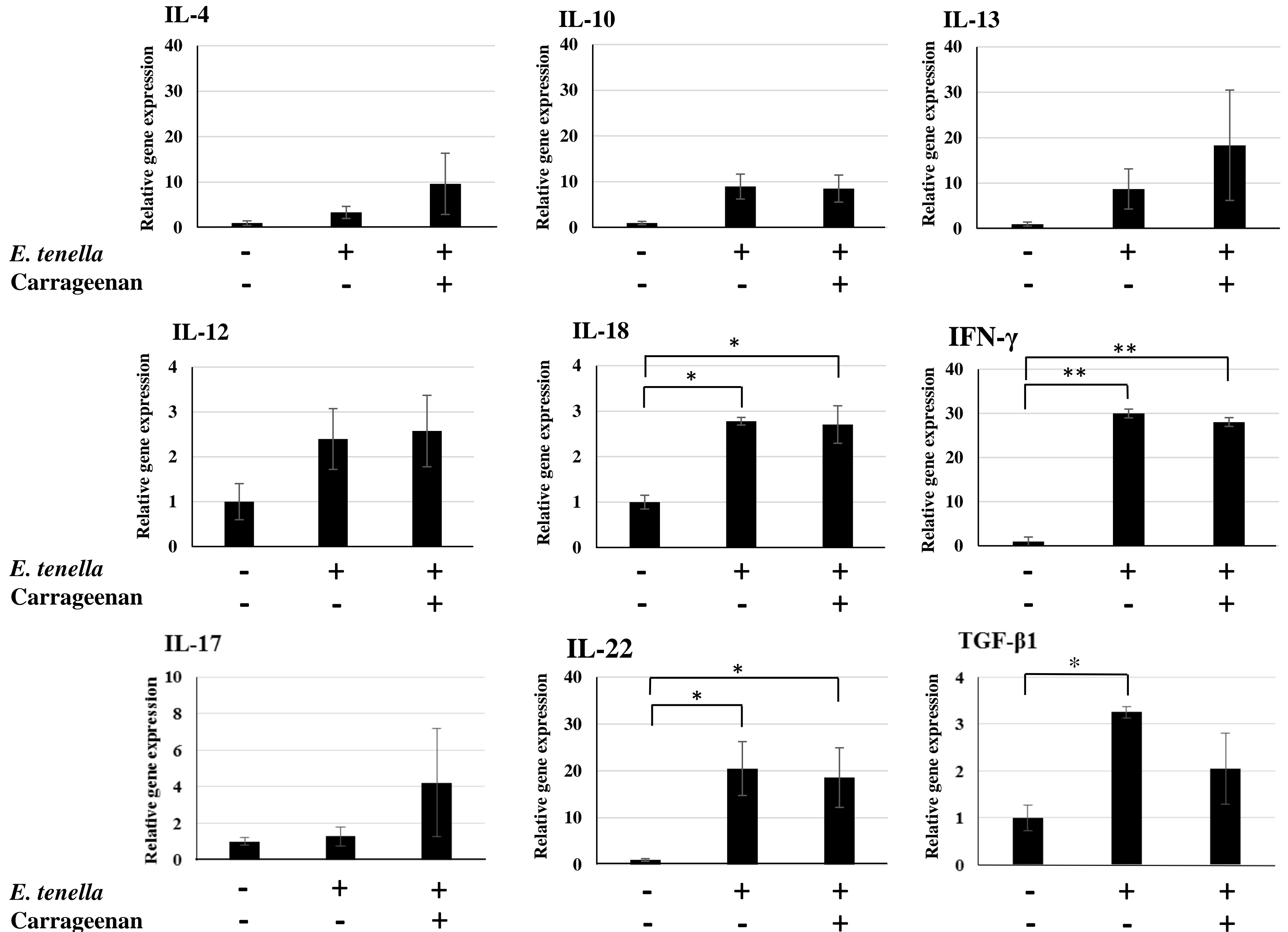


Ho DT et al., Fig. 4

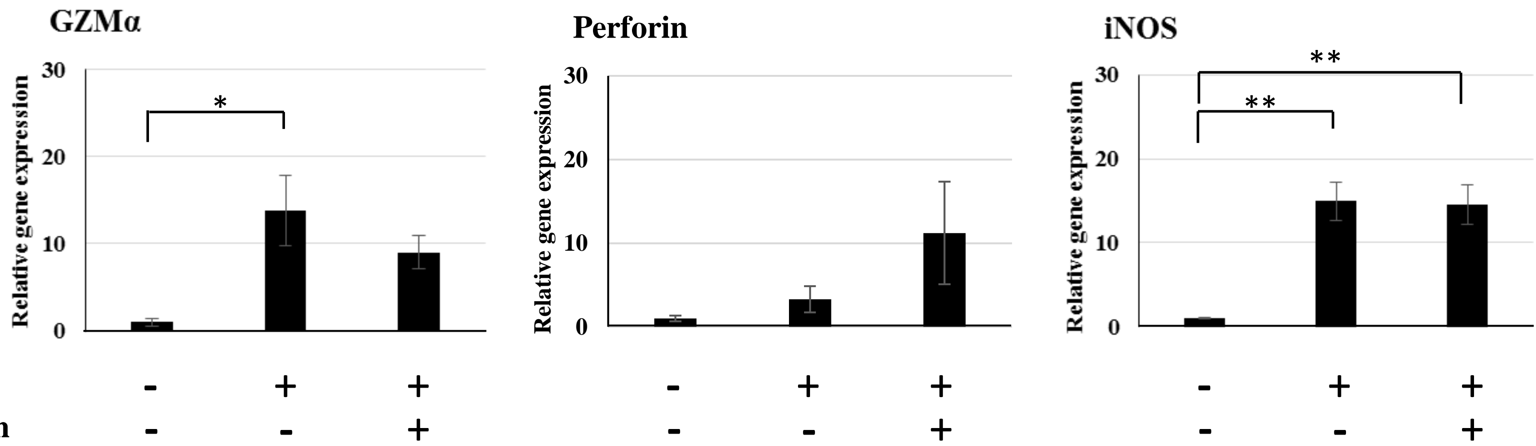


(*: $p < 0.05$)

Ho DT et al., Fig. 5



Ho DT et al., Fig. 6



(*: $p < 0.05$, **: $p < 0.01$)

Table

Table 1. The primer sets for Macrophage Markers

Gene Name	Primer sequences (5' to 3') Forward	Reverse	Accession No.
Macrophage Markers			
CD80	CAGCAAGCCGAACATA	AGCAAACTGGTGGACC	NM_001079739.1
CD83	GCCTACACTCTACTCTTCACCCTG	TATTCTGTCGCCAACTCC	XM_418929.6
MHC-II	GGGGTTTACGACAGCGTCTATT	TTCCGGGTCCCACATCCT	NM_001245061.1
Internal controls			
RPS 17	GGTTGGACAGGCTGCCGAAGT	AAGCTGCAGGAGGAGGAGAGG	NM_204217.1

Table 2. The primer sets for Immune Cell Markers, Cytokines, and Effector Molecules

Gene Name	Primer sequences (5' to 3') Forward	Reverse	Accession No. or References
Immune Cell Markers			
CD3	GGGACCACAGTGACAATCACAT	AGTTTGCACACACTTTGGCAATT	NM_206904
CD4	CAAAAGTGGAGGTGAACGTCGA	ACATGAGCTTCCTCCACGGTAT	NM_204649.1
CD8	CTGCATGGCTCCGACAATGG	ATCGACCACGTCAAGCTGGG	NM_205247.2
Cytokines			
IL-4	GTGCCCACGCTGTGCTTAC	AGGAAACCTCTCCCTGGATGTC	NM_001007079.1
IL-10	GGAGCTGAGGGTGAAGTTTGA	GACACAGACTGGCAGC CAAA	XM_025143715.1
IL-13	CATGACCGACTGCAAGAAGGA	CCGTGCAGGCTCTTCAGACT	NM_001007085
IL-12	ATGGAACTGTGACCTGGACAT	TGGAATCTGAATAGACTGCTCATCA	XM_015293642.2
IL-18	AGGTGAAATCTGGCAGTGGAAT	TGAAGGCGCGGTGGTTT	XM_015297948.2
IFN-γ	CACTGACAAGTCAAAGCCGC	ACCTTCTTCACGCCATCAGG	NM_205149.1
IL-17	TGAAGACTGCCTGAACCA	AGAGACCGATTCCTGATGT	XM_426223.6
IL-22	TCAACTTCCAGCAGCCCTACAT	TGATCTGAGAGCCTGGCCATT	XM_025147965.1
TGF-β1	GAGCATTGCCAAGAAGCACC	TGCGGAAGTCGATGTAGAGC	XM_025144453.1
Effector Molecules			
GZMa	CGCTGTGAAAGTCATTCCCCT	CGTTTCGAGATTTGTCCCCAT	NM_204457.1
Perforin	TGCTGAGAGCCACTTCCACC	ACCTGCCTCTGGTCAGCATG	Xiaoxi et al., 2011
iNOS	ATTCTTATTGGCCCAGGAACAG	GTCACCACCTTTGATCCCTTTC	NM_204961
Internal controls			
GAPDH	CAACCCCAATGTCTC	TCAGCAGCAGCCTTCA	NM_204305.1
RPS 17	GGTTGGACAGGCTGCCGAAGT	AAGCTGCAGGAGGAGGAGAGG	NM_204217.1

1 **Title:** Reduction of macrophages by carrageenan decreases oocyst output and modifies local
2 immune reaction in chick cecum with *Eimeria tenella*
3
4 **Running title:** Roles of macrophages during early *Eimeria tenella* infection
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24 **ABSTRACT**

25 This study aimed to evaluate the disease severity and local immune responses in
26 macrophage-depleted chicks with *Eimeria tenella*. Macrophages were reduced by intraperitoneal
27 injection of a carrageenan solution at 12, 13, and 16 days old, whereas the control group received
28 intraperitoneal phosphate-buffered saline. Both chick groups were orally inoculated with *E. tenella*
29 sporulated oocysts at 14 days old. Feces were collected daily, which were then quantified for
30 oocysts. The chicks were sacrificed on day 5, and the ceca were collected for histopathological
31 observation. The gene expression levels were measured using real-time quantitative reverse
32 transcription-polymerase chain reaction. Macrophage-depleted chicks have been observed to shed
33 a significantly reduced number of fecal oocysts compared to the infected control group. The
34 parasite burden score in cecum specimens of macrophage-depleted chicks was significantly lower
35 than those of infected control on day 5 after infection. Furthermore, macrophage reduction yielded
36 significantly lower cecum histopathological scores and CD4 expression than those of the infected
37 control group. The expression of interleukin (IL)-18, IL-22, interferon-γ, and inducible nitric oxide
38 synthase was also noted to be significantly upregulated in both infected control and macrophage-
39 depleted chicks compared to uninfected chicks. IL-4, IL-13, IL-17, and perforin expressions were
40 also higher with macrophage depletion than in both control groups. These results suggest that
41 macrophages serve as an invasive gate or a transporting vehicle to the site of first merogony.
42 Furthermore, mononuclear phagocytes may play an important role in local immune responses, thus
43 contributing to parasite development during early *E. tenella* infection.

44

45 Keywords: Carrageenan, *Eimeria tenella*, Local immune response, Macrophage.

46

47 **INTRODUCTION**

48 In poultry, avian coccidiosis is considered as the most relevant disease which is caused by
49 intracellular apicomplexan parasites that belong to several different species of *Eimeria* (Dalloul
50 and Lillehoj, 2006). This protozoan parasite exhibits a complex life cycle comprising both
51 extracellular and intracellular stages. After ingesting sporulated oocysts (the exogenous stage of
52 this parasite), sporozoites are released and invade intestinal epithelial cells. The intracellular
53 stages develop inside the host cells, which involves schizogony (asexual reproduction) followed
54 by gametogony (sexual stage). Severe tissue damage may occur after onset of parasite reproduction.
55 As a result diarrhea that may be hemorrhagic in case of *E. tenella* and *E. necatrix* and reduced
56 body weight are observed, resulting in serious economic losses in the poultry industry (Burrell et
57 al., 2019; Daszak, 1999).

58 *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* have been identified as the most
59 frequent species found in chickens (Adriana et al., 2013). Each of these parasites has site
60 specificity; thus, infection occurs in a specific place in the intestines. As for *E. tenella*, it reportedly
61 infects the chick cecum causing severe disease. Immunity to avian coccidiosis can be categorized
62 as a coordinated response of adaptive and innate immunity that involves cell mediated responses
63 (Cornelissen et al., 2009; Hong et al., 2006). Adaptive immunity is specific and is known to
64 regulate antigen-specific responses to prevent the growth of pathogens in the host. Cell-mediated
65 immunity in avian coccidiosis is characterized by antigen-specific or non-specific activation
66 immune cells, such as T cells, natural killer (NK) cells, and macrophages (Lillehoj and Trout,
67 1996). Several researchers have reported that T cells and their associated cytokines play an
68 important role in anticoccidial immunity in chickens (Bumstead et al., 1995; Jeurissen et al., 1996;
69 Lillehoj and Trout, 1996; Vervelde and Jeurissen, 1995). The Th1 immune response seems to be
70 dominant during coccidiosis. Innate immunity is the first line of defense, which includes the

71 recognition of pathogens by phagocytes, such as macrophages and dendritic cells. Immune cells,
72 such as NK cells, dendritic cells, and macrophages are involved in innate immune responses to
73 avian coccidiosis (Dalloul et al., 2007; Shoai et al., 2017). Macrophages and dendritic cells, in
74 particular, are the most important innate immune cells as they can recognize invading pathogens,
75 engulf them, and present antigens to adaptive T cells (Dimier et al., 1998; Shoai et al., 2017).
76 Indeed, extreme macrophage infiltration is observed in the site of parasite reproduction.

77 Several researchers have reported sporozoites in mononuclear cells, including
78 macrophages, in the early infection period of *Eimeria* spp. (Burrell et al., 2019; Challey and Burns,
79 1959; Doran, 1996; Long and Rose, 1976). A study using chicken peritoneal exudate macrophages
80 indicated that sporozoites of *E. tenella* can survive phagocytosis and grow within macrophages
81 until the first schizont generation (Long and Rose, 1976). Based on these observations, it has been
82 hypothesized that macrophages may function as a sporozoite transporter to the lamina propria or
83 epithelial cells located in the intestinal crypt (Challey and Burns, 1959; Doorninck and Becker,
84 1957; Doran, 1996). Other studies have suggested that macrophages may be involved in parasite
85 invasion (Lee and Al-Izzi, 1981; Onago and Ishii 1980; Patton, 1970). Additionally, it remains
86 unknown how the first generation merozoites move or are transported to the lamina propria, the
87 site of second merogony. This study aimed to elucidate the roles of macrophages during *E. tenella*
88 infection by evaluating the disease severity and local immune responses in infected chicks during
89 macrophage depletion.

90

91 **MATERIALS AND METHODS**

92 **Chicks**

93 Eggs (White Leghorn) were purchased from Hisai Poultry Co., Ltd. (Mihara, Hiroshima,
94 Japan). Eggs were then incubated at 37.7 ± 1 °C until hatching. After they hatched, chicks were

95 maintained in a coccidian-free room, given food and water *ad libitum*, and housed at a constant
96 temperature (27 ± 1 °C) with a 12-h dark/light cycle. All procedures were approved by the Animal
97 Care and Use Committee of Okayama University (OKU-2018560) and were conducted in
98 compliance with the Policy on the Care and Use of the Laboratory Animals of Okayama University.

99 **Parasite**

100 The *E. tenella* NIAH strain, which is virulent and maintained at the Laboratory of Animal
101 Physiology, Okayama University (Okayama, Japan), was used herein. *E. tenella* oocysts were
102 purified using the sugar flotation method, sporulated at 28 °C in 2.5 % potassium dichromate, and
103 stored at 4 °C for up to 1 month before use.

104 **Macrophage reduction by carrageenan treatment**

105 Chicks were randomly divided into two groups: carrageenan group and control group
106 [phosphate-buffered saline (PBS)(-) treatment (1 ml/chick)]. ι-Carrageenan was purchased from
107 the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The chicks of each group were reared
108 separately in cages. Carrageenan solution was prepared as described previously (Lee and Al-Izzi,
109 1981). The chicks in the carrageenan group (three chicks) were intraperitoneally inoculated with
110 carrageenan solution (15 mg/ml/chick) at 12, 13, and 16 days old in order to reduce the
111 macrophages. The chicks were then anesthetized and sacrificed by cervical dislocation at 17 days
112 old, and the ceca and spleen were collected. The tissue samples were immediately frozen at -80 °C
113 for gene expression analysis. The total RNA was extracted from the tissues using the RNAzol RT
114 reagent (Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. RNA
115 concentrations were then quantified using a SmartSpec Plus spectrophotometer (Bio-Rad
116 Laboratories, Inc., Tokyo, Japan). Total RNA (1 μg) was subjected to reverse transcription with
117 oligo(dT_{18}) primers using the First Strand cDNA Synthesis kit (Takara Biotechnology, Shiga,
118 Japan) according to the manufacturer's instructions. All cDNA preparations were stored at -20 °C

119 until further use. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-
120 PCR) was conducted in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories) using
121 the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West
122 Cedar Creek, TX). Expression values were then normalized to RPS17 in the same sample and then
123 compared to the PBS(-) control. The sequences of the primer pairs used for qRT-PCR amplification
124 are listed in Table 1. The samples were then heated at 95 °C for 1 min and then subjected to 40
125 cycles of denaturation at 95 °C for 15 s and annealing/elongation for 1 min at 60 °C. Amplifications
126 were performed on four independent samples per group, with triplicate reactions for each sample
127 on the same plate. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and
128 Schmittgen, 2001).

129 **Experimental design**

130 Chicks were randomly divided into three groups: carrageenan (macrophage-depleted)
131 infected, infected control, and uninfected control groups. The chicks in the carrageenan group
132 (eight chicks) were injected with a carrageenan solution as described above. The chicks in the
133 infected control group (eight chicks) were intraperitoneally inoculated with PBS(-) (1 ml/chick).
134 Three uninfected, untreated chicks served as uninfected control. The chicks were orally
135 administered sporulated oocysts of *E. tenella* (1×10^4 oocysts/chick) at 14 days of age and at 5
136 and 10 days post-infection (dpi) ceca were collected. One cecum was immediately frozen at -80 °C
137 for gene expression analysis, the other was separated into three pieces (proximal, medial, and distal
138 regions), each of which was fixed with 10 % formaldehyde for histopathological observation. The
139 experiment was repeated three times.

140 **Fecal collection and oocyst quantification**

141 During 5 to 10 dpi, feces were collected and pooled for each group. Aliquots of 2 g were
142 transferred into 15 ml centrifuge tubes for oocyst quantification (3 tubes for each group). Oocysts

143 per gram of feces (opg) were counted using fecal flotation method with modified McMaster
144 technique (Haug et al, 2006). Briefly, fecal samples (2 g/tube) were mixed thoroughly with 10 ml
145 distilled water, followed by centrifugation at 503 x g for 5 min at room temperature using Sorvall
146 ST8 centrifuge (Thermo Fisher, Rotor No: 7500570). The supernatant was then discarded, and 10
147 ml of a saturated sucrose solution was added to the tubes, mixed thoroughly, and centrifuged at
148 503 x g for 5 min at room temperature. The supernatant was then transferred to other 15 ml
149 centrifuge tubes and mixed well. A drop of the supernatant (10 µl) was placed on a glass slide and
150 covered using cover glass, and the oocysts were counted using light microscopy (three drops per
151 tube). Opg were calculated by the following calculation: opg = n x 500 (n is average number of
152 counted oocyst).

153 **Histopathological observations**

154 The middle part of the formaldehyde-fixed ceca was removed and embedded in paraffin,
155 sectioned at a 6-µm thickness, and deparaffinized (four chicks/group). Sectioned specimens were
156 stained using hematoxylin and eosin (H&E). H&E-stained specimens (six specimens/chick; 200
157 µm intervals) were observed under light microscopy (Olympus FSX100, Olympus, Tokyo, Japan)
158 in order to evaluate the histological score and parasite burden level (eight fields/specimen;
159 magnification, ×200). The ceca inflammation levels were evaluated using a 0 to +4 scoring system
160 as has been described previously (Erben et al., 2014). Parasite burden levels (percentage
161 parasitized epithelium) in the H&E sections were estimated microscopically as described by Allen
162 and Danforth (1998). Histological slides were evaluated by two individual researchers with
163 technical skill in histopathology under blinded conditions.

164 **Gene expression analysis for immune cell markers, cytokines, and effector molecules**

165 At 5dpi ceca were collected (four chicks/*E. tenella*-treated group and three chicks/control
166 group). The procedures for total RNA extraction, cDNA synthesis, and qRT-PCR were conducted

167 in the same way as described above. The expression values were normalized to RPS17 (for immune
168 cell markers) or glyceraldehyde 3-phosphate dehydrogenase (cytokines and effector molecules) in
169 the same sample and then compared to the uninfected control. The sequences of the primer pairs
170 used for qRT-PCR amplification are listed in Table 2. The samples were heated at 95 °C for 1 min
171 and then subjected to 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation for 1
172 min at 60 °C. Amplifications were performed on four independent samples per group, with
173 triplicate reactions for each sample on the same plate. The relative mRNA level was calculated
174 using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

175 **Statistical analysis**

176 Data were expressed as the mean ± standard error of the mean (SEM) or standard deviation
177 (SD) and statistically evaluated by unpaired *t*-test using Kyplot Statistics 5.0 software. $p < 0.05$
178 (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant.

179

180 **RESULTS**

181 **Detection of macrophage reduction**

182 qRT-PCR analysis was used in determining the expression levels of MHC-II, CD80, and
183 CD83 of 16-day-old chicks after carrageenan treatment in the spleen (Fig. 1). As per our findings,
184 the gene expression of MHC-II, CD80, and CD83 was found to be significantly lower in the
185 carrageenan group than in the control group. In particular, MHC-II expression levels in the
186 carrageenan group had about 80 % reduction compared to the PBS(-) control. The results showed
187 that macrophages were successfully suppressed by carrageenan treatment in the chicks.

188 **Fecal oocyst shedding**

189 Fecal oocyst shedding was monitored daily from 5 to 10 dpi (Fig. 2). Oocyst shedding
190 started at 5 dpi in the infected control group and 6 dpi in the carrageenan group. The maximum

191 numbers of fecal oocysts were $2.1 \times 10^6 \pm 1.5 \times 10^5$ oocysts/g feces at 7 dpi for the carrageenan
192 group and $7.1 \times 10^6 \pm 7.3 \times 10^5$ oocysts/g feces at 6 dpi for the infected control group. The number
193 of fecal oocysts in the carrageenan group was found to be significantly lower than that in the
194 infected control group from 5 to 9 dpi ($p < 0.001$).

195 **Histopathological observations**

196 In infected control chicks, infiltrated cells were observed to extend through the mucosa and
197 submucosa, showing marked hyperplasia of the epithelial cells (Fig. 3a). The histological score
198 was lower in the carrageenan group (2.25 ± 0.48) than that in the infected control group (4.00 ±
199 0.00; $p < 0.05$; Fig. 3b). Meanwhile, the parasite burden scores in the carrageenan and infected
200 control groups were 2.25 ± 0.48 and 3.75 ± 0.25 at 5 dpi, respectively. Significant differences arose
201 between parasite burden scores for the carrageenan group and those for the infected control group
202 ($p < 0.05$; Fig. 3c).

203 **Gene expression analysis**

204 qRT-PCR analysis was used to examine the expression of immune cell markers in the ceca
205 among the chick groups (Fig. 4). The gene expression of CD4, but not other immune cell markers
206 examined, was significantly lower in the carrageenan group than in the infected control group.

207 The gene expression levels of cytokines and immune effector molecules are summarized
208 in Figs. 5 and 6. Transcripts of interleukin (IL)-18, IL-22, inducible nitric oxide synthase (iNOS),
209 and interferon-γ (IFN-γ) were significantly higher in both carrageenan and infected control groups
210 compared to uninfected controls (Fig. 5). Transforming growth factor-β1 (TGF-β1; Fig. 5) and
211 granzyme A (Fig. 6) expression was significantly higher in the infected group than in the control
212 group. The expression levels of Th2 cytokines (IL-4 and IL-13) and IL-17 were also higher in the
213 carrageenan group than those of infected control groups but did not reach statistical significance.
214 Perforin was expressed at high levels, albeit not significantly different, in the carrageenan group

215 compared to the infected control group (Fig. 6).

216

217 **DISCUSSION**

218 Mononuclear phagocytes, including macrophages, have been determined as the most
219 important innate immune cells. Doornick et al. have reported the use of macrophages as a vehicle
220 for sporozoite transport in *Eimeria necatrix* infection (Doorninck and Becker, 1957). Another
221 study has indicated that sporozoites of *E. tenella* can survive phagocytosis and grow within
222 macrophages until the first schizont generation (Long and Rose, 1976). However, the role of
223 macrophages in *E. tenella* infection remains to be elucidated. It has been reported that macrophages
224 may be involved in parasite invasion and development in host epithelial cells (Challey, 1959). The
225 data obtained in the present study contribute to the current understanding of macrophage function
226 in *E. tenella* coccidiosis. This study focused on the mononuclear phagocytes, especially
227 macrophages, and evaluated its role during early *E. tenella* infection. Depleting macrophages *in*
228 *vivo* is an adequate strategy to have a first general glimpse on the importance of macrophages in
229 this parasitic disease. Carrageenan was used to deplete macrophages *in vivo* because this chemical
230 is well known to selectively kill about 90 % of the macrophage population 24 h after peritoneal
231 injection in chicken and mice (Ishizaka et al., 1989; Lee and Al-Izzi, 1981). In fact, macrophage
232 markers were significantly reduced in the spleen of carrageenan-treated chicks, proving that
233 macrophages were reduced in the chicks due to carrageenan treatment.

234 The number of shed oocysts and the parasite burden were significantly reduced in
235 carrageenan chicks treated compared to those of the infected control group (Figs. 2 and 3). In the
236 life cycle of *Eimeria* parasites, sporozoites initially invade epithelial cells at the tip of cecal villi
237 and then migrate to the preferred site of development, the crypt epithelium cells, in order to
238 complete their first round of asexual replication (Daszak, 1999; Dimier et al., 2014). However,

239 how the sporozoites reach this first site of reproduction remains unclear as the distances from the
240 ceca fold to crypt epithelial cells are approximately 100 to 200 μm depending on the host (Daszak,
241 1999). A previous report has described the use of macrophages as a vehicle for sporozoite transport
242 in *E. necatrix* infection (Doorninck and Becker, 1957). Our results confirm that macrophages
243 support invasion by *E. tenella* during the early phase of infection and may serve as transport
244 vehicles to the site of first or second merogony.

245 In this study, chickens intraperitoneally inoculated with carrageenan three times (-48, -24,
246 and 48 h after parasite inoculation) showed lower lesion scores and no mortality (data not shown).
247 In contrast, Lee and Al-Izzi (1981) have reported that chickens injected with carrageenan once (at
248 -24, 0, 24, 48, 72, or 96 h after parasite inoculation) or twice (24 h before and 48 h after parasite
249 inoculation) showed higher lesion scores and mortality rates compared to the uninfected control
250 group. The effect of carrageenan on *E. tenella* infection seemed to be greatest when the
251 carrageenan was injected intraperitoneally 24 h before oocyst inoculation, while the effect of
252 treatment after the oocysts were inoculated was deemed minimal or nonexistent. It appears that
253 time of carageenan treatment affects lesion scores and mortality, which may explain differences in
254 observations recorded in our study in comparison to Lee and Al-Izzi (1981). Higher lesion scores
255 and mortality in the latter study may also be due to differences in virulence of the respective
256 parasite strains. Further studies are needed to evaluate these points.

257 T cells are considered as the most essential in terms of protecting against *Eimeria* infection
258 in birds (Lillehoj and Okamura, 2003). Cellular immune responses, especially through Th1 cells
259 and their associated cytokines, play an important role in anticoccidial immunity (Kim et al., 2019).
260 The expression levels of CD4 and Th1 cytokines were significantly higher in the infected control
261 group (Figs. 4 and 5) than those in the macrophage-depleted group and in the control group,
262 although no statistically significant differences between chick groups were observed in other

263 immune cell markers (Fig. 4). These data suggest that the Th1 immune response was induced in
264 infected control chicks by $CD4^+$ T-cell infiltration (Cornelissen, 2009).
265 Furthermore, macrophage depletion induces significantly less inflammation, reduces
266 histological score, and lowers CD4 expression levels compared to the infected control group.
267 Cytokine responses in the ceca of carrageenan-treated chicks appeared to be a mixed Th1/Th2
268 response, and perforin expression was reportedly higher in the carrageenan group than in the
269 infected control group. These results suggest that $CD3^-CD4^-CD8^-$ cells are induced by macrophage
270 depletion and possibly regulate inflammation and local immune response. The γδ T cells have been
271 determined to reside in intraepithelial regions in the mucosa and lymphoid tissue and are associated
272 with CD4 and CD8 double-negative T cells (Fenzl, 2017). These γδ T cells represent up to 50% of
273 peripheral T cells in chickens and are known to expand as a result of pathogen infection (Arstila
274 and Lassila, 1993). In chickens, γδ T cells reside in three populations: $CD8\alpha^-$, $CD8\alpha^-$ low
275 expression, and $CD8\alpha^-$ high expression γδ T cells. Furthermore, γδ T cells are important in the
276 generation of Th2 immunity and as producers of IL-17 and IL-22 (Born et al., 2010; Chien et al.,
277 2014; Martin et al., 2009). In addition, γδ T cells show cytotoxicity against pathogen-infected cells
278 by secreting cytotoxic factors such as perforin (Kristin et al., 2011; Martin et al., 2009). In this
279 study, the level of IL-22 was significantly higher at the site of infection. IL-22 also promotes cell
280 proliferation and plays a role in tissue regeneration (Stange et al., 2012). IL-22 also has a novel
281 antiparasitic role during intestinal intracellular parasite infection in mice (Shoai et al., 2017). In
282 addition, γδ T cells expressing Vγ9 and Vδ2 chains of T-cell receptors represent a nonconventional
283 T-cell subset and control parasite density during malaria infection (Costa, 2011; D'Ombrain et al.,
284 2007). The present results suggest that γδ T cells are induced by macrophage depletion and further
285 regulate parasite reproduction at the site of *E. tenella* infection. Perhaps merozoites released from
286 the first reproductive sites during *E. tenella* infection are killed by γδ T cells, which are induced

287 by macrophage depletion, thereby reducing the parasite burden. Further studies are needed to
288 understand the roles of γδ T cells in the chicks with *E. tenella* under carrageenan treatment
289 conditions.

290 Overall, this study suggests that macrophages serve as a gate for invasion and/or transport
291 vehicle for sporozoites and merozoites to the site of further merogony. Mononuclear phagocytes
292 also play an important role in local immune response, thus contributing to parasite development
293 during early *E. tenella* infection.

294

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301

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396

397 **TABLE CAPTIONS**

398 Table 1. Primer sets for macrophage markers.

399 Table 2. Primer sets for immune cell markers, cytokines, and effector molecules.

400

401 **FIGURE LEGENDS**

402 **Figure 1.** mRNA expression levels of macrophage markers in the spleen of 17 days old chickens
403 treated with carrageenan. Data are presented as mean ± SEM and analyzed by unpaired *t*-test using
404 Kyplot Statistics 5.0 software (*$p < 0.05$; **$p < 0.01$).

405 **Figure 2.** Fecal oocyst shedding during daily from 5 to 10 dpi. Error bars represent SD. ***$p <$
406 0.001 vs. infected control.

407 **Figure 3.** Histopathology of H&E-stained cecum sections at 5 dpi. (a) H&E-stained specimens
408 observed under light microscopy. Arrows indicate intracellular parasites, and arrowheads indicate
409 infiltrating immune cells. (b) Microscopic histopathologic scores (c) Parasite burden score. Data
410 are presented as mean ± SEM. $*p < 0.05$ vs. infected control.

411 **Figure 4.** mRNA expression levels of immune cell markers in the ceca of chicken infected with *E.*
412 *tenella* at 5 dpi. Data are presented as mean ± SEM. $*p < 0.05$.

413 **Figure 5.** mRNA expression levels of cytokines in the ceca of chickens infected with *E. tenella*.
414 Amplifications were performed from four independent samples in each group, with triplicate
415 reactions for each sample. Data are presented as mean ± SEM. ($*p < 0.05$; $**p < 0.01$).

416 **Figure 6.** mRNA expression levels of immune effector molecules in the ceca of chickens infected
417 with *E. tenella*. Amplifications were performed from four independent samples in each group, with
418 triplicate reactions for each sample. Data are presented as mean ± SEM. ($*p < 0.05$; $**p < 0.01$).

1 **Title:** Reduction of macrophages by carrageenan decreases oocyst output and modifies local
2 immune reaction in chick cecum with *Eimeria tenella*
3
4 **Running title:** Roles of macrophages during early *Eimeria tenella* infection
5
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24 **ABSTRACT**

25 This study aimed to evaluate the disease severity and local immune responses in
26 macrophage-depleted chicks with *Eimeria tenella*. Macrophages were reduced by intraperitoneal
27 injection of a carrageenan solution at 12, 13, and 16 days old, whereas the control group received
28 intraperitoneal phosphate-buffered saline. Both chick groups were orally inoculated with *E. tenella*
29 sporulated oocysts at 14 days old. Feces were collected daily, which were then quantified for
30 oocysts. The chicks were sacrificed on day 5, and the ceca were collected for histopathological
31 observation. The gene expression levels were measured using real-time quantitative reverse
32 transcription-polymerase chain reaction. Macrophage-depleted chicks have been observed to shed
33 a significantly reduced number of fecal oocysts compared to the infected control group. The
34 parasite burden score in cecum specimens of macrophage-depleted chicks was significantly lower
35 than those of infected control on day 5 after infection. Furthermore, macrophage reduction yielded
36 significantly lower cecum histopathological scores and CD4 expression than those of the infected
37 control group. The expression of interleukin (IL)-18, IL-22, interferon-γ, and inducible nitric oxide
38 synthase was also noted to be significantly upregulated in both infected control and macrophage-
39 depleted chicks compared to uninfected chicks. IL-4, IL-13, IL-17, and perforin expressions were
40 also higher with macrophage depletion than in both control groups. These results suggest that
41 macrophages serve as an invasive gate or a transporting vehicle to the site of first merogony.
42 Furthermore, mononuclear phagocytes may play an important role in local immune responses, thus
43 contributing to parasite development during early *E. tenella* infection.

44

45 Keywords: Carrageenan, *Eimeria tenella*, Local immune response, Macrophage.

46

47 **INTRODUCTION**

48 In poultry, avian coccidiosis is considered as the most relevant disease which is caused by
49 intracellular apicomplexan parasites that belong to several different species of *Eimeria* (Dalloul
50 and Lillehoj, 2006). This protozoan parasite exhibits a complex life cycle comprising both
51 extracellular and intracellular stages. After ingesting sporulated oocysts (the exogenous stage of
52 this parasite), sporozoites are released and invade intestinal epithelial cells. The intracellular
53 stages develop inside the host cells, which involves schizogony (asexual reproduction) followed
54 by gametogony (sexual stage). Severe tissue damage may occur after onset of parasite reproduction.
55 As a result diarrhea that may be hemorrhagic in case of *E. tenella* and *E. necatrix* and reduced
56 body weight are observed, resulting in serious economic losses in the poultry industry (Burrell et
57 al., 2019; Daszak, 1999).

58 *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* have been identified as the most
59 frequent species found in chickens (Adriana et al., 2013). Each of these parasites has site
60 specificity; thus, infection occurs in a specific place in the intestines. As for *E. tenella*, it reportedly
61 infects the chick cecum causing severe disease. Immunity to avian coccidiosis can be categorized
62 as a coordinated response of adaptive and innate immunity that involves cell mediated responses
63 (Cornelissen et al., 2009; Hong et al., 2006). Adaptive immunity is specific and is known to
64 regulate antigen-specific responses to prevent the growth of pathogens in the host. Cell-mediated
65 immunity in avian coccidiosis is characterized by antigen-specific or non-specific activation
66 immune cells, such as T cells, natural killer (NK) cells, and macrophages (Lillehoj and Trout,
67 1996). Several researchers have reported that T cells and their associated cytokines play an
68 important role in anticoccidial immunity in chickens (Bumstead et al., 1995; Jeurissen et al., 1996;
69 Lillehoj and Trout, 1996; Vervelde and Jeurissen, 1995). The Th1 immune response seems to be
70 dominant during coccidiosis. Innate immunity is the first line of defense, which includes the

71 recognition of pathogens by phagocytes, such as macrophages and dendritic cells. Immune cells,
72 such as NK cells, dendritic cells, and macrophages are involved in innate immune responses to
73 avian coccidiosis (Dalloul et al., 2007; Shoai et al., 2017). Macrophages and dendritic cells, in
74 particular, are the most important innate immune cells as they can recognize invading pathogens,
75 engulf them, and present antigens to adaptive T cells (Dimier et al., 1998; Shoai et al., 2017).
76 Indeed, extreme macrophage infiltration is observed in the site of parasite reproduction.
77 Several researchers have reported sporozoites in mononuclear cells, including
78 macrophages, in the early infection period of *Eimeria* spp. (Burrell et al., 2019; Challey and Burns,
79 1959; Doran, 1996; Long and Rose, 1976). A study using chicken peritoneal exudate macrophages
80 indicated that sporozoites of *E. tenella* can survive phagocytosis and grow within macrophages
81 until the first schizont generation (Long and Rose, 1976). Based on these observations, it has been
82 hypothesized that macrophages may function as a sporozoite transporter to the lamina propria or
83 epithelial cells located in the intestinal crypt (Challey and Burns, 1959; Doorninck and Becker,
84 1957; Doran, 1996). Other studies have suggested that macrophages may be involved in parasite
85 invasion (Lee and Al-Izzi, 1981; Onago and Ishii 1980; Patton, 1970). Additionally, it remains
86 unknown how the first generation merozoites move or are transported to the lamina propria, the
87 site of second merogony. This study aimed to elucidate the roles of macrophages during *E. tenella*
88 infection by evaluating the disease severity and local immune responses in infected chicks during
89 macrophage depletion.
90
91 **MATERIALS AND METHODS**
92 **Chicks**
93 Eggs (White Leghorn) were purchased from Hisai Poultry Co., Ltd. (Mihara, Hiroshima,
94 Japan). Eggs were then incubated at 37.7 ± 1 °C until hatching. After they hatched, chicks were

95 maintained in a coccidian-free room, given food and water *ad libitum*, and housed at a constant
96 temperature (27 ± 1 °C) with a 12-h dark/light cycle. All procedures were approved by the Animal
97 Care and Use Committee of Okayama University (OKU-2018560) and were conducted in
98 compliance with the Policy on the Care and Use of the Laboratory Animals of Okayama University.

99 **Parasite**

100 The *E. tenella* NIAH strain, which is virulent and maintained at the Laboratory of Animal
101 Physiology, Okayama University (Okayama, Japan), was used herein. *E. tenella* oocysts were
102 purified using the sugar flotation method, sporulated at 28 °C in 2.5 % potassium dichromate, and
103 stored at 4 °C for up to 1 month before use.

104 **Macrophage reduction by carrageenan treatment**

105 Chicks were randomly divided into two groups: carrageenan group and control group
106 [phosphate-buffered saline (PBS)(-) treatment (1 ml/chick)]. ι-Carrageenan was purchased from
107 the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The chicks of each group were reared
108 separately in cages. Carrageenan solution was prepared as described previously (Lee and Al-Izzi,
109 1981). The chicks in the carrageenan group (three chicks) were intraperitoneally inoculated with
110 carrageenan solution (15 mg/ml/chick) at 12, 13, and 16 days old in order to reduce the
111 macrophages. The chicks were then anesthetized and sacrificed by cervical dislocation at 17 days
112 old, and the ceca and spleen were collected. The tissue samples were immediately frozen at -80 °C
113 for gene expression analysis. The total RNA was extracted from the tissues using the RNAzol RT
114 reagent (Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer’s instructions. RNA
115 concentrations were then quantified using a SmartSpec Plus spectrophotometer (Bio-Rad
116 Laboratories, Inc., Tokyo, Japan). Total RNA (1 μg) was subjected to reverse transcription with
117 oligo(dT_{18}) primers using the First Strand cDNA Synthesis kit (Takara Biotechnology, Shiga,
118 Japan) according to the manufacturer’s instructions. All cDNA preparations were stored at -20 °C

until further use. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted in the Mini Opticon Real-Time PCR System (Bio-Rad Laboratories) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix Kit (Agilent Technologies, West Cedar Creek, TX). Expression values were then normalized to RPS17 in the same sample and then compared to the PBS(-) control. The sequences of the primer pairs used for qRT-PCR amplification are listed in Table 1. The samples were then heated at 95 °C for 1 min and then subjected to 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation for 1 min at 60 °C. Amplifications were performed on four independent samples per group, with triplicate reactions for each sample on the same plate. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Experimental design

Chicks were randomly divided into three groups: carrageenan (macrophage-depleted) infected, infected control, and uninfected control groups. The chicks in the carrageenan group (eight chicks) were injected with a carrageenan solution as described above. The chicks in the infected control group (eight chicks) were intraperitoneally inoculated with PBS(-) (1 ml/chick). Three uninfected, untreated chicks served as uninfected control. The chicks were orally administered sporulated oocysts of *E. tenella* (1×10^4 oocysts/chick) at 14 days of age and at 5 and 10 days post-infection (dpi) ceca were collected. One cecum was immediately frozen at -80 °C for gene expression analysis, the other was separated into three pieces (proximal, medial, and distal regions), each of which was fixed with 10 % formaldehyde for histopathological observation. The experiment was repeated three times.

Fecal collection and oocyst quantification

During 5 to 10 dpi, feces were collected and pooled for each group. Aliquots of 2 g were transferred into 15 ml centrifuge tubes for oocyst quantification (3 tubes for each group). Oocysts

143 per gram of feces (opg) were counted using fecal flotation method with modified McMaster

144 technique (Haug et al, 2006). Briefly, fecal samples (2 g/tube) were mixed thoroughly with 10 ml

145 distilled water, followed by centrifugation at 503 x g for 5 min at room temperature using Sorvall

146 ST8 centrifuge (Thermo Fisher, Rotor No: 7500570). The supernatant was then discarded, and 10

147 ml of a saturated sucrose solution was added to the tubes, mixed thoroughly, and centrifuged at

148 503 x g for 5 min at room temperature. The supernatant was then transferred to other 15 ml

149 centrifuge tubes and mixed well. A drop of the supernatant (10 µl) was placed on a glass slide and

150 covered using cover glass, and the oocysts were counted using light microscopy (three drops per

151 tube). Opg were calculated by the following calculation: opg = n x 500 (n is average number of

152 counted oocyst).

153 **Histopathological observations**

154 The middle part of the formaldehyde-fixed ceca was removed and embedded in paraffin,

155 sectioned at a 6-µm thickness, and deparaffinized (four chicks/group). Sectioned specimens were

156 stained using hematoxylin and eosin (H&E). H&E-stained specimens (six specimens/chick; 200

157 µm intervals) were observed under light microscopy (Olympus FSX100, Olympus, Tokyo, Japan)

158 in order to evaluate the histological score and parasite burden level (eight fields/specimen;

159 magnification, ×200). The ceca inflammation levels were evaluated using a 0 to +4 scoring system

160 as has been described previously (Erben et al., 2014). Parasite burden levels (percentage

161 parasitized epithelium) in the H&E sections were estimated microscopically as described by Allen

162 and Danforth (1998). Histological slides were evaluated by two individual researchers with

163 technical skill in histopathology under blinded conditions.

164 **Gene expression analysis for immune cell markers, cytokines, and effector molecules**

165 At 5dpi ceca were collected (four chicks/*E. tenella*-treated group and three chicks/control

166 group). The procedures for total RNA extraction, cDNA synthesis, and qRT-PCR were conducted

167 in the same way as described above. The expression values were normalized to RPS17 (for immune
168 cell markers) or glyceraldehyde 3-phosphate dehydrogenase (cytokines and effector molecules) in
169 the same sample and then compared to the uninfected control. The sequences of the primer pairs
170 used for qRT-PCR amplification are listed in Table 2. The samples were heated at 95 °C for 1 min
171 and then subjected to 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation for 1
172 min at 60 °C. Amplifications were performed on four independent samples per group, with
173 triplicate reactions for each sample on the same plate. The relative mRNA level was calculated
174 using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

175 **Statistical analysis**

176 Data were expressed as the mean ± standard error of the mean (SEM) or standard deviation
177 (SD) and statistically evaluated by unpaired *t*-test using Kyplot Statistics 5.0 software. $p < 0.05$
178 (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant.

179

180 **RESULTS**

181 **Detection of macrophage reduction**

182 qRT-PCR analysis was used in determining the expression levels of MHC-II, CD80, and
183 CD83 of 16-day-old chicks after carrageenan treatment in the spleen (Fig. 1). As per our findings,
184 the gene expression of MHC-II, CD80, and CD83 was found to be significantly lower in the
185 carrageenan group than in the control group. In particular, MHC-II expression levels in the
186 carrageenan group had about 80 % reduction compared to the PBS(-) control. The results showed
187 that macrophages were successfully suppressed by carrageenan treatment in the chicks.

188 **Fecal oocyst shedding**

189 Fecal oocyst shedding was monitored daily from 5 to 10 dpi (Fig. 2). Oocyst shedding
190 started at 5 dpi in the infected control group and 6 dpi in the carrageenan group. The maximum

191 numbers of fecal oocysts were $2.1 \times 10^6 \pm 1.5 \times 10^5$ oocysts/g feces at 7 dpi for the carrageenan
192 group and $7.1 \times 10^6 \pm 7.3 \times 10^5$ oocysts/g feces at 6 dpi for the infected control group. The number
193 of fecal oocysts in the carrageenan group was found to be significantly lower than that in the
194 infected control group from 5 to 9 dpi ($p < 0.001$).

195 **Histopathological observations**

196 In infected control chicks, infiltrated cells were observed to extend through the mucosa and
197 submucosa, showing marked hyperplasia of the epithelial cells (Fig. 3a). The histological score
198 was lower in the carrageenan group (2.25 ± 0.48) than that in the infected control group (4.00 ±
199 0.00; $p < 0.05$; Fig. 3b). Meanwhile, the parasite burden scores in the carrageenan and infected
200 control groups were 2.25 ± 0.48 and 3.75 ± 0.25 at 5 dpi, respectively. Significant differences arose
201 between parasite burden scores for the carrageenan group and those for the infected control group
202 ($p < 0.05$; Fig. 3c).

203 **Gene expression analysis**

204 qRT-PCR analysis was used to examine the expression of immune cell markers in the ceca
205 among the chick groups (Fig. 4). The gene expression of CD4, but not other immune cell markers
206 examined, was significantly lower in the carrageenan group than in the infected control group.

207 The gene expression levels of cytokines and immune effector molecules are summarized
208 in Figs. 5 and 6. Transcripts of interleukin (IL)-18, IL-22, inducible nitric oxide synthase (iNOS),
209 and interferon-γ (IFN-γ) were significantly higher in both carrageenan and infected control groups
210 compared to uninfected controls (Fig. 5). Transforming growth factor-β1 (TGF-β1; Fig. 5) and
211 granzyme A (Fig. 6) expression was significantly higher in the infected group than in the control
212 group. The expression levels of Th2 cytokines (IL-4 and IL-13) and IL-17 were also higher in the
213 carrageenan group than those of infected control groups but did not reach statistical significance.
214 Perforin was expressed at high levels, albeit not significantly different, in the carrageenan group

215 compared to the infected control group (Fig. 6).

216

217 **DISCUSSION**

218 Mononuclear phagocytes, including macrophages, have been determined as the most
219 important innate immune cells. Doornick et al. have reported the use of macrophages as a vehicle
220 for sporozoite transport in *Eimeria necatrix* infection (Doorninck and Becker, 1957). Another
221 study has indicated that sporozoites of *E. tenella* can survive phagocytosis and grow within
222 macrophages until the first schizont generation (Long and Rose, 1976). However, the role of
223 macrophages in *E. tenella* infection remains to be elucidated. It has been reported that macrophages
224 may be involved in parasite invasion and development in host epithelial cells (Challey, 1959). The
225 data obtained in the present study contribute to the current understanding of macrophage function
226 in *E. tenella* coccidiosis. This study focused on the mononuclear phagocytes, especially
227 macrophages, and evaluated its role during early *E. tenella* infection. Depleting macrophages *in*
228 *vivo* is an adequate strategy to have a first general glimpse on the importance of macrophages in
229 this parasitic disease. Carrageenan was used to deplete macrophages *in vivo* because this chemical
230 is well known to selectively kill about 90 % of the macrophage population 24 h after peritoneal
231 injection in chicken and mice (Ishizaka et al., 1989; Lee and Al-Izzi, 1981). In fact, macrophage
232 markers were significantly reduced in the spleen of carrageenan-treated chicks, proving that
233 macrophages were reduced in the chicks due to carrageenan treatment.

234 The number of shed oocysts and the parasite burden were significantly reduced in
235 carrageenan chicks treated compared to those of the infected control group (Figs. 2 and 3). In the
236 life cycle of *Eimeria* parasites, sporozoites initially invade epithelial cells at the tip of cecal villi
237 and then migrate to the preferred site of development, the crypt epithelium cells, in order to
238 complete their first round of asexual replication (Daszak, 1999; Dimier et al., 2014). However,

239 how the sporozoites reach this first site of reproduction remains unclear as the distances from the
240 ceca fold to crypt epithelial cells are approximately 100 to 200 μm depending on the host (Daszak,
241 1999). A previous report has described the use of macrophages as a vehicle for sporozoite transport
242 in *E. necatrix* infection (Doorninck and Becker, 1957). Our results confirm that macrophages
243 support invasion by *E. tenella* during the early phase of infection and may serve as transport
244 vehicles to the site of first or second merogony.

245 In this study, chickens intraperitoneally inoculated with carrageenan three times (-48, -24,
246 and 48 h after parasite inoculation) showed lower lesion scores and no mortality (data not shown).
247 In contrast, Lee and Al-Izzi (1981) have reported that chickens injected with carrageenan once (at
248 -24, 0, 24, 48, 72, or 96 h after parasite inoculation) or twice (24 h before and 48 h after parasite
249 inoculation) showed higher lesion scores and mortality rates compared to the uninfected control
250 group. The effect of carrageenan on *E. tenella* infection seemed to be greatest when the
251 carrageenan was injected intraperitoneally 24 h before oocyst inoculation, while the effect of
252 treatment after the oocysts were inoculated was deemed minimal or nonexistent. It appears that
253 time of carageenan treatment affects lesion scores and mortality, which may explain differences in
254 observations recorded in our study in comparison to Lee and Al-Izzi (1981). Higher lesion scores
255 and mortality in the latter study may also be due to differences in virulence of the respective
256 parasite strains. Further studies are needed to evaluate these points.

257 T cells are considered as the most essential in terms of protecting against *Eimeria* infection
258 in birds (Lillehoj and Okamura, 2003). Cellular immune responses, especially through Th1 cells
259 and their associated cytokines, play an important role in anticoccidial immunity (Kim et al., 2019).
260 The expression levels of CD4 and Th1 cytokines were significantly higher in the infected control
261 group (Figs. 4 and 5) than those in the macrophage-depleted group and in the control group,
262 although no statistically significant differences between chick groups were observed in other

263 immune cell markers (Fig. 4). These data suggest that the Th1 immune response was induced in
264 infected control chicks by $CD4^+$ T-cell infiltration (Cornelissen, 2009).

265 Furthermore, macrophage depletion induces significantly less inflammation, reduces
266 histological score, and lowers CD4 expression levels compared to the infected control group.
267 Cytokine responses in the ceca of carrageenan-treated chicks appeared to be a mixed Th1/Th2
268 response, and perforin expression was reportedly higher in the carrageenan group than in the
269 infected control group. These results suggest that $CD3^-CD4^-CD8^-$ cells are induced by macrophage
270 depletion and possibly regulate inflammation and local immune response. The γδ T cells have been
271 determined to reside in intraepithelial regions in the mucosa and lymphoid tissue and are associated
272 with CD4 and CD8 double-negative T cells (Fenzl, 2017). These γδ T cells represent up to 50% of
273 peripheral T cells in chickens and are known to expand as a result of pathogen infection (Arstila
274 and Lassila, 1993). In chickens, γδ T cells reside in three populations: $CD8\alpha^-$, $CD8\alpha^-$ low
275 expression, and $CD8\alpha^-$ high expression γδ T cells. Furthermore, γδ T cells are important in the
276 generation of Th2 immunity and as producers of IL-17 and IL-22 (Born et al., 2010; Chien et al.,
277 2014; Martin et al., 2009). In addition, γδ T cells show cytotoxicity against pathogen-infected cells
278 by secreting cytotoxic factors such as perforin (Kristin et al., 2011; Martin et al., 2009). In this
279 study, the level of IL-22 was significantly higher at the site of infection. IL-22 also promotes cell
280 proliferation and plays a role in tissue regeneration (Stange et al., 2012). IL-22 also has a novel
281 antiparasitic role during intestinal intracellular parasite infection in mice (Shoai et al., 2017). In
282 addition, γδ T cells expressing Vγ9 and Vδ2 chains of T-cell receptors represent a nonconventional
283 T-cell subset and control parasite density during malaria infection (Costa, 2011; D'Ombrain et al.,
284 2007). The present results suggest that γδ T cells are induced by macrophage depletion and further
285 regulate parasite reproduction at the site of *E. tenella* infection. Perhaps merozoites released from
286 the first reproductive sites during *E. tenella* infection are killed by γδ T cells, which are induced

287 by macrophage depletion, thereby reducing the parasite burden. Further studies are needed to
288 understand the roles of γδ T cells in the chicks with *E. tenella* under carrageenan treatment
289 conditions.

290 Overall, this study suggests that macrophages serve as a gate for invasion and/or transport
291 vehicle for sporozoites and merozoites to the site of further merogony. Mononuclear phagocytes
292 also play an important role in local immune response, thus contributing to parasite development
293 during early *E. tenella* infection.

294

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301

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394 cellular compartments stimulated dichotomic immune responses in chickens. J. Immunol. 187,

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396

397 **TABLE CAPTIONS**

398 Table 1. Primer sets for macrophage markers.

399 Table 2. Primer sets for immune cell markers, cytokines, and effector molecules.

400

401 **FIGURE LEGENDS**

402 **Figure 1.** mRNA expression levels of macrophage markers in the spleen of 17 days old chickens

403 treated with carrageenan. Data are presented as mean ± SEM and analyzed by unpaired *t*-test using

404 Kyplot Statistics 5.0 software (*$p < 0.05$; **$p < 0.01$).

405 **Figure 2.** Fecal oocyst shedding during daily from 5 to 10 dpi. Error bars represent SD. ***$p <$

406 0.001 vs. infected control.

407 **Figure 3.** Histopathology of H&E-stained cecum sections at 5 dpi. (a) H&E-stained specimens
408 observed under light microscopy. Arrows indicate intracellular parasites, and arrowheads indicate
409 infiltrating immune cells. (b) Microscopic histopathologic scores (c) Parasite burden score. Data
410 are presented as mean ± SEM. *$p < 0.05$ vs. infected control.

411 **Figure 4.** mRNA expression levels of immune cell markers in the ceca of chicken infected with *E.*
412 *tenella* at 5 dpi. Data are presented as mean ± SEM. *$p < 0.05$.

413 **Figure 5.** mRNA expression levels of cytokines in the ceca of chickens infected with *E. tenella*.
414 Amplifications were performed from four independent samples in each group, with triplicate
415 reactions for each sample. Data are presented as mean ± SEM. (*$p < 0.05$; **$p < 0.01$).

416 **Figure 6.** mRNA expression levels of immune effector molecules in the ceca of chickens infected
417 with *E. tenella*. Amplifications were performed from four independent samples in each group, with
418 triplicate reactions for each sample. Data are presented as mean ± SEM. (*$p < 0.05$; **$p < 0.01$).

Declaration of interests

Manuscript title: Reduction of macrophages by carrageenan decreases oocyst output and modifies local immune reaction in chick cecum with *Eimeria tenella*

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.